ANTIMICROBIAL RESISTANCE IN THE ENVIRONMENT

ANTIMICROBIAL RESISTANCE IN THE ENVIRONMENT

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Patricia dedicates this book to the memory of her parents
Leonard Percy Keen
Margaret Ada Prescott Keen

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PREFACE

It began with Bordeaux. Yes, the wine and yes the fabulous city in France. The eighth annual meeting of the Society of Environmental Chemistry and Toxicology was held in the spring of 1998 in Bordeaux. Keen and Montforts met each other over a glass of Médoc (or was it the Graves?) at the opening wine-tasting social. As environmental professionals and, at the same time, students with a common research focus of veterinary antibiotics as pollutants in the environment, the idea for this book germinated at that very first meeting. Early in our respective academic careers, we were inspired by some of the world's leading scientific authorities on antibiotics and antimicrobial resistance. With time, our respective academic journeys took their course, but the bond that formed during the conversations commiserating over the trials and tribulations of a doctoral study program, eventually morphed into the shared vision to edit a unique book on the subject of our prime research interest.

The past decade has witnessed the sustained growth of concern in the scientific community and the general public regarding the development of antibiotic resistance in bacteria. It is now well accepted that exposure of nontarget microorganisms to antibiotics as a result of human activities can promote resistance in pathogens that can compromise the efficacy of human and veterinary medicine. Health consequences of exposure to methicillin-resistant *Staphylococcus aureus* (MRSA) and other Superbugs now receive regular media attention worldwide, and there is much concern that a portion of the arsenal of antibiotic drugs are losing their ability to successfully combat many critical bacterial infections. There are increasingly frequent reports of scientific evidence that underlines the importance of environmental transport pathways for distribution of antimicrobial-resistant bacteria via surface water, drinking water, and stream networks.

The spread of antibiotic resistance in the environment depends on the presence and transfer of resistance elements among microorganisms, on the genetic mutations that result, and on the selection pressure to retain these genes within the population. In essence, all substances can act as antibiotics—the distinction between those

substances that can be classified as antibiotics and those that act as signaling molecules is governed by the dose. Antibiotic resistance genes are clearly recognized as potential environmental contaminants. The problem, however, with assessing the relative contribution of antibiotic resistance as contaminants is that the complexity and adaptive behavior of bacterial communities in natural ecosystems is not well understood. This intersection of human health risk assessment with ecological and environmental research and risk assessment is becoming more and more evident.

There has been a fundamental shift in thought regarding the role of the environment in the spread of antimicrobial resistance among microorganisms. The perceived boundary between an "ecosystem-based approach" and a "human-health-centered approach" to characterizing antimicrobial resistance as a health risk has somewhat dissolved. The sometimes independent and sometimes overlapping processes in ecosystems serve as both a distribution network and a potential source for the stressors that expose a broad range of microorganism species to antibiotics and antibiotic resistance genetic elements. This duality of the environmental role is an important consideration in the spread of antibiotic resistance given that the resistance mechanisms in some soil-dwelling microorganism mimic those seen in clinically relevant bacteria.

The conceptual reference point of "environment" in exploring the health risk associated with development of antibiotic resistances in organism has expanded. Gradually, the process for risk assessment of health consequences related to development of antimicrobial resistance in bacteria via multiple pathways is adapting to incorporate variability in temporal and spatial scales in the overall evaluation of the risk. The awareness of the importance of antimicrobial resistance as a health risk extends beyond the association with exposures to antimicrobial drugs. For example, the ever-improving biotechnological capabilities in manipulation at the genetic level allow the insertion of antibiotic resistance markers in transgenic plants. This has important implications for introducing opportunities for bi-directional gene flow among soil-dwelling microbes for which the role of antibiotic resistance in the natural ecosystem remains incompletely understood. Recent studies of the effects of antimicrobial properties associated with nanoparticles are also providing evidence that nanotechnology may offer some improvement in treatment of infections. Although several research teams are investigating the environmental risks linked to antibiotic resistance genes as makers in transgenic organisms and the implications for antimicrobial resistance related to use of engineered nanoparticles, these issues are not discussed specifically in this volume.

The overarching theme of this book is that the environment plays a crucial role in the development of antibiotic resistance traits in bacteria and the distribution of antibiotic-resistant microbial species, resistant genetic material, and antibiotic compounds. Resistance genes appear to be everywhere in nature—in pathogens, commensals, and environmental microorganisms. We invite you to gain a broader insight of the role of the environment in the mechanisms of resistance development, the dissemination of antimicrobial-resistant genetic elements, and the transport of antibiotic resistance genes or antibiotics as environmental contaminants through the presentations of our contributing authors in this book.

We would like to express our sincere gratitude to our colleagues for their fantastic contributions to the creation of this book. We appreciate the hard work, the patience, and the friendship that we have shared with this community of researchers during the

completion of the book project. As well, there are many individuals we wish to thank for enabling its completion. The team at Wiley–Blackwell led by Karen Chambers and Anna Ehler have guided us through this evolutionary process with understanding and finesse—for this we are very grateful.

Patricia owes many thanks to several special friends and colleagues who have provided their support throughout this process: Charles Knapp, Teresa Frolek, Lisa Waddell, Debbie Angel, Marco Solinas, Annette Muttray, Heather Slater, David Brownstein, May du Monceau, Katia Freire, Charlene Knapp, Agnes MacDonald, Pablo Trujillo, and Jesse Read. Patricia also thanks her doctoral supervisor Ken Hall and the other members of her research committee, David Graham, Pierre Bérubé, Les Lavkulich, and Bob Hancock, for their inspiration in the preparation of this book. Raphaël Fugère has played important roles in this project as well that have ranged from providing sympathetic critical review to volunteering his technical assistance in field sampling. None of this enterprise could be possible without the unfailing encouragement, patience, love, and technical support of Steve Clark. Thank you.

Patricia is particularly grateful to Daniel Pauly and Rashid Sumaila and many of the fabulous researchers at the University of British Columbia Fisheries Centre for their kindness and support during the time she has shared with them as a visiting scholar. As artists, researchers, teachers, and, of course, friends, Belidson Dias and Philippe Raphanel are Patricia's ever-growing inspiration, and we offer our thanks to Philippe Raphanel for the use of his painting on the cover. The special influences of Henk de Wit and Gérard Faisandier are forever metaphorically etched in the conceptual foundations of most of Patricia's scholarly and creative endeavors.

Mark wishes to acknowledge the support, critiques, and/or inspiration he received from Heike Schmitt, Bent Halling-Sørensen, Tore Midtvedt, Gerard Rijs, Hans Mensink (†), Han de Neeling, Ana Maria de Roda Husman, Saskia Rutjes, Hetty Blaak, Caroline Moermond, Jan Roels, Charles Bodar, Bart Hellings, Sybrand Landman, and literally last but certainly not least, Emily McVey.

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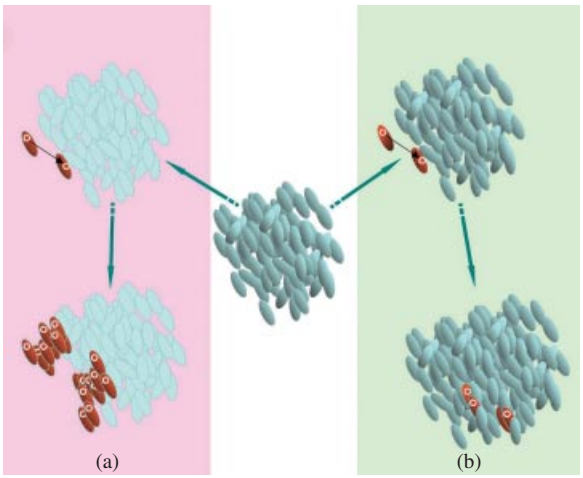

FIGURE 9.1 Second-order selection of transfer of antibiotic resistance genes in the presence of antibiotics. Once an antibiotic resistance gene is present in a gene transfer element, it can be spread among different hosts. Nevertheless, carrying this element will produce a fitness cost, so that in the absence of antibiotics' selective pressure, resistant bacteria, including those that have received the resistance gene by horizontal gene transfer from the original host, will be out competed by the susceptible ones (a). Under circumstances of no selection, acquisition of novel genetic elements is not adaptive. In the presence of antibiotics, however, the growth of susceptible bacteria will be inhibited, and only those containing resistance genes (either the original ones or those that have received the resistance determinant by gene transfer) will be able to grow (b), thus favoring the dissemination of these resistance determinants.

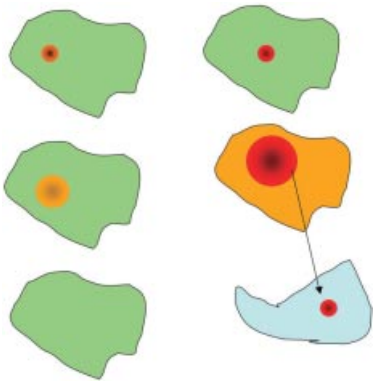

FIGURE 9.2 Different fate of contamination by antibiotics and by antibiotic resistance genes. Antibiotics are chemical compounds that are degraded by time and diluted across space. Their diffusion in natural ecosystems produce a reduction in their concentration and, if there are not more pollution events, the contaminant will disappear sooner or later (a). Contrary to this situation, antibiotic resistance genes are autoreplicative elements, so that their concentration can increase in the presence of antibiotics and eventually can travel across long distances to occupy environments were they were not originally released using wild animals as vectors (b). Antibiotic contamination are shown in orange, and pollution by antibiotic resistance determinants are shown in red.

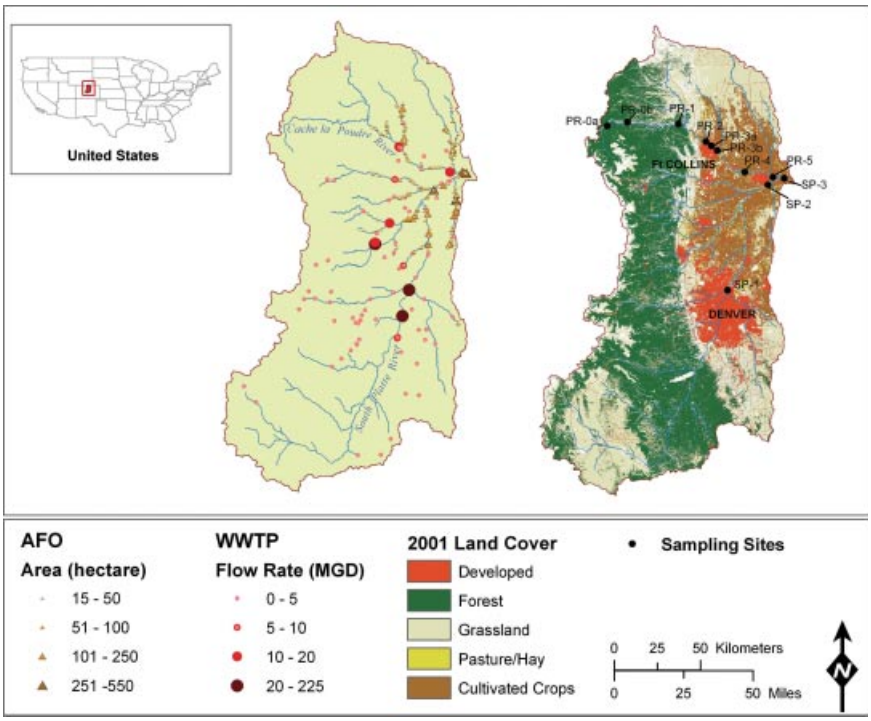

FIGURE 10.1 Land cover classification of South Platte River Watershed, including the South Platte River, the Cache La Poudre River, and their confluence. Sampling sites are indicated in the left panel and wastewater treatment plants (WWTPs) and animal feeding operations (AFOs) are indicated in the right panel.

FIGURE 16.1

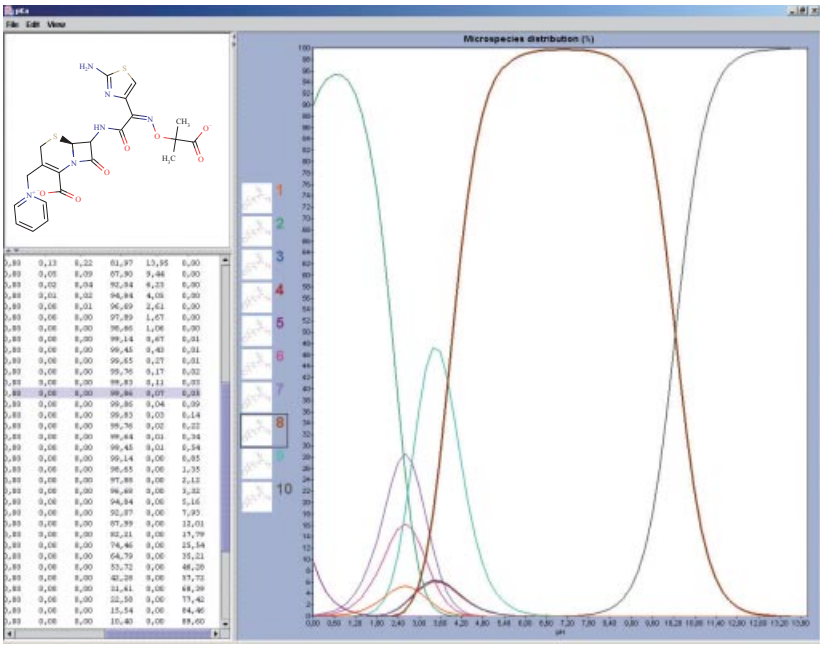

FIGURE 18.1 Internal salt and zwitterionic character of ceftazidime (top). Depending on pH additional different chemical species can be formed by internal protonation, that is, shift of protons between the basic amino functions and acidic carboxyl groups (bottom). (Calculator Plugins were used for structure property prediction and calculation, Marvin 4.1.1, 2006, ChemAxon (http://www.chemaxon.com) (Kümmerer, 2008)

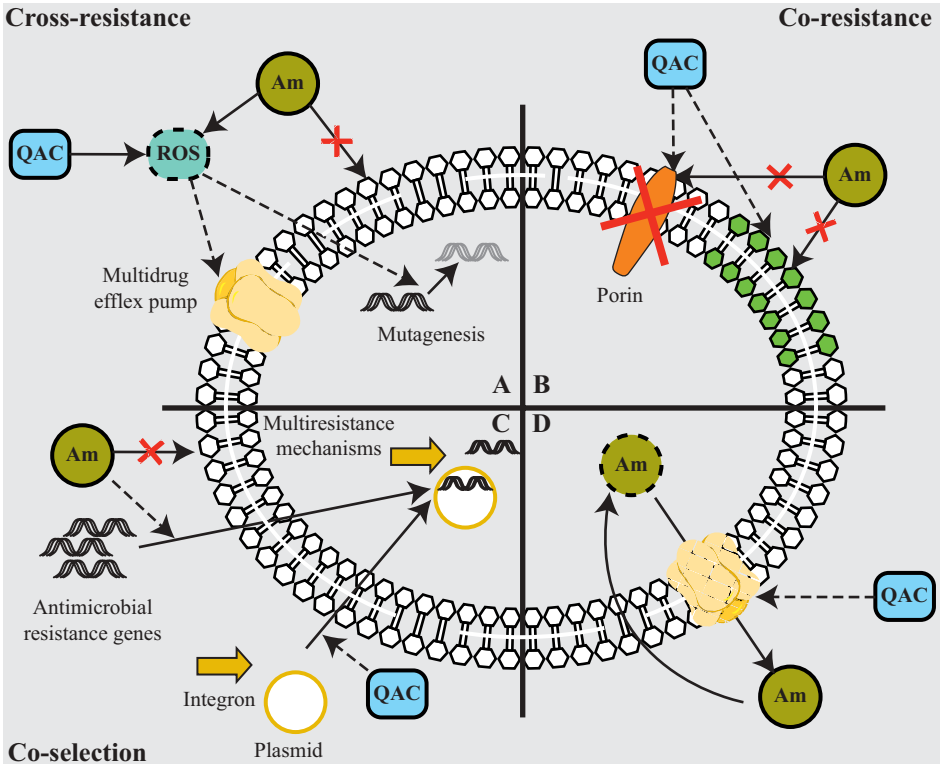

FIGURE 20.11 QAC-induced or selected antibiotic/antimicrobial resistance mechanisms (see descriptions of A, B, C, and D in section 20.6 of chapter 20; Am = antimicrobial agent).

FIGURE 25.2 Sampling of Indian river sediment contaminated with broad-spectrum antibiotics (up to $\sim$1 g ciprofloxacin per kg organic material). Pyrosequencing of the microbial communities within these sediments revealed high levels of resistance and gene transfer elements (Kristiansson et al., 2011).

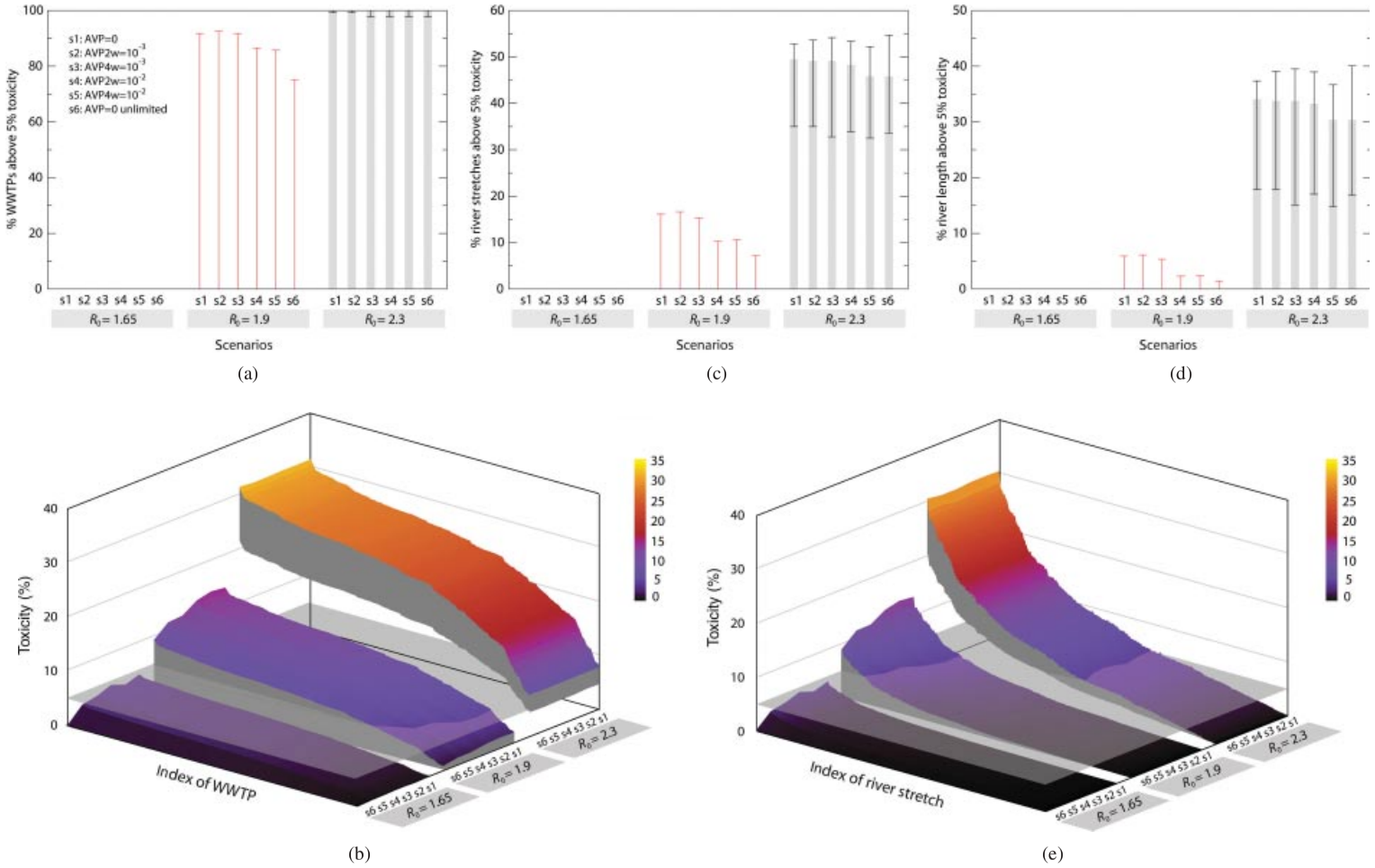

FIGURE 26.4 Predicted toxicity in WWTPs and river stretches.

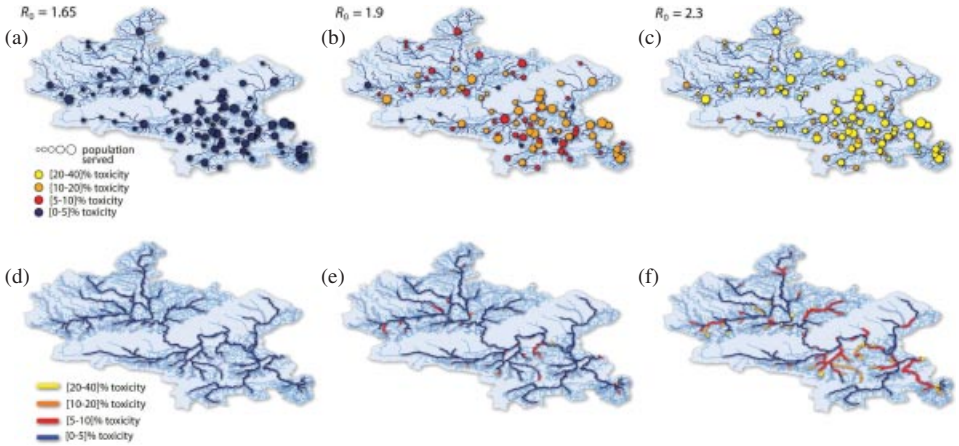

FIGURE 26.5 Predicted toxicity maps.

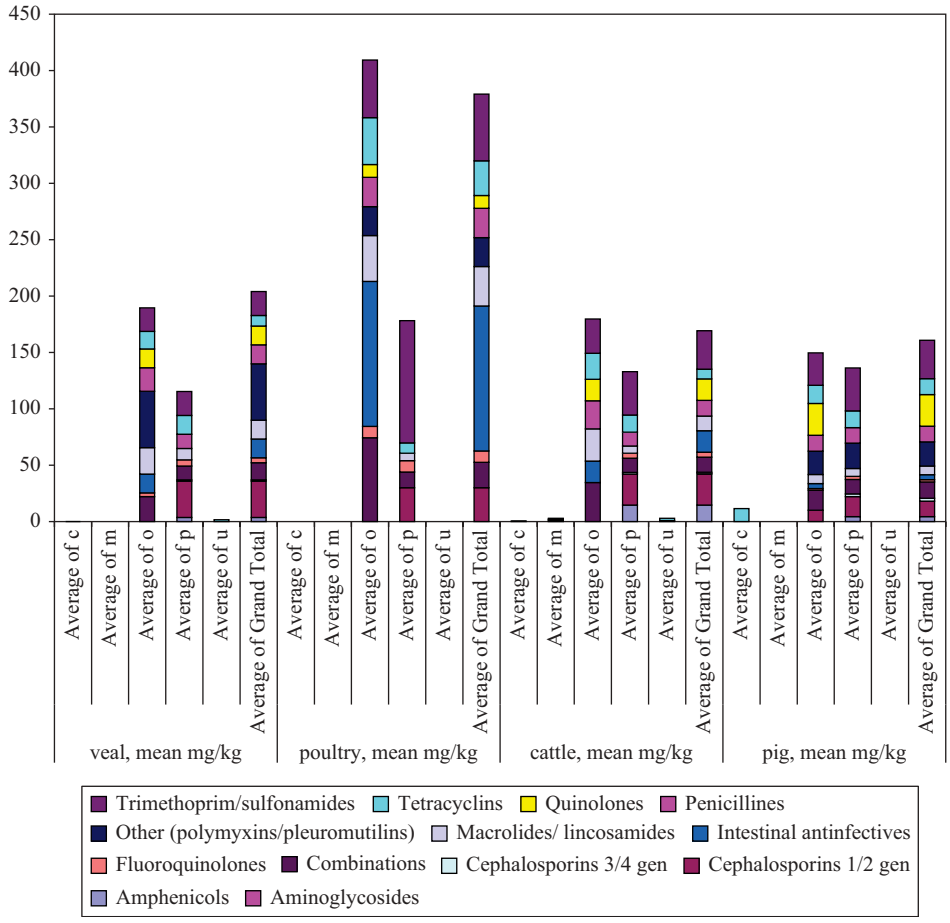

FIGURE 27.1 Calculated doses per pharmacotherapeutic group differentiated per species and by way of administration, based on all for livestock authorized medicinal products in the Netherlands ultimo 2010 (c, cutaneous, m intramammary, o oral, p parenteral, and u intrauterine).

PART I

SOURCES

1

INTRODUCTION

STUART B. LEVY

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With the discovery of antibiotics came the recognition of antibiotic resistance. With time, the concept emerged: wherever antibiotics exist, resistance determinants reside. Thus, the early emergence of antibiotic resistance clinically occurred among patients in hospitals where these new therapeutics were introduced. Resistance appeared initially to sulfa drugs (1930s) and then to penicillin (1940s) (Levy, 2002).

While the early warnings were largely disregarded, resistance increased, continuing to follow mounting antibiotic use. The true breadth of the problem appeared in the 1970s and 1980s, when many different multidrug-resistant (MDR) infectious organisms became evident. Hospitals were no longer the only source of drug resistance. Resistant organisms were appearing among both clinical and nonclinical strains stemming from antibiotic use in the community. The latter microorganisms carried genes for resistance that were shared with other bacteria in the environment.

Over the past several decades, recognition of the environmental load of resistant organisms has increased. Bacteria of little clinical consequence have become health threats because they harbor antibiotic resistance genes that can be transferred to organisms, causing disease in people. The phenomenon has arisen on the heels of increased antibiotic use in communities and among farm animals. Moreover, antibiotics from various sources are being discharged as active drugs into the environment through inadequate waste management. For example, a recent report links finding antibiotics and drug resistance in river sediments to discharge from an antibiotic-producing company in India (Kristiansson et al., 2011). Antibiotics are delivered to crops in manure from animals fed antibiotics for therapy and growth promotion. The presence of both the antibiotic and the resistance genes in the same environment selects for resistant bacteria that are then spread widely. Thus, if for no

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other reason, it should come as no surprise why and how drug resistance has emerged outside the hospital and in the environment.

As this book presents, recognition of resistance is now broad but not necessarily connected with antibiotic use. Resistance may occur in the absence of an antibiotic substrate, particularly in soils where organisms may bear resistance traits to drugs not detected in that environment. Moreover, the environment aids in the spread of resistance through gene transfer.

Antibiotics affect not only the microbial flora of the treated individual or animal, but also the people (and animals) sharing the environment. Thus, the resistance phenomenon can extend well beyond the treated individual, person or animal. These findings illustrate why these therapeutics can be called "societal drugs" (Levy, 2002).

Studies performed by William Cunliffe and associates in London demonstrated that those taking antibiotics for acne selected skin bacteria resistant not only to the acne drug but also to other drugs as well. Importantly, resistant bacteria were found not just on the skin of the treated patients but also on the skin of those sharing the same household (Miller et al., 1996). This kind of "societal" sharing involved an ecologic selection of resistant organisms in the environment of the acne patient. A similar ecologic consequence has been noted on a farm where growth promotion use of oxytetracycline led to selection and spread of resistant fecal *Escherichia coli* among treated and nontreated animals and people (Levy et al., 1976). Chronic use of a single antibiotic led to multidrug resistance (Levy et al., 1976). This phenomenon of antibiotic use and emergence of resistance in nontreated people or animals sharing the same environment is described in other experimental studies as well (Levy, 2002).

In 1986, Dick Novick and I organized a Banbury meeting in Cold Spring Harbor on the epidemiology of antibiotic resistance (Levy and Novick, 1986). Little did we surmise then that in two-and-a-half decades, studies of soil would reveal large numbers of indigenous drug-resistant bacteria. In fact, these organisms can be regarded as "reservoirs" of resistance genes and they far out-number those we face clinically.

In 1989, Bob Miller and I edited a book entitled *Gene Transfer in the Environment* (Levy and Miller, 1989). This volume converged the knowledge of experts in the area of gene transfer with an emphasis on natural environments as locales for gene spread. As cited in the Preface, "gene transfers occur in all ecologic niches." The thrust of both the conference and the book was to illustrate the potential spread of any organism and any resistance gene in the environment, with particular reference to genetically engineered microorganisms. The environment was not the focus of that book. It represented the backdrop for gene exchanges.

What was not defined then but is now more directly addressed in this book is the occurrence of drug resistance within the environment flora. This phenomenon results from production of antimicrobials by the soil organisms themselves, as well as the dispersion of drugs and resistance genes through wastewater from treated animals and people.

Investigations have identified the natural soil environment as a source for both antibiotics and resistance genes. This volume extends that knowledge to many different environments that serve as a source and repository of antibiotic-resistant organisms. Studies detailed in this book stem from findings of a plethora of resistance determinants among soil *Streptomyces* (D'Costa et al., 2006). Other prior studies show evidence that naturally occurring resistance determinants in soil bacteria

degrade the antibiotic, producing nutrients for the growth of these bacteria (Dantas et al., 2008). The presence of resistance genes in the environment has recently been reviewed, addressing the many routes by which resistance genes emerge and spread in the natural environments (Allen et al., 2010).

The concept of intrinsic or naturally occurring resistance among soil organisms, now called the antibiotic resistome, is expanded upon by authors in this volume. Some resistances may be naturally occurring, but others are a result of "pollution." The many avenues/means by which manure and wastewaters, antibiotics, and antibiotic-resistant bacteria are spread into the environment are well documented throughout this volume. The work under discussion includes not only food animals but also the use of antibiotics in fish farms, as reviewed here and elsewhere (Levy, 2002).

This book's strong message is that there exists a broad, general environmental presence of antibiotics and drug-resistant bacteria. They may be concentrated in areas such as hospitals or farms, or spread more widely in the environment in association with people and animals, or found naturally, as demonstrated by the ubiquitous nature of antibiotic resistance genes in the soils. The findings raise a number of interesting questions. What purpose are the naturally present resistance determinants providing? What do they mean to the future discovery and use of antibiotics? Clearly, one can expect resistance emergence no matter how novel the new antimicrobial may be.

Ironically, the relatively high levels of resistance genes in the natural soil environment suggest a reason for our difficulty in finding new antibiotics in soils. Quite clearly, antimicrobials produced in the soil will be subject to inactivation by degrading enzymes that are present there. To salvage the antibiotic, the enzymes would need to be deactivated, for example, by heating, before attempting to find and extract an antimicrobial. Thus, a consequence of finding degradative enzymes among soil microbes is important to the origins of resistance as well as our ability to find new antibiotics (Levy, 2006).

Can we discern a relationship between the environmental reservoir of antibiotic resistance and the potential prediction of resistance emergence in human pathogens? Moreover, what is the level of antibiotics in these environments and does their presence wholly account for the emergence of resistant bacteria? These are naturally occurring microbes for which resistance offers some advantage, not just for survival against other microbes but in defense against discharged waste products, or as signal molecules in microbial community activities.

A Reservoirs of Antibiotic Resistance (ROAR) project, spear-headed by the Alliance for Prudent Use of Antibiotics (www.apua.org), involved experimental systems that demonstrated the transfer of resistance genes among environmental flora. This has led to the development of an online database that includes descriptions of these organisms and their resistance determinants.

A follow-up study, currently undertaken by APUA, examines the kinds of resistance traits genetically and phenotypically among nonclinical bacterial isolates: *E. coli*, *Streptococci*/*Enterococcus*, *Staphylococcus*, *Aeromonas*, *Salmonella*, *Pseudomonas*, *Acinetobacter*, and *Stenotrophomonas* in environments in different areas of the world, including Bangladesh, Georgia, India, Turkey, Uganda, South Africa, South Korea, and Vietnam. The findings so far display large differences in the presence of drug resistances among the same species, but isolated from varied geographic sites.

This book is unique in bringing together a cadre of basic scientists and epidemiologists interested in the presence of antibiotics and resistant bacteria in the environment. It does not focus on organisms affecting disease in people but on the reservoir in nonclinical strains. This is a powerful and comprehensive illustration of change in thought, particularly as we compare what was considered several decades ago and how that has changed over time—from the interest in the environment as a place for gene transfer, to the evaluation of the environment as a reservoir of antibiotics, antibiotic-resistant bacteria, and transferrable resistance genes.

REFERENCES

Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J (2010). Call of the wild: Antibiotic resistance genes in natural environments. *Nat Rev Microbiol* 8:251–259.

Dantas G, Sommer MOA, Oluwasegun RD, Church GM (2008). Bacteria subsisting on antibiotics. *Science* 320:100–103.

D'Costa KM, McGrann DW, Hughes DW, Wright GD (2006). Sampling the antibiotic resistome. *Science* 311:374–377.

Kristiansson E, Fick J, Janzon A, Grabic R, Rutgersson C, Weijdegård B, Söderström H, Larsson DGJ (2011). Pyrosequencing of antibiotic-contaminated river sediments reveals high levels of resistance and gene transfer elements. *PLoS ONE* 6(2):e17038.

Levy SB (2002). *The Antibiotic Paradox: How Misuse of Antibiotics Destroys Their Curative Powers*. Perseus Books, Boston.

Levy SB (2006). Mechanisms for resistance in soil. *Science* 312:529.

Levy SB, Miller RV (Eds.) (1989). *Gene Transfer in the Environment*. McGraw-Hill, New York.

Levy SB, Novick RP (Eds.) (1986). *Antibiotic Resistance Genes: Ecology, Transfer and Expression*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Levy SB, FitzGerald GB, Macone AB (1976). Changes in intestinal flora of farm personnel after introduction of tetracycline-supplemented feed on a farm. *N Engl J Med* 295:583–588.

Miller YW, Eady EA, Lacey RW, Cove JH, Joanes DN, Cunliffe WJ (1996). Sequential antibiotic therapy for acne promotes the carriage of resistant staphylococci on the skin of contacts. *J Antimicob Chem* 38:829–837.

2

PATH TO RESISTANCE

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2.1 INTRODUCTION

There are essentially two modalities of antibiotic resistance (AR) in bacteria, one being mutation of the cellular target of the inhibitor or alteration of its influx process, the other the acquisition and inheritance of exogenous genetic determinants that permit the cell to grow in the presence of the inhibitor (Davies and Davies, 2010). Gene transfer and gene expression within phylogenetically related bacteria occur frequently in nature and have been widely studied; such heterologous expression presumably involves genetic adjustment to assure its efficacy. When DNA (deoxyribonucleic acid) transfer occurs between distantly related bacterial genera, the expression of acquired genes in unrelated cytoplasms may be subject to numerous constraints, and a functional level of expression toward a specific phenotype may require extensive mutational "tailoring." Prior to the present era of metagenomics and whole genome sequencing, AR genes were first identified post facto in clinical isolates. The question raised here is, since AR genes probably originate from microbes that populate different aquatic and terrestrial environments, how do they become established in human and animal pathogens such as *Escherichia coli* or *Staphylococcus aureus*?

2.2 A LITTLE HISTORY: "THE WRITING WAS ON THE WALL"

Apart from plant and other traditional remedies that have been employed for millennia for the treatment of infections, the development of various salts of toxic metals such as arsenic and mercury provided the first extensively used antimicrobials. Little is known about the outcomes, but, given that microbes and heavy-metal salts

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have coexisted for millennia (Silver and Phung, 2005), it is likely that bacterial mutants resistant to these compounds appeared frequently during the course of the "therapy." The identification of microbes as agents of infection during the early days of medical microbiology (1865–1920) subsequently enabled reliable laboratory sensitivity tests. The first antimicrobials of the "modern" age were the sulfonamides, discovered and introduced with considerable success in the mid-1930s; these were also the first to be studied on the basis of structure–activity relationships. However, their use was soon limited by the appearance of resistant strains of streptococci and other previously susceptible Gram-positive pathogens. It was the rediscovery and development of the miraculous drug penicillin after World War II that launched the antibiotic era, when natural products from fungi and bacteria were produced industrially on a large scale and used to control human pathogens (Florey et al., 1949). Extensive use (and misuse) of penicillin led to the appearance of increasingly resistant (or recalcitrant) strains of Gram-positive pathogens, and treatment failures became more frequent. A second major antibiotic, the broad-spectrum streptomycin, was introduced in the 1950s as the desperately needed treatment for tuberculosis (the "white plague" caused by *Mycobacterium tuberculosis*) and became highly successful despite the problems of toxicity (Florey et al., 1949). However, once again resistant strains appeared in parallel with the need for aggressive streptomycin treatment. Tetracycline, chloramphenicol, and erythromycin, discovered and introduced soon afterwards, were successful in the treatment of penicillin-resistant strains, but their cure rates were inevitably compromised by the appearance of resistant pathogens. A good account of the use of antibiotics and concomitant resistance development in the early days of the antibiotic era is given by Maxwell Finland (Finland, 1955).

A vitally important but often ignored factor in antibiotic resistance development is that significant quantities of antibiotics, albeit in crude form, were already being used in the mid-1950s for animal therapy and growth promotion (Jukes and Williams, 1953). While the net merits of this practice are unclear even today, what is certain is that it constitutes an enormous selection pressure on microbes, contributing to the generation of significant reservoirs of resistant strains in the environment that have compromised the use of the same or related antibiotics for treatment of human infections (Salyers and Whitt, 2005). The warning was sounded nearly 60 years ago (McCoy, 1955) and is supported now by the presence of resistance genes in nearly all environments.

Use of semisynthetic antibiotic derivatives to circumvent resistance began in 1959 with the synthesis and production of methicillin, a chemically modified penicillin designed to be active against penicillin-resistant staphylococci. This was a significant step forward in the strategy of antibiotic use. However, bacterial pathogens were up to the challenge, and methicillin-resistant *S. aureus* (MRSA) became evident in the early 1960s. As is well known, MRSA remains a menace: antibiotic resistance does not go away. Even synthetic antimicrobial compounds such as the fluoroquinolones are not safe from the spectre of resistance: spontaneous mutations in the target protein, DNA gyrase, can lead to high levels of resistance against ciprofloxacin.

2.3 MECHANISMS OF ANTIBIOTIC RESISTANCE

Only limited studies of AR mechanisms were carried out during the early stages of antibiotic usage; it was generally assumed that most nonsusceptible bacterial strains

TABLE 2.1 Biochemical Mechanisms of Antibiotic Resistance[a]

Increased efflux	Decreased influx
Enzymatic inactivation	Sequestration
Target modification	Target bypass
Target repair/protection	Target amplification
Biofilm formation	Intracellular localization

[a] The majority of these mechanisms are subject to horizontal gene transfer. Note that a resistant strain may possess multiple mechanisms of resistance to the same antibiotic.

were "variants" with differing levels of susceptibility or the result of simple mutation. Some of these mutations were genetically characterized. Hotchkiss (Kashmiri and Hotchkiss, 1975) and others also identified target amplification as a common mechanism of resistance to sulfonamides in the pneumococci. (Incidentally, antibiotic-resistant mutants subsequently proved invaluable in the seminal experiments demonstrating bacterial gene transfer.)

Target site mutation in the host as a path to antibiotic resistance is now a reasonably well-understood phenomenon. There are nonetheless many physiological and genetic complexities that may accompany the process, with additional mutations required to compensate for any associated reductions in fitness, growth, or pathogenicity. Expression of a resistance phenotype can be dependent on both the bacterial strain and the environment. What happens in petri dishes does not necessarily reflect bacterial behavior in the clinic: it is highly unlikely that clinical AR was based on a single mutation. Indeed, the current use of next-generation sequencing in "bedside" analyses of pathogens has shown that resistance is a complex genotype as a result of multiple, sequential mutations (Mwangi et al., 2007). Pathogens must tolerate/survive many stresses during the course of human infection. Parenthetically, several classes of antibiotics have inherent mutagenic activity and this almost certainly has contributed to their fallibility.

The biochemical mechanisms of resistance in bacterial pathogens currently known are shown in Table 2.1. Some may occur because of alterations in regulatory functions; overexpression of specific functions related to the antibiotic target is common. Unstable AR phenotypes that have properties characteristic of epigenetic changes are often found, but there is as yet no strong experimental evidence to support this supposition (Adam et al., 2008).

2.4 R-FACTORS: PURVEYORS OF EXTRACHROMOSOMAL AR GENES

The perception of antibiotic resistance changed drastically with the discovery of plasmid-borne resistance genes ("R-factors"). The *force majeure* in clinically significant antibiotic resistance is horizontal (or lateral) gene transfer, HGT (or LGT). The initial reports of this phenomenon from Japan in 1959 were met with considerable skepticism, and it was not until after additional reports of R-plasmid resistance in the United Kingdom (1962), Germany (1963), and the United States (1964) that the phenomenon was generally accepted (Davies, 1995). The origins of the plasmid vectors are still unknown, but they are thought to be related to bacterial sex factors or bacteriophages; clinical isolates of bacteria from the preantibiotic era contained

plasmids but no detectable AR genes. The characteristics of transmissible drug resistance are multidrug and multimechanism, and the inheritance of AR plasmids is also associated with genetic changes in the host.

The mechanisms encoded by transmissible AR genes usually result in enzymatic inactivation of the antibiotics, either outside or inside the bacterial cell; for example, since the 1960s, hundreds of β-lactamases and other antibiotic-inactivating enzymes have been characterized (Bush, 2010). However, there are also a multiplicity of other mechanisms of resistance, many of novel function and not derived by mutation (Table 2.1). In many cases, the genes encoding AR are components of an integron, an ingenious bacterial system that in its simplest form consists of a specific recombinase gene flanked by a cognate attachment site (Cambry et al., 2010). An AR gene situated between certain sequences used by the integrase can be site-specifically recombined into the attachment site, and repeated recombination events result in a series of tandemly arranged AR gene cassettes next to the gene for the recombinase. Arrays of AR cassettes appear stable, but the number of cassettes in the array can be increased or reduced, generating a versatile range of phenotypes that are selected in the presence of environmental stresses. Although integrons themselves are not self-mobilizing, an abundance of plasmid-borne mobile genetic elements, such as insertion sequences and transposons, as well as stand-alone transposases can relocate integrons as individuals or groups of AR genes within and among plasmids. Movement of single genes or entire plasmids by well-known bacterial processes (transduction, transformation, conjugation) may further distribute AR genes throughout a bacterial population. Such collections of mobile and multifunctional extrachromosomal AR genes and associated elements are responsible for exacerbating the complexity and intractable nature of many of the widespread clinical multidrug resistance problems of today.

2.5 ORIGINS OF EXTRACHROMOSOMAL RESISTANCE GENES: METAGENOMIC STUDIES OF AR

The AR genes that thwart the treatment of infectious disease ultimately originate from environmental sources: genes for resistance to heavy metals are often found in the same plasmids that confer AR, supporting the notion that R-plasmid resistance came from non-clinical bacteria. Early experiments with soil community DNA demonstrated that microbes in natural environments clearly have the genetic capacity to resist even relatively modern antimicrobials such as the fluoroquinolones (Waters and Davies, 1997) and suggested that further studies might be predictive of clinically significant resistances and thus able to guide antibiotic design. Recent and more comprehensive metagenomics-based analyses for the presence of resistance genes from varied environmental samples have provided strong support for the notion of a global pool of potential resistance genes—"proto-AR genes"—on Earth's surface (Wright, 2007). Complementing this work is a study of antibiotic degradation processes of soil bacteria (Dantas et al., 2008) and subsequently the identification of a large variety of AR gene analogs in the microbiome of the human gastrointestinal (GI) tract (Sommer et al., 2009). Many of the sequences showed good matches with previously identified AR genes of human pathogens. These studies emphasize the findings of earlier work (Shoemaker et al., 2001) that demonstrated extensive

HGT in the gut, indicating that the human GI tract is a major source of AR genes: We may be our own worst enemy.

Of special interest are the β-lactamases that inactivate penicillin and related compounds: These enzymes are chromosomally encoded in many environmental bacteria but are more frequently encoded—in many variations—on plasmids in pathogens. An especially strong relationship has been identified among the CTX-M β-lactamase genes that originated from a soil *Kluyvera* sp. bacterium. The widespread distribution and clinical significance of this family of genes attests to the important role of environmental sources, mobilizable AR, and the facility with which they establish international AR crises (Hawkey and Jones, 2009). The huge β-lactamase family of enzymes is clearly of diverse origin. In addition, the continuing in situ evolution by mutation concurrent with the use of chemically synthesized derivatives of β-lactam antibiotics ensures their omnipresence. Similarly, plasmid-encoded resistance to the fluoroquinolone antimicrobials has increased significantly during the past decade or so, largely due to the appearance of *qnr* genes encoding pentapeptide repeat proteins that bind to DNA and so protect it from fluoroquinolone-induced damage (Strahilevitz et al., 2009). In plasmids, some of these genes have been identified as integron components, while others are associated with transposases, insertion sequence (IS) elements, and other AR genes. Although first detected in association with β-lactam resistance in *Klebsiella* and studied in various clinical isolates, the *qnr* genes in all likelihood have multiple origins, primarily from aquatic bacteria.

There are strongly suggestive relationships between some pathogen-related resistance gene clusters and the soil bacteria used to produce the antibiotic industrially, but direct transfer from environmental strains to pathogen has not been demonstrated (Witte, 2000). The best example is that of vancomycin resistance: a unique biochemical mechanism involving enzymes encoded by a quintuplet operon. The vancomycin resistance cluster (and that of resistance to the closely related glycopeptide teichoplanin) has been identified in the genomes of the producing actinomycete strains. Of greater significance, perhaps, is the presence of the same cluster in environmental Firmicutes, such as *Paenibacillus* sp. (Patel et al., 2000). Curiously, the glycopeptide avoparcin, a vancomycin analog used as a feed additive in pig farming for many years, was shown to be contaminated with chromosomal DNA of the producing organism (Lu et al., 2004). This included DNA of the glycopeptide resistance gene cluster *vanR*; thus, glycopeptide-resistant enterococcal strains could have been created by natural DNA transformation. One can ask if chromosomal or plasmid DNA contamination in crude preparations of antibiotics might contribute to the transmission of resistance genes from producer to pathogen. This may seem improbable, but in each case it has to happen once only, as in the acquisition of the *mecA* gene that confers methicillin-resistance to *S. aureus* (de Lencastre et al., 1994).

The situation with respect to the aminoglycoside antibiotics is somewhat unusual, because the associated resistance mechanisms comprise three types of structural modification catalyzed by three enzyme classes: *O*-phosphotransferases, *O*-adenylyltransferases, and *N*-acetyltransferases. A large number of independent isoforms of these three classes have been identified in pathogens; in addition, hybrid forms of these enzymes have been found (presumably the result of recombination). This plethora of resistance determinants shows that there are many sources for the AG resistance genes, derived from different antibiotic-producing bacteria. There are sequence relationships, with no exact matches between the genes (enzymes) of

different origins, and interesting structural correlations. Cundliffe and Demain (2010) have compiled an exhaustive list of the antibiotic-modifying enzymes found in producing bacteria; the latter include *Bacilli*, *Micromonospora*, *Pseudomonads*, *Saccharopolyspora*, *Streptomycetes*, and other genera. Interestingly, a comprehensive microbiome study (Sommer et al., 2009) found human gut-associated aminoglycoside acetyltransferases but none of the aminoglycoside phosphotransferases that are quite common in soils.

The evidence for acquisition of AR from environmental bacteria is clear in these cases, but what of all the other proto-AR genes in soils and water? There are still relatively few experimental examples of direct transfer of AR from environmental to clinical bacteria, despite the fact that proto-AR genes are unquestionably ubiquitous (although whether or not they are cosmic has yet to be demonstrated!). The aquatic *Vibrio* sp. carry very large superintegrons with upward of 200 cassettes of unknown gene function that are already in mobilizable forms; it has been suggested that these are potential resistance genes (Mazel et al., 1998). There are numerous other interesting correlations; for example, the specific isoleucyl–transfer ribonucleic acid (tRNA) synthetase gene that is the basis for resistance to the antibiotic mupirocin has been shown to be closely related in nucleotide sequence to tRNA synthetase genes found in eukaryotic genomes (yeast and human) (Yanagisawa and Kawakami, 2003). Related genes are also found in pseudomonads and mycobacteria (Sommer et al., 2009); might this AR gene have multiple origins?

2.6 ADAPTING AR GENES

For a proto-AR gene found in an environmental host to be expressed as a resistance function in clinically important bacteria, HGT alone does not suffice. It is essential that the proto-AR genes adapt to the transcriptional and translational rules of the new host. Consider the aminoglycoside phosphotransferase gene present in the antibiotic producer *Streptomyces fradiae*, with a chromosomal DNA guanine + cytosine (G+C%) content of >70% and codon usage and promoter sequence/structure distinct from that of a typical Gram-positive pathogen (<50% G+C). While the *S. fradiae* genes can be cloned and expressed in a low G+C host (under the control of a native promoter), they must function effectively in completely different genetic backgrounds for significant resistance levels to be attained. Perhaps the "tailoring" of AR genes is facilitated because many are plasmid encoded and pass through a variety of bacterial hosts under different selection pressures. If a poorly expressed foreign gene is advantageous for the host in a particular environment, the gene will likely be "adopted." In this regard, it is interesting that in the case of the commonly used fluoroquinolones, an aminoglycoside acetyltransferase gene can undergo mutation conferring a low level of fluoroquinolone resistance at a cost to its ability to detoxify aminoglycosides (Robicsek et al., 2006). In their native hosts the proto-AR genes may have a variety of other functions and resistance determination may be a gratuitous activity. For example, the aminoglycoside phosphotransferases have strong functional relationships with eukaryotic protein kinases (Shakya and Wright, 2010). Are these bacterial AR genes recruited across kingdoms?

There seems little doubt that nature provides the wherewithal for microorganisms (pathogens or not) to generate, exchange, and inherit novel functions resulting in AR

from a vast pool of genetic diversity in the environment (Allen et al., 2010). Could this process be retarded such that antimicrobial agents have a longer life span before falling prey to AR?

2.7 CONCLUDING REMARKS

There have been many articles written in the last decade about the ever-decreasing pipeline—the fact that there are deplorably few new drugs to combat multidrug-resistant microbial pathogens under development by pharmaceutical companies.

Given what has been learned about the origins, development, and dissemination of AR over the past 60 years, prudent stewardship of the compounds in the last lines of defense against infectious agents is required. Use in agriculture should be restrained, exposure of the environment to anthropogenic wastes containing antibiotics should be curtailed, and potential resistance mechanisms should be identified during development of new compounds and their deployment in preclinical trials. It is interesting to note that no transmissible resistance has been detected for the cyclic peptide antibiotic, daptomycin, introduced in 2003 for the treatment of serious Gram-positive infections. This agent has been used only in hospitals and never employed in agriculture or aquaculture. However, potential resistance mechanisms are present in soil microbes (D'Costa et al., 2007). While it may not be possible to prevent the natural traffic of proto-AR genes, it is imperative to minimize the exposure of new compounds to environments where gene selection and tailoring operates in pathogens. Scientists, physicians, industry, government health agencies, as well as the general public must not ignore the writing on the wall this time!

REFERENCES

Adam M, Murali B, Glenn NO, Potter SS (2008). Genetic inheritance based evolution of antibiotic resistance. *BMC Evol Biol* 8:52.

Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J (2010). Call of the wild: Antibiotic resistance genes in natural environments. *Nat Rev Microbiol* 8:251–259.

Bush K (2010). Alarming β-lactamase-mediated resistance in multidrug-resistant Enterobacteriaceae. *Curr Opin Microbiol* 13:558–564.

Cambry G, Guerout AM, Mazel D (2010). Integrons. *Annu Rev Genet* 44:141–166.

Cundliffe E, Demain AL (2010). Avoidance of suicide in antibiotic-producing microbes. *J Ind Microbiol Biotechnol* 37:643–672.

Dantas G, Sommer MOA, Oluwasegun RD, Church GM (2008). Bacteria subsisting on antibiotics. *Science* 320:100–103.

Davies J (1995). Vicious circles: Looking back on resistance plasmids. *Genetics* 139:1465–1468.

Davies J, Davies D (2010). Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev* 74:417–433.

D'Costa VM, Griffiths, E, Wright GD (2007). Expanding the soil antibiotic resistome: Exploring environmental diversity. *Curr Opin Microbiol* 10:481–489.

de Lencastre HB, DeJonge BLM, Matthews PR, Tomasz A (1994). Molecular aspects of methicillin resistance in *Staphylococcus aureus*. *J Antibmicrob Chemother* 33:7–24.

Finland M (1955). Emergence of antibiotic resistant bacteria. *New Engl J Med* 253:909–922, 969–977, 1019–1028.

Florey HW, Chain E, Heatley NG, Jennings MA, Sanders AG, Abraham EP, Florey ME (1949). *Antibiotics*, Vol. II. Oxford University Press, London.

Hawkey PM, Jones AM (2009). The changing epidemiology of resistance. *J Antimicrob Chemother* 64:i3–i10.

Jukes TH, Williams WL (1953). Nutritional effects of antibiotics. *Pharmacol Rev* 5:381–420.

Kashmiri SVS, Hotchkiss RD (1975). Evidence of tandem duplication of genes in a merodiploid region of pneumococcal mutants resistant to sulfonamide. *Genetics* 81:21–31.

Lu K, Asano R, Davies J (2004). Antimicrobial resistance gene delivery in animal feeds. *Emerg Infect Dis* 10:679–683.

Mazel D, Dychinco B, Webb VA, Davies J (1998). A distinctive class of integron in the *Vibrio cholerae* genome. *Science* 280:605–608.

McCoy E (1955). Changes in the host flora induced by chemotherapeutic agents. *Annu Rev Microbiol* 1954;8:257–272.

Mwangi MM, Wu SW, Zhou Y, Sieradzki K, de Lencastre H, Richardson P, Bruce D, Rubin E, Myers E, Siggia ED, Tomasz A (2007). Tracking the *in vivo* evolution of multidrug resistance in *Staphylococcus aureus* by whole-genome sequencing. *Proc Natl Acad Sci* 104:9451–9456.

Patel R, Piper K, Cockerill FR III, Steckelberg JM, Yousten AA (2000). The biopesticide *Paenibacillus popilliae* has a vancomycin resistance gene cluster homologous to the enterococcal VanA vancomycin resistance gene cluster. *Antimicrob Agents Chemother* 44:705–709.

Robicsek A, Strahilevitz J, Jacoby GA, Macielag M, Abbanat D, Park CH, Bush K, Hooper DC (2006). Fluoroquinolone-modifying enzyme: A new adaptation of a common aminoglycoside acetyltransferase. *Nat Med* 12:83–88.

Salyers AA, Whitt DD (2005). *Revenge of the Microbes: How Bacterial Resistance Is Undermining the Antibiotic Miracle*. American Society for Microbiology Press, Washington, DC.

Shakya T, Wright GD (2010). Nucleotide selectivity of antibiotic kinases. *Antimicrob Agents Chemother* 54:1909–1913.

Shoemaker NB, Vlamakis H, Hayes K, Salyers AA (2001). Evidence for extensive resistance gene transfer among *Bacteroides* spp. and among *Bacteroides* and other genera in the human colon. *Appl Environ Microbiol* 67:561–568.

Silver S, Phung LT (2005). A bacterial view of the Periodic Table: Genes and proteins for toxic inorganic ions. *J Indust Microbiol Biotechnol* 32:587–605.

Sommer MOA, Dantas G, Church GM (2009). Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science* 325:1128–1131.

Strahilevitz J, Jacoby GA, Hooper DC, Robicsek A (2009). Plasmid-mediated quinolone resistance: A multifaceted threat. *Clin Microbiol Rev* 22:664–689.

Waters B, Davies J (1997). Amino acid variation in the GyrA subunit of bacteria potentially associated with natural resistance to fluoroquinolone antibiotics. *Antimicrob Agents Chemother* 41:2766–2769.

Witte W (2000). Ecological impact of antibiotic use in animals on different complex microflora: Environment. *Intl J Antimicrob Agents* 14:321–325.

Wright GD (2007). The antibiotic resistome: The nexus of chemical and genetic diversity. *Nat Rev Microbiol* 5:175–186.

Yanagisawa T, Kawakami M (2003). How does *Pseudomonas fluorescens* avoid suicide from its antibiotic pseudomonic acid? Evidence for two evolutionarily distinct isoleucyl-tRNA synthetases conferring self-defense. *J Biol Chem* 278:25887–25894.

3

ANTIBIOTIC RESISTOME: A FRAMEWORK LINKING THE CLINIC AND THE ENVIRONMENT

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3.1 INTRODUCTION

A PubMed search of "antibiotic resistance" reveals a predictable bias in our understanding and study of this issue. The vast majority of the literature over the past half-century has focused on resistance in clinically relevant human pathogens and to a lesser extent bacteria that cause disease in animals. One of the hallmarks of antibiotic use has been that resistance emerges relatively rapidly in pathogens, within a year or as long as a few decades following the deployment of new drugs. The emphasis on resistance in the clinic, and its emergence following the introduction of antibiotics in previously susceptible populations of bacteria, has resulted in an accepted correlation with the phenomenon of resistance as a problem of human and animal pathogens.

However, there is a growing understanding that antibiotic resistance is a natural (and perhaps default) property of virtually all bacteria (Wright, 2007, 2010; Allen et al., 2010). Since environmental bacteria vastly outnumber human pathogens both in shear numbers and in genetic diversity [there are an estimated $\sim 5 \times 10^{30}$ bacteria on the planet and only a relative handful cause human or animal disease (Whitman et al., 1998)], these organisms are potential reservoirs of resistance genes. Indeed there is a growing understanding that environmental microbes are the likely wellspring of much of the resistance encountered in the clinic (Martinez, 2009; Allen et al., 2010; Wright, 2010a).

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TABLE 3.1 Survey of Mechanisms of Antibiotic Resistance[a]

Resistance Mechanism	Example	Antibiotic(s) Affected
Mutation in genes encoding protein targets	Point mutation in *gyrA*, *parC*	Fluoroquinolones
	Point mutations in *rpoB*	Rifamycins
Barrier to antibiotic entry	Gram-negative outermembrane	Many (glycopeptides, macrolides, aminocoumarins etc.)
	Mutation in respiratory quinone biosynthesis	Aminoglycosides
Active efflux	Tripartite resistance nodulation cell division (RND) efflux systems (e.g., AcrA, AcrB, TolC)	Many (fluoroquinolones, phenicols, β-lactams, etc.)
	Multiple transmembrane spanning (MFS) proteins (e.g., NorA, TetA)	Many (fluoroquinolones, phenicols, tetracyclines, etc.)
Target bypass	Production of alternate cell wall	Glycopeptides
	Production of antibiotic-insensitive variants	Trimethoprim
Nonenzymatic protection proteins	Antibiotic-binding proteins	Bleomycin
	Target-binding proteins	Tetracycline
Enzyme-mediated target modification	Methyltransferases	Macrolides, lincosamides, type B streptogramins, aminoglycosides
Enzyme-catalyzed antibiotic modification/inactivation	Hydrolases	β-Lactams
	Kinases	Aminoglycosides, macrolides
	Acetyltransferases	Aminoglycosides, type A streptogramins, fluoroquinolones

[a]The resistance phenotype manifests itself through many different mechanisms. Often multiple mechanisms are found in the same host. The list is this table is not exhaustive, only representative.

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Antibiotic resistance can be manifested through several mechanisms (Table 3.1): (1) mutation in genes encoding antibiotic targets, (2) prevention of antibiotic penetration in the cell, (3) active efflux of antibiotics from the cellular milieu, (4) bypass of antibiotic target, (5) nonenzymatic protection of the target or antibiotic sequestration, (6) enzymatic target modification, or (7) enzyme-mediated antibiotic modification or destruction. The first three mechanisms can spontaneously arise in pathogens, for example, mutations in *gyrA* or *parC* confer resistance to the fluroquinolone antibiotics (Hooper, 1999), the outer membrane of Gram-negative bacteria acts as a natural barrier to many antibiotics [though this could be argued to be antibiotic tolerance rather than resistance (Delcour, 2009)], and constitutive or inducible efflux systems in many organisms confer resistance to antibiotics (Piddock, 2006). These mechanisms can be transferred vertically through bacterial populations following cell division. However, all mechanisms can be acquired horizontally through mobile genetic elements such as plasmids and transposons (Barlow, 2009). The origins of mobile resistance genes has been the subject of debate, but investigation of plasmids from antibiotic naïve strains, for example, from collections predating the antibiotic era, reveal a paucity of resistance elements (Hughes and Datta, 1983). In contrast, plasmids circulating in present-day clinical pathogens often have multiple resistance genes (Bennett, 2008). From where do these genes come?

3.2 THE ANTIBIOTIC RESISTOME

Several years ago, we proposed the concept of a pan-microbial antibiotic "resistome" (D'Costa et al., 2006; Wright, 2007) (Fig. 3.1). We defined the resistome as the

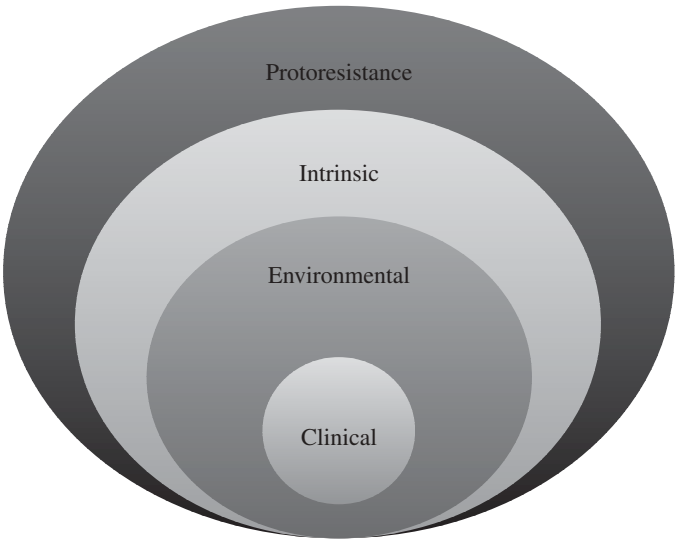

FIGURE 3.1 The Antibiotic resistome. The resistome is a framework that includes all forms of resistance and precursor elements. It does not focus exclusively on resistance in pathogens, rather the resistome includes all bacteria and genes that serve as precursors to resistance elements (protoresistance). (From Wright, 2010b.)

collection of all antibiotic resistance genes in pathogens common to the clinic, opportunistic pathogens from the environment, for example, *Pseudomonas aeruginosa*, nonpathogenic environmental bacteria including antibiotic producers, and the genes that can give rise to resistance—protoresistance elements (Wright, 2010b). To investigate the extent of this resistome, we undertook a systematic study where we isolated ~500 spore-forming bacteria from soils from sites with little human contact, urban areas, and agricultural lands (D'Costa et al., 2006). We then screened these against a panel of 20 antibiotics including natural products, their semisynthetic analogs, and completely synthetic molecules. These antibiotics spanned 50 years of use; from antibiotics in use since the beginning of the antibiotic era (sulfamethoxazole, tetracycline) to the most recently deployed drugs (tigecycline, linezolid). All strains were resistant to at least one antibiotic and on average they were resistant to 7–8 drugs. No antibiotics were sparred, even the latest synthetic drug linezolid. This study concretely demonstrated the extent of the antibiotic resistome in environmental organisms.

Similar results have been reported within the human microbiome, the collection of organisms that colonize all exposed surfaces in and on humans (Sommer et al., 2009). Furthermore, Dantas and colleagues showed that bacteria that can subsist on antibiotics as sole carbon and nitrogen sources are readily sourced from the soil (Dantas et al., 2008). These studies collectively demonstrate that antibiotic resistance is prevalent in bacteria, even in the absence of an obvious human source of antibiotics. The recent identification of resistance elements in deep-sea bacteria (Toth et al., 2010), from bacteria isolated hundreds of meters below the surface (Brown and Balkwill, 2009), and in animals from insects (Allen et al., 2009a) to mammals (Gilliver et al., 1999; Poeta et al., 2009) strengthens the argument that antibiotic resistance genes are prevalent in bacterial communities across the globe and are not restricted to pathogenic organisms.

The sequencing of the genomes of hundreds of bacteria over the past several years has served to support the phenotypic studies described above that suggest a deep reservoir of antibiotic resistance in bacterial populations. Antibiotic resistance genes are found in virtually all sequenced bacteria. Some of these genes are predicted to encode highly specific enzymes such as the chromosomal AmpC β-lactamases of many Gram-negative bacteria (Jacoby, 2009). Others on the other hand are predicted to show broader spectrum in their drug specificities, such as the efflux systems that are ubiquitously found in bacteria that pump out diverse chemical scaffolds (Poole, 2005; Piddock, 2006; Martinez et al., 2009).

The antibiotic resistome concept, therefore, offers a framework for the study of resistance that is not restricted to pathogens in the clinic. As a result, the density of resistance genes available across the globe along with the horizontal movement of genes between microbes serves to predict the emergence of resistance in the clinic. Examples of the relevance of environmental genomes and microbes in the emergence of antibiotic resistance are provided below.

3.3 RESISTANCE IN ANTIBIOTIC PRODUCERS

Most antibiotics in clinical use originate as secondary natural product metabolites of environmental bacteria. The order Actinomycetales has proven to be especially proficient in the production of secondary metabolites, many of which include some

of the most important clinically used antibiotics, for example, the tetracyclines, aminoglycosides, macrolides, glycopeptides, β-lactams, rifamycins, and lipopeptides. Sequencing of actinomycete genomes has revealed an average genome size of roughly 8–12 Mb and the capacity to produce 20–30 small-molecule secondary metabolites (Walsh and Fischbach, 2010). The genetic programs that encode the biochemical machinery to produce secondary metabolites such as antibiotics are generally clustered on the genome in a contiguous array. These biosynthetic gene clusters not only include genes that encode the enzymes that assemble the antibiotics but also genes responsible for self-resistance. These "suicide prevention systems" are integral and essential for the production of antibiotics (Cundliffe, 1989). Furthermore, they often share mechanism and sequence with clinically important resistance elements.

Julian Davies was the first to report the link between aminoglycoside resistance acetyltransferases emerging in the clinic and similar enzymes in antibiotic-producing actinomycetes (Benveniste and Davies, 1973). Aminoglycoside antibiotics are cationic carbohydrate-containing antibiotics that interfere with bacterial translation (Magnet and Blanchard, 2005). The generic mode of action of the aminoglycosides involves binding of the antibiotic to the small subunit of the ribosome, typically through a series of ionic interactions with the 16S ribosomal ribonucleic acid (rRNA), which results in corruption of the fidelity of the codon–anticodon recognition step of translation and the subsequent production of toxic aberrant proteins (Davis, 1987). The aminoglycoside–16S rRNA interaction occurs through a series of ionic interactions, hydrogen bonds, and salt bridges (Carter et al., 2000). Chemical modification of the aminoglycosides, either by acetylation, phosphorylation, or adenylylation, disrupts this complex and results in resistance (Davies and Wright, 1997). Aminoglycoside-producing bacteria encode self-resistance acetyltransferases and phosphotransferases that confer self-resistance. These are highly similar in function and structure to the enzymes found in clinical pathogens.

The paradox of inactivation by chemical modification (and thus inactivation) of an antibiotic during biosynthesis has been nicely resolved by Piepersberg in the study of streptomycin biosynthesis (Piepersberg, 1997). The antibiotic is produced in the nontoxic 6-phospho form. The 6-phospho intermediate has been derived from the activity of a kinase that is highly similar to streptomycin-inactivating kinases found in clinical pathogens such as enterococci (Wright and Thompson, 1999). Following adenosine triphosphate (ATP)-dependent efflux of the 6-phosphostreptomycin by the producing the organism *Streptomyces griseus*, an extracellular phosphatase converts the compound into the active antibiotic (Piepersberg, 1997).

The biosynthesis of glycopeptide antibiotics such as vancomycin offers another example of the link between antibiotic-producing organisms and resistance in the clinic. Glycopeptide antibiotics block cell growth by binding to the D-Ala-Ala termini of growing peptidoglycan chains. The peptidoglycan is a highly crosslinked and uniform extracellular polymer that is a major component of the bacterial cell wall and therefore a vital barrier to cell lysis. By binding to the D-Ala-D-Ala terminus of a peptidoglycan component, the antibiotic prevents interstrand crosslinking and intrastrand growth, resulting in cell lysis. Antibiotic–D-Ala-D-Ala complex formation is the result of the formation of five hydrogen bonds between the drug and the peptidoglycan (Walsh et al., 1996). A key bond is between the amide hydrogen of the D-Ala-D-Ala dipeptide and carbonyl oxygen of the central

phenylglycine residue of the antibiotic. Glycopeptide-producing bacteria incorporate an alternate cell wall biochemistry, terminating their peptidoglycan with the ester D-Ala-D-lactate rather than the amide D-Ala-D-Ala. The switch from amide to ester removes the opportunity to form a critical hydrogen bond with the antibiotic and results in a 1000-fold decrease in affinity for the antibiotic (Bugg et al., 1991). The formation of the D-Ala-D-lactate depsipeptide requires three genes: *vanH*, *vanA*, and *vanX*. The first encodes a D-lactate dehydrogenase that produces D-lactate from ubiquitous pyruvate, the second is an ATP-dependent ligase that links D-Ala and D-lactate. VanX is a highly specific DD-peptidase that cleaves D-Ala-D-Ala but not D-Ala-D-lactate. The *vanHAX* genes are inducible and "normal" D-Ala-D-Ala synthesis is constitutive. The presence of VanX therefore moderates the constitutively produced D-Ala-D-Ala, ensuring that the cell wall is enriched in glycopeptide-resistant D-Ala-D-lactate terminating peptidoglycan precursors.

The *vanHAX* cluster was first discovered in vancomycin-resistant enterococci in the late 1980s and has begun to emerge in drug-resistant staphylococci (Leclercq et al., 1988; Courvalin, 2006). The origin of the cluster was a mystery until the mechanism of self-resistance in glycopeptide producers was elucidated, roughly 10 years later (Marshall et al., 1997, 1998, 1999; Marshall and Wright, 1997, 1998). Sequencing of glycopeptide biosynthetic gene clusters has revealed the presence of a *vanHAX* cluster (Sosio et al., 2000; Pootoolal et al., 2002). Even nonproducing actinomycetes and paenibacilli have since been found to be glycopeptide resistant through the aegis of the *vanHAX* cluster (Patel et al., 2000; Hong et al., 2004; Guardabassi et al., 2005; D'Costa et al., 2006). The *vanHAX* cluster is therefore a highly specialized resistance mechanism that has likely origins in antibiotic-producing and other environmental bacteria.

3.4 RESISTANCE IN THE ENVIRONMENTAL METAGENOME

It is well known that culture-dependent methods significantly underrepresented the microbial diversity in most environments. Estimates that such approaches only sample a few percent of the microbial biodiversity are common. There are several reasons for this. For example, intrinsic differences in growth rates of various species and genera favor fast growing organisms that overgrow slow growing microbes under ordinary laboratory conditions. Specific nutrient requirements are often required, and, in fact, the generous supply of carbon and nitrogen sources in the majority of common laboratory media is detrimental to organisms evolved to grow in nutrient-limited environments. Furthermore, often microbes grow as obligate consortia (mutualist, parasitic, or commensal) and the microbiologist imperative of pure culture biases sampling toward such microbes. While several innovative and successful strategies to improve microbial cell growth and thus increase culture-based diversity have been developed (Gavrish et al., 2008; Burmolle et al., 2009), comprehensive sampling of environmental microbial genomes by culture-based methods remains elusive.

An alternative approach to explore microbial and antibiotic resistance genetic diversity is through metagenomics. In this strategy, total DNA (deoxylibonucleic acid) is isolated from a sample (soil, water, tissue) and either sequenced directly, used as a substrate for probe amplification and subsequent sequencing [e.g., 16S ribosomal

DNA (rDNA) sequences to identify community members (Fierer et al., 2007)], or used to prepare libraries for heterologous gene expression (Rondon et al., 2000). The latter strategy, termed functional metagenomics, is especially powerful to probe resistance gene diversity as the resistance phenotype is readily assayed simply by plating libraries on antibiotic-containing media. The advantage of this approach is that functional genes and their products are identified unbiased by sequence of known resistance elements, thereby increasing the opportunity to identify new genes.

The Handelsman group pioneered the use of functional metagenomics as a means to explore the genetic diversity of antibiotic resistance in unculturable organisms. In a metagenomic library of soil isolated from a Wisconsin oak savanna, untreated with antibiotics, they identified several previously unknown resistance elements including aminoglycoside acetyltransferases (highly abundant), an aminoglycoside kinase, and a tetracycline efflux pump (Riesenfeld et al., 2004). These genes, while similar to known genes, were significantly diverse from previously sequenced genes. Similarly, a soil library from a remote Alaskan location yielded several novel β-lactamases (Allen et al., 2009b). Notably, one of these was a bifunctional enzyme comprised of two distinct domains: an N-terminal Ambler class D enzyme that contributes penicillinase activity and a C-terminal class C cephalosporinase. Bifunctional aminoglycoside-inactivating genes are known in clinically resistant organisms [reviewed in Zhang et al. (2009)] and are thought to emerge as a means to simultaneously coordinate regulation and expression of two activities to provide extended substrate "coverage" in environments where antibiotic use is prevalent. The fact that such an expanded specificity β-lactamase was discovered in a soil sample with no anthropogenic exposure to antibiotics speaks to the myriad of natural products with antibiotic action expressed by bacteria and fungi in such environments that provide a selective pressure for resistance gene evolution.

The functional metagenomics approach is not limited to the soil. For example, it was used to identify resistance genes to the anticancer antibiotic bleomycin from activated sludge (Mori et al., 2008). Additionally, resistant clones to13 diverse antibiotics were readily identified in metagenomic expression libraries from human saliva and feces (Sommer et al., 2009). Functional metagenomics, therefore, offers a robust technique to effectively probe the genetic diversity of complex mixtures of microorganisms and has expanded our understanding of the breadth and depth of resistance in the environment.

3.5 MOVEMENT OF ENVIRONMENTAL RESISTANCE GENES INTO CLINICAL PATHOGENS

The evidence is clear that the environment represents an enormous reservoir of antibiotic resistance genes. Given what we know about the propensity of microbes to mobilize genes horizontally through populations, it stands to reason that there is a link between the environmental resistome and the clinic. However, the similarity of resistance genes, their products and mechanisms in the environment to those found in the clinic does not provide *direct* evidence that there is conduit for environmental genes into pathogens but is rather simply "guilt by association." Nevertheless, there are cases where the recent escape of environmental resistance genes into pathogens is clear.

CTX-M is a family of extended spectrum β-lactamases (ESBLs, so called because of their ability to inactivate the oxyimino-cephalosporins such as ceftazidime and cefotaxime introduced ~30 years ago to overcome emerging resistance to β-lactams) that has become a significant clinical problem across the globe over the past 20 years (Bonnet, 2004). CTX-Ms were named for their ability to hydrolyze cefotaxime and emerged worldwide in the early 1990s in resistant isolates in several different countries. The gene products showed only weak similarity (~40%) to known β-lactamases but were distinguished by their ability to inactivate third-generation cephalosporins. There are now over 100 distinct CTX-M genes reported (http://www.lahey.org/Studies/). Previously reported ESBLS in the clinic were mutants of known enzyme such as TEM and SHV β-lactamases, however, the CTX-M class represented a new class of enzyme. Starting in 2001 several reports emerged demonstrating the similarity of CTX-M β-lactamases with chromosomally encoded β-lactamases from species of *Kluyvera* of environmental origins (Decousser et al., 2001; Humeniuk et al., 2002; Poirel et al., 2002; Olson et al., 2005).

Genes that code for cefotaxime lactamase (CTX-M genes) are found on the chromosome of several *Kluyvera* species including *K. georgiana* (Poirel et al., 2002; Olson et al., 2005) and *K. ascorbata* (Lartigue et al., 2006). The gene products show 100% identity to CTX-M14 and 99% identity to CTX-M8. Furthermore, the genes themselves show near 100% identity to the genes circulating in pathogens. Flanking genes and fragments in pathogens are derived from *Kluyvera* chromosomes, clearly demonstrating their origins (Fig. 3.2). There have, therefore, been multiple contemporary escapes of CTX-M from benign organisms to pathogens. These have spread worldwide and constitute one of the most important resistance mechanisms to the cephalosporins in medicine (Canton and Coque, 2006).

Resistance to the synthetic fluoroquinolone antibiotics such as ciprofloxacin is generally the result of point mutations in target genes such as the DNA gyrase encoding *gyrA* and *parC* that encodes a type IV toposiomerases (Hooper, 1999). Plasmid-mediated fluoroquinolone resistance was first reported in 1998 (Martinez-Martinez et al., 1998) and found to be linked to the expression of Qnr, a pentapeptide

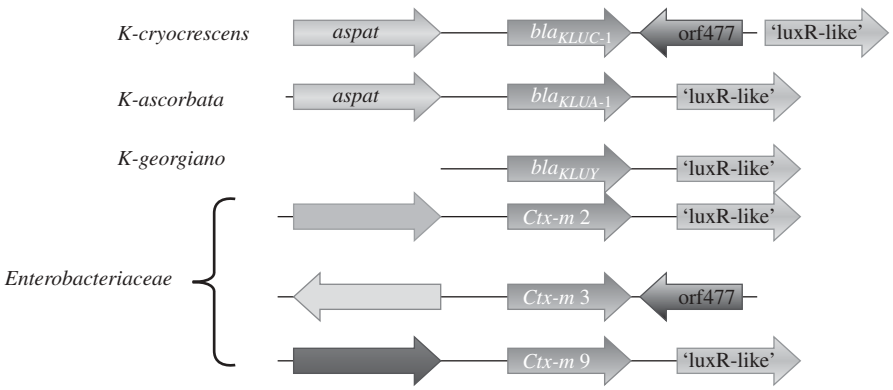

FIGURE 3.2 Organization of CTX-M genes in *Kluyvera* species and pathogens. Chromosomal *bla* genes in *Kluyvera* and on mobile elements in Enterobacteriaceae share sequence identity and gene organization suggesting a direct link between environmental organisms and pathogens. (Data from Olson et al., 2005.)

repeat protein. The mechanism of resistance is linked to the ability of these proteins to bind to topoisomerases, protecting the cell from buildup of toxic topoisomerases–fluroquinolone–DNA complexes. This mechanism is consistent the three-dimensional structures of the Qnr homolog MfpA from *Mycobacterium tuberculosis* (Hegde et al., 2005) and of the chromosomal Qnr from *Enterococcus faecalis* as well as biochemical studies (Hegde et al., 2010). A search for the source of *qnr* genes has found that they are common in aquatic environmental bacteria such as species of *Shewanella* (Poirel et al., 2005), where it is unlikely to play a role in quinolone resistance. Recent studies have shown that the gene in *S. algae* is inducible by cold temperatures, leading to speculation that it serves as topoisomerases regulator during cold stress or adaptation (Kim et al., 2011).

3.6 SUMMARY

The evidence accumulated over the past several years supports the concept of a global resistome that includes not only resistance elements in clinically important pathogens but also genes in nonpathogenic environmental bacteria including antibiotic producers. In fact, the former are clearly in the minority. The genes in benign bacteria may be bone fide resistance elements or may be precursors that can confer resistance given a selective pressure or a different genetic context. As surveys of environmental microbes have shown, antibiotic-resistant bacteria are very easily cultivated. Given the plethora of small molecules that microbes interact with as part of their life cycle in the environment, these mechanisms may be for self-defense, to attenuate or modify chemical signals, or as a means to break down molecules as a food source. The density of the resistome on a global scale dwarfs the elements that have become clinically important.

Given the fact that genes move rapidly through bacterial populations both vertically and horizontally by transformation, phage-mediated transduction and conjugation, and the immensity of the resistome, it is not surprising that resistance emerges quickly following a drug's deployment for medical or agricultural use. There are no "irresistible" antibiotics. Perhaps a better perspective is to question why it takes so long for resistance to emerge? For some antibiotics it is relatively fast, for example, the penicillins, while for others it has been slower, for example, daptomycin, despite a large environmental reservoir of resistance. Understanding these differences will be vital in our continuing efforts to develop new drugs.

REFERENCES

Allen HK, Cloud-Hansen KA, Wolinski JM, Guan C, Greene S, Lu S, Boeyink M, Broderick N, Raffa KF, Handelsman J (2009a). Resident microbiota of the gypsy moth midgut harbors antibiotic resistance determinants. *DNA Cell Biol* 28:109–117.

Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J (2010). Call of the wild: Antibiotic resistance genes in natural environments. *Nat Rev Microbiol* 8(4): 251–259.

Allen HK, Moe LA, Rodbumrer J, Gaarder A, Handelsman J (2009b). Functional metagenomics reveals diverse beta-lactamases in a remote Alaskan soil. *ISME J* 3(2): 243–251.

Barlow M (2009). What antimicrobial resistance has taught us about horizontal gene transfer. *Methods Mol Biol* 532:397–411.

Bennett PM (2008). Plasmid encoded antibiotic resistance: Acquisition and transfer of antibiotic resistance genes in bacteria. *Br J Pharmacol* 153(Suppl 1):S347–357.

Benveniste R, Davies J (1973). Aminoglycoside antibiotic-inactivating enzymes in actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria. *Proc Natl Acad Sci USA* 70:2276–2280.

Bonnet R (2004). Growing group of extended-spectrum beta-lactamases: The CTX-M enzymes. *Antimicrob Agents Chemother* 48(1):1–14.

Brown MG, Balkwill DL (2009). Antibiotic resistance in bacteria isolated from the deep terrestrial subsurface. *Microb Ecol* 57(3):484–493.

Bugg TDH, Wright GD, Dutka-Malen S, Arthur M, Courvalin P, Walsh CT (1991). Molecular basis for vancomycin resistance in *Enterococcus faecium* BM4147: Biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and VanA. *Biochemistry* 30:10408–10415.

Burmolle M, Johnsen K, Abu Al-Soud W, Hansen LH, Sørensen SJ (2009). The presence of embedded bacterial pure cultures in agar plates stimulate the culturability of soil bacteria1. *J Microbiol Methods* 79(2):166–173.

Canton R, Coque TM (2006). The CTX-M beta-lactamase pandemic. *Curr Opin Microbiol* 9 (5):466–475.

Carter AP, Clemons WM, Brodersen DE, Morgan-Warren RJ, Wimberly BT, Ramakrishnan V (2000). Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. *Nature* 407(6802):340–348.

Courvalin P (2006). Vancomycin resistance in gram-positive cocci. *Clin Infect Dis* 42(Suppl 1): S25–34.

Cundliffe E (1989). How antibiotic-producing organisms avoid suicide. *Annu Rev Microbiol* 43:207–233.

Dantas G, Sommer MO, Oluwasegun RD, Church GM (2008). Bacteria subsisting on antibiotics. *Science* 320(5872):100–103.

Davies J, Wright GD (1997). Bacterial resistance to aminoglycoside antibiotics. *Trends Microbiol* 5(6):234–240.

Davies BD (1987). Mechanism of action of aminoglycosides. *Microbiol Rev* 51:341–350.

D'Costa VM, McGrann KM, Hughes DW, Wright GD (2006). Sampling the antibiotic resistome. *Science* 311(5759):374–377.

Decousser JW, Poirel L, Nordmann P (2001). Characterization of a chromosomally encoded extended-spectrum class A beta-lactamase from *Kluyvera cryocrescens*. *Antimicrob Agents Chemother* 45(12):3595–3598.

Delcour AH (2009). Outer membrane permeability and antibiotic resistance. *Biochim Biophys Acta* 1794(5):808–816.

Fierer N, Breitbart M, Nulton J, Salamon P, Lozupone C, Jones R, Robeson M, Edwards RA, Felts B, Rayhawk S, Knight R, Rohwer F, Jackson RB (2007). Metagenomic and small-subunit rRNA analyses reveal the genetic diversity of bacteria, archaea, fungi, and viruses in soil. *Appl Environ Microbiol* 73(21):7059–7066.

Gavrish E, Bollmann A, Epstein S, Lewis K (2008). A trap for in situ cultivation of filamentous actinobacteria. *J Microbiol Methods* 72(3):257–262.

Gilliver MA, Bennett M, Begon M, Hazel SM, Hart CA (1999). Antibiotic resistance found in wild rodents. *Nature* 401(6750):233–234.

Guardabassi L, Perichon B, van Heijenoort J, Blanot D, Courvalin P (2005). Glycopeptide resistance vanA operons in Paenibacillus strains isolated from soil. *Antimicrob Agents Chemother* 49(10):4227–4233.

Hegde SS, Vetting MW, Mitchenall LA, Maxwell A, Blandshard JS (2010). Structural and biochemical analysis of the pentapeptide repeat protein EfsQnr, a potent DNA gyrase inhibitor. *Antimicrob Agents Chemother* 55(1):110–117.

Hegde SS, Vetting MW, Roderick SL, Mitchenall LA, Maxwell A, Takiff HE, Blandshard JS (2005). A fluoroquinolone resistance protein from *Mycobacterium tuberculosis* that mimics DNA. *Science* 308(5727):1480–1483.

Hong HJ, Hutchings MI, Neu JM, Wright GD, Paget MSB, Buttner MJ (2004). Characterization of an inducible vancomycin resistance system in *Streptomyces coelicolor* reveals a novel gene (*vanK*) required for drug resistance. *Mol Microbiol* 52 (4):1107–1121.

Hooper DC (1999). Mechanisms of fluoroquinolone resistance. Drug Resist Updat 2(1):38–55.

Hughes VM, Datta N (1983). Conjugative plasmids in bacteria of the "pre-antibiotic" era. *Nature* 302(5910):725–726.

Humeniuk C, Arlet G, Gautier V, Grimont P, Labia R, Philippon A (2002). Beta-lactamases of *Kluyvera ascorbata*, probable progenitors of some plasmid-encoded CTX-M types. *Antimicrob Agents Chemother* 46(9):3045–3049.

Jacoby GA (2009). AmpC beta-lactamases. *Clin Microbiol Rev* 22(1):161–182.

Kim HB, Park CH, Gavin M, Jacoby GA, Hooper DC (2011). Cold shock induces *qnrA* expression in *Shewanella algae*. *Antimicrob Agents Chemother* 55(1):414–416.

Lartigue MF, Poirel L, Aubert D, Nordmann P (2006). *In vitro* analysis of IS*Ecp1B*-mediated mobilization of naturally occurring beta-lactamase gene *blaCTX-M* of *Kluyvera ascorbata*. *Antimicrob Agents Chemother* 50(4):1282–1286.

Leclercq R, Derlot E, Duval J, Courvalin P (1988). Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. *N Engl J Med* 319(3):157–161.

Magnet S, Blandchard JS (2005). Molecular insights into aminoglycoside action and resistance. *Chem Rev* 105(2):477–498.

Marshall CG, Broadhead G, Leskiw, GD, Wright GD (1997). D-Ala-D-Ala ligases from glycopeptide antibiotic-producing organisms are highly homologous to the enterococcal vancomycin-resistance ligases VanA and VanB. *Proc Natl Acad Sci USA* 94(12):6480–6483.

Marshall CG, Lessard IA, Park, I-S, Wright GD. (1998). Glycopeptide antibiotic resistance genes in glycopeptide-producing organisms. *Antimicrob Agents Chemother* 42(9):2215–2220.

Marshall CG, Wright GD (1997). The glycopeptide antibiotic producer *Streptomyces toyocaensis* NRRL 15009 has both D-alanyl-D-alanine and D-alanyl-D-lactate ligases. *FEMS Microbiol Lett* 157(2):295–299.

Marshall CG, Wright GD (1998). DdlN from vancomycin-producing *Amycolatopsis orientalis* C329.2 is a VanA homologue with D-alanyl-D-lactate ligase activity. *J Bacteriol* 180 (21):5792–5795.

Marshall CG, Zolli M, Wright GD (1999). Molecular mechanism of VanHst, an alpha-ketoacid dehydrogenase required for glycopeptide antibiotic resistance from a glycopeptide producing organism. *Biochemistry* 38(26):8485–8491.

Martinez JL (2009). The role of natural environments in the evolution of resistance traits in pathogenic bacteria. *Proc Biol Sci* 276(1667):2521–2530.

Martinez JL, Sanchez MB, Martinez-Solano L, Hernandez A, Garmendia L, Fajardo A, Alvarez-Ortega C (2009). Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. *FEMS Microbiol Rev* 33(2):430–449.

Martinez-Martinez L, Pascual A, Jacoby G (1998). Quinolone resistance from a transferable plasmid. *Lancet* 351(9105):797–799.

Mori T, Mizuta S, Suenaga H, Miyazaki K. (2008). Metagenomic screening for bleomycin resistance genes. *Appl Environ Microbiol* 74(21):6803–6805.

Olson AB, Silverman M, Boyde DA, McGeer, Willey BM, Pong-Porter V, Daneman N, Mulvey MR (2005). Identification of a progenitor of the CTX-M-9 group of extended-spectrum beta-lactamases from *Kluyvera georgiana* isolated in Guyana. *Antimicrob Agents Chemother* 49(5):2112–2115.

Patel R, Piper K, Cockerill FR, Steckelberg JM, Yousten AA. (2000). The biopesticide *Paenibacillus popilliae* has a vancomycin resistance gene cluster homologous to the enterococcal VanA vancomycin resistance gene cluster. *Antimicrob Agents Chemother* 44 (3):705–709.

Piddock LJ (2006). Multidrug-resistance efflux pumps—Not just for resistance. *Nat Rev Microbiol* 4(8):629–636.

Piepersberg W (1997). *Molecular biology, biochemistry, and fermentation of aminoglycoside antibiotics*. In W Strohl (Ed.), *Biotechnology of industrial antibiotics*. Marcel Dekker, New York, pp. 81–163.

Poeta P, Radhouani H, Pinot L, Martinho A, Rego V, Rodrigues R, Goncalves A, Rodrigues J, Estapa V, Torres C, Igrejas G (2009). Wild boars as reservoirs of extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* of different phylogenetic groups. *J Basic Microbiol* 49(6):584–588.

Poirel L, Kampfer P, Nordmann P (2002). Chromosome-encoded Ambler class A beta-lactamase of *Kluyvera georgiana*, a probable progenitor of a subgroup of CTX-M extended-spectrum beta-lactamases. *Antimicrob Agents Chemother* 46(12):4038–4040.

Poirel L, Rodriguez-Martinez JM, Mammeri H, Liard A, Nordmann P (2005). Origin of plasmid-mediated quinolone resistance determinant QnrA. *Antimicrob Agents Chemother* 49(8):3523–3525.

Poole K (2005). Efflux-mediated antimicrobial resistance. *J Antimicrob Chemother* 56(1): 20–51.

Pootoolal J, Thomas MG, Marshall CG, Neu JM, Hubbard BK, Walsh CT, Wright GD (2002). Assembling the glycopeptide antibiotic scaffold: The biosynthesis of A47934 from *Streptomyces toyocaensis* NRRL15009. *Proc Natl Acad Sci USA* 99(13):8962–8967.

Riesenfeld CS, Goodman RM, Handelsman J (2004). Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. *Environ Microbiol* 6(9):981–989.

Rondon MR, et al. (2000). Cloning the soil metagenome: A strategy for accessing the genetic and functional diversity of uncultured microorganisms. *Appl Environ Microbiol* 66(6): 2541–2547.

Sommer MO, Dantas G, Church GM (2009). Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science* 325(5944):1128–1131.

Sosio M, Bianchi A, Bossi E, Donadio S (2000). Teicoplanin biosynthesis genes in *Actinoplanes teichomyceticus*. *Antonie Van Leeuwenhoek* 78(3–4):379–384.

Toth M, Smith C, Frase H, Mobashery S, Vakulenko S (2010). An antibiotic-resistance enzyme from a deep-sea bacterium. *J Am Chem Soc* 132(2):816–823.

Walsh CT, Fischbach MA (2010). Natural products version 2.0: Connecting genes to molecules. *J Am Chem Soc* 132(8):2469–2493.

Walsh CT, Fisher SL, Park IS, Prahalad M, Wu Z (1996). Bacterial resistance to vancomycin: Five genes and one missing hydrogen bond tell the story. *Chem Biol* 3:21–28.

Whitman WB, Coleman DC, Wiebe WJ (1998). Prokaryotes: The unseen majority. *Proc Natl Acad Sci USA* 95(12):6578–6583.

Wright GD (2007). The antibiotic resistome: The nexus of chemical and genetic diversity. *Nat Rev Microbiol* 5(3):175–186.

Wright GD (2010a). Antibiotic resistance in the environment: A link to the clinic? *Curr Opin Microbiol* 13:589–594.

Wright GD (2010b). The antibiotic resistome. *Expert Opin Drug Discov* 5:779–788.

Wright GD, Thompson PR (1999). Aminoglycoside phosphotransferases: Proteins, structure, and mechanism. *Front Biosci* 4:D9–21.

Zhang W, Fisher JF, Mobashery S (2009). The bifunctional enzymes of antibiotic resistance. *Curr Opin Microbiol* 12(5):505–511.

4

ECOLOGICAL AND CLINICAL CONSEQUENCES OF ANTIBIOTIC SUBSISTENCE BY ENVIRONMENTAL MICROBES

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4.1 INTRODUCTION

Increasing multidrug resistance in clinical pathogens and declining rates of development of new antimicrobials is precipitating a worsening global health crisis (Fischbach and Walsh, 2009). Antibiotic resistance determinants encoded on mobilizable elements can readily transfer between diverse bacteria, allowing the accumulation and dissemination of resistance genes into a variety of interacting microbial communities (D'Costa et al., 2007; Davies, 1994; Davies and Davies, 2010; Wright, 2007). The genetic and biochemical mechanisms that govern the evolution and dissemination of drug resistance can be engineered into or be naturally acquired by many microbial pathogens, effectively annulling our primary chemotherapeutics against these disease-causing agents. For instance, a multidrug-resistant strain of the plague bacterium *Yersinia pestis* was recently isolated that had acquired a mobile genetic element that conferred resistance to six different drugs including tetracycline and chloramphenicol, the two first-line drugs against this pathogen (Pan et al., 2008). Consequently, there is an increasing interest in identifying and characterizing microbes from communities that may be accessible reservoirs of antibiotic resistance machinery (Allen et al., 2010; Wright, 2007).

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4.2 ENVIRONMENTAL ORIGINS OF RESISTANCE: THE PRODUCER HYPOTHESIS

Investigations into reservoirs of resistance are partly inspired by the ecological question of where antibiotic resistance originated or evolved—essentially, if pathogens are the recipient, who are the donors, both currently and over longer evolutionary time scales? Most antibiotics used in the clinic today are structural derivatives of compounds isolated from natural sources. Since antibiotics in nature represent one of the oldest forms of potential biochemical warfare, environmental bacteria would be expected to possess intrinsic mechanisms to detect, resist, and neutralize these threats to their persistence and, hence, represent a reservoir of accessible resistance machinery. Indeed, an intriguing hypothesis, put forward by Julian Davies and colleagues in the late 1970s, postulates that antibiotic-producing soil bacteria are one such primary "originator" of antimicrobial resistance genes (Benvenis and Davies, 1973). The theory intuits that the producers must minimally survive the toxicity of the antimicrobial compounds they produce, and, hence, the genetic antecedents of this self-immunity are de facto resistance genes. Additionally, enzymes involved in the microbial biosynthesis of these chemicals, capable of many specific chemical modifications of these compounds, might be repurposed to catalyze transformations in alternate genomic contexts that modulate or ablate their antimicrobial activity. Interestingly (and unfortunately), antibiotics are used in large quantities at subtherapeutic levels in animal agriculture, but the dosing is usually as crude lysates of the producer organisms, which in addition to the antimicrobial compound also contain genomic material from the producer (Webb and Davies, 1993). Hence, resistance genes natively contained in the producers would be available for lateral gene transfer in animal pathogens. Davies and colleagues' support for the "producer hypothesis" came from biochemical studies showing the activity of aminoglycoside resistance enzymes encoded by producers was identical to those found in pathogens (Benvenis and Davies, 1973). In 2006, Gerry Wright and colleagues provided further compelling evidence for producers being a substantial reservoir of antibiotic resistance genes (which they collectively termed the "resistome"), by phenotypically profiling the resistance of $\sim$400 soil *Streptomycete* isolates against 21 diverse antibiotics, which spanned all bacterial targets and most antibiotic chemical classes. Despite no specific selection for resistance during isolation, they found these microbes were resistant to 7–8 antimicrobials on average, and one microbe in the set was resistant to as many as 15 compounds (D'Costa et al., 2006, 2007). The conundrum with the producer hypothesis, however, comes from the finding that resistance gene sequences identified in the producers are phylogenetically distantly related to those identified in human commensals or pathogens (Marshall et al., 1998). Indeed, it appears these sets of producer versus pathogen resistance genes may have evolutionarily diverged before the anthropogenic antibiotic era (Aminov and Mackie, 2007).

4.3 RESISTOME OF OTHER SOIL BACTERIA: RESPONSE TO THE PRODUCERS?

Antibiotic-producing microbes in the soil may still play a direct role with selection of resistance genes in environmental microbes that may serve as an accessible resistance reservoir for pathogens. The diverse microbial communities that inhabit the soil must

interact with the producers, and many of these bacteria likely respond by developing or acquiring resistance genes to enable coinhabitation of the ecological niches of the producers. Both culture-dependent and culture-independent investigations of soil (and aquatic) communities by numerous researchers have indeed revealed substantial reservoirs of diverse resistance genes (Aarestrup et al., 2001; Cavaco et al., 2008; Demanèche et al., 2008; Enne et al., 2008; Leng et al., 1997). These include areas with high anthropogenic contact, such as agricultural soils, as well as more pristine settings, such as on secluded islands in Alaska (Allen et al., 2009, 2010; Donato et al., 2010; Riesenfeld et al., 2004). From an ecological perspective, while resistance genes work to counteract antimicrobial activity, the biochemical processing of these compounds in the environment is unlikely to end here. Antimicrobials that have been cleaved or modified by a resistance gene could serve distinct cellular roles, such as the ability of anhydrotetracycline (a tetracycline precursor) to activate tetracycline efflux pumps (McCormick et al., 1968; Palmer et al., 2010), or could simply serve as substrates for microbial metabolism. In cases where a metabolic pathway is specific to degradation of the antimicrobial structure, it is even possible that multiple enzymes in that pathway could each serve de facto antimicrobial resistance roles in a new genomic context.

4.4 EARLY REPORTS OF ANTIBIOTIC CATABOLISM BY SOIL BACTERIA

A handful of reports, dating back to the early 1960s, have described soil isolates with the capacity to utilize a few antibiotics as the sole source of carbon and, in some cases, also the sole source of nitrogen. In 1961, Abd-El-Malek et al. reported on a *Streptomyces* sp. capable of utilizing chloramphenicol as a sole carbon and nitrogen source (Abd-El-Malek et al., 1961). Chloramphenicol solutions were percolated in multiple doses through sieved garden soil to enable in situ enrichment of subsisters. The enriched soil was plated on minimal media containing chloramphenicol and ammonium nitrate as the carbon and nitrogen sources, respectively, yielding colonies of a single morphotype after 15 days of incubation at 30°C. Further morphological and metabolic analysis suggested the bacterium was a *Streptomyces* species. The isolate was confirmed to also utilize chloramphenicol as the sole nitrogen source by serial subculture into chloramphenicol minimal media lacking nitrogen. Repeated subculturing was found to decrease the inactivation time and improve growth. The isolate was able to completely inactivate chloramphenicol within 13 days in media containing the compound at concentrations spanning 100 to 600 mg/L, but no growth was detected in 1000 mg/L chloramphenicol after 30 days postinoculation. Also in 1961, Kameda et al. reported on isolation of 8 strains from 4 different Japanese soils with the capacity of utilizing benzylpenicillin as the sole source of carbon (Kameda et al., 1961). The soil isolates (phylotype not described) could use both the parent compound and phenylacetatic acid as the sole carbon source, implicating penicillin acylase as a key step in catabolism. Scarce to no growth was observed with medium lacking ammonium chloride, suggesting that benzylpenicillin could not serve as a sole source of nitrogen. Similarly, 6-aminopenicillanic acid, the other moiety besides phenylacetate formed from penicillin acylase activity, was unable to support substantial growth of the isolates. Interestingly, the isolates seemed to be rod shaped in the phenylacetate medium but formed filaments in the benzylpenicillin medium.

While β-lactamase activity was not directly assayed, it is unlikely to have played a role in benzylpenicillin catabolism by these isolates since the β-lactam ring is contained within the 6-aminopenicillanic acid moiety. Benzylpenicillin degradation was detected after as little as 3 days after inoculation of the isolates. In 1977, Johnsen reported on isolation of a *Pseudomonas fluorescens* strain from the sediment surface of a German lake that could also utilize benzylpenicillin as the sole source of carbon (Johnsen, 1977). This isolate differed from the Kameda strain in a number of ways. It was able to use benzylpenicillin as a sole source of both carbon and nitrogen. It did not possess penicillin acylase activity but did show β-lactamase activity. The degradation pathway proposed by Johnsen involves destruction of the β-lactam ring, to produce benzylpenicilloic acid, followed by decarboxylation to benzylpenilloic acid, which may provide the needed carbon for growth. Interestingly, growth on pure benzylpenicilloic acid by the strain was retarded to 25% of the growth on benzylpenicillin, which might indicate that efficient coexpression of multiple genes required for benzylpenicilloic acid catabolism might require the initial expression of the β-lactamase gene, induced to detoxify the antibiotic activity of benzylpenicillin. In 1979, Beckman and Lessie reported on a number of *Pseudomonas cepacia* (*P. cepacia*), *P. marginata*, and *P. caryophylli* strains with a similar ability to utilize benzylpenicillin as a sole source of carbon, with concomitant expression of high levels of β-lactamase activity (Beckman and Lessie, 1979). These strains exhibited resistance to benzylpenicillin, and its derivatives ampicillin, carbenicillin, and cephalosporin C, but were unable to utilize these other compounds as growth substrates, indicating that cleavage of the β-lactam ring alone was not sufficient for catabolism. Furthermore, other *Pseudomonas* species tested, including strains of the genetically related *P. picketii*, as well as strains of the less related *P. aeruginosa*, *P. putida*, and *P. fluorescens*, were unable to utilize benzylpenicillin as the sole carbon source despite having nearly equal levels of β-lactamase activity and resistance profiles to the various β-lactams. Intriguingly, *P. cepacia* mutants auxotrophic for lysine were also unable to utilize or resist benzylpenicillin, and the utilization phenotype was not rescued with genetic complementation with a β-lactamase containing plasmid from *P. aeruginosa*. Accordingly, the authors speculated that *P. cepacia* genes involved with lysine biosynthesis and for benzylpenicillin resistance and catabolism may reside in an extrachromosomal gene cluster, though attempts to isolate such plasmids were unsuccessful. In 1981, Johnsen reported on isolation of another *Pseudomonas* sp. from activated sludge with the ability to utilize benzylpenicillin as the sole source of carbon (Johnsen, 1981). Unlike the previously reported benzylpencillin-utilizing isolates, this strain was found to express both penicillin acylase and β-lactamase activities. However, much like the Kameda strain, the phenylacetic acid moiety produced by acylase activity served as the carbon source, and the β-lactamase activity in this case may be unrelated to the catabolism phenotype. These early studies demonstrated that soil microbes with antibiotic subsistence phenotypes for a couple of natural antibiotics could be isolated from geographically diverse locations, through expression of diverse catabolic mechanisms.

4.5 THE ANTIBIOTIC SUBSISTOME: WHO AND HOW MUCH?

We recently isolated a few hundred soil strains capable of utilizing 1 of 18 antibiotics as their sole source of carbon (Dantas et al., 2008). Soils were collected from 11 sites

in the United States, with varying degrees of anthropogenic contact, inoculated into a minimal medium containing an antibiotic as the carbon source, and allowed to grow at room temperature over 7 days. Cultures were serially passaged three times, followed by clonal selection on antibiotic–agar plates, and a final passage in liquid antibiotic minimal media. The 18 antibiotics were chosen to target all major bacterial targets, 8 distinct chemical classes, even representations of natural, semisynthetic, and synthetic origins, and a range of ages of clinical deployment. The final growth assays yielded microbes subsisting on antibiotics in 85% of the 11 soil by 18 antibiotic conditional grid (Fig. 4.1), with no statistically significant differences in the ability to culture these microbes from the various soil sources and with the various antibiotics. We refer to the aggregate of all mechanisms to deploy antibiotic as energy source as the antibiotic subsistome. We used 16S ribosomal ribonucleic

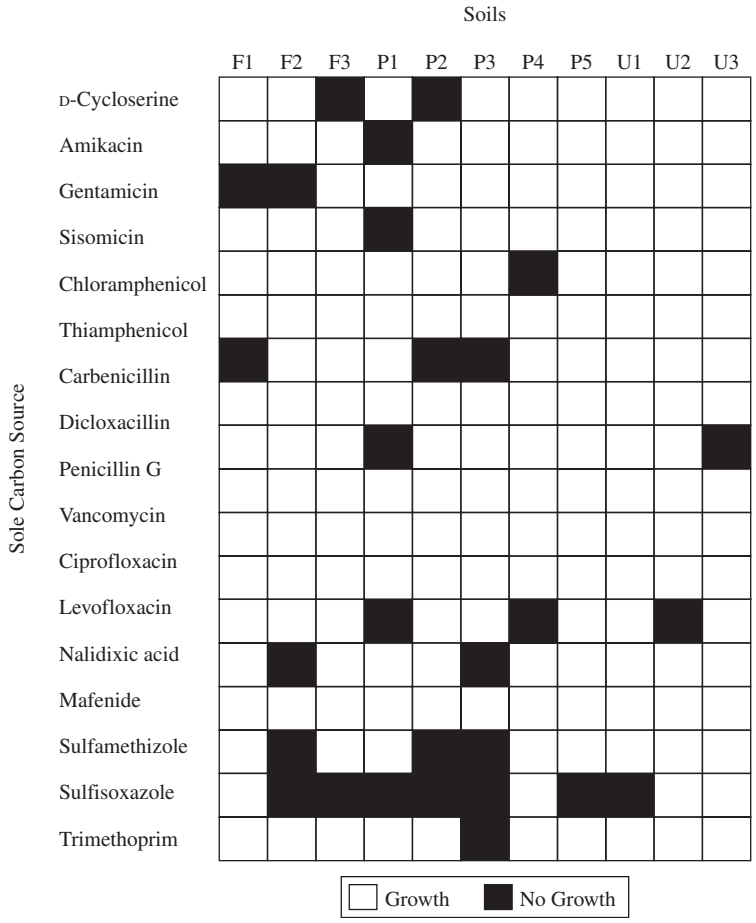

FIGURE 4.1 Clonal bacterial isolates subsisting on antibiotics. Heat map illustrating growth results from all combinations of 11 soils by 18 antibiotics, where white squares represent successful isolation of bacteria from a given soil that are able to utilize that antibiotic as sole carbon source at 1 g/L. Soil samples labeled F1–3 are farm soils and U1–3 are urban soils. Soil samples P1–5 are pristine soils, collected from non urban areas with minimal human exposure over the last 100 years.

acid (rRNA) gene sequencing to determine the phlyogenies of the isolates and found the set included 3 of the 4 major phyla of the soil—dominated by the Proteobacteria (87%), and including isolates from the Actinobacteria (7%) and the Bacteroidetes (6%) (Fig. 4.2). The missing major soil phylum was the Acidobacteria (Janssen, 2006). Interestingly, the 3 observed phyla are also 3 of the 4 major phyla of the human gut microflora, with the Firmicutes not represented (Eckburg et al., 2005; Qin et al., 2010). A set of 75 isolates were then profiled for their resistance to the same 18 antibiotics at both 20 mg/L [approximate minimal inhibitory concentration (MIC) for these antibiotics] and 1 g/L (concentrations used in the subsistence assays). We found these isolates to be extensively multidrug resistant at both sets of concentrations. At the lower concentration, nearly 60% of the set were pan-resistant to the

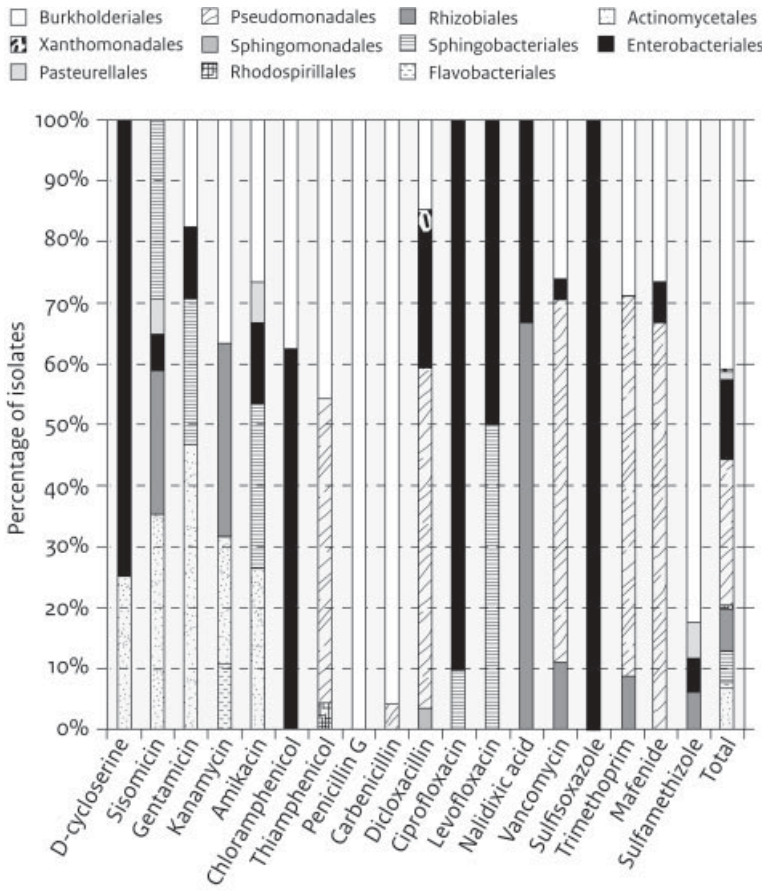

FIGURE 4.2 Phylogenetic distribution of bacterial isolates subsisting on antibiotics. 16S ribosomal DNA (rDNA) was sequenced from antibiotic catabolizing clonal isolates using universal bacterial rDNA primers. High-quality, nonchimeric sequences were classified using Greengenes (DeSantis et al., 2006), with consensus annotations from RDP (Cole et al., 2007) and NCBI taxonomies (Wheeler et al., 2000). Histogram displays the fraction of bacteria subsisting on different antibiotics that belong to particular bacterial orders as determined from the consensus classifications.

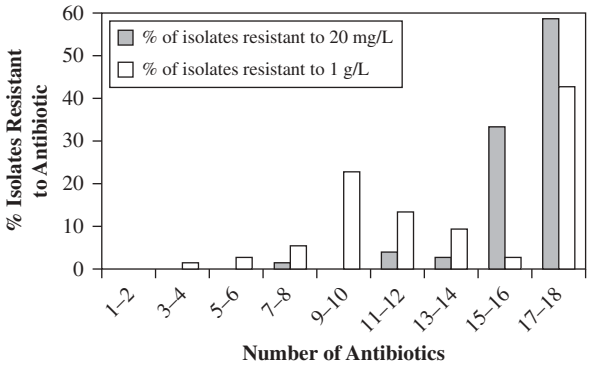

FIGURE 4.3 Antibiotic resistance profiling of 75 representative clonal isolates capable of subsisting on antibiotics. The histogram displays percentage of clonal isolates resistant to each of the 18 antibiotics at 20 mg/L (black bars) and 1 g/L (white bars).

18 antibiotics. At the higher concentration, nearly 40% of the total set were pan-resistant (Fig. 4.3).

Analysis of the degradation products in media of benzylpenicillin-subsisting bacteria using liquid chromatography and mass spectrometry was consistent with a pathway involving first the hydrolysis of the β-lactam ring, followed by a decarboxylation of benzylpenicilloic acid to yield benzylpenilloic acid, similar to the pathway proposed for *P. fluorescens* degradation of penicillin by Johnsen (1977). The proposed catabolic pathway for benzylpenicillin involves the action of a β-lactamase as a first step, providing a potential link between a very common resistance gene seen in pathogenic isolates and antibiotic subsistence by these soil bacteria (Davies and Davies, 2010).

4.6 ANTIBIOTIC SUBSISTENCE AS A SCAVENGER PHENOTYPE

We and others have demonstrated that bacteria subsisting on both synthetic and natural product antibiotics can be identified from a variety of sources. Since many of the antibiotics that can sustain growth of bacteria are produced by soil-dwelling bacteria and can be found at up to milligram per gram quantities in the soil (Thiele-Bruhn, 2003), it is not surprising that certain bacteria have evolved the capacity to subsist on these naturally occurring antibiotics as they represent a carbon source that can be utilized. The fact that organic molecules present in nature are typically biotically recycled could explain the catabolism of natural product antibiotics; however, we see almost equal microbial catabolism of human-made synthetic antibiotics such as the sulfonamides (Fig. 4.1). One explanation for the widespread catabolism of synthetic antibiotics may relate to their organic substructures, which are found in nature. Metabolic mechanisms exist for processing those substructures and may allow for the utilization of the parent synthetic antibiotic molecule. However, it should be noted that more than half of the bacterial isolates we identified belong to the orders Burkholderiales and Pseudomonadales (Fig. 4.2). These bacterial orders often have large genomes 6–10 Mb, which has been suggested

to be positively correlated to their metabolic diversity and multiple antibiotic resistance (Projan, 2007). Indeed *Burkholderiales* and *Pseudomonadales* can be thought of as scavengers, capable of utilizing a large variety of single carbon sources as food.

We hypothesize that some enzymatic antibiotic resistance mechanisms may have originated from such metabolically diverse scavenging organisms, which are capable of subsisting on a variety of naturally occurring molecular substructures. As these organisms were confronted with antibiotics in their habitat, they were likely able to repurpose those parts of their diverse catabolic arsenal that recognized the natural substructures, to allow them to utilize this new carbon source. This catabolic capability would give the scavengers a substantial selective advantage over other microbes, since they would be both tolerant to these microbial toxins while simultaneously cornering a specialized catabolic environmental niche. Lateral transfer of even a single gene involved in antibiotic enzymatic processing (and consequent inactivation) to other organisms could allow for the conferral of antibiotic resistance on the recipient organism disseminating the antibiotic resistance genes from the bacteria subsisting on antibiotics.

4.7 ECOLOGICAL CONSEQUENCES OF THE ANTIBIOTIC SUBSISTOME

While we have observed bacterial antibiotic subsistence in a variety of environmental samples, we have yet to determine whether microbes with this phenotype have evolved specifically for this purpose or if this is simply a by-product of a diverse and plastic metabolism. Nevertheless, having this machinery distributed in the environment has some interesting ecological consequences. In the case where these toxins can accumulate to inhibitory concentrations, perhaps the most obvious consequence is detoxification of the microenvironment inhabited by these bacteria, which could additionally benefit other susceptible microbes sharing that microenvironment. Given the complex and poorly understood interactions between the complex communities in the soil, such altruistic actions by one bacterium might enable survival of otherwise susceptible bacterial communities upon which the antibiotic-subsisting bacterium relies. In this way, antibiotic subsistence may represent an alternative population-based resistance mechanism, as was recently demonstrated in lab evolution experiments with *Escherichia. coli* by Collins and colleagues (Lee et al., 2010). Another consequence of antibiotic subsistence may be signal degradation or interception, as antibiotics at subinhibitory concentrations have been hypothesized to serve as signaling molecules (Davies et al., 2006; Yim et al., 2007). Efficient signaling requires signal degradation on appropriate time scales, and bacteria subsisting on antibiotics may serve such roles through interactions with producers. Alternatively, subsisters may also simply hijack or "eavesdrop" on signaling between producers by utilizing their signaling molecules. Finally, the ability to subsist on compounds that are toxic to most other microbes has the consequence of reduced competition for this specialized food niche. Since the above-described scenarios are by no means mutually exclusive, it is quite likely that all of these ecological roles may be at play in different environmental niches and community interactions.

4.8 INVESTIGATING CONNECTIONS BETWEEN SUBSISTOMES AND RESISTOMES

Thus far, the genes involved in antibiotic subsistence for any compound have not been identified; however, the identity and relationships of these genes to common resistance genes will illuminate the relationship between soil subsistence and antibiotic resistance. It should be noted that over a quarter of the isolates capable of subsisting on antibiotics have human pathogenic isolates as their closest relative based on 16S profiling (Dantas et al., 2008). Considering that lateral gene transfer occurs more readily between closely related species, the bacteria subsisting on antibiotics could indeed serve as an accessible reservoir of antibiotic resistance genes to human pathogenic isolates.

4.9 METAGENOMIC FUNCTIONAL SELECTIONS FOR DISCOVERING GENES ENABLING ANTIBIOTIC SUBSISTENCE AND RESISTANCE

A promising approach for discovery of antibiotic degradation genes is *metagenomic functional selections*, wherein genomic or metagenomic DNA (deoxyribonucleic acid) from a microbial strain or community can be shotgun cloned in an expression system in a bacterial host lacking the phenotype of interest (in this case, antibiotic catabolism), followed by subjecting the metagenomic transformants to a survival selection (Sommer et al., 2009, 2010). In the case of antibiotic subsistence, only genetic fragments enabling utilization of the antibiotic would survive on media containing the antibiotic as a sole carbon source. We use the phrase metagenomic functional selections to specifically refer to the idea of subjecting metagenomic DNA to an experimental functional assay, generally through shotgun expression in a heterologous host, in an attempt to distinguish this approach from broader definitions of *functional metagenomics*, which include sophisticated computational approaches for annotation of functions in shotgun metagenomic sequence data, but without direct experimental validation (Dinsdale et al., 2008). Metagenomic functional selections have been successfully applied to identify a variety of enzymatic functions from cultured and uncultured microbes, including genes and pathways for degradation of or resistance toward numerous xenobiotics [recently reviewed in Uchiyama and Miyazaki (2009)]. Since we postulate that antibiotic catabolism in at least some of our isolates involves the initial expression of resistance genes, we highlight below the power of metagenomic functional selections by describing our application of this method to investigate the antibiotic resistome of human intestinal microflora (Sommer et al., 2009).

It is quite possible that environmental resistomes may use the commensal human microflora as a conduit for eventual dissemination of resistance genes to pathogens, especially considering the enrichment of resistomes in the microflora of food animals through extensive use of antimicrobials in agriculture (Aarestrup et al., 2001). The microbes that inhabit the human body are likely the most directly accessible reservoir of resistance genes for pathogens, due to their high likelihood of genetic interaction during disease progression. Accordingly, we recently investigated the resistome harbored by the intestinal microflora of two healthy adult humans who had been

free of antibiotic therapy for over one year prior to sampling (Sommer et al., 2009). We applied a metagenomic functional selection approach to capture and sequence hundreds of antibiotic resistance genes from genomic DNA of aerobic bacteria cultured from the individuals' fecal samples, as well as from direct culture-independent metagenomic sampling of the same samples (Sommer et al., 2009). The genes from cultured isolates were closely related to genes previously described, including many that were identical to resistance genes described in human pathogens. These include the CTX-M-15 β-lactamase, recently identified in epidemic plasmids in disease isolates from around the globe. This work confirmed that resistance gene exchange between commensals and pathogens has likely occurred in our recent past (Salyers et al., 2004). In stark contrast, the genes we uncovered with culture-independent sampling were largely novel, with less than 65% average nucleotide identity to any genes in the National Center for Biotechnology Information (NCBI) nonredundant gene database. Phylogenetic analysis of these uncultured genes confirmed their genomic sources as the Bacteroidetes and Firmicutes, the dominant members of the human microflora, which are genetically distinct from the proteobacterial host (*E. coli*) used from functional selection. These results highlighted the severe previous undersampling of the resistome of the human microbiota due to reliance on culturing. However, they also demonstrated the utility of this method for capturing fully functional genes for xenobiotic resistance from phylogenetically diverse sources. We are currently applying these methods to interrogate the genetic antecedents of antibiotic utilization and resistance in the antibiotic subsisters.

4.10 ANTIBIOTIC SUBSISTENCE BY PATHOGENIC BACTERIA

Antibiotic subsistence on a variety of distinct antibiotics was recently identified in several hundred isolates of the pathogenic proteobacterium *Salmonella* (Barnhill et al., 2010). These strains included multiresistant and antibiotic-sensitive isolates derived from various food animals, in clinical, nonclinical, and food samples. Almost a third of the isolates were *Salmonella enterica* subspecies *enterica* serovar Typhimurium, which harbors the Salmonella genomic island 1 (SGI1) integron encoding multidrug resistance. The authors speculated a connection between the subsistence phenotype and the SGI1 integron, since more than half the isolates harboring the integron exhibited an antibiotic subsistence phenotype. Of the 572 isolates profiled, nearly a quarter subsisted on at least one antibiotic, while about 7% could utilize more than one antibiotic. Of the 12 antibiotics tested, only tetracycline was unable to support subsistence for any of the isolates profiled. Intriguingly, the authors found several cases in which it appeared that the ability to subsist on an antibiotic was unrelated to the mechanism of resistance. For instance, most isolates capable of subsisting on sulfisoxazole contained the *sul1* gene, which encodes a nonsusceptible version of dihydropteroate synthase. This resistance gene confers high-level resistance to sulfonamide antibiotics and would not be expected to be involved in the catabolism of sulfisoxazole. This work represents the first ever demonstration of antibiotic subsistence in a human pathogen, and highlights the importance of this phenotype, initially identified in the environment, in clinical settings. The fact that *Salmonella* is primarily a food-borne pathogen is particularly troubling when one considers that the amounts of antibiotics used in food animals, largely for nontherapeutic reasons,

outweigh human therapeutic use by manyfold (Silbergeld et al., 2008). Hence, mechanistic elucidation of antibiotic catabolism by these *Salmonella* isolates and their relationship to resistance requires urgent attention. Since *Salmonella* is closely related to *E. coli,* metagenomic functional selections in *E. coli* should be ideally suited to identify the catabolic pathways from these isolates, which would enable a more clear view of how this phenotype has been acquired by pathogenic *Salmonella* isolates.

4.11 CONCLUDING REMARKS

Investigations into microbial antibiotic subsistence and degradation are still clearly in their infancy, with substantial work to be done on the mechanisms that enable these phenotypes, and the potential evolutionary advantages these may confer on the microbes that harbor them. The role these phenotypes might play in microbial community ecology remain a complete mystery, both in environments like the soil where antibiotic producers exist in appreciable numbers, as well as within animal and human microflora, where there is a heightened potential for genetic exchange with pathogens. New systemswide molecular methods are allowing researchers to systematically investigate antibiotic resistance as a property exchanged within and between diverse communities. Application of these methods to the subsistome will allow us to answer the fascinating ecological and clinical questions that the discovery of these antibiotic subsistence phenotypes have posed.

REFERENCES

Aarestrup FM, Seyfarth AM, Emborg H-D, Pedersen K, Hendriksen RS, Bager F (2001). Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark. *Antimicrob Agents Chemother* 45:2054–2059.

Abd-El-Malek Y, Monib M, Hazem A (1961). Chloramphenicol, a simultaneous carbon and nitrogen source for a *Streptomyes* sp. from Egyption soil. *Nature* 189:775–776.

Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J (2010). Call of the wild: Antibiotic resistance genes in natural environments. *Nat Rev Microbiol* 8: 251–259.

Allen HK, Moe LA, Rodbumrer J, Gaarder A, Handelsman J (2009). Functional metagenomics reveals diverse beta-lactamases in a remote Alaskan soil. *ISME J* 3:243–251.

Aminov RI, Mackie RI (2007). Evolution and ecology of antibiotic resistance genes. *FEMS Microbiol Lett* 271:147–161.

Barnhill AE, Weeks KE, Xiong N, Day TA, Carlson SA (2010). Identification of multiresistant *Salmonella* isolates capable of subsisting on antibiotics. *Appl Environ Microbiol* 76:2678–2680.

Beckman W, Lessie TG (1979). Response of *Pseudomonas cepacia* to beta-lactam antibiotics: Utilization of penicillin G as the carbon source. *J Bacteriol* 140:1126–1128.

Benvenis R, Davies J (1973). Aminoglycoside antibiotic-inactivating enzymes in actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria. *Proc Nat Acad Sci. USA* 70:2276–2280.

Cavaco LM, Frimodt-Moller N, Hasman H, Guardabassi L, Nielsen L, Aarestrup FM (2008). Prevalence of quinolone resistance mechanisms and associations to minimum inhibitory concentrations in quinolone-resistant *Escherichia coli* isolated from humans and swine in Denmark. *Microbiol Drug Resist* 14:163–169.

Cole JR, Chai B, Farris RJ, Wang Q, Hulam-Syed-Mohideen AS, McGarrell DM, Bandela AM, Cardenas E, Garrity GM, Tiedje JM (2007). The ribosomal database project (RDP-II): Introducing myRDP space and quality controlled public data. *Nucleic Acids Res* 35:D169–D172.

Dantas G, Sommer MO, Oluwasegun RD, Church GM (2008). Bacteria subsisting on antibiotics. *Science* 320:100–103.

Davies J (1994). Inactivation of antibiotics and the dissemination of resistance genes. *Science* 264:375–382.

Davies J, Davies D (2010). Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev* 74:417–433.

Davies J, Spiegelman GB, Yim G (2006). The world of subinhibitory antibiotic concentrations. *Curr Opin Microbiol* 9:445–453.

D'Costa VM, Griffiths E, Wright GD (2007). Expanding the soil antibiotic resistome: Exploring environmental diversity. *Curr Opin Microbiol* 10:481–489.

D'Costa VM, McGrann KM, Hughes DW, Wright GD (2006). Sampling the antibiotic resistome. *Science* 311:374–377.

Demanèche S, Sanguin H, Poté J, Navarro E, Bernillon D, Mavingui P, Wildi W, Vogel TM, Simonet P (2008). Antibiotic-resistant soil bacteria in transgenic plant fields. *Proc Natl Acad Sci USA* 105:3957–3962.

DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Anderson GL (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* 72:5069–5072.

Dinsdale EA, Edwards RA, Hall D, Angly F, Breitbart M, Brulc JM, Furlan M, Desnues C, Haynes M, Li LL, McDaniel L, Moran MA, Nelson KE, Nilsson C, Olson R, Paul J, Brito BR, Ruan YJ, Swan BK, Stevens R, Valentine DL, Thurber RV, Wegley L, White BA, Rohwer F (2008). Functional metagenomic profiling of nine biomes. *Nature* 452:629–632.

Donato JJ, Moe LA, Converse BJ, Smart KD, Berklein FC, McManus PS, Handelsman J (2010). Metagenomic analysis of apple orchard soil reveals antibiotic resistance genes encoding predicted bifunctional proteins. *Appl Environ Microbiol* 76:4396–4401.

Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Relman DA (2005). Diversity of the human intestinal microbial flora. *Science* 308:1635–1638.

Enne VI, Cassar C, Sprigings K, Woodward MJ, Bennett PM (2008). A high prevalence of antimicrobial resistant *Escherichia coli* isolated from pigs and a low prevalence of antimicrobial resistant *E. coli* from cattle and sheep in Great Britain at slaughter. *FEMS Microbiol Lett* 278:193–199.

Fischbach MA, Walsh CT (2009). Antibiotics for emerging pathogens. *Science* 325:1089–1093.

Jaseen PH (2006). Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Appl Environ Microbiol* 72:1719–1728.

Johnseen J (1977). Utilization of benzylpenicillin as carbon, nitrogen and energy source by a *Pseudomonas fluorescens* strain. *Arch Microbiol* 115:271–275.

Johnseen J (1981). Presence of beta-lactamase and penicillin acylase in a *Pseudomonas* sp utilizing benzylpenicillin as a carbon source. *J Gen Appl Microbiol* 27:499–503.

Kameda Y, Toyoura E, Kimura Y, and Omori T (1961). A method for isolating bacteria capable of producing 6-aminopenicillanic acid from benzylpenicillin. *Nature* 191:1122–1123.

Lee HH, Molla MN, Cantor CR, Collins JJ (2010). Bacterial charity work leads to population-wide resistance. *Nature* 467:82–85.

Leng Z, Riley DE, Berger RE, Krieger JN, Roberts MC (1997). Distribution and mobility of the tetracycline resistance determinant tetQ. *J Antimicrob Chemother* 40:551–559.

Marshall CG, Lessard IAD, Park I-S, Wright GD (1998). Glycopeptide antibiotic resistance genes in glycopeptide-producing organisms. *Antimicrob Agents Chemother* 42:2215–2220.

McCormick JR, Jensen ER, Johnson S, Sjolander NO (1968). Biosynthesis of the tetracyclines. IX. 4-Aminodedimethylaminoanhydrodemethylchlortetracycline from a mutant of *Streptomyces aureofaciens*. *J Am Chem Soc* 90:2201–2202.

Palmer AC, Angelino E, Kishony R (2010). Chemical decay of an antibiotic inverts selection for resistance. *Nat Chem Biol* 6:105–107.

Pan JC, Ye R, Wang H-Q, Xiang H-Q, Zhang W, Yu X-F, Meng D-M, He Z-S (2008). *Vibrio cholerae* O139 multiple-drug resistance mediated by *Yersinia pestis* pIP1202-like conjugative plasmids. *Antimicrob Agents Chemother* 52:3829–3836.

Pan SJ (2007). (Genome) size matters. *Antimicrob Agents Chemother* 51:1133–1134.

Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez F, Yamada T, Mende DR, Li J, Xu J, Li S, Li D, Cao J, Wang B, Liang H, Zheng H, Xie Y, Tap J, Lepage P, Bertalan M, Batto JM, Hansen T, Le Paslier D, Linneberg A, Nielsen HB, Pelletier E, Renault P, Sicheritz-Ponten T, Turner K, Zhu H, Yu C, Li S, Jian M, Zhou Y, Li Y, Zhang X, Li S, Qin N, Yang H, Wang J, Brunak S, Doré, Guarner F, Kristiansen K, Pedersen O, Parkhill J, Weissenbach J, MetaHIT Consortium, Bork P, Ehrlich SD, Wang J (2010). A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 464:59–65.

Riesenfeld CS, Goodman RM, Handelsman J (2004). Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. *Environ Microbiol* 6:981–989.

Salyers AA, Gupta A, Wang Y (2004). Human intestinal bacteria as reservoirs for antibiotic resistance genes. *Trends Microbiol* 12:412–416.

Silbergeld EK, Graham J, Price LB (2008). Industrial food animal production, antimicrobial resistance and human health. *Annu Rev Public Health* 29:151–169.

Sommer MO, Dantas G, Church GM (2009). Functional characterization of the antibiotic resistance reservoir in human microflora. *Nature* 325:1128–1131.

Sommer MO, Church GM, Dantas G (2010). A functional metagenomic approach for expanding the synthetic biology tollbox for biomass conversion. *Mol Syst Biol* 6:360.

Thiele-Bruhn S (2003). Pharmaceutical antibiotic compounds in soils—A review. *J Plant Nutrition Soil Sci* 166:145–167.

Uchiyama T, Miyazaki K (2009). Functional metagenomics for enzyme discovery: Challenges to efficient screening. *Curr Opin Biotechnol* 20:616–622.

Webb V, Davies J (1993). Antibiotic preparations contain DNA: A source of drug resistance genes? *Antimicrob Agents Chemother* 37:2379–2384.

Wheeler DL, Chappey C, Lash AE, Leipe DD, Madden TL, Schuler GD, Tatusova TA, Rappa BA (2000). Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res* 28:10–14.

Wright GD (2007). The antibiotic resistome: The nexus of chemical and genetic diversity. *Nat Rev Microbiol* 5:175–186.

Yim G, Wang HMH, Davies J (2007). Antibiotics as signalling molecules. *Philos Trans R Soc B Biol Sci* 362:1195–1200.

5

IMPORTANCE OF ADAPTIVE AND STEPWISE CHANGES IN THE RISE AND SPREAD OF ANTIMICROBIAL RESISTANCE

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5.1 INTRODUCTION

One of the major problems we are facing today in the context of infectious diseases is the relentless increase and spread of antimicrobial resistance. However, the origins of this phenomenon can be traced to well before the clinical antibiotic era. Compounds with antimicrobial activity are known to be produced by practically all living organisms, including bacteria, fungi, plants, and animals. Consequently, microorganisms, especially antibiotic producers themselves, have evolved mechanisms to protect themselves against the toxicity of these compounds. Also, existing cellular components with a totally different function can, in many cases, confer resistance to a given antimicrobial, thus acquiring a new function. All these traits would often be associated with a reduction in fitness and even virulence when the antimicrobial agent is not present. For pathogens, this means that maintaining these determinants would only be beneficial under antibiotic pressure. The corresponding genetic markers would otherwise be lost as part of the natural selection process. Nevertheless, this changed drastically with the use of antibiotics as therapeutics. In the beginning, antibiotics were very effective in clearing the infections, but shortly afterward resistant organisms started to arise. The high selective pressure caused by intensive utilization of these compounds would inevitably lead to the selection of those

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microbes able to withstand the concentrations administered to the patients. Indeed, there have been reports of resistance toward practically all antibiotics available today. Alarmingly, there is a group of microorganisms, named Superbugs, that are resistant to most antimicrobials currently prescribed, and these include some of society's most prevalent causes of hospital infections including methicillin-resistant *Staphylococcus aureus* (MRSA), multidrug-resistant *Pseudomonas aeruginosa*, *Enterococcus* sp., and the like. Overall, antibiotic resistance represents a serious threat to modern medicine as we might one day be left helpless to combat bacterial infections. It is therefore imperative to tackle this problem and minimize the rise of resistance as well as develop novel and more efficient therapeutics. A further problem is that antimicrobial agents are not only used in human medicine but also in veterinary medicine, agriculture, industry, and the like. As a result, the levels of antibiotics found in many different settings, including areas adjacent to farms and sometimes natural ecosystems, can be fairly high. These niches have now become reservoirs of resistance markers that could be later transferred to human pathogens. This occurrence also needs to be taken into account if we want to succeed in the battle against antibiotic resistance.

There are three principal types of antibiotic resistance, namely intrinsic, acquired, and adaptive. Intrinsic resistance comprises all the natural, underlying characteristics of a particular species or strain that make it immune to the action of one or more types of drugs. Resistance determinants can also be acquired via horizontal transfer or as a result of mutations. This acquired resistance may lead to a high-level decrease in susceptibility, although in many cases, in particular in the case of mutational resistance, only a low-level (stepwise) increase is observed. Finally, microorganisms can become adaptively resistant to antibiotics in response to certain changes in the environmental conditions. In contrast to intrinsic and acquired mechanisms of resistance, which are permanent and stable, adaptive resistance is characterized by its transient nature. For a long time, clinical research focused on resistance phenotypes with a high impact and of a permanent nature. For this reason, phenomena such as stepwise and adaptive resistance have been largely neglected and are, as a result, poorly understood. Nonetheless, a growing number of studies highlight the importance that these mechanisms might have in the rise and geographical expansion of antimicrobial resistance. In fact, the ability of microorganisms to survive at concentrations around the minimal inhibitory concentration (MIC) facilitates the subsequent acquisition of high-level resistance markers (Baquero, 2001). Furthermore, there is more and more evidence that the decrease in susceptibility of a particular species does not necessarily occur in a single large step but rather in a gradual and steady manner. Thus, it is now clear that the baseline MICs are creeping, that is, slowly rising over time. Stepwise and adaptive resistance are good candidates to play a role in this MIC creep. Interestingly, it has transpired that some of the mechanisms responsible for these two different types of resistance are overlapping or related to some extent.

One of the best studied microorganisms in terms of resistance mechanisms is the opportunistic human pathogen *P. aeruginosa*. This ubiquitous bacterium can live in numerous habitats including soil, water, plants, and animals. Moreover, it is one of the leading causative agents of hospital-acquired infections as well as morbidity and mortality in cystic fibrosis (CF) patients. One constant characteristic of *P. aeruginosa* infections is that they are very difficult to eradicate. This pathogen is not only

intrinsically resistant to antibiotics, but it too can easily become less susceptible through the acquisition of new traits or adaptation via complex regulatory pathways. All these reasons make *Pseudomonas* an excellent and intriguing model to describe what we currently know about adaptive and stepwise acquired resistance. Specifically with reference to this book, *Pseudomonas* is found throughout the environment as well as being a prominent opportunistic pathogen of humans and animals, causing more than 160,000 infections annually in the United States. Nonetheless, we will also include some examples corresponding to other microorganisms. This chapter intends to summarize the most recent findings on the adaptive and stepwise types of resistance, as well as illustrate how these may contribute to the overall decrease in susceptibility to antimicrobials in bacteria.

5.2 TRANSCRIPTIONAL RESPONSES TO ANTIMICROBIALS

The use of novel molecular techniques in microbiology has permitted the observation of changes experienced in the transcriptome or proteome of microbes under different environmental conditions. One such technique is microarray analysis, which reveals the differences in gene expression between two samples. Particular attention has been paid to the responses displayed by bacteria upon exposure to sublethal and lethal doses of antimicrobial compounds. The results obtained to date in these experiments have revealed interesting hints about the mechanisms involved in antibiotic activity and antibiotic resistance. Moreover, these data suggest that antibiotics actually have complex modes of action, thereby challenging the traditional view of antibiotics having a single target in the bacterial cell. Brazas and Hancock (2005a) described the existence of gene signatures specific to a given antimicrobial compound that should be distinguished when interpreting microarray data. These genes may, according to the authors, be classified in four groups: genes dysregulated as a direct or indirect effect of the mechanism of action of the drug are, respectively, groups 1 and 2; group 3 comprises those genes altered as a downstream effect of target inhibition but that do not participate in the antibiotic activity or in antibiotic resistance. Finally, the genes included in group 4 are specific to a particular species, strain, or antibiotic and are termed bystander effects. An example can be provided by analyzing the transcriptional profile of *P. aeruginosa* cells exposed to ciprofloxacin (Brazas and Hancock, 2005b), which is summarized in Table 5.1. Fluoroquinolones interfere with DNA (deoxyribonucleic acid) replication resulting in DNA damage. The direct effect of this is the induction of the SOS response genes, which could be classified into group 1. The *Pseudomonas* SOS response involves the upregulation of the genes in the R2/F2 pyocin region. Interestingly, mutation of these genes resulted in a considerable decrease in susceptibility to ciprofloxacin. This seems to indicate that the higher expression of pyocins in the presence of this antibiotic is contributing to its killing action. It is likely that these pyocins constitute a phage lytic system that leads to cell lysis upon its activation (Nakayama et al., 2000). Genes in group 2 include those involved in metabolic changes, general stress responses, and antibiotic resistance mechanisms, which are the indirect result of the target inhibition. For instance, exposure to ciprofloxacin induces the expression of the efflux pump MexAB-OprM and the sigma factor *algU*, which coordinates an environmental stress response. In turn, the upregulation of *algU* results in a higher expression of genes involved in

TABLE 5.1 Examples of Genes Included in the Four Groups of the Signature Response to Ciprofloxacin[a]

	Characteristics	Examples
Group 1	**Direct effects** of target inhibition	SOS response genes
Group 2	**Indirect effects** elicited by target inhibition aimed at compensating for the damage caused by the antibiotic	Efflux pump *mexAB-oprM; algU* (cell wall stress response)
Group 3	**Secondary effects** of target inhibition that are not related to antibiotic activity or contribute to antibiotic resistance	Alginate production genes (*algR*, *algB*, etc.)
Group 4	**Bystander effects**, unrelated to target inhibition but constant for a particular microorganism or antibiotic	Motility and attachment genes (*pilG*, *fimV*, etc.)

[a]These examples have been taken from the microarray data published by Brazas and Hancock (2005a).

alginate biosynthesis, such as *algB*, which can be considered an example of group 3. Finally, exposure to ciprofloxacin leads to a downregulation of genes involved in motility and attachment. These could be described as bystander effects and would belong to group 4.

These experiments have also revealed intriguing results that might be linked to the ecological role of antibiotics. It is of particular interest that subinhibitory concentrations of antibiotics can sometimes induce the expression of virulence-related factors. A good example of this phenomenon is provided by Linares et al. (2006) who studied the transcriptome of *P. aeruginosa* in the presence of sublethal doses of three antibiotics belonging to different classes, namely tobramycin, ciprofloxacin, and tetracycline. This study found, for instance, that tobramycin upregulates the expression of genes involved in pyoverdine synthesis, whereas tetracycline induces pyochelin biosynthesis and type III secretion (T3S) genes. Moreover, the effect of subinhibitory antibiotics on some virulence-related phenotypes was also determined. Thus, all three antibiotics induced biofilm formation, but only tobramycin and tetracycline promoted swimming and swarming motility. In contrast, ciprofloxacin seemed to inhibit motility phenotypes. A previous study by Marr et al. (2007) had previously shown that subinhibitory tobramycin induces the expression of motility-related genes. Also, the presence of subinhibitory tetracycline increased cytotoxicity in *P. aeruginosa*, which is in good agreement with the induction of T3S. The T3S genes were also upregulated in the presence of subinhibitory concentrations of the macrolide azithromycin (Nalca et al., 2006). However, this antibiotic appeared to downregulate the expression of other quorum sensing-regulated genes such as *lasA* and *rhlB*. The adaptability of *P. aeruginosa*, whereby it is able to live in many different niches including soil, water, as well as human and animal hosts, affords a good model to try to pinpoint the responses of bacteria to antibiotics in nature. As mentioned previously, antibiotics are widespread in the natural environment. However, the concentrations detected in this context are well below those used in therapy, although within antibiotic-intensive farming operations concentrations may be higher. For this reason, it is thought that the role of these compounds cannot be

the inhibition of the growth of competitor organisms and is unlikely to directly select for high-level acquired resistance. In fact, the results discussed above provide significant evidence that antibiotics might be signaling molecules in a similar way to quorum sensing signals (Linares et al., 2006). Indeed, in *P. aeruginosa*, quorum sensing regulators such as the *Pseudomonas* quinolone signal (PQS) and the homoserine lactones display antimicrobial activity at high doses (Dubern and Diggle, 2008; Kaufmann et al., 2005; Wells, 1952).

In the context of antibiotic resistance, microarray analyses give very helpful information about possible mechanisms activated in bacteria to fight against the toxic effects of antimicrobials. Overall, high-throughput molecular techniques prove that microorganisms can easily alter their expression profile and adapt to the presence of antibiotics, thereby becoming more resistant. Furthermore, these experiments show how small changes in gene expression and small changes in resistance can be determinants in increasing bacterial fitness in the presence of an antimicrobial insult. This tactic would allow certain cells in the population to survive, multiply, and, in many cases, have a greater probability of attaining stable determinants of high-level resistance. Without these novel techniques, we probably would not have even begun to comprehend the relevance or to understand the underlying mechanisms of the types of resistance described in this chapter. Characterization of the expression patterns of known antibiotics can also be very useful in the design and development of new antimicrobials. Thus, comparison of the gene signatures between a novel and a well-described antibiotic can give information about possible cell targets, induction of adaptive resistance mechanisms, and the like. This will allow for a more efficient selection of the best candidates for further development and subsequent introduction into the clinic.

5.3 STEPWISE INCREASE IN RESISTANCE (INTRINSIC RESISTOME AND MUTATIONAL RESISTOME)

In clinical settings it is well known that pathogens have, throughout the years, increasingly become resistant to the different antibiotics currently available. Thus, the initial susceptibility observed when an antibiotic was first introduced in the market gave way relatively soon to phenotypes with varying degrees of resistance. One example would be the rise of resistance to the aminoglycoside tobramycin in *P. aeruginosa*. Originally, *Pseudomonas* strains were susceptible with an MIC $< 2\,\mu g/mL$ (Dibb et al., 1983), but some time after administration became frequent, resistance increased constantly. Indeed, right now we are facing the problem that resistant isolates with an MIC of $16\,\mu g/mL$ are isolated on a frequent basis, and sometimes strains with an MIC $> 128\,\mu g/mL$ are identified (MacLeod et al., 2000). These highly resistant strains represent a serious threat, especially because they are often resistant to more than one antibiotic. Multidrug-resistant bacteria, including *Pseudomonas*, are extremely difficult to eradicate and have become a major problem in the treatment of infections. As mentioned previously, such organisms are also referred to as Superbugs.

It was originally thought that these increases in antibiotic resistance happened suddenly. However, recent studies demonstrate that the "baseline" MIC actually shows a stepwise rise over time. This phenomenon has been clearly shown by

Steinkraus et al. (2007) in the case of susceptible MRSA isolates. A stepwise increase in MIC in the years 2001–2005 was observed for originally susceptible clinical MRSA isolates. The geometric mean MIC for vancomycin shifted from 0.62 to 0.94 μg/mL. This change is small; however, it is of concern as it can lead to a higher level of antibiotic resistance over time. Little attention has been paid to these small changes, which are often unnoticed in clinical screenings. However, over the last years it has become clear that a subtle increase in the MIC could actually contribute to the high-level resistance that leads to clinical failure. The phenomenon of low-level antibiotic resistance and its impact on high-level antibiotic resistance has been extensively reviewed by Baquero (2001). This review describes clearly how several compounds can select for low-level resistance and explains how these phenotypes may lead to high-level resistance. For instance, even though a single mutation might lead to just a small increase in the MIC, the occurrence of additional mutations in the same strain would inevitably result in a more dramatic change (El'Garch et al., 2007). These authors showed that a mutation in *galU*, *nuoG*, *mexZ*, or *rplY* led to a twofold increase in resistance to aminoglycosides. For example, the MIC for tobramycin was 1 μg/mL for all the single mutants, whereas the MIC of the wild-type was 0.5 μg/mL. Interestingly, an increase in resistance could be observed once double, triple, and quadruple mutants were tested. A *galU*/*nuoG*/*mexZ*/*rplY* quadruple mutant exhibits a tobramycin MIC of 8 μg/mL. This clearly shows the increase in resistance for additional mutations.

Although the mechanisms involved in low-level resistance have just started to be understood, different authors already suggest that they are far more complex than originally anticipated (Alvarez-Ortega et al., 2010, Breidenstein et al., 2008, Dötsch et al., 2009; Schurek et al., 2008). Recently, several screening studies revealed that many different mutations can lead to low-level resistance to antibiotics in *P. aeruginosa*. Moreover, while some mutations lead only to low-level resistance to one antibiotic class, several others result in low-level resistance to different antibiotic classes at the same time. The antibiotics used for these screens belonged to different classes and included fluoroquinolones, aminoglycosides, β-lactams, tetracycline, and sulfonamide. In this section, we will discuss the results obtained in these screenings with particular focus on the mutations that lead to decreased susceptibility and, therefore, to low-level resistance (mutational resistance). Nonetheless, we will also briefly mention some of the identified genes that led to increased susceptibility when mutated (involved in intrinsic resistance). Most of the studies were carried out using the comprehensive Harvard library (Liberati et al., 2006), which has been created in a PA14 strain background and comprises an array of more than 4500 individual mutants in separate genes. However, some studies also used the PAO1 miniTn5- *luxCDABE* library (Lewenza et al., 2005), and due to strain specificity, certain differences were observed (Brazas et al., 2007; Fajardo et al., 2008). Also, one has to keep in mind that the screens were performed on libraries of nonredundant genes and all essential genes are missing. Therefore, the gene list is not comprehensive and it is possible that more genes have an impact on resistance. Interestingly, all of these studies confirm the view that nonclassical antibiotic resistance genes participate in resistance, an idea that has developed in recent years. A better knowledge of these genes may play an important role in identifying new antimicrobial targets.

The ciprofloxacin resistome was analyzed in two independent studies by Brazas et al. (2007) and Breidenstein et al. (2008), which used the PAO1 and PA14

backgrounds, respectively. These screens found many genes that, once inactivated, led to altered susceptibility. Most times, the fold changes observed were only twofold, and such small changes can be easily missed in the clinic or environment. However, the large number of different genes that can lead to a small increase or decrease in susceptibility is important as it reflects a very large reservoir of mechanisms by which bacteria can become resistant. The ciprofloxacin resistome is not only large but also diverse in that a variety of genes involved in different cellular functions has an impact on ciprofloxacin resistance. Cross resistance between ciprofloxacin and other fluoroquinolones, such as nalidixic acid and levofloxacin, could also be observed. Breidenstein et al. (2008) discovered that 114 out of the approximately 4500 transposon mutants screened showed an altered susceptibility to ciprofloxacin. Thus, 35 mutants had an increase and 79 mutants had a decrease in susceptibility. As mentioned above, most of these showed only a twofold change, and many of these genes are distinct from those traditionally found to be involved in antibiotic resistance. Indeed, many genes from different functional classes are involved including those involved in transport of small molecules, membrane proteins, energy metabolism, DNA replication and recombination, cell division, hypothetical proteins, phage proteins, and the like. Interestingly, prior microarray analysis revealed that 43 out of the 114 identified resistance genes were dysregulated after exposure to 0.3× or 1× MIC of ciprofloxacin in the wild-type strain (Brazas and Hancock, 2005b). This overlap between global gene expression and resistance determinants highlights the fact that *Pseudomonas* can activate certain defense mechanisms in order to combat the bactericidal activity of ciprofloxacin.

Of particular interest among the mutants that led to increased susceptibility to ciprofloxacin, reflecting intrinsic resistance mechanisms, were those with mutations involved in DNA replication and repair. These genes included the Holliday junction helicases *ruvA* and *ruvB*, the recombinase *xerD*, the site-specific recombinase *sss*, the adenosine-triphosphate-(ATP)-dependent helicase *recG*, the ATP-dependent protease *lon*, DNA topoisomerase I *topA*, and DNA binding protein *fis* (Breidenstein et al., 2008; Dötsch et al., 2009). For example, the inactivation of *lon* leads to a four to eight fold change in increased susceptibility, making *lon* very important for ciprofloxacin resistance. The cell division protein *ftsK*, when mutated, also showed an eightfold increase in susceptibility. Other studies showed that inactivated *ftsK* also had a supersusceptible phenotype to different classes of antimicrobials represented by levofloxacin, ceftazidime, imipenem, meropenem, ertapenem, and cefotaxime (Alvarez-Ortega et al., 2010; Dötsch et al. 2009). The major intrinsic efflux pump *mexAB-oprM*, which is known to be involved in antibiotic resistance, was also demonstrated to have an increased susceptibility to all tested antibiotic classes (fluoroquinolones, β-lactams, tetracycline, and sulfonamide) upon mutation. It is therefore clear that the antibiotic resistance mechanisms are not specific for one antibiotic class as significant overlap exists (Fig. 5.1). The intrinsic ciprofloxacin resistome was thus shown to involve a huge gene pool.

On the other hand, several genes that when mutated led to mutational resistance and MIC creep over time, were identified. These included phage-related genes, Hydrogenated nicotinamide adenine dinucleotide (NADH) dehydrogenases, DNA mismatch repair proteins *mut*, the *mexCD-oprJ* efflux regulator *nfxB* as well as genes involved in iron transport. Interestingly, Brazas and Hancock (2005b) previously observed, using the PAO1 mutant library, that mutants in the bacteriophage-like R2/F2 pyocins

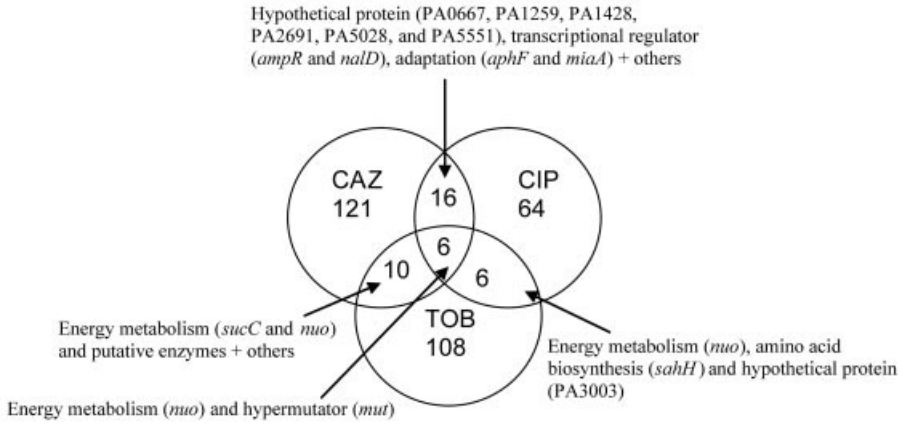

FIGURE 5.1 Analysis of the mutants that showed increased resistance to three antibiotics, representing different antibiotic classes, found in several screens (Alvarez-Ortega et al., 2010; Breidenstein et al., 2008; Dötsch et al., 2009; Schurek et al., 2008). The figure represents the number of resistant mutants to each antibiotic as well as the overlaps between the different antibiotics. Examples of the overlapping genes are given for each group. The abbreviations are as follows: CAZ, ceftazidime, CIP, ciprofloxacin, and TOB, tobramycin.

(PA0613-PA0648) are resistant to ciprofloxacin. These genes are also highly upregulated upon ciprofloxacin exposure, making them a susceptibility determinant.

Likewise, a study of the aminoglycoside resistome indicated that free radicals may impact on antibiotic killing. Thus, mutation of genes involved in aerobic respiration, such as cytochrome and NADH reduction genes, showed a decrease in tobramycin susceptibility (Schurek et al., 2008). Kindrachuk et al. (2011) demonstrated that these genes are downregulated in wild-type *P. aeruginosa* upon lethal exposure to tobramycin under anaerobic and aerobic conditions. In contrast, studies in *Escherichia coli* carried out by Kohanski et al. (2007, 2008) showed that the components of the tricarboxylic acid cycle (TCA) and electron-transport chain are upregulated upon exposure to aminoglycosides (gentamicin and kanamycin), suggesting possible mechanistic differences in these two species. The study by Schurek et al. (2008) also identified many other determinants of low-level tobramycin resistance in *Pseudomonas*. In fact, 135 genes leading to a twofold change upon mutation were identified. These genes are thought to contribute to the gradual low-level increase in tobramycin resistance. These small changes were also validated for selected genes in the PAO1 background. Mutated genes leading to low-level tobramycin resistance are primarily involved in energy metabolism (cytochrome, *nuo*, *nos*, and *nqr*), DNA replication and repair (*mut*, *mic*, *uvr*, and *radA*), and lipopolysaccharide (LPS) biosynthesis (*wbp*). Generally, aminoglycosides cross the cytoplasmic membrane via an energy-dependent process involving the electron-transport chain (Hancock, 1981). Therefore, it is not surprising that energy metabolism mutants exhibit an increase in resistance. This resistance can be easily explained by an alteration of the membrane potential that causes a decrease in aminoglycoside uptake (Mates et al., 1982). In contrast, a different study by Dötsch et al. (2009) did not observe the acquisition of resistance to tobramycin after inactivation of the *nuo* operon (NADH oxidoreductase). These mutants did,

however, display resistance to piperacillin, piperacillin-tazobactam, cefepime, cefotaxime, ceftazidime, and ciprofloxacin. Schurek et al. (2008) further observed that mutants in genes involved in the assembly of the A-band of LPS exhibit increased resistance to tobramycin (*wbpZ*, *wbpY*, *wzt*, *wzm,* and *wbpW*). By using the 1-*N*-phenylnaphthylamine (NPN) assay (Loh et al., 1984), the interaction between tobramycin and the outer membrane could be determined for the wild-type and the resistant LPS mutants. The authors could clearly demonstrate that tobramycin is less able to permeabilize the outer membrane of the LPS mutants, therefore enabling them to become more resistant. The tobramycin resistome analysis demonstrated for the first time that aminoglycoside resistance is affected by a wide range of different factors. The variety of identified genes that, when mutated, lead to minor changes in the MIC indicates that it is highly possible that if more mutations occur they would have an additive effect. This accumulation phenomenon had previously been suggested by Baquero (2001) and later demonstrated by El'Garch et al. (2007). Therefore, the observation that hundreds of mutations can lead to tobramycin resistance is of great importance, especially in the context of clinical settings. CF patients often undergo aminoglycoside treatment in order to treat *Pseudomonas* infections in their lungs; however, it is known that increasingly resistant strains are selected in the lungs. Thus, antibiotic therapy failure can be partly explained by the occurrence of tobramycin resistance mutations, such as those investigated in the study by Schurek et al. (2008).

Another important antibiotic class for the treatment of *Pseudomonas* infections is the β-lactams, which includes cephalosporins, carbapenems, and penicillins. β-lactams bind to penicillin-binding proteins (PBP) leading to peptidoglycan synthesis blockage (Walsh, 2003). Resistant mutants are known to involve mutations in membrane proteins, efflux, and the enzyme β-lactamase (Poole, 2004). However, the independent low-level β-lactam resistance studies by Dötsch et al. (2009) and Alvarez-Ortega et al. (2010) revealed that far more genes lead to an increase in β-lactam resistance if mutated. The study of Alvarez-Ortega et al. (2010) focused on the cephalosporin ceftazidime and 2 carbapenems, namely imipenem and meropenem, while Dötsch et al. (2009) included the penicillins. Although the overall structure of the β-lactams is similar, distinct differences between the tested antibiotics could be observed. In total, Alvarez-Ortega et al. (2010) found 78 mutants with an alteration in susceptibility. Of these, 41 mutants showed reduced susceptibility and 37 showed an increase in susceptibility toward at least one antibiotic. Most mutants leading to a low-level increase in resistance were observed for ceftazidime (37 ceftazidime/9 imipenem/14 meropenem resistant mutants). Only one hypothetical mutant, PA0908, exhibited a 2- to 3-fold increase in MIC toward all 3 antibiotics tested, while 14 mutants showed reduced susceptibility to 2 antibiotics. This small overlap indicates somewhat specific resistance mechanisms depending on the particular compound. The fact that the largest number of resistant mutants appeared in the ceftazidime screen highlights the easier development of ceftazidime-resistant mutants through mutation. This is very important for clinical treatment as all three antibiotics are currently used for antipseudomonal therapy. However, one has to keep in mind that some cross resistance to other antibiotic classes was observed through the different screens and, therefore, overlap between the resistant mechanisms can exist. Based on these observations, it would be important to reconsider the administration regimes of certain β-lactams in the light of their potential for

resistance. Albeit the changes are modest, they correlate with the low-level resistance phenotype described by Baquero (2001) and in combination could lead to significant antibiotic resistance and play a part in MIC creep over time. Regarding ceftazidime, the isolated mutants were involved in efflux as well as cell wall and LPS biosynthesis; some exhibited increased β-lactamase production. In the aminoglycoside resistome analysis described above, mutants involved in LPS biosynthesis were also identified. However, these were not identical to the ones found in the β-lactam screen. LPS biosynthesis mutants in *wapR*, *wpmM*, *wbpL*, *wspE*, *galU*, and the operon PA5001-5005 exhibited an increase in resistance to cephalosporins, penicillins, and sometimes to carbapenems (Alvarez-Ortega et al., 2010; Dötsch et al., 2009). The basis for this phenomenon might be a decrease in antibiotic penetration, although any such effect would be indirect as, unlike the aminoglycosides, β-lactams pass through the channels of outer membrane porins. β-lactam resistance also occurs due to an overexpression of efflux systems, such as MexAB-OprM, which occurs when the negative regulator, *nalC*, is mutated. Several other mutations leading to ceftazidime resistance could be identified including mutants in the following genes: *ampR*, *ampD*, *dacB* and *mpl*. These strains exhibited different degrees of *ampC* β-lactamase overproduction (6.4- to 55-fold increase) which would make these mutants more resistant to ceftazidime. Interestingly, *ampR* mutants were also shown to be more resistant to almost all other tested antibiotics, except tobramycin and carbapenems. In the case of the carbapenems (meropenem and imipenem), the most important mechanism of attaining increased resistance via mutation is the inactivation of the OprD porin through which carbapenems are known to enter the cell.

A comparison of the results obtained in a variety of different screens revealed that some genes are involved in resistance to more than one antibiotic class (Fig. 5.1). These include *mutS* and *mutL*, also known as hypermutators. If either one of these two genes is mutated, the spontaneous mutation rate increases dramatically (100- to 1000-fold) (Oliver et al., 2000). The result of this is that they exhibit an accumulation of secondary mutations, and this makes these strains rapidly acquire high resistance to several antibiotics. These same hypermutators are commonly found in CF patients (Ciofu et al., 2005) and help reduce the effectiveness of the antibiotic therapy administered for chronic lung infections. At an early stage of the infection, weak hypermutators are isolated from patients with CF (Kenna et al., 2007). Some of these weak hypermutators in *P. aeruginosa* were identified by Wiegand et al. (2008). A mutator phenotype is generally related to a disruption in DNA repair genes and, therefore, it was not surprising to find a modest increase in mutation frequency for mutants in *mutT*, *mutY*, and *mutL*. Two other gene deletions led to an increase in mutation frequency, namely *radA* (PA4609) and PA3959. The DNA repair protein RadA, once mutated, enhances the mutation frequency by 15-fold, whereas PA3958, belonging to the endonuclease/exonuclease/phosphatase family, increases the mutational frequency by only 3.4-fold. Thus it is evident that there are a variety of hypermutator mutants that could play a role in the early infection stages of CF patients. The investigation of these new hypermutator strains emphasizes the antibiotic treatment problem in the clinic as well as the need to find new strategies for overcoming these resistance problems.

Overall, the identification of hundreds of genes that when mutated contribute to antibiotic resistance provides a new perspective on resistance. The large number of mutations leading to low-level antibiotic resistance might provide an explanation for

the clinical phenomenon of MIC creep, and ultimately lead to high-level antibiotic resistance. Indeed, such mutations might occur in environmental situations and, while significant, be easily missed.

5.4 MECHANISMS OF ADAPTIVE RESISTANCE

The induction of resistance determinants by certain environmental cues is known as adaptive resistance. This type of resistance is transient and, in general, the initial levels of susceptibility can be restored after removing the inducing signal. In some cases, however, several passages under noninducing conditions are required (Mawer and Greenwood, 1978). Some of these triggering signals correspond to environmental factors in the milieu surrounding the microorganism such as anaerobiosis, altered pH, and decreased levels of nutrients including ions or particular carbon sources. This means that when the pathogen is in certain ecological niches or mediating an infection, it might exhibit greater resistance to antimicrobial agents than predicted when grown on typical laboratory nutrient-rich medium. This is further complicated by the fact that the presence of antibiotics, in particular at subinhibitory concentrations, can also make the bacteria able to withstand a subsequent challenge with an otherwise lethal dose. In addition, when bacterial cells form multicellular structures such as biofilms or swarming colonies, they are also less susceptible to antibiotics. As biofilms are currently regarded as one of the most common forms that bacteria use to live in natural environments and within the host, this is of particular significance. Furthermore, all of these factors can potentiate one another. For instance, exposure of biofilms to antibiotics can increase their level of resistance even more (Bagge et al., 2004; Pamp et al., 2008).

Microbiologists have known for decades that the exposure of bacterial cells to sublethal concentrations of an antimicrobial drug in the laboratory increases the microbe's ability to resist a subsequent antibiotic insult. For example, the existence of adaptive resistance to aminoglycosides in *P. aeruginosa* was described by Barber and Waterworth (1966). Interestingly, strains adapted to high levels of gentamicin in vitro showed a loss in virulence compared to their parent strain (Weinstein et al., 1971). This would make them less effective when infecting the host. Therefore, it was considered at the time that adaptive resistance would not be important in the clinical context. However, as early as 1978, an intriguing study demonstrated that after inducing adaptation with gentamicin or tobramycin at lower concentrations, *E. coli* exhibited a modest adaptive resistance phenotype that did not significantly reduce virulence or growth (Mawer and Greenwood, 1978). This led to decreased susceptibility toward several aminoglycosides and not just the one used in the initial exposure. A consequence of this is that if bacteria carrying a gentamicin-specific high-level resistance marker get exposed to gentamicin, they acquire increased resistance to other aminoglycosides, for example, tobramycin, preventing it from being used later in the treatment. In spite of these data, the phenomenon of adaptive resistance was still deemed irrelevant by many. It was not until the 1990s that the development of adaptive resistance to aminoglycosides was taken into account in order to improve the effectiveness of administration regimes. At that time several studies recommended the use of higher doses and longer intervals between doses within the range allowed by the toxicity associated with this class of antibiotics (Barclay et al., 1996; Daikos et al., 1991).

As mentioned above, we now know that adaptive resistance is induced in the presence of many different environmental cues, including subinhibitory (sub-MIC) concentrations of antimicrobials. However, its impact and the specific underlying mechanisms are just starting to be understood. One of the main problems in evaluating the clinical impact of adaptive resistance is that it is not a stable phenotype, and, as a result, it cannot be easily detected with traditional antimicrobial resistance screening methods. Nevertheless, it seems very likely that adaptation to host conditions or to antibiotic exposure during treatment is one of the reasons for clinical failure of antibiotic therapy. One implication of this type of resistance is that the susceptibility profile of a pathogen might be completely different under in vitro and in vivo conditions. Furthermore, such adaptations sometimes confer protection from, that is, cross resistance to, several antimicrobial agents, including those from different classes. Another cause of concern is that, like stepwise resistance, the development of adaptive resistance can be associated with a greater probability of evolution toward a high-level resistance phenotype (Driffield et al., 2008; Hausner and Wuertz, 1999; Molin and Tolker-Nielsen, 2003).

Albeit insufficient, the knowledge we currently have seems to indicate that adaptive resistance is a complex and tightly regulated phenomenon. In fact, many advances in this field were made through understanding transcriptional regulation. In the following sections we will summarize the different tactics employed by bacteria in acquiring adaptive resistance and provide examples for each of them.

5.4.1 Efflux and Influx

One of the most common mechanisms used by bacteria to become resistant to an antimicrobial compound is by limiting its accumulation inside the cell. There are two different ways of achieving that, namely limiting the entrance of the drug into the cell or actively pumping it out of the cell. In many cases, these mechanisms are intrinsic and are characteristic of a particular species. For example, *P. aeruginosa* possesses a low permeability outer membrane and as a result antibiotics cannot penetrate easily into the bacterium. Indeed, it is believed that it is the synergy between this reduced permeability and active efflux (as well as the above-mentioned enzymes like β-lactamase) that makes *Pseudomonas* an intrinsically highly resistant species. The genes encoding efflux pumps, porins, and the like are sometimes only expressed at a very low level under normal growth conditions and require the presence of a specific environmental cue to upregulate their expression. In these situations, one can observe clear examples of adaptive resistance.

Active extrusion of toxic compounds, including antibiotics, out of the bacterial cell is mediated by transport proteins called efflux pumps. Five families of efflux pumps have been identified in bacteria to date (Schweizer, 2003). Among these, four obtain energy from the proton motive force. These are the RND (resistance–nodulation–division), the MF (major facilitator), the SMR (small multidrug resistance), and the MATE (multidrug and toxic compound extrusion) families. In contrast, the energy source for the ABC (ATP-binding cassette) family is ATP hydrolysis. Some of these transporters are specific and can only export one compound, whereas others have a broad range of substrates, thus participating in the development of multidrug resistance. Constitutive expression of efflux pumps may have deleterious effects on bacterial fitness and even virulence. This was

described for the *P. aeruginosa nalB* and *nfxB* multidrug-resistant mutants, which overexpress the RND efflux pumps MexAB-OprM and MexCD-OprJ, respectively (Sanchez et al., 2002). These mutants showed impairments in different virulence-related phenotypes such as phenazine and protease production or virulence in a *Caenorhabditis elegans* model, but not in biofilm formation. They also showed reduced fitness in water or on dry surfaces, environments that reflect potential ecological reservoirs of this pathogen. As a result of these potential disadvantages, there is a tight control of the transcription of the genes encoding pumps so that they are only upregulated when they provide an advantage to the cell. Some well-described cases have related this induction with the presence of antimicrobial compounds. *P. aeruginosa* possesses multiple efflux pumps, including 12 putative candidates belonging to the RND family, although not all of them have yet been characterized (Stover et al., 2000). Each pump has a different range of exported compounds. Likewise, specific types of antimicrobials tend to induce only certain pumps. For example, aminoglycosides are known to induce the MexXY efflux pump, which accounts for a significant part of adaptive resistance to this class of antibiotics (Hocquet et al., 2003). In fact, mutants in *mexXY* do not acquire as great an increase in resistance upon exposure to aminoglycosides as does the parent strain (Hocquet et al., 2003). In a similar manner, part of the adaptive resistance triggered by subinhibitory ciprofloxacin is due to the upregulation of the efflux pump MexAB-OprM (Brazas and Hancock, 2005b). Due to the ability of some of these pumps to extrude multiple drugs, a worrying consequence of this is that exposure to one antibiotic would induce the expression of the corresponding efflux pump. This would then make the cells less susceptible to several classes of antibiotics. Figure 5.2 provides an example of this phenomenon based on the upregulation of *mexAB-oprM* by ciprofloxacin. Also of concern is the existence of cross resistance between biocides and antibiotics. Biocides are chemical substances generally used as disinfectants, antiseptics, and antifouling agents in diverse human activities. These include hospitals, households, industry, and agriculture. As a result, the possibility that exposure to these compounds is inducing resistance not only to biocides themselves but also to clinical drugs is alarming indeed. One of the predicted mechanisms for this phenomenon is the induction by biocides of efflux pumps (Chuanchuen et al., 2001; Hegstad et al., 2010; McMurry et al., 1998).

The upregulation of efflux pumps has also been observed in biofilms. For instance, Zhang and Mah (2008) identified an MF efflux pump in *P. aeruginosa* that participates in increased resistance to tobramycin and ciprofloxacin in the biofilm state but not in a planktonic culture. Transcriptional analysis showed that this pump, encoded by the operon PA1874-77, was upregulated during biofilm formation. Also, the resistance of *Pseudomonas* biofilms to azithromycin was found to require the presence of MexCD-OprJ (Gillis et al., 2005). Another interesting occurrence is the induction of the genes *mexAB-oprM* upon exposure of the cells in the upper layers of *Pseudomonas* biofilms to colistin (Pamp et al., 2008). In contrast to biofilms, the increased resistance of *P. aeruginosa* swarming cells does not seem to involve the participation of any of the well-characterized efflux pumps (Lai et al., 2008).

Of note, *P. aeruginosa* mutants showing an overexpression of efflux pumps are commonly isolated from patients after antibiotic therapy. These isolates show a reduced susceptibility toward multiple antibiotics. For example, Westbrock-Wadman et al. (1999) reported the overexpression of *mexXY* in aminoglycoside-resistant

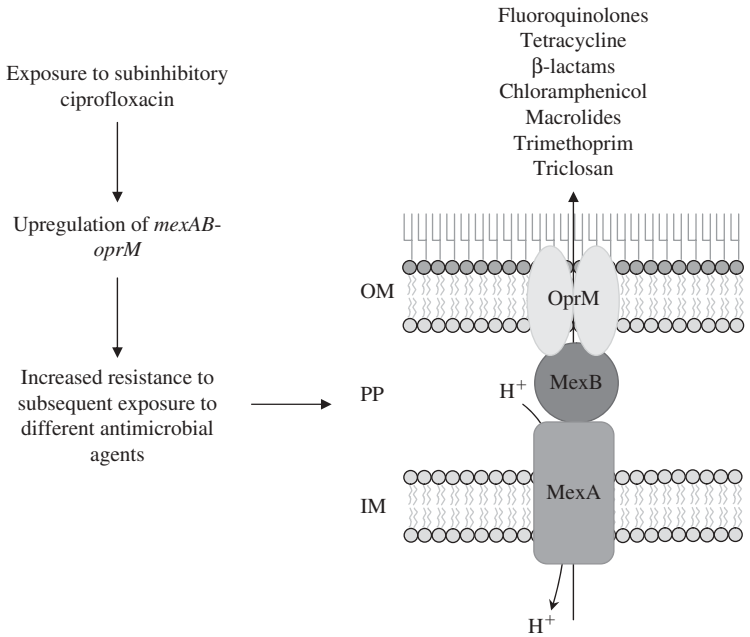

FIGURE 5.2 Example of the acquisition of adaptive resistance via the upregulation of efflux pumps. The presence of subinhibitory concentrations of ciprofloxacin induces the transcription of the operon encoding MexAB-OprM. As a result of this, the bacterium becomes more resistant to a wide range of antimicrobials from different classes because the cells will pump the drugs out more efficiently. Some examples of the known substrates of MexAB-OprM are indicated (Poole, 2001). The following abbreviations are used: OM, outer membrane; PP, periplasm, IM, inner membrane.

isolates, and another multiresistant clinical isolate displayed simultaneous overexpression of MexAB-OprM and MexEF-OprN (Pumbwe and Piddock, 2000). Additionally, a study reported that fluoroquinolone resistance in CF isolates was due to overexpression of MexCD-OprJ and MexEF-OprN in the majority of cases (Jalal et al., 2000), whereas in strains isolated from wounds and urine samples the principal mechanism is the mutation of *gyrA* and *parC* (Jalal and Wretlind, 1998).

Besides increased efflux, another mechanism to achieve a greater level of resistance is by reducing the uptake and, as a result, the accumulation of the antibiotic in the cytoplasm. In 1982, Gerber and Craig described a low-level aminoglycoside resistance phenotype in *P. aeruginosa* that they designated "impermeability type of resistance." Some years later, Gilleland et al. (1989) observed that this phenotype could be reproduced by in vitro exposure to increasing concentrations of aminoglycosides, starting with a dose equaling the MIC for each individual antibiotic. This suggested that it was actually adaptive resistance and not mutant selection that led to the decreased susceptibility trait. Around the same time, another study demonstrated that cytoplasmic accumulation of aminoglycosides requires the proton motive force as well as a functional aerobic respiration pathway (Taber et al., 1987). For this reason, the fact that subinhibitory aminoglycosides upregulate genes involved in the anaerobic pathway could well contribute to the development of adaptive resistance to

these compounds. Examples of this are *anr* and *denA*, which code for a regulatory protein and a nitrite reductase, respectively, and whose expression is induced by tobramycin (Karlowsky et al., 1997). In fact, *P. aeruginosa* cells are considerably more resistant to aminoglycosides when grown under anaerobic conditions Kindrachuk et al. (2011). Thus, the concentration required to produce similar killing rates is at least 10 times higher under anaerobic conditions.

The limited capacity of the antibiotic to access cells is also a characteristic of biofilms. In this case, it is the extracellular matrix rather than the cell envelope that hinders the access of the antibiotic molecules to their cell targets. This polymeric matrix is constituted of polysaccharides, proteins, and DNA, and it is thought to retard the penetration of the antimicrobial agents. One example is the aminoglycosides that, because of their cationic nature, would interact with the negatively charged polymers (Kumon et al., 1994) and perhaps with anionic DNA molecules present in the extracellular matrix (Mulcahy et al., 2008). However, it is not very clear if this delayed penetration of antibiotics is sufficient to result in a significant level of resistance. Furthermore, this reduced penetration into the biofilm has been observed only for β-lactams and aminoglycosides and not all reports agree on that (Hoyle et al., 1992; Shigeta et al., 1997; Yasuda et al., 1993). In contrast, studies regarding other antibiotic classes seem to indicate that the antimicrobial drugs can diffuse sufficiently into the biofilm, thereby suggesting that other mechanisms must account for the increased resistance of these communities (Shigetta et al., 1997; Suci et al., 1994; Vrany et al., 1997).

5.4.2 Modifications of the Cell Envelope

One of the mechanisms of bacterial resistance to antibiotics is the alteration of the cell envelope such that interactions with the antimicrobial molecules are limited. A well-studied example of this is the modification of the lipopolysaccharide (LPS), which leads to increased resistance to polymyxins and other cationic antimicrobial peptides. The most important among these modifications is the addition of 4-aminoarabinose to the lipid A portion of LPS. This inhibits the interaction with LPS and consequent self-promoted uptake of cationic molecules like the aforementioned peptides. Aminoarabinose addition in *P. aeruginosa* is carried out by the products of the LPS modification (*arn*) operon, which is a homolog of the equivalent *Salmonella* operon. The upregulation of this operon is under tight control, and it generally entails the participation of two-component regulatory systems (TCS). TCS regulators generally consist of two proteins, a sensor histidine kinase, which spans the cytoplasmic membrane, and a cytoplasmic DNA-binding response regulator (Stock et al., 2000). The kinase senses changes in the surrounding milieu via a periplasmic loop. Then, it autophosporylates and transduces the stimulus to the cytoplasmic response regulator, also by phosphorylation. The final result of this process is the binding of the phosphorylated response regulator to a recognition sequence in the promoter of multiple genes and the consequent up- or down-regulation of the target genes involved in responding to the environmental stress. *P. aeruginosa* possesses one of the largest collections of TCSs known so far, which comprises 64 sensor kinases and 63 response regulators (Gooderham and Hancock, 2009; Stover et al., 2000). As in *Salmonella*, the *Pseudomonas arn* operon is induced at low concentrations (in the μM range) of divalent cations (Mg^{2+}, Ca^{2+}). Under these conditions, two TCS regulators are activated independently, namely PmrAB and

PhoPQ, and their response regulators independently increase the transcription of the *arn* operon (Macfarlane et al., 1999; McPhee et al., 2003). The overall result is a greater resistance to antimicrobial peptides under suboptimal Mg^{2+}or Ca^{2+} growth conditions. However, the level of divalent cations in the human body is not limiting (about 1–2 mM), and therefore it seems unlikely that this pathway plays a significant role in the clinical context. Nevertheless, modifications akin to those induced in vitro in a medium with a low content of divalent cations have been observed in *P. aeruginosa* isolated from CF patients (Ernst et al., 1999, 2007). In their article, McPhee et al. (2003) revealed a finding that hinted at the possible explanation behind this intriguing dilemma. This study demonstrated that the induction of the LPS modification operon could be achieved by exposing the cells to subinhibitory concentrations of the antimicrobial peptide CP11CN, a derivative of the bovine cathelicidin indolicidin. Although the authors observed that the operon containing the *pmrAB* genes was upregulated under these conditions, neither PmrAB nor PhoPQ was essential for peptide-dependent adaptation. Thus, strains with mutations in these two two-component regulators showed induction levels similar to those obtained for the parent strain. Recently, Fernández et al. (2010) identified the system ParRS, which is necessary for the upregulation of the *arn* operon elicited by indolicidin as well as the bacterial peptides polymyxin B and colistin. In contrast, other peptides like CP28 or polyphemusin induced similar levels of adaptive resistance in the *parRS* mutants and the parent strain. Therefore, it is very likely that additional two-component systems participate in sensing the presence of antimicrobial peptides. Of note, the human host defense peptide LL37 does not significantly induce the transcription of the *arn* operon (Fernández et al., 2010; Overhage et al., 2008a).

Altogether these studies seem to indicate that cationic peptides are good candidates for the induction of aminoarabinose modification of the lipid A in the CF airways. Indeed, the polymyxin colistin is a common therapeutic agent in CF, and the bacterial cells might also come in contact with host defense peptides during the infection. Alarmingly, Fernández et al. (2010) showed that the LPS modification observed in the presence of indolicidin and polymyxins increased resistance to tobramycin and gentamicin, as well as to antimicrobial peptides, probably due to the positive charge of these molecules. Like colistin, tobramycin is very frequently used in CF treatment. This makes it imperative to understand how adaptations leading to resistance to both antibiotics can be induced throughout the course of the infection. Moreover, study of clinical polymyxin-resistant isolates revealed the acquisition of altered expression patterns of the genes involved in adaptive resistance to peptides (Schurek et al., 2009).

5.4.3 Stress Responses

The ability to adapt rapidly to changes in their surrounding milieu is paramount for bacteria, both in their ecological niches and inside the host, as they usually live in dynamic environments. Thus, chemical and physical parameters such as pH, temperature, nutrients, and oxygen concentration are constantly varying. This is especially important when these changes are drastic and potentially compromise the survival of the cells. As a result, bacteria have evolved stress responses that allow them to rapidly modify their transcriptional and proteomic profiles until the conditions return to their normal range. In addition to the conditions listed above, antibiotics are also a source of stress, in particular when they approach lethal levels.

Fluoroquinolones are synthetic antibiotics that interfere with DNA synthesis by inhibiting the activity of DNA gyrase and topoisomerase IV. Therefore, fluoroquinolones can be classified as DNA-damaging agents. In bacteria, DNA damage triggers the so-called SOS response. The SOS network detects and repairs the DNA damage, but, if this is unsuccessful, it will lead to error-prone repair resulting in mutagenesis and/or death of the bacterium. The topoisomerase inhibitor ciprofloxacin is known to upregulate the expression of the genes involved in the SOS response in several microorganisms, including *P. aeruginosa* (Brazas and Hancock, 2005b; Cirz et al., 2006; Hastings et al., 2004). This fluoroquinolone can induce double-strand breaks in the DNA. Therefore, exposure to this antibiotic, even at subinhibitory concentrations, switches on the SOS network in order to repair the damage and increase the survival chances of the cells. In that sense, the fluoroquinolone-mediated induction of this response makes the cells less susceptible to antibiotic challenge. Another consequence of this upregulation is the appearance of adaptive point mutations that may confer protection not only from fluoroquinolones but also from other antibiotic classes. This phenomenon is the consequence of the aforementioned error-prone repair systems, such as the SOS mutator DNA polymerase IV (McKenzie et al., 2001). Wiegand et al. (2008) showed how the mutation of genes with mutator or antimutator activity may lead to resistance to several antibiotic classes. Several studies on *E. coli* have also demonstrated that the SOS response can be triggered by the presence of β-lactams, thereby contributing to enhanced survival to lethal antibiotic doses (Miller et al., 2004). In this case, it is thought that the activating signal is the inactivation of the penicillin binding protein 3, encoded by *ftsI*, which is sensed by the two-component system DpiBA. This would interrupt cell division and result in an increased tolerance to antibiotic exposure.

Another vital stress response is the one triggered by heat shock, which has been mostly characterized in *E. coli* (Guisbert et al., 2008). This regulatory network is generally initiated by a temperature shift. The temperature rise results in protein misfolding, which once detected will increase the cytoplasmic levels of active $\sigma^{32}(\sigma^{H})$ transcription factor. This, in turn, will upregulate a regulon mainly constituted of genes encoding proteases and chaperones that will either degrade or refold the damaged proteins. This system is fairly conserved among bacteria. In addition to high temperature, there are other signals that can trigger this response. One of them is the exposure to aminoglycosides. These compounds interfere with protein synthesis and cause errors during translation, which increases the level of misfolded proteins in the cytoplasm and activates the heat shock response. This has been observed in bacteria such as *E. coli* (Shaw et al., 2003) and *Bacillus subtilis* (Lin et al., 2005). In *P. aeruginosa*, the heat shock response has not yet been studied in great depth. However, we do know that there are homologs of the proteins described in *E. coli*, including the heat shock sigma factor, which in *Pseudomonas* is called RpoH (Potvin et al., 2008). Furthermore, recent evidence indicates that exposure to lethal tobramycin induces the heat shock network in *P. aeruginosa* Kindrachuk et al. (2011). Interestingly, Schurr and Deretic (1997) found that the regulation of mucoidy conversion and the heat shock response is coordinated in *P. aeruginosa*.

Also induced by tobramycin is the two-component system AmgRS, which regulates an envelope stress response in *P. aeruginosa* (Lee et al., 2009). This response contributes to increased resistance to the antibiotic challenge as mutants in the *amgRS* operon are significantly more susceptible to aminoglycosides. This system is

a homolog of *E. coli* CpxRA, which detects the stress caused by the presence of misfolded proteins in the membrane (Shimohata et al., 2002). The authors suggested the possible utilization of this two-component regulator as a target of new drugs. This could potentiate the action of aminoglycosides, thus permitting the use of smaller doses of these toxic antimicrobials.

5.4.4 Production of Inducible Enzymes

The production of enzymes able to degrade or inactivate antibiotic compounds is very widespread among bacteria. One of the best known examples is the group of the β-lactamases, which are the major resistance mechanism to β-lactams. The mechanism of action is by hydrolyzing the β-lactam ring, which results in deactivation of the antibiotic. Because β-lactams can be used to treat a wide range of infections and do not represent a high toxicity risk for the patient, resistance to this type of antibiotics is of great concern (Livermore, 1996). β-lactamases are ancient proteins that existed well before the clinical antibiotic era. In fact, evolutionary studies on these enzymes appear to indicate that they probably existed more than 2 billion years ago (Hall and Barlow, 2004; Garau et al., 2005). β-lactamases can be plasmid or chromosomally encoded. The chromosomal β-lactamase gene, *ampC*, is present in numerous species. In some bacteria, this gene is constitutively expressed, whereas in others its expression is inducible by β-lactams (Normark et al., 1986). *P. aeruginosa* is an example of the latter. Thus, in *Pseudomonas*, exposure to subinhibitory levels of many β-lactams significantly upregulates the transcription of *ampC* through the activation of the LysR-type transcriptional regulator AmpR (Hanson and Sanders, 1999). The consequence of this would be the acquisition of adaptive resistance to different β-lactams (Lindberg and Normark, 1986; Livermore, 1987). However, not all β-lactams induce *ampC* expression to the same degree and not all β-lactams are deactivated by this β-lactamase. In that sense, the utilization of drugs, like cefepime, that do not upregulate *ampC* is recommended (Sanders, 1993), especially since the development of adaptive resistance to certain β-lactams, such as ceftazidime, penicillin, and cefotaxime, during the course of an infection has been associated with clinical failure (Pai et al., 2004).

Although not described in *P. aeruginosa*, it is worth noting the fact that, in some cases, the exposure to subinhibitory concentrations of antibiotics induces not only a drug-inactivating enzyme but also the mobilization of the genetic element carrying it. This is the case of tetracycline resistance genes from *Enterococcus faecalis* (Celli and Trieu-Cuot, 1998) and *Bacteroides fragilis* (Privitera et al., 1979), which are carried by transposon Tn916 and a plasmid, respectively.

Accumulation of antibiotic-degrading or modifying enzymes has been observed in the extracellular matrix of biofilms. This would significantly reduce the amount of antibiotic able to penetrate the biofilm and reach the bacterial cells. This phenomenon has been observed for a β-lactamase of *Klebsiella pneumoniae* (Anderl et al., 2000).

5.4.5 Multicellular Behaviors

Although we tend to consider microorganisms as unicellular entities, it is clear that under specific conditions they can associate and exhibit social behaviors. These

activities are under tight control of cell-to-cell signals such as the quorum sensing molecules. Such bacterial communities have been attracting growing interest among microbiologists. In particular, the most well-known examples, namely biofilms and swarming motility, are known to be involved in antibiotic resistance, virulence, and even evasion of the host defense mechanisms (Overhage et al., 2008b; Verstraeten et al., 2008). The study of microbial multicellular behavior is also interesting from an evolutionary perspective, as it might provide hints about the evolution to a true pluricellular state. Finally, recent research indicates that biofilms and swarming colonies are probably very common growth forms in natural, clinical, and industrial environments, as well as during the course of bacterial infections (Davey and O'Toole, 2000; Overhage et al., 2008b).

The formation of dense microbial aggregates attached to surfaces has been known for centuries, but it was only about three decades ago that studies on biofilms began. Communities known as biofilms can be constituted by one or more species of microorganisms. Biofilm cells display considerable differences in the transcriptome and proteome compared to planktonic cells. Indeed, it could be said that they show similarities with the behavior of cells from a multicellular organism, including the differentiation of cells in the community. In the clinical setting, biofilms are a serious cause of concern due to their high level of resistance to antimicrobial agents. Indeed, some authors have demonstrated that biofilm cells can be 100- or 1000-fold more resistant than planktonic cells (Hoyle and Costerton, 1991). Furthermore, biofilms usually show increased resistance to multiple drugs, which further complicates the implementation of an adequate treatment regime. In the case of *P. aeruginosa*, biofilms have been observed in prosthetic materials (Donlan, 2001) as well as in the lungs of CF patients (Bjarnsholt et al., 2009) and in chronic wounds (Kirketerp-Møller et al., 2008). Already in 1985, a *P. aeruginosa* isolate from a urinary tract infection displayed a 1000-fold decrease in susceptibility to the aminoglycoside tobramycin when the cells were forming a biofilm (Nickel et al., 1985). Once the cells were taken out of the biofilm and grown in a liquid culture, they reverted to their original degree of resistance. This confirmed the adaptive nature of biofilm-associated resistance to antibiotics.

Biofilms show increased resistance by upregulation or accumulation of known mechanisms of antibiotic resistance, such as enzymes or efflux pumps, as well as by delayed penetration of the antibiotic through the extracellular matrix. However, there are other characteristics specific to this multicellular state that also contribute to the reduction in susceptibility. One major trait of biofilm communities is that they are very heterogeneous. That is, cells forming part of the biofilm, even if they are of the same species, may be in totally different growth states. The explanation for this cell differentiation is that not all cells have equal access to oxygen and nutrients, and this will shape their metabolic profile (Fig. 5.3). Thus, the cells in the deep layers of the biofilm must adapt to a nutrient-deprived and nearly anaerobic environment, whereas the cells in the upper layer will have greater oxygen and nutrient availability. As a result, some cells will be in a slow growth mode while others will show a high metabolic activity. Heterogeneity in itself is an advantage as cells throughout the biofilm will show different responses to certain classes of antibiotics. For instance, if a given antimicrobial kills metabolically active cells, as is the case of β-lactams, ciprofloxacin and tetracycline, then the center of the biofilm will not be eradicated and will regrow once therapy is interrupted

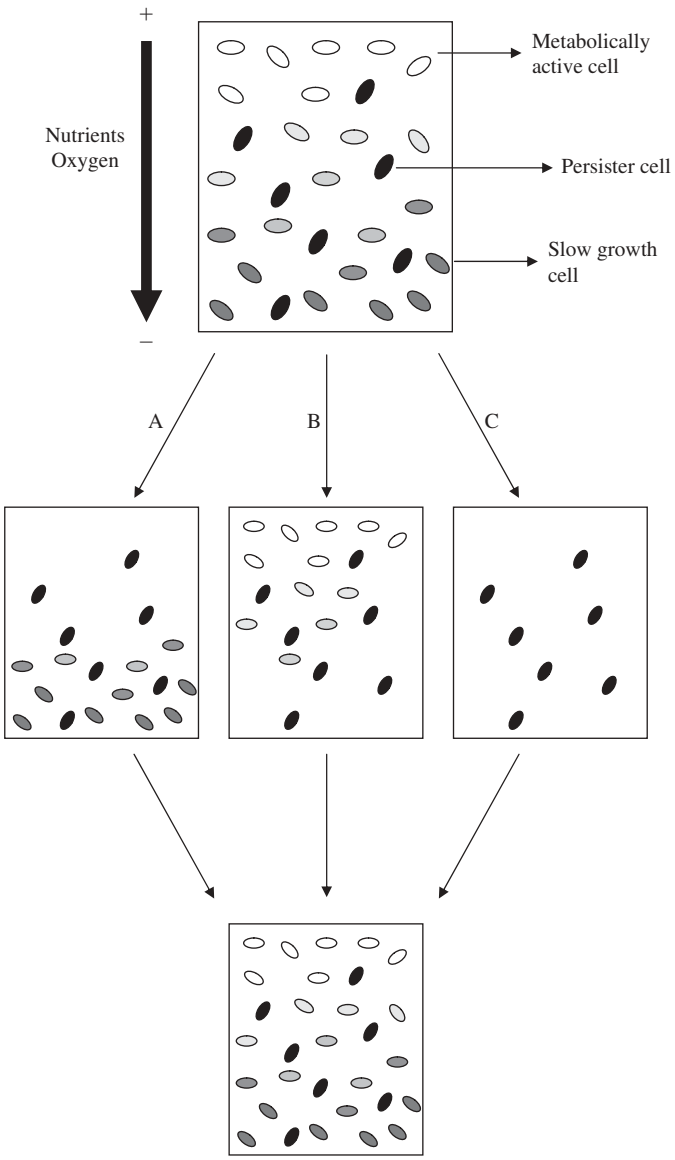

FIGURE 5.3 Schematic representation of cases in which biofilms show resistance to three different antibiotic treatments. A, exposure to an antimicrobial that kills metabolically active cells; B, elimination of slow growth cells from the deeper layers of the biofilm while the more active ones acquire adaptive resistance and survive; and C, treatment that eradicates both the active and the inactive cells but allows for the survival of persisters. The result of all three scenarios is the regeneration of a mature biofilm with identical structure and resistance profile as the original one.

(Pamp et al., 2008). In contrast, other antibiotics such as colistin kill the cells in a deficient metabolic state but are not as effective against more active cells that are able to acquire tolerance to the antibiotic, for example, through upregulation of

the expression of the LPS modification operon and the MexAB-OprM efflux pump, as mentioned above (Pamp et al., 2008). However, in most cases, increased resistance in biofilms is due to the less active cells (Brown et al., 1988; Gilbert et al., 2002). In fact, anaerobic growth conditions favor the development of tolerance toward diverse antibiotics, including ciprofloxacin, carbenicillin, tobramycin, chloramphenicol, ceftazidime, and tetracycline in *P. aeruginosa* biofilms (Borriello et al., 2004). Finally, it is important to mention that biofilms have a greater fraction of persister cells than planktonic cultures (Lewis, 2008). Persistence is a dormant state during which cells show greater tolerance to the action of antimicrobial agents. Upon removal of the toxic compound, these cells can start growing again and give rise to a new population with identical levels of antibiotic resistance to those of the original one (Fig. 5.3). Alarmingly, a high rate of persisters has been observed in samples from CF patients (Mulcahy et al., 2010; Smith et al., 2006).

Swarming is a social type of motility, which requires complex intercellular communication and involves the fast and coordinate movement of the cells over a semisolid surface (Fraser and Hughes, 1999). Overhage et al. (2008b) proposed that swarming could be the state adopted by some *P. aeruginosa* cells when moving across the thickened mucus covering the lung epithelial surface of CF patients. This makes it important to understand the characteristics of this distinct physiological state. Recent studies have demonstrated that swarmer cells show a higher level of expression of virulence determinants. In *Pseudomonas*, Overhage et al. (2008b) observed the upregulation of the genes involved in the type III secretion system, alkaline protease, pyochelin, and pyoverdin, all of which have been implicated in pathogenicity. Furthermore, like biofilms, swarming colonies have a greater resistance to antimicrobial compounds (Lai et al., 2008; Overhage et al., 2008b). This has also been demonstrated for other species such as *Salmonella*, *Serratia marcescens*, *E. coli*, and the like. In all cases, the vegetative levels of resistance could be restored by incubating the cells from a swarming colony under nonswarming conditions, such as a liquid culture (Kim et al., 2003; Lai et al., 2008; Overhage et al., 2008b). Despite being a clear adaptive response, the recovery of the initial susceptibility depends on the antibiotic and the bacterium. Thus, *Salmonella* swarming cells reverted to planktonic levels of resistance to polymyxin B after one pass in a liquid medium, while resistance to kanamycin only decreased gradually (Kim and Surette, 2003). The mechanisms of adaptive resistance during swarming are yet to be identified. Studies in *Salmonella* have reported the possible participation of the LPS modification (*pmr*/*arn*) operon (Kim et al., 2003) and the CysB regulon (Turnbull and Surette, 2008). However, a more recent article argued that it was not the physiological adaptations during swarming but the elevated cell density and the mobility of swarming cells that led to increased resistance (Butler et al., 2010). However, it is possible that this is not so in *Pseudomonas*. In fact, swarming in *Pseudomonas* is accompanied by a highly complex transcriptional response (Overhage et al., 2008b), whereas in *Salmonella* there were not many significant differences in the expression pattern (Wang et al., 2004). The precise molecular mechanisms of swarming adaptive resistance in *Pseudomonas* remain, however, elusive, and more studies need to be done to understand this interesting multicellular state.

5.5 CONCLUSION

The generalized misuse and overuse of antimicrobial compounds over the last decades have resulted in an accelerated evolutionary process leading to bacterial resistance. Microorganisms are being exposed to relatively high doses of these compounds due to human activity not only in the clinic, households, and industrial settings but also in natural environments.

Throughout this chapter, we have shown how bacteria such as *P. aeruginosa* can easily acquire a low-level increase in resistance, usually via mutation, or transiently become less susceptible to an otherwise lethal antibiotic challenge. Moreover, some of the molecular mechanisms responsible for these two types of antibiotic resistance are shared or at least related to some degree. For instance, the same gene may be downregulated under specific environmental conditions or carry a point mutation in a particular strain, resulting in both cases in reduced susceptibility to an antimicrobial agent. These phenomena are worrying enough because they might hinder the efforts to treat an infection or to eradicate the bacteria from a specific environment. In fact, it is now clear that antibiotic resistance during an infection is quite different from what is predicted on the basis of laboratory tests. Moreover, the dangers related to the acquisition of stepwise or adaptive resistance do not end there. Indeed what is really alarming is that, over time, these apparently irrelevant types of resistance might lead to a permanent high-level resistance phenotype. For example, several low-impact mutations can accumulate in the same strain, and/or the cells will be more likely to horizontally acquire high-level resistance determinants from other bacteria due to their ability to resist lethal antibiotic concentrations, even if only in a temporary manner.

A better understanding of the possible triggers and mechanisms of stepwise and adaptive resistance is essential in order to plan new treatment strategies, specifically conceived to circumvent these issues. One such example is the design of novel therapeutics. For instance, new drugs could be developed that do not significantly induce adaptive resistance or that target specific molecules involved in adaptive responses (e.g., prevent quorum sensing or disaggregate biofilms). Also important are the use of combination therapy and rational planning of more adequate programs in terms of dose and timing. In that sense, it is very important to avoid the exposure of the pathogens to subinhibitory concentrations of antimicrobials during treatment.

In conclusion, the application of novel molecular technologies has opened the door for us to comprehend in greater depth the gradual process of antibiotic resistance acquisition. This gives hope for the development of new more effective antimicrobials and administration regimes, which might slow down the relentless increase in resistance of bacterial pathogens.

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REFERENCES

Alvarez-Ortega C, Wiegand I, Olivares J, Hancock REW, Martinez JL (2010). Genetic determinats involved in the susceptibility of *Pseudomonas aeruginosa* to beta-lactam antibiotics. *Antimicrob Agents Chemother* 54(10):4159–4167.

Anderl JN, Franklin MJ, Stewart PS (2000). Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob Agents Chemother* 44(7):1818–1824.

Bagge N, Schuster M, Hentzer M, Ciofu O, Givskov M, Greenberg EP, Høiby N (2004). *Pseudomonas aeruginosa* biofilms exposed to imipenem exhibit changes in global gene expression and beta-lactamase and alginate production. *Antimicrob Agents Chemother* 48(4):1175–1187.

Barber M, Waterworth PM (1966). Activity of gentamicin against *Pseudomonas* and hospital staphylococci. *Br Med J* 1(5481):203–205.

Barclay ML, Begg EJ, Chambers ST, Thornley PE, Pattemore PK, Grimwood K (1996). Adaptive resistance to tobramycin in *Pseudomonas aeruginosa* lung infection in cystic fibrosis. *Antimicrob Chemother* 37(6):1155–1164.

Baquero F (2001). Low-level antibacterial resistance: A gateway to clinical resistance. *Drug Resist Updat* 4(2):93–105.

Bjarnsholt T, Jensen PØ, Fiandaca MJ, Pedersen J, Hansen CR, Andersen CB, Pressler T, Givskov M, Høiby N (2009). *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. *Pediatr Pulmonol* 44(6):547–558.

Borriello G, Werner E, Roe F, Kim AM, Ehrlich GD, Stewart PS (2004). Oxygen limitation contributes to antibiotic tolerance of *Pseudomonas aeruginosa* in biofilms. *Antimicrob Agents Chemother* 48(7):2659–2664.

Brazas MD, Hancock REW (2005a). Using microarray gene signatures to elucidate mechanisms of antibiotic action and resistance. *Drug Discov Today* 10(18):1245–1252.

Brazas MD, Hancock REW (2005b). Ciprofloxacin induction of a susceptibility determinant in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 49(8):3222–3227.

Brazas MD, Breidenstein EBM, Overhage J, Hancock REW (2007). Role of lon, an ATP-dependent protease homolog, in resistance of *Pseudomonas aeruginosa* to ciprofloxacin. *Antimicrob Agents Chemother* 51(12):4276–4283.

Breidenstein EBM, Khaira BK, Wiegand I, Overhage J, Hancock REW (2008). Complex ciprofloxacin resistome revealed by screening a *Pseudomonas aeruginosa* mutant library for altered susceptibility. *Antimicrob Agents Chemother* 52(12):4486–4491.

Brown MR, Allison DG, Gilbert P (1988). Resistance of bacterial biofilms to antibiotics: A growth-rate related effect? *J Antimicrob Chemother* 22(6):777–780.

Butler MT, Wang Q, Harshey RM (2010). Cell density and mobility protect swarming bacteria against antibiotics. *Proc Natl Acad Sci USA* 107(8):3776–3781.

Celli J, Trieu-Cuot P (1998). Circularization of Tn916 is required for expression of the transposon-encoded transfer functions: Characterization of long tetracycline-inducible transcripts reading through the attachment site. *Mol Microbiol* 28(1):103–117.

Chuanchuen R, Beinlich K, Hoang TT, Becher A, Karkhoff-Schweizer RR, Schweizer HP (2001). Cross-resistance between triclosan and antibiotics in *Pseudomonas aeruginosa* is mediated by multidrug efflux pumps: Exposure of a susceptible mutant strain to triclosan selects *nfxB* mutants overexpressing MexCD-OprJ. *Antimicrob Agents Chemother* 45(2):428–432.

Ciofu O, Riis B, Pressler T, Poulsen H, Hoiby N (2005). Occurrence of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis patients is associated with the oxidative stress caused by chronic lung inflammation. *Antimicrob Agents Chemother* 49(6):2276–2282.

Cirz RT, O'Neill BM, Hammond JA, Head SR, Romesberg FE (2006). Defining the *Pseudomonas aeruginosa* SOS response and its role in the global response to the antibiotic ciprofloxacin. *J Bacteriol* 188(20):7101–7110.

Daikos GL, Lolans VT, Jackson GG (1991). First-exposure adaptive resistance to aminoglycoside antibiotics in vivo with meaning for optimal clinical use. *Antimicrobial Agents Chemother* 35(1):117–123.

Davey ME, O'Toole GA (2000). Microbial biofilms: From ecology to molecular genetics. *Microbiol Mol Biol Rev* 64(4):847–867.

Dibb WL, Asphaug Kjellevold V, Digranes A (1983). *Pseudomonas aeruginosa* and *Acinetobacter calcoaceticus*: In vitro susceptibility of 150 clinical isolates to five beta-lactam antibiotics and tobramycin. *Chemotherapy* 29(5):332–326.

Donlan RM (2001). Biofilms and device-associated infections. *Emerg Infect Dis* 7(2):277–281.

Dötsch A, Becker T, Pommerenke C, Magnowska Z, Jänsch L, Häussler S (2009). Genome-wide identification of genetic determinants of antimicrobial drug resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 53(6):2522–2531.

Driffield K, Miller K, Bostock JM, O'Neill AJ, Chopra I (2008). Increased mutability of *Pseudomonas aeruginosa* in biofilms. *J Antimicrob Chemother* 61(5):1053–1056.

Dubern JF, Diggle SP (2008). Quorum sensing by 2-alkyl-4-quinolones in *Pseudomonas aeruginosa* and other bacterial species. *Mol Biosyst* 4(9):882–888.

El'Garch F, Jeannot K, Hocquet D, Llanes-Barakat, Plesiat P (2007). Cumulative effects of several nonenzymatic mechanisms on the resistance of *Pseudomonas aeruginosa* to aminoglycosides. *Antimicrob Agents Chemother* 51(3):1016–1021.

Ernst RL, Yi EC, Guo L, Lim KB, Burns JL, Hackett M, Miller SI (1999). Specific lipopolysaccharide found in cystic fibrosis airway *Pseudomonas aeruginosa*. *Science* 286(5444):1561–1565.

Ernst RK, Moskowitz SM, Emerson JC, Kraig GM, Adams KN, Harvey MD, Ramsey B, Speert DP, Burns JL, Miller SI (2007). Unique lipid A modifications in *Pseudomonas aeruginosa* isolated from the airways of patients with cystic fibrosis. *J Infecti Dis* 196(7):1088–1092.

Fajardo A, Martinez-Martin N, Mercadillo M, Galan JC, Ghysels B, Matthijs S, Cornelis P, Wiehlmann L, Baquero F, Martinez JL (2008). The neglected intrinsic resistome of bacterial pathogens. *PloS ONE* 3(2):e1619.

Fernández L, Gooderham WJ, Bains M, McPhee JB, Wiegand I, Hancock REW (2010). Adaptive resistance to the "last hope" antibiotics polymyxin B and colistin in *Pseudomonas aeruginosa* is mediated by the novel two-component regulatory system ParR-ParS. *Antimicrob Agents Chemother* 54(8):3372–3382.

Fraser GM, Hughes C (1999). Swarming motility. *Curr Opin Microbiol* 2(6):630–635.

Garau G, Di Guilmi AM, Hall BG (2005). Structure-based phylogeny of the metallo-β-lactamases. *Antimicrob Agents Chemother* 49(7):2778–2784.

Gerber AU, Craig WA (1982). Aminoglycoside-selected subpopulations of *Pseudomonas aeruginosa*: Characterization and virulence in normal and leukopenic mice. *J Lab Clin Med* 100(5):671–681.

Gilbert P, Maira-Litran T, McBain AJ, Rickard AH, Whyte FW (2002). The physiology and collective recalcitrance of microbial biofilm communities. *Adv Microb Physiol* 46:202–256.

Gilleland LB, Gilleland HE, Gibson JA, Champlin FR (1989). Adaptive resistance to aminoglycoside antibiotics in *Pseudomonas aeruginosa*. *J Med Microbiol* 29(1):41–50.

Gillis RJ, White KG, Choi KH, Wagner VE, Schweizer HP, Iglewski BH (2005). Molecular basis of azithromycin-resistant *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother* 49(9):3858–3867.

Gooderham WJ, Hancock REW (2009). Regulation of virulence and antibiotic resistance by two-component regulatory systems in *Pseudomonas aeruginosa*. *FEMS Microbiol Rev* 33 (2):279–294.

Guisbert E, Yura T, Rhodius VA, Gross CA (2008). Convergence of molecular, modeling, and systems approaches for an understanding of the *Escherichia coli* heat shock response. *Microbiol Mol Biol Rev* 72(3):545–554.

Hall BG, Barlow M (2004). Evolution of the serine β-lactamases: Past, present and future. *Drug Resist Updat* 7(2):111–123.

Hancock REW (1981). Aminoglycoside uptake and mode of action—with special reference to streptomycin and gentamicin. I. Antagonists and mutants. *J Antimicrob Chemother* 8(4):429–445.

Hanson ND, Sanders CC (1999). Regulation of inducible AmpC beta-lactamase expression among Enterobacteriaceae. *Curr Pharm Des* 5(11):881–894.

Hastings PJ, Rosenberg SM, Slack A (2004). Antibiotic-induced lateral transfer of antibiotic resistance. *Trends Microbiol* 12(9):401–404.

Hausner M, Wuertz S (1999). High rates of conjugation in bacterial biofilms as determined by quantitative in situ analysis. *Appl Environ Microbiol* 65(8):3710–3713.

Hegstad K, Langsrud S, Lunestad BT, Scheie AA, Sunde M, Yazdankhah SP (2010). Does the wide use of quaternary ammonium compounds enhance the selection and spread of antimicrobial resistance and thus threaten our health? *Microb Drug Resist* 16(2):91–104.

Hocquet D, Vogne C, El Garch F, Vejux A, Gotoh N, Lee A, Lomovskaya O, Plésiat P (2003). MexXY-OprM efflux pump is necessary for adaptive resistance of *Pseudomonas aeruginosa* to aminoglycosides. *Antimicrob Agents Chemother* 47(4):1371–1375.

Hoyle BD, Costerton JW (1991). Bacterial resistance to antibiotics: The role of biofilms. *Prog Drug Res* 37:91–105.

Hoyle BD, Alcantara J, Costerton JW (1992). *Pseudomonas aeruginosa* biofilm as a diffusion barrier to piperacillin. *Antimicrob Agents Chemother* 36(9):2054–2056.

Jalal S, Wretlind B (1998). Mechanisms of quinolone resistance in clinical strains of *Pseudomonas aeruginosa*. *Microb Drug Resist* 4(4):257–261.

Jalal S, Ciofu O, Hoiby N, Gotoh N, Wretlind B (2000). Molecular mechanisms of fluoroquinolone resistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *Antimicrob Agents Chemother* 44(3):710–712.

Karlowsky JA, Hoban DJ, Zelenitsky SA, Zhanel GG (1997). Altered *denA* and *anr* gene expression in aminoglycoside adaptive resistance in *Pseudomonas aeruginosa*. *J Antimicrob Chemother* 40(3):371–376.

Kaufmann GF, Sartorio R, Lee SH, Rogers CJ, Meijler MM, Moss JA, Clapham B, Brogan AP, Dickerson TJ, Janda KD (2005). Revisiting quorum sensing: Discovery of additional chemical and biological functions for 3-oxo-*N*-acylhomoserine lactones. *Proc Natl Acad Sci USA* 102(2):309–314.

Kenna DT, Doherty CJ, Foweraker J, Macaskill L, Barcus VA, Govan JR (2007). Hypermutability in environmental *Pseudomonas aeruginosa* and in populations causing pulmonary infection in individuals with cystic fibrosis. *Microbiology* 153(Pt6):1852–1859.

Kim W, Surette MG (2003). Swarming populations of *Salmonella* represent a unique physiological state coupled to multiple mechanisms of antibiotic resistance. *Biol Proced Online* 5:189–196.

Kim W, Killam T, Sood V, Surette MG (2003). Swarm-cell differentiation in *Salmonella enterica* serovar Typhimurium results in elevated resistance to multiple antibiotics. *J Bacteriol* 185(10):3111–3117.

Kindrachuk KN, Fernández L, Bains M, Hancock REW (2011). Involvement of an ATP-dependent protease, PA0779/AsrA, in inducing heat shock in response to tobramycin in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 55(5):1874–1882.

Kirketerp-Møller K, Jensen PØ, Fazli M, Madsen KG, Pedersen J, Moser C, Tolker-Nielsen T, Høiby N, Givskov M, Bjarnsholt T (2008). Distribution, and ecology of bacteria in chronic wounds. *J Clin Microbiol* 46(8):2717–2722.

Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ (2007). A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* 130(5):797–810.

Kohanski MA, Dwyer DJ, Wierzbowski J, Cottarel G, Collins JJ (2008). Mistranslation of membrane proteins and two-component system activation trigger antibiotic-mediated cell death. *Cell* 135(4):679–690.

Kumon H, Tomochika K, Matunaga T, Ogawa M, Ohmori H (1994). A sandwich cup method for the penetration assay of antimicrobial agents through *Pseudomonas* exopolysaccharides. *Microbiol Immunol* 38(8):615–619.

Lai S, Tremblay J, Déziel E (2008). Swarming motility: A multicellular behaviour conferring antimicrobial resistance. *Environ Microbiol* 11(1):126–136.

Lee S, Hinz A, Bauerle E, Angermeyer A, Juhaszova K, Kaneko Y, Singh PK, Manoil C (2009). Targeting a bacterial stress response to enhance antibiotic action. *Proc Natl Acad Sci USA* 106(34):14570–14575.

Lewenza S, Falsafi RK, Winsor G, Gooderham WJ, McPhee JB, Brinkman FS, Hancock REW (2005). Construction of a mini-Tn5-luxCDABE mutant library in *Pseudomonas aeruginosa* PAO1: A tool for identifying differentially regulated genes. *Genome Res* 15(4):583–589.

Lewis K (2008). Multidrug tolerance of biofilms and persister cells. *Curr Top Microbiol Immunol* 322:107–131.

Liberati NT, Urbach JM, Miyata S, Lee DG, Drenkard E, Wu G, Villanueva J, Wei T, Ausubel FM (2006). An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. *Proc Natl Acad Sci USA* 103(8):2833–2838.

Lin JT, Connelly MB, Amolo C, Otani S, Yaver DS (2005). Global transcriptional response of *Bacillus subtilis* to treatment with subinhibitory concentrations of antibiotics that inhibit protein synthesis. *Antimicrob Agents Chemother* 49(5):1915–1926.

Linares JF, Gustafsson I, Baquero F, Martinez JL (2006). Antibiotics as intermicrobial signaling agents instead of weapons. *Proc Natl Acad Sci USA* 103(51):19484–19489.

Lindberg F, Normark S (1986). Contribution of chromosomal β-lactamases to β-lactam resistance in *enterobacteria*. *Rev Infect Dis* 8(Suppl. 3):S292–304.

Livermore DM (1987). Clinical significance of β-lactamase induction and stable derepression in gram-negative rods. *Eur J Clin Microbiol* 6(4):439–445.

Livermore DM (1996). Are all beta-lactams created equal? *Scand J Infect Dis Suppl* 101: 33–43.

Loh B, Grant C, Hancock REW (1984). Use of the fluorescent probe 1-*N*-phenylnaphthylamine to study the interactions of aminoglycoside antibiotics with the outer membrane of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 26(4):546–551.

Macfarlane EL, Kwasnicka A, Ochs MM, Hancock REW (1999). PhoPQ homologues in *Pseudomonas aeruginosa* regulate expression of the outer-membrane protein OprH and polymyxin B resistance. *Mol Microbiol* 34(2):305–316.

MacLeod DL, Nelson LE, Shawar RM, Lin BB, Lockwood LG, Dirks JE, Miller GH, Burns JL, Garber RL (2000). Aminoglycoside-resistance mechanisms for cystic fibrosis *Pseudomonas aeruginosa* isolates are unchanged by long-term, intermittent, inhaled tobramycin treatment. *J Infect Dis* 181(3):1180–1184.

Marr AK, Overhage J, Bains M, Hancock REW (2007). The Lon protease of *Pseudomonas aeruginosa* is induced by aminoglycosides and is involved in biofilm formation and motility. *Microbiology* 153(2):474–482.

Mates SM, Eisenberg ES, Mandel LJ, Patel L, Kaback HR, Miller MH (1982). Membrane potential and gentamicin uptake in *Staphylococcus aureus*. *Proc Natl Acad Sci USA* 79(21):6693–6697.

Mawer SL, Greenwood D (1978). Specific and non-specific resistance to aminoglycosides in *Escherichia coli*. *J Clin Pathol* 31(1):12–15.

McKenzie GJ, Lee PL, Lombardo M-J, Hastings PJ, Rosenberg SM (2001). SOS mutator DNA polymerase IV functions in adaptive mutation and not adaptive amplification. *Mol Cell* 7(3):571–579.

McMurry LM, Oethinger M, Levy SB (1998). Overexpression of *marA*, *soxS* or *acrAB* produces resistance to triclosan in *Escherichia coli*. *FEMS Microbiol Lett* 166(2):305–309.

McPhee JB, Lewenza S, Hancock REW (2003). Cationic antimicrobial peptides activate a two-component regulatory system, PmrAB. That regulates resistance to polymyxin B and cationic antimicrobial peptides in *Pseudomonas aeruginosa*. *Mol Microbiol* 50(1):205–217.

Miller C, Thomsen LE, Gaggero C, Mosseri R, Ingmer H, Cohen SN (2004). SOS response induction by β-lactams and bacterial defense against antibiotic lethality. *Science* 305(5690):1629–1631.

Molin S, Tolker-Nielsen T (2003). Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. *Curr Opin Biotechnol* 14(3): 255–261.

Mulcahy H, Charron-Mazenod L, Lewenza S (2008). Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *PLoS Pathogens* 4(11): e1000213.

Mulcahy LR, Burns JL, Lory S, Lewis K (2010). Emergence of *Pseudomonas aeruginosa* strains producing high levels of persister cells in patients with cystic fibrosis. *J Bacteriol* 192(23):6191–6199.

Nakayama K, Takashima K, Ishihara H, Shinomiya T, Kageyama M, Kanaya S, Ohnishi M, Murata T, Mori H, Hayashi T (2000). The R-type pyocin of *Pseudomonas aeruginosa* is related to P2 phage, and the F-type is related to lambda phage. *Mol Microbiol* 38(2): 213–231.

Nalca Y, Jänsch L, Bredenbruch F, Geffers R, Buer J, Häussler S (2006). Quorum-sensing antagonistic activities of azithromycin in *Pseudomonas aeruginosa* PAO1: A global approach. *Antimicrob Agents Chemother* 50(5):1680–1688.

Nickel JC, Wright JB, Ruseska I, Marrie TJ, Whitfield C, Costerton JW (1985). Antibiotic resistance of *Pseudomonas aeruginosa* colonizing a urinary catheter in vitro. *Eur J Clin Microbiol* 4(2):213–218.

Normark S, Lindquist S, Lindberg F (1986). Chromosomal beta-lactam resistance in enterobacteria. *Scand J Infect Dis Suppl* 49:38–45.

Oliver A, Canton R, Campo P, Baquero F, Blazquez J (2000). High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science* 288(5469):1251–1254.

Overhage J, Campisano A, Bains M, Torfs EC, Rehm BH, Hancock REW (2008a). Human host defense peptide LL-37 prevents bacterial biofilm formation. *Infect Immun* 76(9):4176–4182.

Overhage J, Bains M, Brazas MD, Hancock REW (2008b). Swarming of *Pseudomonas aeruginosa* is a complex adaptation leading to increased production of virulence factors and antibiotic resistance. *J Bacteriol* 190(8):2671–2679.

Pai H, Kang CI, Byeon JH, Lee KD, Park WB, Kim HB, Kim EC, Oh MD, Choe KW (2004). Epidemiology and clinical features of bloodstream infections caused by AmpC-

type-β-lactamases-producing *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 48(10): 3720–3728.

Pamp SJ, Gjermansen M, Johansen HK, Tolker-Nielsen T (2008). Tolerance to the antimicrobial peptide colistin in *Pseudomonas aeruginosa* biofilms is linked to metabolically active cells, and depends on the *pmr* and *mexAB-oprM* genes. *Mol Microbiol* 68(1): 223–240.

Poole K (2001). Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. *J Mol Microbiol Biotechnol* 3(2):255–264.

Poole K (2004). Resistance to β-lactam antibiotics. *Cell Mol Life Sci* 61(17):2200–2223.

Potvin E, Sanschagrin F, Levesque RC (2008). Sigma factors in *Pseudomonas aeruginosa*. *FEMS Microbiol Rev* 32(1):38–55.

Privitera G, Sebald M, Fayolle F (1979). Common regulatory mechanism of expression and conjugative ability of a tetracycline resistance plasmid in *Bacteroides fragilis*. *Nature* 278(5705):657–659.

Pumbwe L, Piddock LJV (2000). Two efflux systems expressed simultaneously in multidrug-resistant *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 44(10):2861–2864.

Sánchez P, Linares JF, Ruiz-Díez B, Campanario E, Navas A, Baquero F, Martínez JL (2002). Fitness of in vitro selected *Pseudomonas aeruginosa nalB* and n*f*xB multidrug resistant mutants. *J Antimicrob Chemother* 50(5):657–664.

Sanders CC (1993). Cefepime: The next generation? *Clin Infect Dis* 17(3):369–379.

Schurek KN, Marr AK, Taylor PK, Wiegand I, Semenec L, Khaira BK, Hancock REW (2008). Novel genetic determinants of low-level aminoglycoside resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 52(12):4213–4219.

Schurek KN, Sampaio JL, Kiffer CR, Sinto S, Mendes CM, Hancock REW (2009). Involvement of *pmrAB* and *phoPQ* in polymyxin B adaptation and inducible resistance in non-cystic fibrosis clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 53(10):4345–4351.

Schurr MJ, Deretic V (1997). Microbial pathogenesis in cystic fibrosis: Co-ordinate regulation of heat-shock response and conversion to mucoidy in *Pseudomonas aeruginosa*. *Mol Microbiol* 24(2):411–420.

Schweizer HP (2003). Efflux as a mechanism of resistance to antimicrobials in *Pseudomonas aeruginosa* and related bacteria: Unanswered questions. *Genet Mol Res* 31;2(1):48–62.

Shaw KJ, Miller N, Liu X, Lerner D, Wan J, Bittner A, Morrow BJ (2003). Comparison of the changes in global gene expression of *Escherichia coli* induced by four bactericidal agents. *J Mol Microbiol Biotechnol* 5(2):105–122.

Shigeta M, Tanaka G, Komatsuzawa H, Sugai M, Suginaka H, Usui T (1997). Permeation of antimicrobial agents through *Pseudomonas aeruginosa* biofilms: A simple method. *Chemotherapy* 43(5):340–345.

Shimohata N, Chiba S, Saikawa N, Ito K, Akiyama Y (2002). The Cpx stress response system of *Escherichia coli* senses plasma membrane proteins and controls HtpX, a membrane protease with a cytosolic active site. *Genes to Cells* 7(7):653–662.

Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, D'Argenio DA, Miller SI, Ramsey BW, Speert DP, Moskowitz SM, Burns JL, Kaul R, Olson MV (2006). Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *Proc Natl Acad Sci USA* 30;103(22):8487–8492.

Steinkraus G, White R, Friedrich L (2007). Vancomycin MIC creep in non-vancomycin-intermediate *Staphylococcus aureus* (VISA), vancomycin-susceptible clinical methicillin-resistant *S. aureus* (MRSA) blood isolates from 2001–2005. *J Antimicrob Chemother* 60(4): 788–794.

Stock AM, Robinson VL, Goudreau PN (2000). Two-component signal transduction. *Annu Rev Biochem* 69:183–215.

Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FS, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrock-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Smith K, Spencer D, Wong GK, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock REW, Lory S, Olson MV (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 406(6799):959–964.

Suci PA, Mittelman MW, Yu FP, Geesey GG (1994). Investigation of ciprofloxacin penetration into *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother* 38(9):2125–2133.

Taber HW, Mueller JP, Miller PF, Arrow AS (1987). Bacterial uptake of aminoglycoside antibiotics. *Microbiol Rev* 51(4):439–457.

Turnbull AL, Surette MG (2008). L-Cysteine is required for induced antibiotic resistance in actively swarming *Salmonella enterica* serovar Typhimurium. *Microbiology* 154(11): 3410–3419.

Verstraeten N, Braeken K, Debkumari B, Fauvart M, Fransaer J, Vermant J, Michiels J (2008). Living on a surface: Swarming and biofilm formation. *Trends Microbiol* 16(10): 496–506.

Vrany JD, Stewart PS, Suci PA (1997). Comparison of recalcitrance to ciprofloxacin and levofloxacin exhibited by *Pseudomonas aeruginosa* biofilms displaying rapid-transport characteristics. *Antimicrob Agents Chemother* 41(6):1352–1358.

Walsh C (2003). Antibiotics that act on cell wall biosynthesis. In C Walsh (Ed.), *Antibiotics: Actions, Origins, Resistance*. ASM Press, Washington, DC, pp. 23–49.

Wang Q, Frye JG, McClelland M, Harshey RM (2004). Gene expression patterns during swarming in *Salmonella typhimurium*: Genes specific to surface growth and putative new motility and pathogenicity genes. *Mol Microbiol* 52(1):169–187.

Weinstein MJ, Drube CG, Moss EL Jr, Waitz JA (1971). Microbiologic studies related to bacterial resistance to gentamicin. *J Infect Dis* 124:S11–17.

Wells IC (1952). Antibiotic substances produced by *Pseudomonas aeruginosa*; syntheses of Pyo Ib, Pyo Ic, and Pyo III. *J Biol Chem* 196(1):331–340.

Westbrock-Wadman S, Sherman DR, Hickey MJ, Coulter SN, Zhu YQ, Warrener P, Nguyen LY, Shawar RM, Folger KR, Stover CK (1999). Characterization of a *Pseudomonas aeruginosa* efflux pump contributing to aminoglycoside impermeability. *Antimicrob Agents Chemother* 43(12):2975–2983.

Wiegand I, Marr AK, Breidenstein EBM, Schurek KN, Taylor P, Hancock REW (2008). Mutator genes giving rise to decreased antibiotic susceptibility in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 52(10):3810–3813.

Yasuda H, Ajiki Y, Koga T, Kawada H, Yokota T (1993). Interaction between biofilms formed by *Pseudomonas aeruginosa* and clarithromycin. *Antimicrob Agents Chemother* 37(9):1749–1755.

Zhang L, Mah TF (2008). Involvement of a novel efflux system in biofilm-specific resistance to antibiotics. *J Bacteriol* 190(13):4447–4452.

6

ENVIRONMENTAL RESERVOIRS OF RESISTANCE GENES IN ANTIBIOTIC-PRODUCING BACTERIA AND THEIR POSSIBLE IMPACT ON THE EVOLUTION OF ANTIBIOTIC RESISTANCE

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6.1 INTRODUCTION: ANTIBIOTIC BIOSYNTHESIS IN NATURAL ENVIRONMENTS

Antibiotic production is a common attribute among soil bacteria, and examples can be found in the Gram-negative groups of pseudomonads and *Erwinia* strains, in Gram-positive bacteria within actinobacteria and myxobacteria, and in low guanine–cytosine (G+C) gram-positive groups such as *Bacillus* species. The actinobacteria are often regarded as notable antibiotic producers, as members of the *class Actinobacteria* synthesize some of the most economically important antibiotics still used clinically, including drugs such as erythromycin, gentamicin, tetracycline, the antitumor agent daunorubicin, the immunosuppressant rapamycin, and the anti-helminthic agent avermectin (Paradkar et al., 2003). In addition to these well-known antibiotics, members of one particular genus, *Streptomyces,* synthesize thousands more secondary metabolites. A screen of the Antibiotic Literature Database (ABL) indicated that *Streptomyces* strains were responsible for the biosynthesis of 32.1% of over 23,000 microbial products possessing some biological activity (Lazzarini et al., 2000). Genes involved in antibiotic biosynthesis are clustered and regulated by a

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variety of mechanisms, including signaling molecules. The production of signaling molecules can relate to environmental cues in streptomycetes (Takano et al., 2001; Bibb and Hesketh, 2009) and quorum sensing in Gram-negatives such as carbapenem production by *Erwinia carotovora* (Bainton et al., 1992). Clearly, the selection for resistance to all these potentially antagonistic compounds will depend on the extent of their production in natural environments and impact on competing populations. Detection of antibiotics in soil is problematic due to low-level production and adsorption to clays. Thiostrepton production in sterile soil was determined within the range of 30–50 ng/g (Wellington et al., 1993). The importance of antibiotic production in biological pest control has frequently been reported (Diallo et al., 2011), and the direct detection of phenazine in the wheat rhizosphere after inoculated and incubation with *Pseudomonas fluorescens* and *Pseudomonas aureofaciens* proved the significance of antibiotic production in control of Take-all disease caused by *Gaeumannomyces graminis* var. tritici (Brisbane and Rovira, 1988). A *luxAB* reporter gene was used to monitor the expression of the antibiotic phenazine-1-carboxylic acid (PCA) by Seveno et al. (2001). The *luxAB* from *Vibrio harveyi* was inserted in the *phzB* gene of the phenazine operon, and transcription was monitored by measurement of luminescence in liquid culture, on nutrient agar, on sterile wheat seedlings, and in sterile bean rhizosphere. Production of phenazine was confirmed both in liquid culture and on solid media, but it could not be detected on wheat seedlings or in bean plant root rhizosphere despite the fact that transcription of the *phzB::luxAB* reporter gene occurred on the bean plant root. Further indirect evidence for production in situ in natural environments involved the detection of messenger ribonucleic acid (mRNA) of an antibiotic production gene (Anukool et al., 2004); streptothricin (ST) production by *Streptomyces rochei* F20 was monitored in liquid culture, soil, and the rhizosphere of spring wheat. The ST resistance gene (*sttR*) and ST biosynthesis gene (*sttA*) coding for peptide synthetase of *S. rochei* were selected for detection targets using Polymerase chain reaction (PCR) and, as expected, the resistance gene expression was more readily detected than the biosynthesis gene. The *sttR* mRNA was detected both in sterile and nonsterile rhizospheres, but neither *sttR* nor *sttA* transcripts were detected in the rhizoplane. Antibiotic resistance genes appeared to be widespread in isolates of streptomycetes obtained from soil (Phillips et al., 1992; Wiener et al., 1998), and these are not always associated with antibiotic production. *Streptomyces coelicolor* produces a number of antibiotics but not vancomycin nor any other similar glycopetide antibiotic but contains a cluster of seven genes that confer inducible, high-level vancomycin resistance (Hong et al., 2004). This may be in response to naturally produced vancomycin in soil produced by other actinobacteria, and, once mobilized, as a distinct resistance mechanism such genes could be transferred to other bacteria. The *vanH, A*, and *X* resistance genes found in *Enterococcus* species have orthologs in vanomycin producers *Streptomyces toyocaensis* and *Amycolatopsis orientalis* (Marshall et al., 1998).

Bacteria resistant to a range of antibiotics including chloramphenicol, streptomycin, and tetracycline were isolated from Siberian permafrost sediments dating back 3 million years, long before antibiotics were in clinical use (Mindlin et al., 2008). Thus, it is important to appreciate that resistance in soil bacteria is common. Our own studies focused on trying to establish evidence for specific reservoirs of antibiotic resistance genes in actinobacteria, which could occupy the same niche in soil as those capable of antibiotic production (Huddleston et al., 1997; Egan et al., 1998, 2001;

Tolba et al., 2002; Nikolakopoulou et al., 2005; Laskaris et al., 2010). This provided evidence for the horizontal gene transfer (HGT) of both antibiotic biosynthesis gene clusters and individual resistance genes originating from such clusters. Most of this work has focused on streptomycin production and resistance as this is a highly prevalent gene cluster in nature. The ability to produce streptomycin in vitro is widespread within actinobacteria, but predominantly *Streptomyces* species were used for commercial-scale production in the 1950s and 1960s. Approximately 1% of randomly screened soil actinobacteria can synthesize streptomycin (Baltz, 2006). Few antibiotics can match its prevalence; only streptothricin is found at a frequency of 10^{-1}, while actinomycin D and tetracycline are in the 10^{-2}–10^{-3} range. Most antibiotic producers are much less prevalent; for example, daptomycin production required the screening of nearly 10^{7} actinobacteria to be discovered (Baltz, 2008). There are exponentially more antibiotics present at lower frequencies; in one study about 200 were discovered at frequencies of around 4×10^{-7}, about 800 at around 2×10^{-7}, and more than 1000 at around 1×10^{-7} (Baltz, 2005). The production of streptomycin is both common and highly widespread, as it appears to have an almost global distribution. Producers belonging to the *S. griseus* species have been isolated from soils in diverse countries such as the United States, Mexico, Japan (Gordon and Horan, 1968), Germany (Tolba et al., 2002), and Brazil (Huddleston et al., 1997). The wide distribution and high frequency of streptomycin production indicates that this phenotype provides a significant advantage to the organism possessing it, even when compared to most other antibiotics whose producers are normally found in lower numbers in soil.

The widespread prevalence of streptomycin producers would have applied significant evolutionary pressure on other soil-inhabiting susceptible bacteria. A number of resistance mechanisms thus exist (Davies and Wright, 1997), and a survey of deoxyribonucleic acid (DNA) extracted from diverse soils, sediments, and manure proved that streptomycin resistance genes disabling the antibiotic were prevalent in all samples where all known mechanisms could be detected (van Overbeek et al., 2002).

6.2 AVOIDING SUICIDE, CLUSTERING OF ANTIBIOTIC BIOSYNTHESIS GENES, EXPRESSION AND REGULATION: STREPTOMYCIN A CLASSIC EXAMPLE

Streptomycin is a basic aminocyclitol aminoglycoside antibiotic. It was the first aminoglycoside to be discovered and the first antibiotic approved for the treatment of tuberculosis following human trials in 1947 (Anonymous, 2008). It is no longer widely used due to its toxicity in the peripheral and central nervous system at higher doses and hypersensitivity reactions (Kamal et al., 2008), as well as its ototoxicity and nephrotoxicity (Mingeot-Leclercq and Tulkens, 1999). Aminoglycosides as a whole account for only about 3% of the total of all antibiotics produced and used (Madigan and Martinko, 2005). However, streptomycin is still used in agriculture where it is applied for control of *Erwinia amylovora*, which causes fire blight in apple and pear trees, though it is also used to treat other bacterial infections (McManus et al., 2002).

The effects of streptomycin on a bacterial cell can be divided into two stages. In the first stage, the positively charged streptomycin molecules bind in an energy-independent

manner to the negatively charged moieties of phospholipids, lipopolysaccharides, and outer membrane proteins in Gram-negative bacteria or to the phospholipids and teichoic acids in Gram-positive bacteria (Taber et al., 1987). This displaces Mg^{2+} and Ca^{2+} ions that link adjacent lipopolysaccharide molecules, which destabilizes the outer membrane and enhances its permeability (Hancock, 1984; Martin and Beveridge, 1986). This is followed by the energy-dependent uptake of streptomycin, which requires a threshold transmembrane potential generated by a membrane-bound respiratory chain. As a result, anaerobic organisms or bacteria with malfunctioning electron-transport mechanisms can show resistance to streptomycin (Vakulenko and Mobashery, 2003). In *Escherichia coli* it is taken up by the oligopeptide transport system, though other transport mechanisms may also be involved (Kashiwagi et al., 1998). Uptake by a transport system is necessary, as streptomycin molecules are too large and polar to passively diffuse through porins (Mingeot-Leclercq et al., 1999). At this stage, the levels of the antibiotic are too low to arrest protein synthesis. However, streptomycin binds to the A site [aminoacyl–transport RNA (tRNA) binding site] on the 16S ribosomal RNA(rRNA), causing a deformation in the 16S tertiary structure that significantly reduces the rate at which peptidyl–tRNA is translocated from the A to the P site (peptidyl–tRNA binding site) and destabilizes the binding of peptidyl–tRNA to the P site (Karimi and Ehrenberg, 1996). This induces a 25-fold or greater reduction in proofreading accuracy (Karimi and Ehrenberg, 1994), which results in the production of defective proteins that are incorporated in the cell membrane leading to the loss of membrane integrity (Vakulenko and Mobashery, 2003). This causes the second killing phase during which large amounts of the antibiotic cross the compromised cell membrane, which accumulates rapidly within the cell as it binds electrostatically to anionic groups of macromolecules or is sequestered by the degradation products of mistranslated proteins (Busse et al., 1992; Piepersberg and Distler, 1997). It then irreversibly saturates all the cell's ribosomes, arresting protein synthesis and causing cell death (Vakulenko and Mobashery, 2003).

Streptomycin is also capable of interacting with RNA sequences other than the 16S rRNA such as the group I introns (Wallace and Schroeder, 1998). Group I introns are self-splicing ribozymes that catalyze their own excision from mRNA, tRNA, and rRNA precursors and are found in bacteria and some eukaryotes (Stahley and Strobel, 2006). Group I intron splicing requires the binding of an exogenous guanosine via its guanidino group to the catalytic core of the intron. Streptomycin possesses a guanidino group and thus acts as a competitive inhibitor, preventing the ribozyme's activation (Wallace and Schroeder, 1998). In addition streptomycin appears to also inhibit the ribozyme by binding to an RNA structural motif on the group I intron similar to the one it binds to on the 16S rRNA (von Ahsen and Noller, 1993). These structures are composed of a hairpin loop that is base paired with an adjoining RNA loop and are termed pseudoknots (Powers and Noller, 1991; Piepersberg and Distler, 1997). Streptomycin is also capable of inhibiting eukaryotic nuclear pre-mRNA splicing in vitro. This appears to be due to the nonspecific binding of streptomycin to the mRNA, which prevents the folding of the pre-mRNA into a splicing-compatible tertiary structure (Hertweck et al., 2002).

There is also some preliminary evidence that streptomycin may regulate growth and peptidoglycan formation in some streptomycete strains, as it has been detected bound to a cell wall precursor unit (Szabo et al., 1989) where it activated lytic

enzymes in the cell wall (Szabo et al., 1990). The streptomycin gene cluster is composed of 27 genes and occupies a region 32.6 kb in length (Fig. 6.1) (Tomono et al., 2005).

The cluster genes encoding enzymes for the synthesis of the streptomycin subunits are not found in subpathway-specific operons. The genes are instead found in mixed operons, which may indicate the need for strictly coordinated expression of the cluster genes (Piepersberg and Distler, 1997). The *Streptomyces glaucescens* hydroxystreptomycin cluster has a number of genes that are homologous to the streptomycin gene cluster; however, their sequence homology ranges from 80% to less than 60% and the gene order of the two clusters differs as well (Distler et al., 1992). Streptomycin is created by the condensation of three moieties that are all synthesized from glucose-6-phosphate (Fig. 6.2). The enzymes StrO, StrI, StsC, StsE, StrB1, StsB, StsA and StrB1 or StrB2 produce streptidine-6-phosphate. StrN, StrD, StrE, StrM, and StrL synthesize dTDP-dihydrostreptose. StrN, StrQ, StrP, StrS, StsG, StrF, StrG, and StrX produce NDP-*N*-methyl-L-glycosamine (Flatt and Mahmud, 2007). The streptidine-6-phosphate and dTDP-dihydrostreptose are condensed by StrH followed by NDP-*N*-methyl-L-glycosamine to produce dihydrostreptomycin-6-phosphate (Flatt and Mahmud, 2007). A dehydrogenase (possibly StrU) then converts it into streptomycin-6-phosphate, which is then exported via a transmembrane complex formed by StrV and StrW. Once outside the cell membrane the inactive form of the antibiotic is dephosphorylated by StrK to produce biologically active streptomycin (Piepersberg and Distler, 1997), but this can reenter the cell, so genes coding for resistance must be expressed prior to the onset of biosynthesis to avoid suicide.

Three possible types of resistance have been described: The first resistance mechanism, prevention of antibiotic uptake, requires the reduction or elimination of the electrical potential generated by a membrane-bound respiratory chain. As a

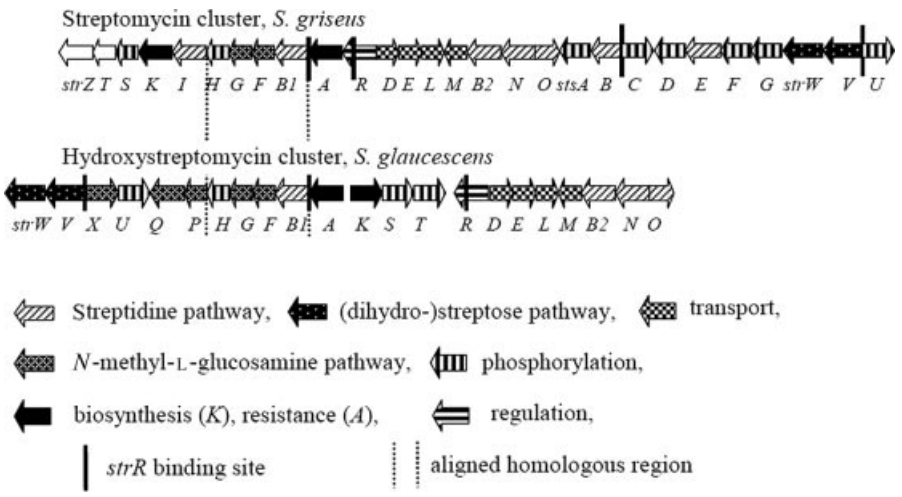

FIGURE 6.1 Streptomycin gene clusters involved in biosynthesis of streptomycin and closely related products. Streptomycin gene cluster from *Streptomyces griseus*, hydroxystreptomycin cluster from *S. glaucescens*. [Adapted from Tolba (2004), after (Piepersberg and Distler, 1997).]

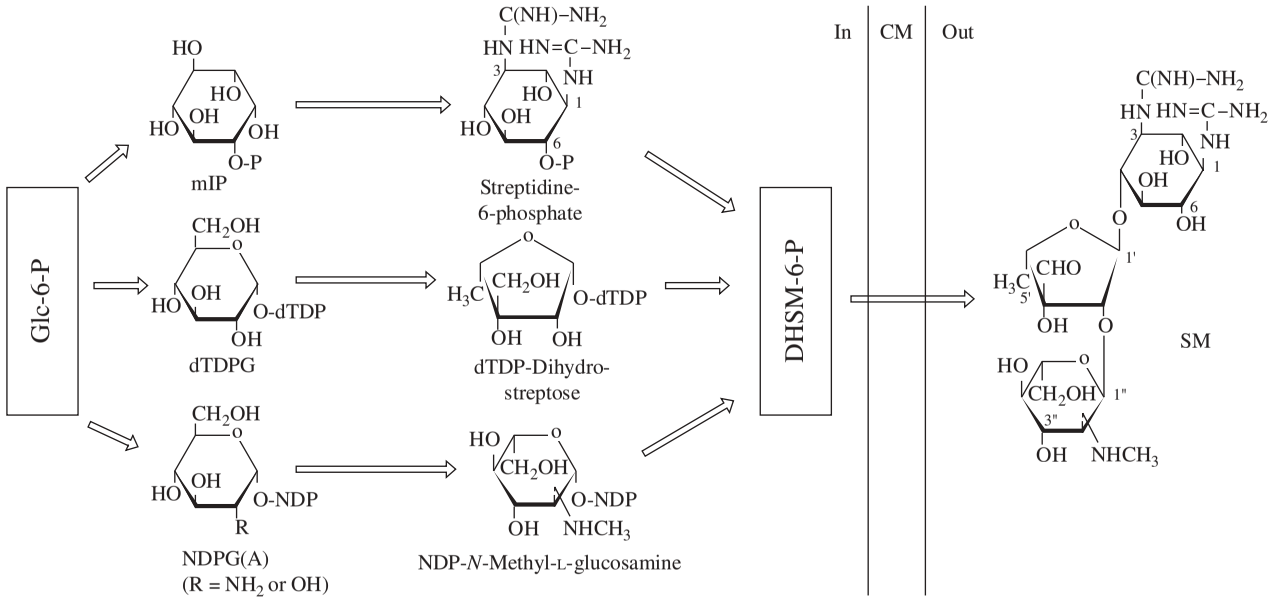

FIGURE 6.2 Outline of the streptomycin biosynthetic pathway. Glucose-6-phosphate (Glc-6-P) is used to synthesize myo-inosytolphosphate (mIP), deoxythymidinediphosphate-glucose (dTDPG) and nucleosidediphosphate-glucose (or glycosamine) [NDPG(A)], which are processed and condensed into dihydro-streptomycin-6-phosphate (DHSM-6-P), which is oxidized and dephosphorylated to streptomycin (SM) during or after transport through the cytoplasmic membrane (CM). [Taken from (Piepersberg and Distler, 1997].

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result, bacteria with a deficient electron-transport system can become resistant (Vakulenko and Mobashery, 2003). This mechanism does not occur in producers such as *S. griseus*, but reduced streptomycin uptake occurs in *Pseudomonas* and other nonfermenting Gram-negative bacilli. It is likely to be due to membrane impermeabilization, although the molecular mechanisms causing it are largely unknown (Mingeot-Leclercq et al., 1999). *Pseudomonas aeruginosa* can display adaptive resistance to aminoglycosides, which is not due to mutations but rather to the upregulation of genes involved in anaerobic metabolism (Karlowsky et al., 1997). A switch to anaerobic metabolism can provide protection against streptomycin as streptomycin can no longer enter the cell via energy-dependent uptake. It has been suggested that *Mycobacterium gordonae*, *M. szulgai*, and *M. avium* are resistant to streptomycin because their cell envelope acts as a permeability barrier because they have no mutations in their *rpsL* gene (Honore and Cole, 1994); however, there is no direct evidence supporting this hypothesis.

The second resistance mechanism, enzymatic modification of the antibiotic, can be performed by three groups of enzymes on aminoglycosides: acetyl CoA-dependent *N*-acetyltransferases, adenosine triphosphate (ATP)-dependent *O*-adenyltransferases, and ATP-dependent *O*-phosphoryltransferases (Wright and Thompson, 1999), though only the latter two have been documented to inactivate streptomycin (Davies and Wright, 1997). The *S. griseus* streptomycin producers possess two resistance genes: *strA* (*aphD*), classified as APH(6)-Ia, which phosphorylates streptomycin on the hydroxyl group found on the 6′ end of the molecule (Cundliffe, 1989), and *aphE* [APH(3″)-Ia], which targets the 3″ end hydroxyl group and which may have originated from *S. fradiae* (Heinzel et al., 1988). The inactivated antibiotic can be exported from the cell using the StrV/StrW ABC transporter. It is theorized that the APH resistance genes evolved from enzymes involved in antibiotic biosynthesis (Piepersberg and Distler, 1997). The streptomycin biosynthetic enzymes StrN and StsE display peptide motifs similar to those of phosphate transfer (Pissowotzki et al., 1991) and may, therefore, share a common ancestor with StrA. The *sph* gene [APH(6)-Ib] from the hydroxystreptomycin gene cluster in *S. glaucescens* can also provide low-level resistance against streptomycin due to the structural similarity of the two target molecules (Vogtli and Hutter, 1987). Genes homologous to StrA are also present outside producers; the *str* [APH(6)-Ic] gene in the transposon Tn05 and the *strB* [APH(6)-Id] plasmid RSF1010, found in enterobacteria, may be derived from *strA* as they have a 51% amino acid homology to the *S. griseus* and *S. glaucescens* resistance genes (Shaw et al., 1993). The *strB* gene, located on plasmids such as RSF1010 next to an APH(3″)-Ib streptomycin resistance gene that is confusingly also called *strA*, is widely distributed in the environment indicating that it is readily transferrable between organisms (Sundin and Bender, 1996; Wright and Thompson, 1999). *Mycobacterium fortuitum* also possesses a streptomycin resistance gene [APH(3″)-Ic] that is homologous to the 3″ APH genes from *S. griseus*, and it can be assumed that RSF1010 arrived to this species via HGT (Ramon-Garcia et al., 2006). Unlike phosphoryltransferases, adenyltransferases are not found in streptomycin producers. ANT(3″)-I genes are very varied, displaying between 59 and 95% amino acid sequence identity to one another (Vakulenko and Mobashery, 2003), and are widespread, having been found in plasmids, integrons, and transposons from Gram-negative (Fling et al., 1985; Chinault et al., 1986; Levesque et al., 1995) as well as in Gram-positive bacteria (Courvalin and

Fiandt, 1980; Nesvera et al., 1998). The ANT(3″)-Ia gene was detected in more than 90% of streptomycin-resistant clinical isolates (Shaw et al., 1991). The *ant(6)-Ia* gene is also widespread, found in over 80% of enterococcal and staphylococcal clinical isolates tested in a European study (Ounissi et al., 1990) and almost 50% of *Enterococcus faecalis* and *Enterococcus faecium* isolates in Japan (Kobayashi et al., 2001). The gene *aadK*, a 6′-adenylyltransferase that provides low-level streptomycin resistance present in the chromosome of *Bacillus subtilis* 168 (Noguchi et al., 1993), has a 58% amino acid sequence identity to *ant(6)-Ia*, while a homolog from *Bacillus halodurans* had 41% sequence identity to *ant(6)-Ia* and even less to *aadK* (Vakulenko and Mobashery, 2003). The origin of *O*-adenyltransferases is uncertain, as they have no close homolog. A sequence that forms part of the ATP and Mg^{2+} binding sites is similar to that of enzymes that catalyze nucleoside-monophosphate transfer-generating pyrophosphates, which suggests that ANTs may have evolved from existing metabolic enzymes such as DNA polymerases (Davies and Wright, 1997).

The third resistance mechanism, alteration of the site of antimicrobial action, can be achieved by mutation of *rrs*, encoding the 16S rRNA, *rpsL*, encoding the S12 ribosomal protein, or *rsmG*, encoding a 16S rRNA methyltransferase. Mutations that disrupt the 530 loop of the 16S rRNA in *E. coli* (Melancon et al., 1988) or its homolog in other bacteria such as *Mycobacterium tuberculosis* (Meier et al., 1994) or chloroplasts (Yeh et al., 1994) can generate a resistance phenotype. The 530 region is the most conserved area of the 16S rRNA (Noller, 1984); mutations at position 530 are lethal for the cell (Powers and Noller, 1990). Mutations at position 912, part of the 900 stem region, of the *E. coli* 16S rRNA (Frattali et al., 1990) or its homolog in other organisms (Honore and Cole, 1994; Gregory and Dahlberg, 2009) do the same. Both the 530 and the 900 region interact with the S12 ribosomal protein (Stern et al., 1988) and play a vital role in both translational accuracy control and tRNA binding (Wang et al., 1999). These two regions are located adjacent to one another in secondary structure models (Ramaswamy and Musser, 1998), and the resistance phenotype is due to these mutations disrupting the pseudoknot to which streptomycin binds (Moazed and Noller, 1987; Powers and Noller, 1991). Due to the presence of multiple *rss* copies in most bacterial species, a mutation on a single gene is unlikely to provide resistance as most of the cell's ribosomes will still be vulnerable. 16S mutations are therefore an important source of resistance in organisms with one or two *rss* genes, such as *Mycobacterium* species, but are unlikely to play a significant role in other bacteria (Musser, 1995). All bacterial species, however, appear to have a single copy of the *rpsL* gene encoding for ribosomal protein S12, and its mutations are therefore more commonly responsible for streptomycin resistance. This highly conserved protein is part of the 30S ribosomal subunit and is located close to the codon–anticodon interaction site (Rodnina and Wintermeyer, 2001) where it is involved in determining the fidelity of protein synthesis (Yates, 1979). The most common mutation is at codon 43, where AAG changes to AGG (Lys to Arg) though an AAG to ACG (Lys to Thr) can also occur less frequently (Ramaswamy and Musser, 1998). Mutations in codon 88 such as AAG to AGG (Lys to Arg) or AAG to CAG (Lys to Gln) can also grant streptomycin resistance (Sreevatsan et al., 1996). Mutations in the *rrs* (Honore et al., 1995), the *rpsL* (Ito and Wittmann, 1973), or a combination of *rpsL* and *miaA* (leading to loss of a tRNA hypermodification) (Diaz et al., 1986) genes can render the bacterium not only resistant but also dependent on streptomycin for its transcription. This is because proofreading becomes excessively

intense and streptomycin is required to suppress the proofreading function of the ribosome. Without the increase in translational efficiency brought about by streptomycin, the dependent cells cannot manufacture proteins fast enough to survive (Diaz et al., 1986). Mutations that inactivate the *rsmG* gene can provide *S. coelicolor* (Nishimura et al., 2007) and *Thermus thermophilus* (Gregory et al., 2009) with low-level streptomycin resistance. The RsmG methyltransferase methylates base G527 of the 16S rRNA (Gregory et al., 2009). As the streptomycin molecule comes into contact with the bases C526 and G527 (Carter et al., 2000), that methyl group may be necessary for the efficient binding of the antibiotic. *Streptomyces griseus* and other producers do not display mutations in the *rpsL* or *rsmG* genes, but Tanaka et al. (Tanaka et al., 2009) demonstrated that mutations in these genes conferring streptomycin resistance could be generated in *S. griseus,* although high-level phenotypic resistance already occurred due to streptomycin modifying enzymes (minimal inhibitory concentration (MIC) 70 μg/mL). Spontaneous mutants were isolated on plates containing 70 to 2000 μg/mL streptomycin, and mutation in the *rsmG* gene but not the *rpsL* gene resulted in enhanced streptomycin production. The latter was attributed to increased transcription of the regulatory genes *metK* and *strR*, and this resulted in enhanced transcription of biosynthetic genes *strB1, strF,* and *strD*.

Streptomycin production in *S. griseus* NBRC 13350 is controlled by the signaling molecule termed A-factor, which is an example of the γ-butyrolactones that have been identified in playing a regulatory role in the switch-on of antibiotic production (Horinouchi, 2007; Bibb and Hesketh, 2009). AfsA synthesizes A-factor (2-isocapryloyl-3R-hydroxymethyl-γ-butyrolactone) in a growth-dependent manner (Horinouchi, 2002). When the concentration of A-factor passes a threshold, at or near the middle of exponential growth, it binds to ArpA (A-factor receptor protein) and causes it to dissociate from the promoter of *adpA* (Ohnishi et al., 1999). The removal of the repressor allows the transcription and translation of AdpA, which in turn activates a large number of genes involved in secondary metabolism and differentiation (Horinouchi, 2007). One of the sites it binds to is on the streptomycin gene cluster, where it induces the expression of the streptomycin regulatory gene *strR* (Vujaklija et al., 1993) (see binding sites in Fig. 6.1) as well as of the resistance gene *strA* due to readthrough (Tomono et al., 2005). StrR, in turn, further induces its own expression in addition to the expression of the biosynthetic and resistance genes in the streptomycin cluster (Retzlaff and Distler 1995). In addition to the A-factor cascade, the transcriptional activator AtrA-g, which binds upstream of the *strR* gene, appears to act as a tuner for the expression of the streptomycin gene cluster. Δ*artA-g S. griseus* mutants produce slightly smaller amounts of streptomycin than the wild type under certain growth conditions (Hirano et al., 2008).

6.3 COEVOLUTION OF ANTIBIOTIC BIOSYNTHESIS AND COUNTERRESISTANCE IN NATURAL POPULATIONS OF SOIL BACTERIA

Examination of a large collection of streptomycete strains freshly isolated from soil revealed that many possessed streptomycin resistance, and, in a number of strains, the genes coding for streptomycin-inactivating enzymes were detected (Tolba et al.,

2002). Members of the same taxon might be expected to compete for similar niches, and, therefore, highly bioactive strains of *S. griseus* producing streptomycin should give rise to "cheaters," which would be highly streptomycin-resistant strains that had lost the capacity to produce streptomycin (a metabolic cost). This has been well studied in bacteria that produce virulence factors, including antibiotics in response to signaling molecules as part of the quorum-sensing (QS) regulatory circuits. Evolutionary theory predicts that the cost of performing a cooperative behavior leaves a population vulnerable to social cheating (Lehmann and Keller, 2006; West et al., 2006). Cheaters are individuals that cease (or reduce) the production of shared metabolites and benefit from the cooperative actions of others. Therefore, QS populations are at risk of invasion by either signal-negative cheaters that do not produce signals or by signal-blind cheaters that avoid production of QS-controlled extracellular factors. Further analysis revealed this to be the case where closely related strains of *S. griseus* contained *strA* but no biosynthetic cluster, lacked regulatory genes, and showed differences in the mode of expression with no involvement of a signaling factor. The *strA* resistance-only gene could have theoretically either evolved from a primary metabolism gene, as the structure of phosphotransferases is highly conserved, or originated from an aminoglycoside cluster. The *S. griseus str* resistance-only genes all clustered in the same clade as *strA* genes from aminoglycoside biosynthetic clusters, so the most plausible explanation for their origin was that they were derived from a resistance gene that was involved in self-protection rather than from a phosphotransferase that served as a housekeeping gene. The precise origins of the sole *strA* gene were less clear, the high sequence divergence indicated that *strA* originated from an *str* cluster other than that of *S. griseus* and was retained by the close relatives of streptomycin producers because it granted them a selective advantage. The flanking regions of the sole *strA* gene did not indicate a similar position to that occupied by the *str* cluster (Fig. 6.3), but the cluster appeared to have been acquired by *S. grisues* in an HGT event as comparison to *S. coelicolor* revealed shared flanking regions (Tomono et al., 2005) as depicted in Figure 6.3.

The low level of expression of *strA* in resistance-only strains suggests that a reduced amount of enzyme is responsible for the low resistance of these strains. This was supported by the fact that the active sites in resistance-only StrA were all conserved. It should be noted though that the *strA* homolog from the *S. glaucescens* hydroxystreptomycin cluster also provides low streptomycin resistance and has

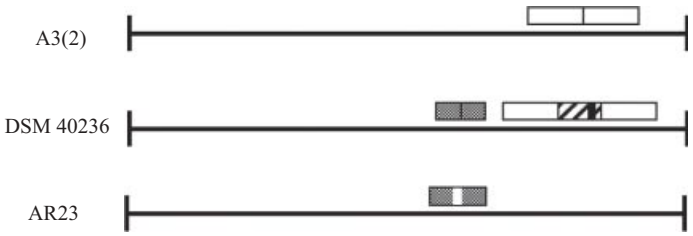

FIGURE 6.3 Comparison of locations of flanking regions to the streptomycin cluster and resistance gene in *S. coelicolor* A3(2), *S. griseus* DSM 40236 str producer, and *S. griseus* AR23 sole str resistance strain. strA shown as a black block.

conserved active sites despite the fact that it must presumably be highly expressed in order to protect *S. glaucescens* from the hydroxystreptomycin that it produces. Therefore, alterations in locations other than the active sites might also influence the specificity of the enzyme. This would not be surprising, as single amino acid changes can suffice to cause large variations in the substrate specificity of aminoglycoside acetyltransferases (Rather et al., 1992).

The distribution of streptomycin and related aminoglycoside clusters on a concatenated housekeeper phylogenenetic tree strongly indicated that the whole *str* cluster has undergone HGT, as the alternative would require the independent loss of the cluster in a large number of clades (Laskaris et al., 2010). Furthermore, the fact that the cluster from a strain of *S. platensis* was almost identical in structure to the *S. griseus* cluster when strains more closely related to *S. griseus,* such as *S. humidus or S. glaucescens*, had *str* clusters that were considerably more diverged was also indicative of HGT. The discovery of transposase remnants flanking both sides of the *S. platensis str* cluster was indicative that the streptomycin gene cluster has spread via a transposon, as have other clusters such as the one involved in andrimid biosynthesis. The absence of transposon remnants in *S. griseus* is probably indicative of a more ancient transposition event, which has allowed the transposon sequences to be completely eliminated. The fact that the streptomycin cluster in *S. griseus* is located between two genes that are adjacent to one another in the *S. coelicolor* A3(2) genome is strongly indicative of an insertion event (Tomono et al., 2005). The deletion of *strB2* in CR50 is an example of the streptomycin gene cluster evolving via the loss of one of its tailoring enzymes.

6.4 RESISTANCE IN GRAM-NEGATIVE BACTERIA: ANTIBIOTIC RESISTANCE IN THE RHIZOSPHERE

Many modern β-lactam antibiotics are secondary metabolites of *Streptomyces* species, and the majority of streptomycetes and other actinobacteria have β-lactamase genes with a high G+C content (≥70%). However, β-lactamase genes found in human and animal pathogens have G+C contents in the order of 45 to 65% suggesting a Gram-negative origin (Gaze et al., 2008). There are a number of Gram-negative antibiotic producers, including plant pathogens and rhizobacteria, such as *Erwinia, Serratia*, *Flavobacterium, Pseudomonas, Chromobacterium*, and *Agrobacterium* sp., which produce carbapenems, β-lactams, and moncocyclic β-lactams (Jensen and Demain, 1995). Those producing carbapenemases include *Erwinia carotovora, Erwinia herbicola*, and a *Serratia* sp. (Coulthurst et al., 2005), although it is suggested that, at least in *E. carotovora,* there is little likelihood for cross resistance against clinical antibiotics due to the specificity of the resistance mechanism. However, there is now firm evidence that progenitors of clinically important resistance genes such as the extended spectrum β-lactamases (ESBLs). CTX-Ms, a clinically important group of ESBLs, originated in rhizosphere-associated bacteria (Bonnet, 2004; Gaze et al., 2008). To confer resistance in human-associated bacteria, HGT must occur. Genes can be transferred individually, in clusters, or in large islands containing many different genes. DNA can be mobilized randomly but more significantly via transfer of mobile genetic elements (MGEs) such as plasmids, pathogenicity islands, internal sequencing (IS) elements, transposons, and integrons. Genes encoded on MGEs control mobilization

and also include additional genes, many of which confer adaptive traits (Ochman et al., 2000). The horizontal gene pool contains genes involved in pathogenicty, antibiotic and disinfectant resistance, and metabolic traits. Less well understood is which mobile genes are present in bacteria in the environment; and, in the case of antibiotic resistance genes, are they the same as those found in the clinic, and what selects for their maintenance in the absence of clinical antibiotics? It has been suggested that by increasing our understanding of environmental reservoirs of mobile adaptive genes, we will be able to determine the types of genes that exist including the kinds of resistance genes that may emerge clinically (D'Costa et al., 2006).

In addition to direct selection by exposure to antibiotics produced by indigenous bacteria, selection may also occur in antibiotic-contaminated environments such as soils amended with slurry from intensively reared livestock (Byrne-Bailey et al., 2009, 2011). Kümmerer has produced comprehensive reviews on the fate of antibiotics in the environment and their impact on resistance, illustrating that antibiotic residues are present in diverse ecosystem compartments (Kümmerer, 2009a, 2009b). Co-selection may also occur where genes conferring resistance to heavy metals or biocides and detergents are situated on the same mobile genetic element as antibiotic resistance determinants. Co-selection for class 1 integrons carrying antibiotic resistance genes may be produced by the presence of quaternary ammonium compound (QAC) resistance genes on the same elements. QACs are used in a wide range of domestic and industrial products, including detergents and biocides. Integrons are recombination and expression systems that capture genes as part of a genetic element known as a gene cassette (Recchia and Hall, 1995). Most cassettes of known function confer antibiotic or QAC resistance. Research by the authors demonstrated that QAC resistance was higher in isolates from environmental samples exposed to QAC pollution, and class 1 integron prevalence was significantly higher in populations preexposed to QACs (Gaze *et al*., 2005). Further work demonstrated that metagenomic DNA from QAC-contaminated sample sites contained large numbers of class 1 integrons and showed high QAC resistance gene carriage (Gaze et al., 2011), illustrating that in the absence of antibiotics, QACs are the major driver of selection for these clinically important genetic elements. These findings mirror those of other researchers studying *qac* gene carriage in aquatic systems (Gillings et al., 2009a, 2009b). Metagenomic approaches reveal the existence of diverse resistance genes in soil organisms, including novel ESBLs (Allen et al., 2009). Research by the authors also demonstrated that novel β-lactamases conferring resistance to third-generation cephalosporins are present in sewage sludge and sludge-amended soil, including genes associated with insertion sequences responsible for mobilization (unpublished data). Waste management practices introduce complex mixtures of chemicals and bacteria into the environment where, in combination with antibiotics from producers, selection for new combinations of resistance genes can occur, recruiting novel genes from the environmental resistome into human-associated bacteria, which may subsequently emerge in the clinic.

6.5 CONCLUSIONS

Despite alternative hypotheses concerning the function of antibiotics, there is still considerable evidence indicating that antibiotics act antagonistically toward other

microorganisms in the environment. One suggested explanation for the distribution of secondary metabolites, they are common in organisms lacking an immune system and rare in organisms possessing one, is that their production increases the organism's fitness for survival by acting as an alternative defense mechanism (Maplestone et al., 1992). The failure to detect antibiotics in soil is due both to nutrient limitations placing constraints on bacterial growth and secondary metabolite production (Anukool et al., 2004) and because many antibiotics strongly adsorb onto soil or clay particles, making them difficult to extract and detect (Sarmah et al., 2006). Antibiotics can remain biologically active even when tightly bound to soil particles (Chander et al., 2005). Antibiotics can therefore retain their bactericidal properties whether free or adsorbed and are thus biologically relevant in soil antagonism even when they cannot be detected. A number of the transcriptional effects triggered by subinhibitory concentrations of antibiotics can be interpreted as an attempt by the microorganisms to ready themselves for a higher and potentially lethal amount of antibiotic. In *B. subtilis* sublethal concentrations of chloramphenicol and erythromycin (inhibitors of translation elongation) induced the synthesis of the stringently controlled ribosomal proteins and elongation factors while gentamicin (which interferes with ribosomal translation accuracy and does not affect elongation) induced the expression of ribosomal proteins and not elongation factors (Lin et al., 2005). Antibiotic production by biocontrol bacteria has long been implicated as an important characteristic in the control of soil-borne plant pathogens. Haas and Keel (2003) reviewed the evidence for production in situ based on chemical extraction and expression of antibiotic biosynthesis genes. There was convincing evidence for the production by pseudomonads of antifungal antibiotics including phenazines, 2,4-diacetylphloroglucinol, and pyoluteorin, and antibiotic-negative mutants give reduced protection against fungi. A number of *Streptomyces* species are also used for biocontrol (Raaijmakers et al., 2002) and can reduce the extent of infection caused by the potato pathogen *S. scabies*. This is due at least in part to the antibiotics produced by the suppressive strains, because *S. scabies* mutants resistant to some of these antibiotics are able to readily infect potato tubers (Neeno-Eckwall et al., 2001). The rhizosphere, therefore, is a niche for which there is convincing evidence of antibiotics acting as inhibitors against other competitors. Another niche where antibiotics have been confirmed to be present in lethal concentrations is on the bodies of fungus-farming insects. These insects create fungus gardens on which they feed and can combat infections by parasitic fungal species in their gardens through the use of antimicrobial compounds derived from bacteria grown on specialized regions of their own bodies (Mueller and Gerardo, 2002). Interestingly, strains of *S. griseus* are associated with the fungus-cultivating Ambrosia beetle (Grubbs et al., 2011). It seems very likely that the beetle is taking advantage of the prolific antibiotic metabolic capacity of the streptomycetes.

REFERENCES

Allen HK, et al. (2009). Functional metagenomics reveals diverse beta-lactamases in a remote Alaskan soil. *ISME J* 3(2):243–251.

Anonymous (2008). Streptomycin. *Tuberculosis (Edinb)* 88(2):162–163.

Anukool U, et al. (2004). In situ monitoring of streptothricin production by *Streptomyces rochei* F20 in soil and rhizosphere. *Appl Environ Microbiol* 70(9):5222–5228.

Bainton NJ, et al. (1992). A general role for the lux autoinducer in bacterial cell signalling: Control of antibiotic biosynthesis in Erwinia. *Gene* 116(1):87–91.

Baltz RH (2005). Antibiotic discovery from actinomycetes: Will a renaissance follow the decline and fall. *SIM News* 55:186–196.

Baltz RH (2006). Marcel Faber Roundtable: Is our antibiotic pipeline unproductive because of starvation, constipation or lack of inspiration? *J Ind Microbiol Biotechnol* 33(7):507–513.

Baltz RH (2008). Renaissance in antibacterial discovery from actinomycetes. *Curr Opin Pharmacol* 8(5):557–563.

Bibb M, Hesketh A (2009). Chapter 4. Analyzing the regulation of antibiotic production in streptomycetes. *Methods Enzymol* 458:93–116.

Bonnet R (2004). Growing group of extended-spectrum beta-lactamases: The CTX-M enzymes. *Antimicrob Agents Chemother* 48(1):1–14.

Brisbane PG, Rovira AD (1988). Mechanisms of inhibition of *Gaeumannomyces graminis* var. *tritici* by fluorescent pseudomonads. *Plant Pathol* 37:104–111.

Busse HJ, et al. (1992). The bactericidal action of streptomycin: Membrane permeabilization caused by the insertion of mistranslated proteins into the cytoplasmic membrane of *Escherichia coli* and subsequent caging of the antibiotic inside the cells due to degradation of these proteins. *J Gen Microbiol* 138(3):551–561.

Byrne-Bailey KG, et al. (2009). Prevalence of sulfonamide resistance genes in bacterial isolates from manured agricultural soils and pig slurry in the United Kingdom. *Antimicrob Agents Chemother* 53(2):696–702.

Byrne-Bailey KG, et al. (2011). Integron prevalence and diversity in manured soil. *Appl Environ Microbiol* 77(2):684–687.

Carter AP, et al. (2000). Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. *Nature* 407(6802):340–348.

Chander Y, et al. (2005). Antibacterial activity of soil-bound antibiotics. *J Environ Qual* 34(6): 1952–1957.

Chinault AC, et al. (1986). Characterization of transferable plasmids from *Shigella flexneri 2a* that confer resistance to trimethoprim, streptomycin, and sulfonamides. *Plasmid* 15(2): 119–131.

Coulthurst SJ, et al. (2005). Regulation and biosynthesis of carbapenem antibiotics in bacteria. *Nat Rev Microbiol* 3(4):295–306.

Courvalin P, Fiandt M (1980). Aminoglycoside-modifying enzymes of *Staphylococcus aureus*; expression in *Escherichia coli*. *Gene* 9(3–4):247–269.

Cundliffe E (1989). How antibiotic-producing organisms avoid suicide. *Annu Rev Microbiol* 43:207–233.

D'Costa VM, et al. (2006). Sampling the antibiotic resistome. *Science* 311(5759):374–377.

Davies J, Wright GD (1997). Bacterial resistance to aminoglycoside antibiotics. *Trends Microbiol* 5(6):234–240.

Diallo S, et al. (2011). Mechanisms and recent advances in biological control mediated through the potato rhizosphere. *FEMS Microbiol Ecol* 75(3):351–364.

Diaz I, et al. (1986). How do combinations of rpsL- and miaA- generate streptomycin dependence? *Mol Gen Genet* 202(2):207–211.

Distler J, et al. (1992). Streptomycin biosynthesis and its regulation in Streptomycetes. *Gene* 115(1–2):105–111.

Egan S, et al. (1998). Transfer of streptomycin biosynthesis gene clusters within streptomycetes isolated from soil. *Appl Environ Microbiol* 64(12):5061–5063.

Egan S, et al. (2001). Phylogeny of *Streptomyces* species and evidence for horizontal transfer of entire and partial antibiotic gene clusters. *Antonie Van Leeuwenhoek* 79(2):127–133.

Flatt PM, Mahmud T (2007). Biosynthesis of aminocyclitol-aminoglycoside antibiotics and related compounds. *Nat Prod Rep* 24(2):358–392.

Fling ME, et al. (1985). Nucleotide sequence of the transposon Tn7 gene encoding an aminoglycoside-modifying enzyme, 3″(9)-*O*-nucleotidyltransferase. *Nucleic Acids Res* 13(19):7095–7106.

Frattali AL, et al. (1990). Effects of mutagenesis of C912 in the streptomycin binding region of *Escherichia coli* 16S ribosomal RNA. *Biochim Biophys Acta* 1050(1–3):27–33.

Gaze WH, et al. (2005) Incidence of class 1 integrons in a quaternary ammonium compound-polluted environment. *Antimicrob Agents Chemother* May;49(5):1802–1807.

Gaze W, et al. (2008). Antibiotic resistance in the environment, with particular reference to MRSA. *Adv Appl Microbiol* 63:249–280.

Gaze WH, Zhang L, Abdouslam NA, Hawkey PM, Calvo-Bado L, Royle J, Brown H, Davis S, Kay P, Boxall ABA, Wellington EMH (2011). Impacts of anthropogenic activity on the ecology of class 1 integrons and integron-associated genes in the environment. *ISME J* Aug;5(8):1253–1261.

Gillings MR, et al. (2009a). Evidence for dynamic exchange of qac gene cassettes between class 1 integrons and other integrons in freshwater biofilms. *FEMS Microbiol Lett* 296(2):282–288.

Gillings MR, et al. (2009b). Gene cassettes encoding resistance to quaternary ammonium compounds: A role in the origin of clinical class 1 integrons? *ISME J* 3(2):209–215.

Gordon RE, Horan AC (1968). A piecemeal description of *Streptomyces griseus* (Krainsky) Waksman and Henrici. *J Gen Microbiol* 50(2):223–233.

Gregory ST, Dahlberg AE (2009). Genetic and structural analysis of base substitutions in the central pseudoknot of *Thermus thermophilus* 16S ribosomal RNA. *RNA* 15(2):215–223.

Gregory ST, et al. (2009). Structural and functional studies of the *Thermus thermophilus* 16S rRNA methyltransferase RsmG. *RNA* 15(9):1693–1704.

Grubbs KJ, et al. (2011). The complete genome sequence of *Streptomyces cf. griseus* (XyelbKG-1), an ambrosia beetle-associated actinomycete. *J Bacteriol* 193:2890–2891.

Haas D, Keel C (2003). Regulation of antibiotic production in root-colonizing *Peudomonas* spp. and relevance for biological control of plant disease. *Annu Rev Phytopathol* 41:117–153.

Hancock RE (1984). Alterations in outer membrane permeability. *Annu Rev Microbiol* 38:237–264.

Heinzel P, et al. (1988). A second streptomycin resistance gene from *Streptomyces griseus* codes for streptomycin-3″-phosphotransferase. Relationships between antibiotic and protein kinases. *Arch Microbiol* 150(2):184–192.

Hertweck M, et al. (2002). Inhibition of nuclear pre-mRNA splicing by antibiotics in vitro. *Eur J Biochem* 269(1):175–183.

Hirano S, et al. (2008). Conditionally positive effect of the TetR-family transcriptional regulator AtrA on streptomycin production by *Streptomyces griseus*. *Microbiology* 154(Pt 3): 905–914.

Hong HJ, et al. (2004). Characterization of an inducible vancomycin resistance system in *Streptomyces coelicolor* reveals a novel gene (vanK) required for drug resistance. *Mol Microbiol* 52(4):1107–1121.

Honore N, Cole ST (1994). Streptomycin resistance in mycobacteria. *Antimicrob Agents Chemother* 38(2):238–242.

Honore N, et al. (1995). Novel mutation in 16S rRNA associated with streptomycin dependence in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 39(3):769–770.

Horinouchi S (2002). A microbial hormone, A-factor, as a master switch for morphological differentiation and secondary metabolism in *Streptomyces griseus*. *Front Biosci* 7:d2045–2057.

Horinouchi S (2007). Mining and polishing of the treasure trove in the bacterial genus streptomyces. *Biosci Biotechnol Biochem* 71(2):283–299.

Huddleston AS, et al. (1997). Molecular detection of streptomycin-producing streptomycetes in Brazilian soils. *Appl Environ Microbiol* 63(4):1288–1297.

Ito T, Wittmann HG (1973). Amino acid replacements in proteins S5 and S12 of two *Escherichia coli* revertants from streptomycin dependence to independence. *Mol Gen Genet* 127(1):19–32.

Jensen SE, Demain AL (1995). Beta-lactams. *Biotechnology* 28:239–268.

Kamal A, et al. (2008). Efforts towards the development of new antitubercular agents: Potential for thiolactomycin based compounds. *J Pharm Pharm Sci* 11(2):56s–80s.

Karimi R, Ehrenberg M (1994). Dissociation rate of cognate peptidyl-tRNA from the A-site of hyper-accurate and error-prone ribosomes. *Eur J Biochem* 226(2):355–360.

Karimi R, Ehrenberg M (1996). Dissociation rates of peptidyl-tRNA from the P-site of *E. coli* ribosomes. *EMBO J* 15(5):1149–1154.

Karlowsky JA, et al. (1997). Altered denA and anr gene expression in aminoglycoside adaptive resistance in *Pseudomonas aeruginosa*. *J Antimicrob Chemother* 40(3):371–376.

Kashiwagi K, et al. (1998). Relationship between spontaneous aminoglycoside resistance in *Escherichia coli* and a decrease in oligopeptide binding protein. *J Bacteriol* 180(20):5484–5488.

Kobayashi N, et al. (2001). Distribution of aminoglycoside resistance genes in recent clinical isolates of *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus avium*. *Epidemiol Infect* 126(2):197–204.

Kümmerer, K. (2009a). Antibiotics in the aquatic environment—A review—Part I. *Chemosphere* 75(4):417–434.

Kümmerer, K. (2009b). Antibiotics in the aquatic environment—A review—Part II. *Chemosphere* 75(4):435–441.

Laskaris P, et al. (2010). Coevolution of antibiotic production and counter-resistance in soil bacteria. *Environ Microbiol* 12(3):783–796.

Lazzarini A, et al. (2000). Rare genera of actinomycetes as potential producers of new antibiotics. *Antonie Van Leeuwenhoek* 78(3–4):399–405.

Lehmann L, Keller L (2006). The evolution of cooperation and altruism—A general framework and a classification of models. *J Evol Biol* 19(5):1365–1376.

Levesque C, et al. (1995). PCR mapping of integrons reveals several novel combinations of resistance genes. *Antimicrob Agents Chemother* 39(1):185–191.

Lin JT, et al. (2005). Global transcriptional response of *Bacillus subtilis* to treatment with subinhibitory concentrations of antibiotics that inhibit protein synthesis. *Antimicrob Agents Chemother* 49(5):1915–1926.

Madigan MT, Martinko JM (2005). *Brock Biology of Microorganisms*. Prentice Hall, Upper Saddle River, NJ 6–26.

Maplestone RA, et al. (1992). The evolutionary role of secondary metabolites—A review. *Gene* 115(1–2):151–157.

Marshall CG, et al. (1998). Glycopeptide antibiotic resistance genes in glycopeptide-producing organisms. *Antimicrob Agents Chemother* 42(9):2215–2220.

Martin NL, Beveridge TJ (1986). Gentamicin interaction with *Pseudomonas aeruginosa* cell envelope. *Antimicrob Agents Chemother* 29(6):1079–1087.

McManus PS, et al. (2002). Antibiotic use in plant agriculture. *Annu Rev Phytopathol* 40:443–465.

Meier A, et al. (1994). Genetic alterations in streptomycin-resistant *Mycobacterium tuberculosis*: Mapping of mutations conferring resistance. *Antimicrob Agents Chemother* 38(2): 228–233.

Melancon P, et al. (1988). A mutation in the 530 loop of *Escherichia coli* 16S ribosomal RNA causes resistance to streptomycin. *Nucleic Acids Res* 16(20):9631–9639.

Mindlin SZ, et al. (2008). Isolation of antibiotic resistance bacterial strains from Eastern Siberia permafrost sediments. *Russian J Genet* 44(1):27–34.

Mingeot-Leclercq MP, Tulkens PM (1999). Aminoglycosides: Nephrotoxicity. *Antimicrob Agents Chemother* 43(5):1003–1012.

Mingeot-Leclercq MP, et al. (1999). Aminoglycosides: Activity and resistance. *Antimicrob Agents Chemother* 43(4):727–737.

Moazed D, Noller HF (1987). Interaction of antibiotics with functional sites in 16S ribosomal RNA. *Nature* 327(6121):389–394.

Mueller UG, Gerardo N (2002). Fungus-farming insects: Multiple origins and diverse evolutionary histories. *Proc Natl Acad Sci USA* 99(24):15247–15249.

Musser JM (1995). Antimicrobial agent resistance in mycobacteria: Molecular genetic insights. *Clin Microbiol Rev* 8(4):496–514.

Neeno-Eckwal EC, et al. (2001). Competition and antibiosis in the biological control of potato scab. *Can J Microbiol* 47(4):332–340.

Nesvera J, et al. (1998). An integron of class 1 is present on the plasmid pCG4 from gram-positive bacterium *Corynebacterium glutamicum*. *FEMS Microbiol Lett* 169(2):391–395.

Nikolakopoulou TL, et al. (2005). PCR detection of oxytetracycline resistance genes otr(A) and otr(B) in tetracycline-resistant streptomycete isolates from diverse habitats. *Curr Microbiol* 51(4):211–216.

Nishimura K, et al. (2007). Mutations in rsmG, encoding a 16S rRNA methyltransferase, result in low-level streptomycin resistance and antibiotic overproduction in *Streptomyces coelicolor* A3(2). *J Bacteriol* 189(10):3876–3883.

Noguchi N, et al. (1993). Genetic mapping in *Bacillus subtilis* 168 of the aadK gene which encodes aminoglycoside 6-adenylyltransferase. *FEMS Microbiol Lett* 114(1):47–52.

Noller HF (1984). Structure of ribosomal RNA. *Annu Rev Biochem* 53:119–162.

Ochman H, et al. (2000). Lateral gene transfer and the nature of bacterial innovation. *Nature* 405(6784):299–304.

Ohnishi Y, et al. (1999). The A-factor regulatory cascade leading to streptomycin biosynthesis in *Streptomyces griseus*: Identification of a target gene of the A-factor receptor. *Mol Microbiol* 34(1):102–111.

Ounissi H, et al. (1990). Gene homogeneity for aminoglycoside-modifying enzymes in gram-positive cocci. *Antimicrob Agents Chemother* 34(11):2164–2168.

Paradkar A, et al. (2003). Streptomyces genetics: A genomic perspective. *Crit Rev Biotechnol* 23(1):1–27.

Phillips L, Wellington EMH, Rees SB, King GP (1992). The distribution of DNA sequences homologous to antibiotic production and resistance genes within type strains and wild isolates of *Streptomyces* species. *J Antibiotics* 45:1481–1491.

Piepersberg W, Distler J (1997). Aminoglycosides and sugar components in other secondary metabolites. In H Kleinkauf and H von Dohren (Eds.), *Biotecnology: Products of Secondary Metabolism*, Vol. 7. Verlag Chemie, Weinheim.

Pissowotzki K, et al. (1991). Genetics of streptomycin production in *Streptomyces griseus*: Molecular structure and putative function of genes strELMB2N. *Mol Gen Genet* 231(1): 113–123.

Powers T, Noller HF (1990). Dominant lethal mutations in a conserved loop in 16S rRNA. *Proc Natl Acad Sci USA* 87(3):1042–1046.

Powers T, Noller HF (1991). A functional pseudoknot in 16S ribosomal RNA. *EMBO J* 10(8): 2203–2214.

Raaijmakers JM, et al. (2002). Antibiotic production by bacterial biocontrol agents. *Antonie Van Leeuwenhoek* 81(1–4):537–547.

Ramaswamy S, Musser JM (1998). Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. *Tuber Lung Dis* 79(1):3–29.

Ramon-Garcia S, et al. (2006). Novel streptomycin resistance gene from *Mycobacterium fortuitum*. *Antimicrob Agents Chemother* 50(11):3920–3922.

Rather PN, et al. (1992). Genetic analysis of bacterial acetyltransferases: Identification of amino acids determining the specificities of the aminoglycoside 6′-*N*-acetyltransferase Ib and IIa proteins. *J Bacteriol* 174(10):3196–3203.

Recchia GD, Hall RM (1995). Gene cassettes: A new class of mobile element. *Microbiology* 141(Pt 12):3015–3027.

Retzlaff L, Distler J (1995). The regulator of streptomycin gene expression, StrR, of *Streptomyces griseus* is a DNA binding activator protein with multiple recognition sites. *Mol Microbiol* 18:151–162.

Rodnina MV, Wintermeyer W (2001). Ribosome fidelity: tRNA discrimination, proofreading and induced fit. *Trends Biochem Sci* 26(2):124–130.

Sarmah AK, et al. (2006). A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere* 65(5):725–759.

Seveno NA, et al. (2001). Growth of *Pseudomonas aureofaciens* PGS12 and the dynamics of HHL and phenazine production in liquid culture, on nutrient agar, and on plant roots. *Microb Ecol* 41(4):314–324.

Shaw KJ, et al. (1991). Correlation between aminoglycoside resistance profiles and DNA hybridization of clinical isolates. *Antimicrob Agents Chemother* 35(11):2253–2261.

Shaw KJ, et al. (1993). Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiol Rev* 57(1):138–163.

Sreevatsan S, et al. (1996). Characterization of rpsL and rrs mutations in streptomycin-resistant *Mycobacterium tuberculosis* isolates from diverse geographic localities. *Antimicrob Agents Chemother* 40(4):1024–1026.

Stahley MR, Strobel SA (2006). RNA splicing: Group I intron crystal structures reveal the basis of splice site selection and metal ion catalysis. *Curr Opin Struct Biol* 16(3): 319–326.

Stern S, et al. (1988). Interaction of ribosomal proteins S5, S6, S11, S12, S18 and S21 with 16S rRNA. *J Mol Biol* 201(4):683–695.

Sundin GW, Bender CL (1996). Dissemination of the strA–strB streptomycin-resistance genes among commensal and pathogenic bacteria from humans, animals, and plants. *Mol Ecol* 5(1):133–143.

Szabo I, et al. (1990). Effect of aminoglycoside antibiotics on the autolytic enzyme of *Streptomyces griseus*. *Arch Microbiol* 155(1):99–102.

Szabo I, et al. (1989). Production of a streptomycin–Park nucleotide complex by *Streptomyces griseus*. *Antimicrob Agents Chemother* 33(1):58–62.

Taber HW, et al. (1987). Bacterial uptake of aminoglycoside antibiotics. *Microbiol Rev* 51(4): 439–457.

Takano E, et al. (2001). A complex role for the gamma-butyrolactone SCB1 in regulating antibiotic production in *Streptomyces coelicolor* A3(2). *Mol Microbiol* 41(5):1015–1028.

Tanaka Y, et al. (2009). Activation of secondary metabolite-biosynthetic gene clusters by generating rsmG mutations in *Streptomyces griseus*. *J Antibiot (Tokyo)* 62(12):669–673.

Tolba S. (2004). Distribution of streptomycin resistance and biosynthesis genes in streptomycetes recovered from different soil sites and the role of horizontal gene transfer in their dissemination. PhD thesis, University of Warwick.

Tolba S, et al. (2002). Distribution of streptomycin resistance and biosynthesis genes in streptomycetes recovered from different soil sites. *FEMS Microbiol Ecol* 42(2):269–276.

Tomono A, et al. (2005). Transcriptional control by A-factor of strR, the pathway-specific transcriptional activator for streptomycin biosynthesis in *Streptomyces griseus*. *J Bacteriol* 187(16):5595–5604.

Vakulenko SB, Mobashery S (2003). Versatility of aminoglycosides and prospects for their future. *Clin Microbiol Rev* 16(3):430–450.

van Overbeek LS, et al. (2002). Prevalence of streptomycin-resistance genes in bacterial populations in European habitats. *FEMS Microbiol Ecol* 42(2):277–288.

Vogtli M, Hutter R (1987). Characterisation of the hydroxystreptomycin phosphotransferase gene (sph) of *Streptomyces glaucescens*: Nucleotide sequence and promoter analysis. *Mol Gen Genet* 208(1–2):195–203.

von Ahsen U, Noller HF (1993). Footprinting the sites of interaction of antibiotics with catalytic group I intron RNA. *Science* 260(5113):1500–1503.

Vujaklija D, et al. (1993). Detection of an A-factor-responsive protein that binds to the upstream activation sequence of strR, a regulatory gene for streptomycin biosynthesis in *Streptomyces griseus*. *J Bacteriol* 175(9):2652–2661.

Wallace ST, Schroeder R (1998). In vitro selection and characterization of streptomycin-binding RNAs: Recognition discrimination between antibiotics. *RNA* 4(1):112–123.

Wang R, et al. (1999). Three-dimensional placement of the conserved 530 loop of 16S rRNA and of its neighboring components in the 30 S subunit. *J Mol Biol* 286(2):521–540.

Wellington EMH, Marsh P, Toth I, Cresswell N, Huddleston L, Schilhabel M (1993). The selective effects of antibiotics in soils. In R Guerrero and C Pedros-Alio (Eds.), *Trends in Microbial Ecology*. Spanish Society for Microbiology, Barcelona, Spain, pp. 331–336.

West SA, et al. (2006). Social evolution theory for microorganisms. *Nat Rev Microbiol* 4 (8):597–607.

Wiener P, et al. (1998). Evidence for transfer of antibiotic-resistance genes in soil populations of streptomycetes. *Mol Ecol* 7(9):1205–1216.

Wright GD, Thompson PR (1999). Aminoglycoside phosphotransferases: Proteins, structure, and mechanism. *Front Biosci* 4:D9–21.

Yates JL (1979). Role of ribosomal protein S12 in discrimination of aminoacyl-tRNA. *J Biol Chem* 254(22):11550–11554.

Yeh KC, et al. (1994). Point mutations in the chloroplast 16S rRNA gene confer streptomycin resistance in *Nicotiana plumbaginifolia*. *Curr Genet* 26(2):132–135.

7

MECHANISMS OF BACTERIAL ANTIBIOTIC RESISTANCE AND LESSONS LEARNED FROM ENVIRONMENTAL TETRACYCLINE-RESISTANT BACTERIA

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7.1 MECHANISM OF BACTERIAL RESISTANCE

Historic evidence for antibiotic-resistant bacteria being a product of human activity is suggested by the study of Hughes and Datta (1983), which found that from a collection of Enterobacteriaceae, isolated between 1917 and 1954, 24% carried conjugative plasmids but only 2% were tetracycline resistant and all isolates were from the genera *Proteus*. None of the *Salmonella*, *Shigella*, *Escherichia*, or *Klebsiella* isolates were positive for tetracycline resistance (Tc^r) (Hughes and Datta, 1983). However, by the mid-1950s Tc^r and multidrug-resistant *Escherichia coli* and *Shigella* were described, which was later determined to be due to the presence of plasmid-mediated antibiotic resistance (Watanabe, 1963). A lack of tetracycline resistance genes was also found in early enterococci (Atkinson et al., 1997) and *Neisseria gonorrhoeae* (Cousin et al., 2003). These studies suggest that antibiotic resistance genes were acquired as a result of increased antibiotic use by humans in the last 60 years.

There are a number of different ways that bacteria can become resistant to antibiotics. The first mechanism is due to random chromosomal mutations that lead to changes in the gene product that altered or eliminated the expression of a protein. A second mechanism is by acquisition of new DNA (deoxyribonucleic acid) that is

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available to a limited number of bacteria that are naturally transformable. These bacteria have receptors that allow them to take up DNA from related strains or species and integrate this foreign DNA, which may be parts of genes, complete genes or even defined elements into their genome. The integration of new pieces of a gene creates a mosaic gene composed of the host's and foreign DNA, and this mosaic protein is able to reduce the antibiotic susceptibility of the host bacteria. Some species of bacteria are able to acquire foreign DNA by transduction, which uses bacteria phage for transmission of the DNA. This occurs during phage replication and is due to packaging host bacterial DNA into the phage protein coat, which, when injected into a new host bacterium, allows the foreign DNA to be incorporated into the host's genome (Di Luca et al., 2010). However, the most common way bacteria become antibiotic resistant is by acquisition of new genes associated with mobile elements (plasmids, transposons, and integrons). These mobile elements may carry genes for metal resistance, use of alternative carbon sources, and/or classical virulence genes as well as a variety of different antibiotic resistance genes. Mobile elements are the main driving force in horizontal gene transfer between strains, species, and genera. They are normally responsible for the rapid spread of particular elements throughout bacterial communities around the world. Studying these mobile elements has increased our understanding of some of the mechanisms by which bacteria adapt to their changing environment.

7.2 MUTATION

Mutations occur during normal replication and generally result in a low-to-moderate increase in the level of antibiotic resistance. Mutations can also occur due to exposure to ultraviolet light, chemicals and/or insertion of mobile elements, or a simple error that alters the DNA composition of a gene resulting in an alteration of the amino acid composition of the protein. Point mutation changes are due to DNA base pair nucleotide substitution. Point mutations can lead to silent mutations, where, because of code redundancy, the mutation codes for the same or closely related amino acid and thus does not alter its function. Other alterations result in mis-sense mutations that are due to substitution of a very different amino acid resulting in alterations in the protein's function. Some changes result in non-sense mutation because the change creates a stop codon, and thus the altered gene results in the production of truncated protein that is not functional. Mutations due to insertions and/or deletions also occur and can be caused by insertion or deletion of mobile elements such as transposons. These changes often result in greatly altered or nonfunctional proteins and in some cases may be lethal to the host. Mutations are passed on to daughter cells during cell division and rarely by transformation and/or transduction. Mutations can occur in specific structural proteins such as penicillin-binding proteins (PBPs), in RNA (ribonucleic acid) molecules such as the 23S ribosomal RNA (rRNA) or 16S rRNA and/or in genes that regulation expression of other proteins. Mutations in any of these genes may alter susceptibility to single or multiple classes of antibiotics. However, for most classes of antibiotics multiple mutations are required to confer clinically relevant levels of resistance. Subtherapeutic levels of antibiotics as given in animal feed to promote growth provide selective pressure for the accumulation of mutations in exposed bacteria.

7.3 TRANSFORMATION AND TRANSDUCTION

Some species of bacteria are naturally transformable, which enables individual bacterium to take up exogenous DNA through the cell wall from the environment. Normally foreign DNA from the same or related species is incorporated into the bacteria's own genome. Naturally transformable bacteria contain multiple copies of short DNA uptake signal sequences in their genome (Smith et al., 1999). The Gram-positive bacteria take up DNA from any source but incorporate only homologous DNA from related species into their chromosomes. In contrast, Gram-negative bacteria preferentially uptake related DNA only. Sometimes, only partial genes are replaced, which create mosaic proteins, while in other cases complete genes are added. In either case, some transformation may confer increased antibiotic resistance.

The famous Avery et al. (1944) experiments demonstrated that transformation can occur in vivo. The experiment mixed a nonpathogenic noncapsulated *Streptococcus pneumoniae* and a heat-killed virulent encapsulated strain together and injected the mixture into mice. Only encapsulated *S. pneumoniae* are able to kill the mice and surprisingly some of the mice died. When the bacteria were isolated from these animals, encapsulated *S. pneumoniae* were recovered. The isolates had the same capsule as was found in the heat-killed strain, suggesting that something from this mixture was incorporated into the live *S. pneumoniae* allowing them to make capsule.

Since these early experiments, a number of other naturally transformable bacteria have been identified including other Gram-positive species of *Streptococcus*, *Bacillus subtilis*, cyanobacterium *Synechocystis* spp. and Gram-negative *Actinobacillus actinomycetemcomitans*, *Acinetobacter* spp., *Campylobacter jejuni*, *Haemophilus influenzae*, *Helicobacter pylori, Klebsiella oxytoca*, *N. gonorrhoeae*, *N. meningitidis*, *Pseudomonas stutzeri*, and *Thermus thermophilus* (Chen and Dubnau, 2004; Smith et al., 1999). Interestingly, *A. actinomycetemcomitans*, *H. influenzae*, *H. pylori*, *Neisseria* spp., and *S. pneumoniae* are associated with humans while the other species are environmental bacteria, which are defined as those species and genera that are primarily found outside of humans and animals rather than in or on animals or humans even if, on occasion, they cause infections. It is likely that other naturally transformable species and/or strains will be identified over time.

Transduction transfers bacterial DNA from one bacterium to another via bacteriophage. The phage infects a bacterial cell, and during normal replication some of the host chromosomal or small plasmid DNA is packaged in the phage head, which is then released. When this phage head infects the next bacterial host, it releases the bacterial DNA that may then become part of the new host's genome. Transduction is limited to closely related strains that share common phage. *Staphylococcus aureus* plasmids, carrying a variety of different antibiotic resistance genes, and perhaps some of the *mecA* elements that code for an alternative PBP and confer resistance to all staphylococcal β-lactams, are thought to be spread between strains by transduction. More recently the *mef*(A)/*tet*(O) element has been shown to be transferred by phage among group A streptococci (Di Luca et al., 2010).

7.4 CONJUGATION

Conjugative gene exchange is the main process of horizontal gene transfer between bacteria strains, species, and genera. It is a key element in bacterial evolution and the

spread of antibiotic resistance genes within and between different ecosystems and their associated bacteria. Conjugation is primarily responsible for the lateral transfer of most antibiotic resistance genes. Conjugation can lead to a rapid dissemination of antibiotic resistance genes within and between bacterial communities and between different ecosystems.

Antibiotic resistance in most bacteria is due to the acquisition of new genes via conjugal transfer, which provides new proteins to the host bacterium. Conjugation allows DNA to be directly transferred from one living bacterium to another by direct cell-to-cell contact. Conjugation can occur between the same or different strains, species, and/or genera of bacteria, and it is clear that once a gene is in a bacterial community it can be spread to many unrelated ecosystems. Conjugation can also occur between bacteria and eukaryotic cells (Waters, 2001).

The *tet* genes listed in Table 7.1 are associated with conjugative, nonconjugative, and moblizable plasmids, transposons, and conjugative transposons. It is currently assumed that mobility and type of element that a specific *tet* gene is associated with directly influences the *tet* genes host range and ability to spread to new genera and multiple ecosystems. Yet some *tet* genes [such as the *tet*(E) gene, which is not associated with mobile elements] are found in multiple genera from a variety of different ecosystems (Chopra and Roberts, 2001; Roberts, 1997; see below). One advantage of all these elements, regardless of whether we can demonstrate mobility under laboratory conditions, is that it allows the *tet* genes to be linked with a large number of different antibiotic resistance genes and other types of genes and may keep them in bacterial communities even without selective pressure as occurred with the *tet*(X) gene, which is not functional in its *Bacteroides* spp. hosts (see below).

TABLE 7.1 Mechanism of Resistance of *tet* and *otr* Genes

Efflux (27)	Ribosomal Protection (12)	Enzymatic (3)	Unknown[a]
tet(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E)	*tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(32),	*tet*(X)[c]	*tet*(U)
tet(G), *tet*(H), *tet*(J), *tet*(V), *tet*(Y)	*tet*(Q), *tet* (T), *tet*(36)	*tet*(34)	
tet(Z), *tet*(30), *tet*(31), *tet*(33)	*otr*(A), *tetB*(P)[b], *tet*(44), *tet*	*tet*(37)[c]	
tet(35)[d]			
tet(39), *tet*(41)			
tet(K), *tet*(L), *tet*(38)			
tetA(P), *tet*(40), *tet*(42), *tet*(43)			
otr(B), *otr*(C), *tcr3*			

[a] *tet* (U) has been sequenced but does not appear to be related to either efflux or ribosomal protection proteins.

[b] *tetB*(P) is not found alone and *tetA*(P) and *tetB*(P) are counted as one operon.

[c] *tet*(X) and *tet*(37) are unrelated but both are NADP-requiring oxidoreductases.

[d] Not related to other *tet* efflux genes.

7.5 MOBILE ELEMENTS

Mobile elements include plasmids, conjugative transposons, transposons, and integrons. All of these elements have been involved in lateral gene exchange between strains, species, genera, from one ecosystem to another, and between humans and the environment and vice versa. These elements are able to carry a variety of different antibiotic resistance genes, as well as genes that confer resistance to disinfectants, heavy metals, genes that produce toxins, virulence factors, and/or genes for using alternative energy sources. All of the genes carried on one of these elements may influence the carriage of the entire mobile unit and/or help maintain the genes in the bacterial population as has occurred with the *tet*(X) gene in *Bacteroides* (Speer et al., 1991). Because the various *tet* genes are found in all of these different genetic elements (except for integrons), they have been linked to a variety of other antibiotic-resistant genes, genes coding for heavy-metal resistance as well as specific mobile elements. A representative list of genes linked to various *tet* genes are provided in Table 7.2. Only representative closely linked genes have been included in the table, though not every linkage described in the literature is represented. It is likely that new mobile elements will continue to be described over time allowing different *tet* genes to be linked with other resistance.

Linkage between antibiotic resistance genes on mobile elements may be a mechanism for nonfunctional genes to become stable in the bacterial population without selective pressure. A good example of this is the presence of the *tet*(X), gene, which codes for an enzyme that breaks down tetracycline in the presence of oxygen but is nonfunctional in its *Bacteroides* host. It is thought to have entered linked to the *erm*(F) gene, which codes for macrolide-lincosamide-streptogramin B and is functional in anaerobes and beneficial in protecting the bacterial host in the presence of clindamycin therapy (Speer et al., 1991). This may also explain why other antibiotic resistance genes that code for antibiotics that are no longer or rarely used (such as aminoglycosides and/or chloramphenicol) are still found in the bacterial populations. Brief descriptions of the major mobile elements that carry antibiotic resistance gene are provided below. For a more in-depth information on all types of transposable genetic elements see the review by Roberts et al. (2008).

7.5.1 Plasmids

Plasmids were first described the 1950s and are mostly circular DNA molecules that range in size and can replicate independently of the bacterial chromosome. Watanabe (1963) linked the presence of these mobile elements to antibiotic resistance genes. Plasmids can integrate into the chromosome as illustrated by the integration of the staphylococcal plasmid pT181 carrying the *tet*(K) gene within the SSC*mecA* type III 67 kb element (Grimdmann et al., 2006). Plasmids may include transposons and/or integrons and carry a variety of different antibiotic and/or heavy-metal resistance genes, genes that code for toxins, genes that code for the degradation of various compounds as alternative carbon sources, and/or genes that code for other virulence factors. All the genes within a mobile element normally move as a unit from bacterium to bacterium (Chopra and Roberts, 2001). In general, the 27 *tet* genes that code for efflux proteins are associated with plasmids. The first Tc^r bacteria were identified in Japan and carried conjugative plasmids with multiple resistance genes (Watanabe, 1963).

TABLE 7.2 Distribution of *tet* Resistance Genes Among Gram-Negative and Gram-Positive Bacteria, *Mycobacterium*, *Mycoplasma*, *Nocardia*, *Streptomyces*, and *Ureaplasma*

One Gene		Two or More Genes	
Efflux			
Gram-Negative			
***n* = 13**		***n* = 14**	
Aggregatibacter	*tet*(B)	*Alcaligenes*	*tet*(A)(E)(39)
Agrobacterium	*tet*(30)	*Bordetella*	*tet*(A)(C)
Alteromoas	*tet*(D)	*Brevundimonsa*	*tet*(B)(G)
Brevundimonas	*tet*(39)	*Halomonas*	*tet*(C)(D)
Chlamydia	*tet*(C)	*Mannheimia*	*tet*(B)(G)(H)(L)
Chryseobacterium	*tet*(A)	*Morganella*	*tet*(D)(J)(L)
Erwinia	*tet*(B)	*Moraxella*	*tet*(B)(H)
Francisella	*tet*(C)	*Ochrobactrum*	*tet*(G)(L)
Histophilus	*tet*(H)	*Plesiomonas*	*tet*(A)(B)(D)
Laribacter	*tet*(A)	*Roseobacter*	*tet*(B)(C)(E)(G)
Rahnella	*tet*(L)	*Salmonella*	*tet*(A)(B)(C)(D)(G)(L)
Sporosarcins	*tet*(L)	*Stenotrophomonas*	*tet*(35)(39)
Treponema	*tet*(B)	*Variovorax*	*tet*(A)(L)
		Yersinia	*tet*(B)(D)
Gram-positive			
***n* = 8**		***n* = 1**	
Cellulosimicrobium	*tet*(39)	*Nocardia*	*tet*(K)(L)
Geobacillus	*tet*(L)		
Lysinibacillus	*tet*(39)		
Micrococcus	*tet*(42)		
Oceanobacillus	*tet*(L)		
Pediococcus	*tet*(L)		
Vagococcus	*tet*(L)		
Virgibacillus	*tet*(L)		
Ribosomal Protection and/or Efflux/Enzymatic			
Gram-negative			
***n* = 12**			***n* = 37**
Acidaminococcus	*tet*(W)	*Acinetobacter*	*tet*(A)(B)(G)(H)(L)(M)(39)
Brachybacterium	*tet*(M)	*Actinobacillus*	*tet*(B)(H)(L)(O)
Eikenella	*tet*(M)	*Aeromonas*	*tet*(A)(B)(C)(D)(E)(M)(Y)(31)
Capnocytophaga	*tet*(Q)	*Anaerovibrio*	*tet*(O)(Q)
Chryseobacterium	*tet*(A)	*Bacteroides*	*tet*(M)(Q)(W)(X)(36)
Hafnia	*tet*(M)	*Butyrivibrio*	*tet*(O)(W)
Kingella	*tet*(M)	*Campylobacter*	*tet*(O)(44)
Lawsonia	*tet*(M)	*Citrobacter*	*tet*(A)(B)(C)(D)(L)(M)(O)(S)(W)
Pseudoalteromonas	*tet*(M)	*Edwardsiella*	*tet*(A)(D)(M)
Ralstonia	*tet*(M)	*Enterobacter*	*tet*(A)(B)(C)(D)(G)(L)(M)(39)
Rhanela	*tet*(M)	*Escherichia*	*tet*(A)(B)(C)(D)(E)(G)(L)(M)(W)(Y)
Spingobacterium	*tet*(X)	*Flavobacterium*	*tet*(A)(E)(L)(M)
		Fusobacterium	*tet*(G)(L)(M)(O)(Q)(W)
		Gallibacterium	*tet*(B)(H)(K)(L)(31)
		Haemophilus	*tet*(B)(K)(M)

One Gene		Two or More Genes	
Ribosomal Protection and/or Efflux/Enzymatic			
Gram-negative			
***n* = 12**		***n* = 37**	
		Klebsiella	*tet*(A)(B)(C)(D)(M)(S)(W)
		Kurthia	*tet*(L)(M)
		Megasphaera	*tet*(O)(W)
		Mitsuokella	*tet*(Q)(W)
		Neisseria	*tet*(B)(M)(O)(Q)(W)
		Pantoea	*tet*(B)(M)
		Paenibacillus	*tet*(M)(L)(42)
		Pasteurella	*tet*(B)(D)(H)(G)(L)(M)(O)
		Porphyromonas	*tet*(Q)(W)
		Prevotella	*tet*(M)(Q)(W)
		Providencia	*tet*(B)(E)(G)(M)[j](39)
		Photobacterium	*tet*(B)(D)(M)(Y)
		Pseudomonas	*tet*(A)(B)(C)(E)(G)(M)(34)(L)(X)(42)
		Psychrobacter	*tet*(H)[l](M)(O)
		Proteus	*tet*(A)(B)(C)(E)(G)(L)(J)(M)
		Selenomonas	*tet*(M)(Q)(W)
		Serratia	*tet*(A)(B)(C)(E)(M)(34)(41)
		Shewanella	*tet*(D)(G)(M)
		Shigella	*tet*(A)(B)(C)(D)(M)
		Subdolgranulum	*tet*(Q)(W)
		Veillonella	*tet*(A)(L)(M)(S)(Q)(W)
		Vibrio	*tet*(A)(B)(C)(D)(E)(G)(M)(34)(35)
Gram-Positive/Cell-Wall-Free/Others			
***n* = 15**		***n* = 23**	
Abiotrophia	*tet*(M)	*Actinomyces*	*tet*(L)(M)(W)
Afipia	*tet*(M)	*Aerococcus*	*tet*(M)(O)
Anaerococcus	*tet*(M)	*Arthrobacter*	*tet*(M)(33)
Arcanobacterium	*tet*(W)	*Bacillus*	*tet*(K)(L)(M)(W)(39)(42) *otr*(A)
Amycolatopsis	*tet*(M)	*Bifidobacterium*	*tet*(L)(M)(O)(W)
Bacterionema	*tet*(M)	*Clostridium*	*tet*(K)(L)(M)(O)(P)(Q)(W)(36)(40)(44)
Brachybacterium	*tet*(M)	*Corynebacterium*	*tet*(M)(Z)(33)
Catenibacterium	*tet*(M)	*Enterococcus*	*tet*(K)(L)(M)(O)(S)(T)(U)
Erysipelothrix	*tet*(M)	*Eubacterium*	*tet*(K)(M)(O)(Q)(32)
Granulicatella	*tet*(M)	*Gardnerella*	*tet*(M)(Q)
Finegoldia	*tet*(M)	*Gemella*	*tet*(M)(O)
Mycoplasma	*tet*(M)	*Granulicatella*	*tet*(M)(O)
Roseburia	*tet*(W)	*Lactobacillus*	*tet*(K)(L)(M)(O)(S)(Q)(W)(Z)(36)
Ruminococcus	*tet*(Q)	*Lactococcus*	*tet*(M)(S)
Sporosarcina	*tet*(M)	*Listeria*	*tet*(K)(L)(M)(S)
Ureaplasma	*tet*(M)	*Microbacterium*	*tet*(M)(42)
		Mobiluncus	*tet*(O)(Q)
		Mycobacterium	*tet*(K)(L)(M)(V) *otr*(A)(B)
		Paenibacillus	*tet*(L)(M)(42)
		Peptostreptococcus	*tet*(K)(L)(M)(O)(Q)
		Staphylococcus	*tet*(K)(L)(M)(O)(S)(U)(W)(38)(42)
		Streptococcus	*tet*(K)(L)(M)(O)(Q)(T)(U)(W)(32)
		Streptomyces	*tet*(K)(L)(M)(W) *otr*(A)(B)(C) *tcr3 tet*

Association of *tet* genes with plasmids provides flexibility to be linked with a large number of different genes (Table 7.3). It also enables the linked genes to be added or subtracted over time. Møller et al. (1977) found that patients treated with low oral doses of tetracycline, for acne, had an increase in the number of Tc^r and multidrug-resistant *E. coli*. The level of multidrug-resistant *E. coli* increased from none to 50% of the patients by the fourth week posttreatment. In contrast, many of the 12 *tet* genes that code for ribosomal protection are found on conjugative transposons. This may explain why the *tet*(M) gene is so widespread since conjugative transposons usually have a wider host range and can be transferred to both Gram-positive and Gram-negative bacteria while plasmids have a more restricted host range (see below). Gram-positive *tet* genes are found in both Gram-positive and Gram-negative bacteria while the Gram-negative *tet* genes are not found naturally in Gram-positive bacteria (Table 7.2). This is not unique to the *tet* genes since similar

TABLE 7.3 Tetracycline Antibiotic Resistance Genes Linked to Other Genes or Elements

Gene	Linkage	Phenotype/Element
Efflux		
tet(A)	*bla*$_{TEM}$	β-lactamase
	strA, strB	Streptomycin
	sul2	Sulfamethoxazole
	floR	Florfenicol/chloramphenicol
	SGI1	*Salmonella* genomic island 1
	mer operon	Mercury
	Tn*21,* Tn*1721*	Transposon
tet(B)	*bla*$_{TEM}$	β-lactamase
	catA	Chloramphenicol
	tet(M)	Tetracycline
	strA, strB	Streptomycin
	sul1, *sul2*	Sulfamethoxazole
	mer operon	Mercury
	int1	Class 1 integron
	Tn*10*	Transposon carrying *bla*$_{TEM}$
	SGI1	*Salmonella* genomic island 1
tet(G)	*aadA2*, *aadB*	Aminoglycoside
	dfrA	Trimethoprim
	floR	Florfenicol/chloramphenicol
	sul1	Sulfamethoxazole
	cmlA9	Chloramphenicol
	SGI1	*Salmonella* genomic island 1
	*qacE*Δ*1*	detergent resistance
tet(H)	*sul2*	Sulfamethoxazole
	strA, *strB*	Streptomycin
tet(K)	*mecA*	Methicillin
	dfrK	Trimethoprim
	mer operon	*Mercury*
	pT181	*S. aureus* plasmid
	pI258	*S. aureus* plasmid with *mer* operon

Gene	Linkage	Phenotype/Element
	SCC*mec* element III	One of the characterized *mecA* elements
	Tn*554*	Transposon carrying *erm*(A) [MLS_B]
tet(L)	*dfrK*	Trimethoprim
tet(33)	*aadA9*	Aminoglycoside
	IS*6100*	Insertion sequence
tet(40)	*tet*(O/32/O)	Tetracycline (mosaic gene)
Ribosomal Protection		
tet(M)	*erm*(B)	MLS_B
	mef(A), *msr*(D)	Macrolide
	aphA-3	Kanamycin
	tet(B)	Tetracycline
	mer operon	Mercury
	Tn*917*	Transposon carrying *erm*(B)
	Tn*916*-Tn*1545*	Transposon family
tet(O)	*mef*(A), *msr*(D)	Macrolide
tet(Q)	*erm*(B), (F), (G)	MLS_B
	mef(A), *msr*(D)	Macrolide
	rteABC	excision
	CTnDOT, Tn*4351*, Tn*4400*	*Bacteroides* conjugative transposons
tet(S)	Tn*916S*	transposon
tet(W)	Tn*B1230*	*Bifidobacterium* transposon
	ATE-1,-2,-3	*Arcanobacterium* transposon
Enzymatic		
tet(X)	*erm*(F)	MLS_B

distributions are found with genes conferring antibiotic resistance to other classes of antibiotics such as macrolides (Roberts, 2008).

Plasmids normally carry entry exclusion gene(s), which reduces the host cell ability of acquiring other plasmids with the same origin of replication as the resident plasmid. Thus two plasmids with the same origin of replication are considered incompatible with each other whether they are conjugative or mobilizable (Garcillan-Barcia and De la Cruz, 2008). Incompatibility of plasmids may limit the spread of some of plasmids and their associated *tet* genes between species and/or genera and partially explain the differences in distribution found among the different *tet* genes (Table 7.2). Some *S. aureus* isolates have dealt with plasmid incompatibility by integrating their plasmids into their chromosome where the plasmid incompatibility does not function. This strategy allows the host to acquire multiple plasmids with the same origin of replication (Gillespie et al., 1986). In contrast, plasmids with different origins of replication can coexist within a single cell. Today, single plasmids may carry multiple different *tet* genes, and/or an isolate may have multiple different *tet* genes on different plasmids or some *tet* genes on plasmid(s) and other *tet* genes in the chromosome.

The *tet* plasmids occur in a variety of sizes, from the small 4.45-kb *tet*(K) positive *S. aureus* pT181 plasmid to large plasmids of $\geq$ 300 kb. Many of the large *tet* plasmids are conjugative or capable of mobilization, however, the *tet*(E) gene has

been found on large plasmids ~170 kb, which do not demonstrate these tendencies under laboratory conditions (DePaola and Roberts, 1995; Sorum et al., 1992). However, the *tet*(E) gene has been identified in 10 different genera, many of which are associated with water and/or aquaculture (Table 7.2). How the *tet*(E) gene has spread between bacteria in nature around the world is a mystery and indicates that we cannot yet reproduce all the possible mechanisms of gene exchange that occur in nature under laboratory conditions (DePalo and Roberts 1995; Sorum et al., 1992).

7.5.2 Transposons

Transposons are discrete pieces of DNA that are able to move from one location in the bacterial genome to another location. Transposons are flanked by terminal inverted or direct repeats. Insertion sequences (IS elements) are a family of small elements that range from 768 to 1426 bp in size and flank composite transposons, while unit transposons do not carry insertion sequences. Transposons vary in the genes they carry, including the genes they use for insertion and excision into the bacterial genome, and in the antibiotic resistance genes present or other genes for heavy-metal resistance. As with plasmids, there transposons continue to evolve due to their ability to recombine and exchange their genes with other elements. The typical composite transposon carries a gene that codes for an enzyme, such as a site-specific recombinase or resolvase, which is involved in excision and integration within the host genome and one or more other genes that code for antibiotic resistance and/or other genes that are flanked IS sequences.

The best characterized of the transposons is Tn*10*, which carries the *tet*(B) gene that codes for the most widely distributed Gram-negative tetracycline efflux protein (Table 7.1) (Lawley et al., 2000). The Tn*10* transposon is most frequently associated with Gram-negative plasmids but can also be found in the chromosome of Gram-negative bacteria such as *H. influenzae* (Marshall et al., 1984). Transposons are found on plasmids and multiple copies may be distributed throughout the bacterial chromosome. The ability to be on the chromosome and/or plasmids allows flexibility as well as stability for these genes. Insertion of a transposon within the genome may lead to mutations, the loss of gene function, or modification of gene expression.

7.5.3 Conjugative Transposons [CTns]

Conjugative transposons are self-transmissible integrating elements that can have broad host ranges and carry all the genes required to move from one bacterial cell to another by cell-to-cell contact. Conjugative transposons have fewer restrictions in moving between unrelated bacteria than do plasmids because they lack incompatibility exclusion systems. As a result, multiple copies of the same or related conjugative transposons can be found within a single bacterium (Norgren and Scott, 1991). The first conjugative transposons were identified in the 1980s and were members of the Tn*916*–Tn*1545* transposons family, which normally carries the *tet*(M) gene coding for a ribosomal protection gene. This family is the most promiscuous of the conjugative transposons described and is one of the best characterized. The Tn*916*–Tn*1545* transposons family integrates site specifically in some species and relatively nonspecifically in other species (Rice, 2007). The Tn*916* transposon is 18 kb and has relatively few restriction sites. Some investigators have

suggested that the surrounding flanking regions represent ancient structures evolved for broad-host range transfer, and the presence of the *tet*(M) gene in the Tn*916* transposon is relatively recent in the evolutionary progression of this element (Rice, 2007). Low-dose exposure to tetracycline promotes the conjugal transfer of these transposons to neighboring bacterial cells (Facinelli et al., 1993). The Tn*916* can also mobilize co-resident plasmids.

The Tn*916*–Tn*1545* conjugative transposons are adaptable and able to form composite elements by integration of one transposon within another transposon. Both transposons encode for their own transfer, and the complete composite element may be transferred to another bacterium, or the embedded transposon can be transferred separately. Composite transposons may have multiple mobile elements, various types of IS elements as well as regions from plasmids and genes from different genera of bacteria. For example, the 65-kb composite Tn*5385* transposon carries resistance genes for penicillin, erythromycin, gentamicin, streptomycin, tetracycline, and mercury and has genetic elements related to those found in three different transposons isolated from enterococci, streptococci, and staphylococci (Rice, 2007).

New conjugative elements carrying *tet* genes continue to evolve as illustrated by the recent characterization of an $\sim$ 60-kb conjugative transposon were the *tet*(O) gene was linked to an efflux *mef*(A)-*msr*(D) conjugative element (Giovanetti et al., 2003). In recent years, these transposons have acquired an increasing number of different antibiotic resistance genes and genes for heavy-metal resistance (Table 7.3) (Lancaster et al., 2004; Soge et al., 2008; Soge et al., 2009). The *tet*(S) gene has been identified on a Tn*916*-like element Tn*916*S in a single strain of *Streptococcus intermedius* (Roberts and Mullany, 2009). The *tet*(W) genes are associated with two different transposons, one of which is found in anaerobes such as *Bifidobacterium* and in aerobic *Arcanobacterium pyogenes* (Billington et al., 2002; Billington and Jost, 2006). *Bacteroides* spp. have conjugative transposons ranging in size from 65 to $>$ 150 kb. One of the best studied is the 65-kb CTnDOT, which is 65 kb [carrying the *tet*(Q) gene and normally linked to the *erm*(F) gene] that codes for a rRNA methylase and confers resistance to macrolides, lincosamides, and streptogramin B (Table 7.3). In the presence of tetracycline, frequency of conjugal transfer increases and the CTnDOT is able to mobilize co-resident nonconjugative transposons, mobilizable plasmids, and unlinked integrated nonreplicating *Bacteroides* units (NBUs). CTnDOT-like elements have been identified in a variety of different Gram-negative and Gram-positive genera (Chung et al., 1999).

7.5.4 Integrons

Integrons are two-component systems that include an integrase (*int1*) and an *attI* sequence, which is the site for integration of cassettes containing different antibiotic, heavy-metal, and/or disinfectant resistance genes (Recchia and Hall, 1995; Vo et al., 2007). Integrons are found as part of plasmids, chromosomes, and transposons. The *tet* genes are not normally part of integrons but can be found between integrons and were part of the recently characterized mosaic genomic 86-kb chromosomal antibiotic resistance island, which includes integrons and nonintegron regions carried in the *Acinetobacter baumannii* strain AYE genome (Fournier et al., 2006). This element carried a large number of different genes coding for antibiotic and

heavy-metal resistance and two copies of the *tet*(A) gene. The homologous region in the antibiotic-susceptible *A. baumannii* has transposases but no antibiotic resistance genes, suggesting that this structure was present prior to acquisition of the integrons and resistance genes. At this time, it is unknown if the 86-kb element can be moved as a single unit between strains, but data from Adams et al. (2010) demonstrated variability in the composition of the resistance island between various *A. baumannii* strains.

7.5.5 Tetracycline Resistance Genes

Tetracyclines are one of the oldest classes of antibiotics used and the first broad-spectrum class of antibiotics. Tetracyclines interact with the bacterial ribosomes by reversibly attaching to the ribosome that blocks protein synthesis. Tetracyclines are active against a wide range of Gram-positive, Gram-negative, anaerobic, and aerobic bacteria, cell-wall-free microbes, intercellular bacteria, and protozoan parasites. Tetracyclines are relatively safe, and the older compounds are inexpensive and have been widely used in clinical, veterinary, and agricultural purposes for 60 years (Roberts, 2005). For this chapter, Gram-positive bacteria will also include cell-wall-free *Mycoplasma*, *Ureaplasma*, as well as *Mycobacterium*, *Nocardia,* and *Streptomyces* (Table 7.2). The first Tc^r bacteria were identified in isolates from the 1950s (Watanabe, 1963). Bacteria may become resistant to tetracyclines, by mutation, while the majority of bacteria become tetracycline resistant because they acquire new genes that (a) pump tetracycline out the cell (efflux); (b) protect the ribosome from the action of tetracyclines; or (c) enzymatically deactivate tetracyclines (Table 7.1).

The modern era for tetracycline resistance genes began with the study by Mendez et al. (1980) where the heterogeneity among the Tc^r bacteria was described and the first evidence for distinctive tetracycline resistant (*tet*) genes was identified in Gram-negative bacteria. Different types of *tet* genes were grouped together by the definition of having $\leq$79% amino acid identity with all previous characterized *tet* genes because, at the time, this was the level of discrimination that could be achieved with the technology of the day. Two genes were considered part of the same type/class and given the same gene designation when they shared at least 80% amino acid sequence identity over the entire length of the protein.

The nomenclature system proposed in 1999 (Levy et al., 1999) is still followed today. For a *tet* gene to be assigned a new designation, it must be completely sequenced and its amino acid composition compared with all currently known classes of *tet* genes. It is then submitted to the *tet* nomenclature clearing house run by Dr. Stuart Levy and Ms. McMurry in Boston, which will assign a new *tet* resistance gene name. Before a potential gene can be given a new designation, experiments to demonstrate that the gene confers Tc^r are required. It is inadequate to demonstrate that a gene sequence has characteristics in common with previously characterized *tet*/*otr* genes. Thus, simple data mining of genomes that look for DNA and amino acid similarities to known *tet*/*otr* genes is not adequate for recognition. The "new" gene must be shown to confer tetracycline resistance to the bacterial host before it can be recognized as a valid tetracycline resistance gene. Obtaining the new *tet* gene name should be done prior to submitting the sequence to GenBank or a manuscript for publication (Levy et al., 1999). A website (http://faculty.washington.edu/marilynr/) with the characterized and archived tetracycline resistance genes was established and

TABLE 7.4 Tetracycline Resistance Genes Unique to Environmental Bacteria[a]

Efflux 12/27 (44%)	Ribosomal Protection 3/12 (25%)	Enzymatic 2/3 (66%)
tetA(P), *tet*(V), *tet*(30), *tet*(35), *tet*(33), *tet*(39), *tet*(41), *tet*(42), *tet*(43), *otr*(B), *otr*(C), *tcr3*	*tetB*(P), *otr*(A), *tet*	*tet*(X)[b], *tet*(34)

[a]Species and genera which are primarily found outside of humans and animals rather than in or on animals or humans, even if on occasion they cause infections.

[b]The *tet*(X) is only functional in environmental (aerobic) *Spingobacterium* spp. though it is also found in (anaerobic) *Bacteroides* spp.

provides tables that list the mechanisms and distribution of the *tet*/*otr* genes among different Gram-negative and Gram-positive genera and representative GenBank numbers for each sequenced *tet*/*otr* gene. The information on the website and the tables provided in this chapter are taken from the work of Dr. Roberts' laboratory as well as from published articles and abstracts presented at scientific meetings. From the latter two sources, the data may not have been independently verified. The Gram-negative *tet* genes are found exclusively in Gram-negative bacteria, the Gram-positive *tet* genes may be carried by Gram-negative and *Streptomyces*, while the *otr* and Gram-positive *tet* genes are found in *Mycobacterium,* and *Streptomyces* (Table 7.2).

There are 43 different *tet*/*otr* genes listed in Table 7.1 that confer resistance to tetracyclines. The *otr* genes were originally found in antibiotic-producing *Streptomyces*, and their designation has not been changed although the *otr* genes are now found in *Mycobacterium* spp. (Table 7.2). The *otr* and *tcr* genes are unique to environmental bacteria (Table 7.4). Twenty-seven genes code for energy-dependent efflux proteins, 12 genes code for ribosomal protection proteins, 3 genes that code for inactivating enzymes, and 1 gene has an unknown mechanism of resistance (Table 7.1).

7.6 EFFLUX

The first tetracycline-resistant efflux proteins were identified in the 1950s in Japan where they were later hypothesized to be located on conjugative plasmids (Watanaba, 1963). Today there are 27 genetically distinct efflux genes characterized coding for drug-H^+ energy-dependent transmembrane sequence (TMS) proteins that span the lipid bilayer of the inner cell membrane 9–14 times. These proteins have been divided into 7 different groups based on the number of TMS present (9–14), the G+C% (guanine–cytosine) of the gene, and similarities to other *tet* efflux genes (Thaker et al., 2010). These efflux proteins normally export tetracycline and doxycycline but not minocycline or tigecycline (a newer glycylcycline) out of the cell. The one exception is the Gram-negative *tet*(B) gene that exports tetracycline, doxycycline, and minocycline and confers resistance in the host bacterium to all three tetracyclines. The efflux proteins are able to reduce intracellular concentrations of tetracycline that allows the majority of the ribosomes to continue to function and the bacterium to grow in the presence of tetracycline. The efflux proteins work by exchanging a proton for the tetracycline–cation complex against a concentration

gradient and require intact cells to function. The efflux genes are the most commonly found *tet* genes in aerobic and facultative Gram-negative bacteria (Table 7.2). Twelve (41%) of the efflux genes [*tetA*(P), *tet*(V), *tet*(Z), *tet*(30), *tet*(33), *tet*(35), *tet*(39), *tet*(41), *tet*(42), *otr*(B), *otr*(C), *tcr*] are unique to environmental bacteria (Table 7.4). Eight of the efflux genes have been shown to be linked to other genes (Table 7.3). This is likely an underestimation, and over time more linkages with different *tet* efflux genes are likely to be identified. Eight of the efflux genes have been linked to other genes, transposons, and/or specific plasmids, although more linkages should be identified in the future as elements continue to exchange DNA (Table 7.3).

Upstream of the structural efflux gene is a divergently transcribed repressor gene that produces a protein that binds to the palindromic operator in the promoters for both the repressor and structural *tet* gene resulting in prevention of the initiation of transcription. At $\sim$1 nM tetracycline, a tetracycline-divalent cation complex interacts with the repressor protein, releasing it from the DNA, and thus transcription of both genes occurs. A more detailed description of mechanism of tetracycline resistance due to the efflux proteins can be found in Palm et al. (2008).

Tetracycline-resistant genes coding for efflux proteins are the most commonly found *tet* genes among Gram-negative aerobic and facultative bacteria. Fifty-five Gram-negative and 25 Gram-positive genera carry these genes (Table 7.2). Of the 76 Gram-negative genera known to carry tetracycline resistance genes, 27 (36%) of these genera carry only efflux genes, of which 13 carry a single efflux gene and 14 carry multiple efflux genes. Of the 47 Gram-positive genera, only 9 (19%) carry efflux genes with 8 carrying a single efflux gene and *Nocardia* carrying 2 efflux genes (Table 7.2). The *tet*(B) gene is the most common efflux gene among Gram-negative genera and has been identified in 31 genera, while the *tet*(A) gene is found in 20, *tet*(C) gene in 10, *tet*(D) gene in 16, *tet*(E) gene in 10, *tet*(G) gene in 13, the *tet*(H) gene in 8, and the *tet*(35) in two Gram-negative genera. The *tet*(K) gene is found in 12 Gram-positive genera and the *otr*(B) gene is found in *Mycobacterium* and *Streptomyces*. The *tet*(L) gene is found in 14 Gram-negative and 19 Gram-positive genera, the *tet*(39) gene is found in 4 Gram-negative and 3 Gram-positive genera, while the *tet*(42) gene is found in 4 Gram-positive and 2 Gram-negative genera (Table 7.2). Twelve (44%) of the efflux genes including the *tet*(J), *tetA*(P) *tet*(V), *tet*(Y), *tet*(Z), *tet*(30), *tet*(31), *otr*(C), *tcr, tet*(33), *tet*(40), and *tet*(41) are found in a single genera (Table 7.2). The *tet*(43) gene has been isolated from metagenomic DNA library and has yet to be identified in a specific species or genus (see below).

The *tet*(C) gene is usually associated with plasmids, though it is located in the chromosome in Tc^r *Chlamydia suis*, an obligate intracellular bacteria from the intestinal tract of pigs in Europe and the United States (Suchland et al., 2009). The Tc^r *C. suis* isolates contained a13-kb segment of foreign DNA including a truncated repressor gene, *tetR*(C), and a functional *tet*(C) gene with a 13-kb region that has a high degree of identity with a pRAS3.2 plasmid from *Aeromonas salmonicida*, a microbe that does not grow in pigs (L'Abbe-Lund and Sorum, 2002). This is the first report of a known acquired *tet* gene in an obligate intracellular bacterium and the degree to which the *tet* genes have spread through the bacterial populations and different ecosystems.

Prior to this work, it had been hypothesized that intracellular bacteria such as *Chlamydia* spp. were unlikely to participate in conjugation with other genera because of the requirement for actively growing donor and recipient bacteria to occupy the

same eukaryotic cell. Therefore, the only way an obligate intracellular *Chlamydia* could have acquired the *tet*(C) gene is to assume that another bacteria carrying the 13-kb region with the *tet*(C) gene co-infected the same eukaryotic cell as did the *C. suis*. *A. salmonicida* has an optimal growth temperature below 20°C, and since the *C. suis* and *A. salmonicida* have different optimal growth temperatures, it is highly unlikely that the *A. salmonicida* was directly involved in the genetic transfer of the *tet* (C) gene to the *C. suis*. Dugan et al. (2004) suggested that the *tet*(C) gene and surrounding sequences may be on a mobilizable element that was transferred from *A. salmonicida* into one or more other bacterial species, which were able to exist within the pig intestinal tract, and was the donor of the DNA into the *C. suis* within the pig. Suchland et al. (2009) have demonstrated that co-infection between Tc^r *C. suis* and susceptible *Chlamydia trachomatis* resulted in Tc^r *C. trachomatis*, which contain the 13-kb*tet*(C) region inserted within the ribosomal cluster of the chromosome. These experiments provide support for the hypothesis that the 13-kb region is a mobile element. The presence of *tet*(C)-positive Tc^r *C. suis* represens the first example of horizontal transfer of an antibiotic resistance gene by an obligate intracellular bacteria and also represents the first time a *tet* gene has been moved from one intracellular bacteria to another. It is likely that other *tet* genes and different antibiotic resistance genes may be introduced and become established in other obligate intracellular bacteria.

The most recently identified efflux gene, the *tet*(43) gene was isolated from metagenomic DNA, and which species or genus carries this gene remains unknown; thus it is not listed in Table 7.2.

7.7 RIBOSOMAL PROTECTION

Twelve ribosomal protection genes have been characterized, of which three (25%) [*tetB*(P), *otr*(A), *tet*] are unique to environmental bacteria (Tables 7.1 and 7.4). The genes have been divided into three base groups related to their amino acid sequences rather than G+C% content as is done with the efflux genes (Thaker et al., 2010). The ribosomal protection genes code for cytoplasmic proteins of $\sim$ 72.5 kDa in size that protect the ribosomes from the action of tetracycline in vitro and in vivo. Unlike the efflux genes, the ribosomal protection genes confer resistance to tetracycline, doxycycline, and minocycline but not tigecycline (Roberts, 2005). The proteins have sequence similarity to the ribosomal elongation factors, EF-G and EF-TU, and are grouped in the translation factor super family of GTPase (Leipe et al, 2002). A model based on Tet(O)-mediated Tc^r biochemical and structural data for both Tet (M) and Tet(O) proteins has been proposed. In this model, the ribosomes without tetracycline function normally, and, when tetracycline is added to the growth media, it binds to the ribosomes, altering their conformational state, which interrupts the elongation cycle and protein synthesis stops. The ribosomal protection proteins are thought to interact with the base of the h34 ribosomal protein, causing allosteric disruption of the primary tetracycline binding site(s), which releases the bound tetracycline. The ribosome returns to its normal conformational state and resumes protein synthesis. What is not clear is whether the *tet* ribosomal proteins actively prevent tetracycline from rebinding once it has been released or if the released tetracycline is able to rebind to the same or a different ribosome. Further details can

be found in other publications (Connell et al., 2003a, b). It has been assumed that all 12 ribosomal protection proteins in this group have similar mechanisms of action.

The majority of Gram-positive (82%) and Gram-negative (64%) genera carry either ribosomal protection genes alone or in combination with efflux/enzymatic genes as illustrated in Table 7.2. The ribosomal protection genes predominate in Tc^r oral, anaerobic, and urogenital Gram-negative bacteria while they are less common among enteric Gram-negative bacteria. The *tet*(M) gene had been identified in clinical *Enterococcus* spp. isolated between 1954 and 1955, which is approximately the same time as the first Gram-negative *tet* efflux genes were identified; however, the early *Enterococcus* spp. study was not published until 1997, although the Gram-negative studies were published 30 years earlier (Atkinson et al., 1997; Watanabe, 1963). Thus both the *tet* efflux and *tet* ribosomal protection genes have been in the bacterial population for >50 years.

Forty-nine Gram-negative genera have been characterized that carry at least one ribosomal protection *tet* gene(s). Of these, 12 (24%) Gram-negative genera carry a single ribosomal protection gene, while the remaining genera carry multiple or ribosomal protection or efflux and ribosomal protection genes (Table 7.2). Thirty-eight Gram-positive genera carry ribosomal protection genes, of which 15 carry a single gene and 23 carry one or more ribosomal protection and/or both ribosomal protection and efflux *tet* genes (Table 7.2). The *tet*(M), *tet*(O), *tet*(Q), *tet*(S), *tet*(W), and *tet*(36) ribosomal protection genes are found in both Gram-positive and Gram-negative genera (Table 7.2). The *tet*(T), *tet*(32), and *tet*(44) genes have been found in 2 Gram-positive genera each, the *otr*(A) gene was found in 3 Gram-positive genera, the *tet* gene found in *Streptomyces,* and the *tetB*(P) found in one Gram-positive genera (Table 7.2).

Nine of the ribosomal protection genes have a G+C% ranging between 30 and 40% and are thought to be of Gram-positive origin, while the *tet*(W) gene has a G+C % between 50 and 55% and its origin is unclear. The *Streptomycetes tet* and *otr*(A) genes have G+C% ranging from 68–78% and their origin is thought to be *Streptomycetes*. Despite the difference in G+C%, the Tet(M) protein shares 68–72% amino acid identity with the Tet(O), Tet(S), Tet(W), Tet(32), and Tet(36) proteins are grouped together. The Tet(Q) proteins share 60% amino acid identity and the Tet(T) is grouped together, while the *tetB*(P), *otr*(A), and *tet* are grouped together and are unique to environmental bacteria (Table 7.4) (Thaker et al., 2010).

The *tet*(32) gene is found in *Eubacterium* and *Streptococcus* and from the oral metagenome of Northern European children (Lancaster *et al.,* 2005; Warburton et al., 2009). The *tet*(36) gene has been identified in Gram-negative *Bacteroides* and Gram-positive *Clostridium* and *Lactobacillus* spp., as well as from metagenomic DNA isolated from manure pits (Whittle et al., 2003). The *tet*(T) gene has been associated with *Enterococcus* and *Streptococcus*, while the *tet* and *otr*(A) genes are found in *Streptomycetes* and *Mycobacterium* or *Streptomycetes*, respectively. The *tet*(W) gene has been identified in 15 Gram-negative and 10 Gram-positive genera and is the only *tet* gene identified in Gram-positive *Arcanobacterium* and *Roseburia* and Gram-negative *Acidaminococcus* (Table 7.2). The *tet*(Q) gene is found in 12 Gram-negative and 10 Gram-positive genera. It is the only *tet* gene identified in Gram-negative *Capnocytophaga* and Gram-positive *Ruminococcus*. The *tet*(O) gene is the only *tet* gene currently found in Gram-negative *Campylobacter* but is also found

in 7 other Gram-negative and 12 Gram-positive genera. The *tet*(S) gene is found in 4 Gram-positive and 3 Gram-negative genera. The *tet*(M), *tet*(Q), and *tet*(W) are usually associated with conjugative transposons, while the *tet*(O) and *tet*(S) genes have commonly been associated with conjugative and nonconjugative plasmids.

There have been more papers written about the *tet*(M) gene than any other ribosomal protection *tet* gene. The *tet*(M) gene is commonly found in oral, urogenital, aerobic, and anaerobic Gram-positive and Gram-negative nonenteric bacteria, while it is less common in enteric genera (Table 7.2). The *tet*(M) positive bacteria have been isolated from a variety of different species across the bacterial spectrum and from multiple, different ecosystems. Some variability at the base pair level is found, and different *tet*(M) genes may have a variation of their base pairs of at least 11%. The *tet*(M) gene has been identified in 36 Gram-negative genera and in 8 of these genera, it is the only *tet* gene found. In 35 Gram-positive genera, it is the only *tet* gene identified in 13 of these genera (Table 7.2).

The *tet*(M) gene is part of a conjugative transposon that is often in the bacterial chromosome. In some *Clostridium perfringens* isolates, the *tet*(M) gene is found in the chromosome on an incomplete element and cannot move, while in other *C. perfringens* isolates the *tet*(M) gene is on complete transposons and can be conjugally transferred between isolates (Soge et al., 2008). One exception of the chromosomal location for the *tet*(M) gene was found in the genus *Neisseria*. In *N. gonorrhoeae,* the *tet*(M) gene was located on 25.2 MDa conjugative plasmids with the first *tet*(M) positive *N. gonorrhoeae* collected in 1983. These same *tet*(M)-positive 25.2-MDa conjugative plasmids have since been found in Tc^r *N. meningitidis, Kingella denitrificans*, and *Eikenella corrodens* strains (Roberts and Knapp, 1988a, b). The *tet*(M) plasmids confer high levels of Tc^r (minimal inhibitory concentration $\geq 16\,\mu g/mL$) and are closely related to the 24.5-MDa indigenous *N. gonorrhoeae* conjugative plasmids (Roberts, 1989). Interestingly, the Tc^r plasmids have a wider host range than the ancestral 24.5-MDa *N. gonorrhoeae* conjugative plasmid but, like the ancestral plasmid, were able to facilitate the transfer of the small mobilizable gonococcal β-lactamase plasmids from one bacterium to another (Roberts, 1989; Roberts and Knapp, 1988b). Only part of the *tet*(M) Tn*916*-like transposon is found on the 25.2-MDa Tc^r *Neisseria* plasmids, while the complete *tet*(M) transposon was integrated into a Tc^r *Haemophilus ducreyi* conjugative plasmid (Roberts, 1989). The *tet*(M) gene is also found naturally in commensal *Neisseria* spp. but here the *tet*(M) genes are located in the chromosome. Today, in some geographical locations, such as Asia, nearly 50% of the *N. gonorrhoeae* isolated carry these Tc^r plasmids.

The *tetB*(P) gene has been found only in the genus *Clostridium*. It is unique among the ribosomal protection genes because all isolates that carry this gene also carry a *tetA*(P) gene that codes for an inducible efflux protein. The two genes are transcribed from a single promoter that is located 529 bp upstream of the *tetA*(P) start codon and the *tetB*(P) gene overlaps the *tetA*(P) gene by 17 nucleotides (Johanesen et al., 2001). The *tetA*(P) gene has been found alone where it does confer Tc^r to the bacterial host, while the *tetB*(P) gene has not. When the *tetB*(P) gene was cloned away from the *tetA*(P) gene and introduced into *C. perfringens* (the natural host) and *E. coli* recipients, the resultant transformants had low-level Tc^r. Thus, it is not clear if the *tetB*(P) gene contributes to the natural host's Tc^r phenotype. Both the *tetA*(P) and *tetB*(P) genes are often associated with conjugative and nonconjugative plasmids.

7.8 MOSAIC

Mosaic *tet* genes consist of regions from two known *tet* genes with a descriptive designation such as *tet*(O/W) representing a hybrid between the *tet*(O) at one end and *tet*(W) at the other end of the gene (Stanton and Humphrey, 2003). A *tet*(W/O/W) designation would represent a hybrid between the *tet*(O) and *tet*(W) genes with a partial *tet*(O) sequence between the ends of the *tet*(W) gene. Mosaic genes can only be determined by sequencing the complete gene and at this time, the number of different genera known to have them is very limited. Three different hybrid genes have been sequenced from *Megasphaera elsdenii*, and the amino acids coded by these three genes share 95.8, 89, and 91.9% identity with the Tet W protein with 13–43% of their sequences at the ends of the gene related to *tet*(O) genes. All three of the mosaic genes had G+C% between 50 and 55 similar to that of other *tet*(W) genes. A new name was suggested for designating hybrid genes that coded for proteins made of more than 50 amino acid residues in a single stretch that are from different genes (Levy et al., 2005). The various mosaic genes identified are *tet*(O/W), *tet*(O/W/O), *tet*(O32/O), and *tet*(O/W/32/W/O).

The gene originally designated *tet*(32) from a *Clostridium*-like strain has been sequenced, and from bp 0 to 243 it had 100% identity with the same region in the *tet*(O) genes. The 158-bp noncoding region upstream of the structural gene showed 98% sequence homology with the upstream regions of the *tet*(O) genes from *Streptococcus mutans* and *C. jejuni*, GenBank # M20925 and M18896, respectively. The sequences at the end of the gene (1262 – 1782 bp) had a 98.8% sequence homology with the *tet*(O) gene. However, the sequences between bp 244 and1263 share $< 70\%$ similarity with any other known *tet* gene, and since the overall DNA homology of the gene was $< 80\%$ to the *tet*(O) or any other *tet* gene, it was given a new designation *tet*(O/32/O) (Stanton et al., 2005). The original work of Melville et al. (2001) found by polymerase chain reaction (PCR) that 6 of 9 rumen sheep samples and 8 of 11 pig fecal samples were positive for *tet*(32). However, in light of the more recent data regarding its hybrid nature, it is unclear if these positive samples actually contained the *tet*(O/32/O) sequence or different genes. More recently, mosaic *tet* genes from *Bifidobacterium* and *Lactobacillus* have been identified that combined sequences from *tet*(O), *tet*(W), and *tet*(32) genes (Van Hoek et al., 2008).

7.9 ENZYMATIC

Three genes that code for inactivating enzymes have been identified, *tet*(X) (*Bacteroides*, *Pseudomonas*, *Spingobacterium*), *tet*(34) (*Pseudomonas, Serratia*, *Vibrio*), and *tet*(37) (metagenomic). These three *tet* genes are found only in Gram-negative species. Six of the seven genera that carry one of these inactivating *tet* genes may carry efflux and/or ribosomal protection *tet* genes, thus their contribution to bacterial Tc^r compared to the efflux and ribosomal protection *tet* genes is unclear (Table 7.2). Perhaps as more environmental bacteria are characterized, more genera carrying one of these *tet* genes may be found and/or other inactivating *tet* genes will be identified.

The *tet*(X) gene encodes for a flavin nicotinamide adenine dinucleotide phosphate-dependent monooxygenase that inactivates by regioselectively adding a hydroxyl

group to the C-11a position of the antibiotic. This action requires oxygen and confers resistance to tetracycline, doxycycline, minocycline, and tigecycline (Moore et al., 2005; Yang et al., 2004). Related proteins have been identified in the chromosomes of *Cytophaga* and *Streptomycetes coelicolor* but they do not inactivate tetracycline (Thaker et al., 2010). The *tet*(X) gene was originally found in a strict anaerobe, *Bacteroides* spp., where it was linked to an rRNA methylase gene [(*erm*(F)] that confers resistance to macrolides, lincosamides, and streptogramin B and are part of the conjugative transposons CTnDOT, Tn*4351*, and Tn*4400* (Whittle et al., 2003). Because the TetX protein requires oxygen to degrade the tetracycline, the *tet*(X) gene does not confer Tc^r in *Bacteroides* spp. where it was first identified (Speer et al., 1991). The *erm*(F) and *tet*(X) genes have a G+C% content of 36 and 37%, respectively, suggesting that these genes did not originate in the *Bacteroides* spp. identified for which the G+C% content ranged between 40 and 48%. This suggested that the various transposons were inserted within the *Bacteroides* spp. chromosome where the *tet*(X) gene has been maintained but not expressed while the *erm*(F) gene is functional (Speer et al., 1991). It was previously hypothesized that a functional *tet*(X) gene might be found in an environmental species. This hypothesis has since been proven to be correct (Ghosh and LaPara, 2007; Ghosh et al., 2009), and thus it is listed in Table 7.4 as a unique environmental *tet* gene.

The *tet*(37) gene codes for a second NADP-dependent monooxygenase that is unrelated to the *tet*(X) gene but has a similar G+C% content of 37.9% and shares homology with other flavoproteins, oxidoreductases, and NADP-requiring enzymes (Diaz-Torres et al., 2003). The way this protein inactivates tetracycline is not clear. The *tet*(37) gene has only been cloned from the oral metagenome and no specific bacteria have been identified that carry this gene.

The *tet*(34) gene was first described in *Vibrio* spp. and codes for an enzyme that is similar to a xanthine–guanine phosphoribosyl transferase rather than a NADP-dependent monooxygenase (Nonaka and Suzuki, 2002; Nonaka et al., 2000). The *tet*(34) gene has been found in three Gram-negative genera (*Vibrio*, *Pseudomonas,* and *Serratia*) and is also unique to environmental bacteria (Table 7.4) (Nonaka and Suzuki, 2002; Miranda et al., 2003).

7.10 UNKNOWN

The *tet*(U) gene produces a small protein (105 amino acids) that confers low-level tetracycline resistance (Chopra and Roberts, 2001). The TetU protein has 21% similarity over its length to the TetM protein, but it does not include the consensus GTP-binding sequences, which are thought to be very important for tetracycline resistance in ribosomal protection proteins. The *tet*(U) gene has been identified in a vancomycin- and tetracycline-resistant *S. aureus* strain that did not carry the *tet*(K), *tet*(L), *tet*(M), or *tet*(O) genes. From the same patient, vancomycin-resistant enterococci were cultured that carried both the *tet*(U) and *tet*(L) genes and a few isolates also carried the *tet*(K) and/or *tet*(M) genes (Weigel et al., 2004). The *tet*(U) gene has also been identified in *Enterococcus* spp. The importance of the *tet*(U) gene is unclear since both *Enterococcus* and *Staphylococcus* isolates are able to carry a variety of efflux and ribosomal protection *tet* genes (Table 7.2).

7.11 THE ENVIRONMENT

Among the culturable bacteria, some genera are found only in the environment, while others are found in the environment, in animals, and/or humans. For this discussion, environmental bacteria are defined as those species and genera that are primarily found outside of humans and animals rather than in or on animals or humans even if, on occasion, they cause infections. In addition, some of the *tet* genes have been found in environmental species even though other species within the same genera are associated with animals and/or humans. It has, in the past, been assumed that most environmental bacteria were not well adapted to live in humans or animals. However, this idea is changing as progress in medical science allows severely immunocompromised patients to live in the community. In addition, people who have foreign objects permanently present in their bodies and various means of occupational exposure have provided new opportunities for environmental bacteria to cause disease (Rowlinson et al., 2006; Trujillo and Goodfellow, 2003). Other factors have also made the distinction between environmental and nonenvironmental bacteria and their associated *tet* genes more difficult because the mixing of the two has become increasingly common. This has resulted in very few ecosystems left that have not been touched by the waste associated with the activities of human civilization—whether it is in the polar regions or the Amazon jungle. As a result, there is continual mixing of environmental and nonenvironmental bacteria that provides multiple opportunities for horizontal genetic exchange of antibiotic resistance genes.

Both antibiotics and antibiotic-resistant bacteria are moved by water and wind as well as by transportation of goods and people around the world. One result of this has been the spread of specific strains around the world such as recently identified *Clostridium difficile* NAP1/027/BI (Gould and Limbago, 2010). Originally *C. difficile* was thought to be a nosocomial disease associated with the hospital setting, but today *C. difficile* is considered a food-borne and community pathogen. Similarly, 25 years ago *A. baumannii* was a rare pathogen, and *Acinetobacter* spp. were thought to primarily be found in the environment where it is well adapted to grow at a variety of different temperatures and pH values, use a variety of carbon and energy sources, and persist in both moist and dry places for extended time periods. However, today *A. baumannii* is an opportunistic pathogen that has been of major concern for military trauma patients (Abbo et al., 2005).

Antibiotics are used for both human and agricultural activities for prevention and treatment of infections, as well as food additives and growth promoters in food production in some part of the world and include animal husbandry, aquaculture, fruit crops, and bee-keeping. All of these activities contaminate the environment, which provides selective pressure on the resident environmental bacteria to become antibiotic resistant and in some cases increases transfer of specific *tet* genes (Facinelli et al., 1993). Antibiotics can be found in domestic animal manure, which may be transferred when this manure is applied to fields or stored in lagoons. Antibiotics are sprayed onto crops, which contaminate the surrounding soil, sediment, and groundwater. Antibiotics may be incorporated into the food given to farm animals and fish, which will, in turn, contaminate the surrounding area. Antibiotics from human therapeutic use, especially from hospital effluents, is a continual source of pollution and are considered part of the "emerging contaminants" in municipal waste with

concentrations of tetracycline varying from nanogrames to micrograms per liter (Verlicchi et al., 2010). At these levels, an antibiotic may select for Tc^r environmental bacteria, which may, once present, persist for extended time periods in the environment and become a reservoir for *tet* genes.

Antibiotic-resistant bacteria from human activity may contaminate the environment either directly, as occurs when manure is applied to enrich agricultural fields, or indirectly due to sewage contamination. The first description of the *tet*(M) gene in *Bacillus* spp. and Tc^r *Bacillus cereus* strains carrying the *tet*(M) gene, on a functional Tn*916* element, were found in animal manure and in the fields where the manure was spread. These results suggest that the presence of the *tet*(M) carrying *B. cereus* in the fields was a direct result of manure application to the soil. Whether the *tet*(M) carrying *B. cereus* would be able to act as a donor and transfer the *tet*(M) gene to related *Bacillus anthracis*, and/or *Bacillus thuringiensis* is unknown, however, toxin-encoding plasmids are shared between the three species (Agersø et al., 2002).

An example of how human wastes can increase Tc^r bacteria is illustrated by a 1980s study that dealt with the observation of three groups of wild baboons in Kenya (Rolland et al., 1985). Of the three groups of baboons that were examined, the two groups that lived in their natural habitat with limited or no human contact, had low levels of antibiotic and Tc^r Gram-negative enteric bacteria. The third group of baboons lived close to a tourist lodge that provided opportunities for daily contact with unprocessed human refuse, and from these animals, high levels of antibiotic-resistant Gram-negative enteric bacteria were identified with $> 90\%$ Tc^r. This study suggests that contact with human refuse greatly increased the carriage of Tc^r bacteria in these wild animals (Rolland et al., 1985). Unfortunately, the surrounding environmental bacteria were not sampled in any of these studies, but one could speculate that the level of Tc^r bacteria was most likely higher than in the areas where the two other baboon groups lived. Human and animal Tc^r bacteria share the majority of the efflux and ribosomal protection genes with environmental bacteria (Tables 7.2 and 7.4). More recently, other studies have shown carriage of Tc^r bacteria from arctic and subarctic seals (Glad et al., 2010), wild boars (Poeta et al., 2009), and wild rabbits (Silva et al., 2010), though the sources of the Tc^r *E. coli* bacteria carrying the known *tet*(A) and/or *tet*(B) genes in these other studies were not determined. The presence of human/animal Tc^r bacteria in the environment provides ample opportunity for drug-resistant intestinal bacteria to transfer their *tet* genes to environmental bacteria where they may share these genes, along with the other genes carried by mobile elements, with other environmental bacteria within and between ecosystems.

Tetracyclines have been extensively used in aquaculture and Tc^r bacteria, including fish pathogens and environmental bacteria associated with fin fish aquaculture settings from around the world, have been characterized (Akinobowale et al., 2007; DePaola et al. 1995; Furushita et al., 2003; Jacobs et al., 2007; Nawaz et al., 2008). Tc^r bacteria can be found in fish feed, the sediment under the fish pens, as well as the water entering and leaving the freshwater ponds (Kerry et al., 1995; Miranda and Zemelman, 2002). Some of the greatest diversity in Tc^r genes carried has been identified in the aquaculture environment. In one of our studies, we found that 40% of the Tc^r bacteria isolated from Chilean salmon fish farms carried unidentified Tc^r genes, suggesting diversity in the types of *tet* resistance genes present in this ecosystem, which is higher then routinely found in collections from humans or

food animals (Miranda et al., 2003). We also identified new genera carrying previously characterized *tet* genes. Many of these *tet* genes were not readily transferred under laboratory conditions, thereby raising the question of how some of the genes were being transferred to bacteria across the world and from very different environments (Miranda et al., 2003). The diversity of type and number of Tc^r bacteria found in the aquaculture setting suggests that this may be one environment where there is rapid evolution of Tc^r bacteria.

Because the environments throughout the world are physically diverse, many of the associated bacteria are different from those normally found in animals and/or humans. Most of these environmental bacteria have not been characterized, and it is estimated that less than 1% of the total number of bacteria present in many different environments have been cultured (Kümmerer, 2004). Therefore, any antibiotic resistance genes associated with these unculturable bacteria may only be identified by molecular methods such as those used to isolate the *tet*(43) gene. This gene along with a variety of other antibiotic resistance genes, was isolated from metagenomic analysis of soil from an apple orchard which had had repeated treatment with streptomycin (Donato et al., 2010). One can assume that using this approach with other environmental samples will identify additional novel tetracycline resistance genes in the future, however, we may never know what bacteria carry these genes.

7.12 GENES FOUND IN ENVIRONMENTAL BACTERIA

Currently, there are 77 Gram-negative genera and 47 Gram-positive genera that have been identified that naturally carry at least 1 tetracycline resistance gene(s) (Table 7.2). Seven to 10 different *tet* genes have been characterized in 11 (14%) of the Gram-negative genera and 11 (15%) of the Gram-positive genera, and 9 (81%) Gram-negative genera and 4 (57%) Gram-positive genera are found in the environment or found in both the environment and in humans/animals. Over the last few years, the number of genera identified with known and new efflux *tet* genes has greatly increased, primarily due to the characterization of environmental isolates from soil and water sites (Table 7.2) (Roberts, 2005). Recently, a *tet*(X) positive aerobic Gram-negative Tc^r *Sphingobacterium* sp., isolated from agricultural soil, was identified that degraded tetracycline, indicating that the *tet*(X) gene is functional in this aerobic host (Ghosh and LaPara, 2007). A 12-kb region that included the *tet*(X) gene and the up and downstream flanking regions was sequenced. The *Sphingobacterium* sp. *tet*(X) region shared organizational features and genes with the *Bacteroides tet*(X) gene and surrounding sequences found in the conjugative transposon CTnDOT (Ghosh et al., 2009; Shoemaker et al., 2001; Whittle et al., 2001). However, unlike the *Bacteroides tet*(X) gene, the *Sphingobacterium tet*(X) gene was not linked to an *erm*(F) gene, though it did have features suggestive of those found in mobilizable transposons. However, we were unable to show conjugal transfer of the *tet*(X) gene under laboratory conditions (Ghosh et al., 2009). In addition, the chromosome of *Sphingobacterium* sp. and the *tet*(X) gene had similar 37% G+C, suggesting this or a related bacteria could be the ancestral source of the *tet*(X) gene now found in *Bacteroides* spp. This is the first report of the presence of the *tet*(X) gene in an aerobic bacterium. Whether the *tet*(X) gene is commonly carried by

bacteria from agricultural soil or other environmental ecosystems is unknown but suggests that this gene may have first appeared in environmental species.

The tetracycline genes originally identified in the genus *Streptomyces* [*otr*(A), *otr*(B), *otr*(C), *tcr3*, *tet*] account for 29% of the genes listed in Table 7.4. However, the *otr*(A) and *otr*(B) are now found in *Bacillus* and *Mycobacterium* and may be associated with other related environmental genera. Many *Clostridium* spp. are found in the environment, though they are also associated with the intestinal tract of humans and animals. However, for this discussion the *tetA*(P) and *tetB*(P) genes appear to be unique to *Clostridium* spp. and are considered unique to environmental bacteria. Other environmental genes include:

- The *tet*(V) gene that has been found in *Mycobacterium smegmatis* (primarily an environmental bacteria) (De Rossi et al., 1998).
- The *tet*(30) gene in *Agrobacterium* (Luo and Farrand 1999).
- The *tet*(33) gene has been found in environmental *Arthrobacter* and *Corynebacterium* spp. (Agersø and Sandvang, 2005; Tauch et al., 2002).
- *The tet*(35) gene in *Vibrio* and *Stenotrophomonas* spp. (Teo et al. 2002).
- The *tet*(41) gene in *Serratia* spp. (Thompson et al., 2007).
- The *tet*(42) gene in *Bacillus, Microbacterium, Micrococcus*, *Paenibacillus*, and *Pseudomonas* spp. (Brown et al., 2008).
- The *tet*(34) gene was first described in *Vibrio spp*. and more recently identified in *Pseudomonas* and *Serratia* and is listed in Table 7.4.
- The *tet*(43) gene was identified from metagenomic analysis of soil from an apple orchard, and it is unknown what species or genus carries this gene. Interestingly, the *tet*(43) gene that was isolated from the metagenomic sample has a 65.1% G+C content, which is similar to that found in other unique resistance genes *tet* (Z) (63.1%), *tet*(41) (67.2%), *tet*(42) (68.4%), *otr*(B) (71.3%) and *otr*(C) (69.6%) found in environmental hosts.

For this discussion, I have designated the *tet*(39) gene as unique to environmental bacteria because it has been characterized in *Acinetobacter* spp. isolated from integrated fish farms in Thailand (Agersø and Petersen, 2007). *Acinetobacter* spp. can be isolated from drinking and surface water, soil, sewage, different types of foods, and from some healthy people where *Acinetobacter* spp. is associated with the skin. Thus, the genus *Acinetobacter,* like many other genera, includes both environmental and nonenvironmental isolates and species. *Acinetobacter baumannii–A. calcoaceticus* isolates carrying the *tet*(39) gene have been identified from traumatic wound infections where the environment was the likely source of the infecting bacterium (Akers et al., 2009). In another study of Tc^r bacteria from a polluted river in southwestern Nigeria, three Gram-positive (*Bacillus*, *Cellulosimicobium*, and *Lysinibacillus*) and five Gram-negative genera (*Alcaligenes*, *Brevundimonas*, *Enterobacter, Providencia*, and *Stenotrophomonas*) carrying the *tet*(39) gene were isolated (Adelowo and Fagade, 2009). However, *Cellulosimicobium cellulans*, which is usually found in soil and decaying plant material, has been on occasion associated with infections in an immunocompromised host or in patients with foreign bodies (Rowlinson et al., 2006), illustrating the blurring of the line between strictly environmental and nonenvironmental bacteria.

7.13 CONCLUSION

Tc^r bacteria are widely distributed throughout the world. They have been isolated from deep subsurface trenches, in wastewater, surface and ground water, sediments and soils, and places that are relatively untouched by human civilization such as penguins in Antarctica and seals from the Arctic (Donato et al., 2010; Glad et al., 2010; Kümmerer, 2004; Rahman et al., 2008). Seventeen (39%) of the 43 known *tet* genes including 12 (44%) of the efflux, 3 (25%) of the ribosomal protection, and 2 (66%) of the enzymatic *tet* genes are assigned to unique to environmental bacteria (Table 7.4). Whether this represents a true separation between *tet* genes that are "unique" to environmental bacteria or because these 17 genes have not been used in surveillance studies of animal/human bacteria is unclear. It is possible that over time, these "unique *tet* genes" will move into bacteria associated with animals and/or humans as the *tet*(X) gene suggests. To a large extent what is in the environmental bacterial population remains largely unexplored. However, over time and as more of these environments are explored, it is clear that the number of new environmental genera of bacteria carrying *tet* genes will continue to increase, as will the number of *tet* genes identified from metagenomic DNA preparations from all types of ecosystems. Unfortunately, it is likely that human activities will continue to pollute the environment, which will make distinction between environmental and nonenvironmental impacts increasingly difficult. The level at which the environmental reservoir of Tc^r bacteria will influence the continued use of tetracyclines as therapeutic agents in humans, animals, and plants remains unclear and studies addressing this issue are needed.

REFERENCES

Abbo A, Navon-Venezia S, Hamemer-Muntz O, Krichali T, Siegman-Igra Y, Carmeli Y (2005). Multidrug-resistant *Acinetobacter baumannii*. *Emerg Infect Dis* 11:22–29.

Adams MD, Chan ER, Neil D, Molyneaux ND, Robert A, Bonomo RA (2010). Genomewide analysis of divergence of antibiotic resistance determinants in closely related isolates of *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 54:3569–3577.

Adelowo OO, Fagade OE (2009). The tetracycline resistance gene *tet39* is present in both Gram-negative and Gram-positive bacteria from a polluted river, Southwestern Nigeria. *Lett Environ Microb* 48:167–172.

Agersø Y, Petersen A (2007). The tetracycline resistance determinant Tet 39 and the sulphonamide resistance gene *sulI* are common among resistant *Acinetobacter* spp. isolated from integrated fish farms in Thailand. *J Antimicrob Chemother* 59:23–27.

Agersø Y, Sandvang D (2005). Class 1 integrons and tetracycline resistance genes in *Alcaligenes, Arthrobacter*, and *Pseudomonas* spp. *Appl Environ Microb* 71:7941–7947.

Agersø Y, Jensen LB, Givskov M, Roberts MC (2002). The identification of a tetracycline resistance gene *tet*(M), on a Tn*916*-like transposon, in the *Bacillus cereus* group. *FEMS Microb Lett* 214:251–256.

Akinbowale OL, Peng H, Barton MD (2007). Diversity of tetracycline resistance genes in bacteria from aquaculture sources in Australia. *J Appl Micrbiol* 103:2016–2025.

Akers KS, Mende K, Yun HC, Hospenthal DR, Beckius MK, Yu X, Murray CK (2009). Tetracycline susceptibility testing and resistance genes in isolates of *Acinetobacter baumannii-Acinetobacter calcoaceticus* complex from a U.S. military hospital. *Antimicrob Agents Chemother* 53:2693–2695.

Atkinson BA, Abu-Al-Jaibat A, LeBlanc DJ (1997). Antibiotic resistance among enterococci isolated from clinical specimens between 1953 and 1954. *Antimicrob Agents Chemother* 41:1598–1600.

Avery OT, MacLeod CM, McCarty M (1944). Studies on the chemical nature of the substance inducing transformation of pneumococcal types. *J Exp Med* 79:137–158.

Billington SJ, Jost BH (2006). Multiple genetic elements carry the tetracycline resistance gene *tet*(W) in the animal pathogen *Arcanobacterium pyogenes*. *Antimicrob Agents Chemother* 50:3580–3587.

Billington SJ, Songer JG, Jost BH (2002). Widespread distribution of a Tet W determinant among tetracycline-resistant isolates of the animal pathogen *Arcanobacterium pyogenes*. *Antimicrob Agents Chemother* 46:1281–1287.

Brown MG, Mitchell EH, Balkwill DL (2008). Tet 42, a novel tetracycline resistance determinant isolated form deep terrestrial subsurface bacteria. *Antimicrob Agents Chemother* 52:4518–4521.

Chen I, Dubnau D (2004). DNA uptake during bacterial transformation. *Nat Rev Microb* 2:241–249.

Chopra I, Roberts MC (2001). Tetracycline antibiotics: Mode of action, applications, molecular biology and epidemiology of bacterial resistance. *Microb Mol Biol Rev* 65:232–260.

Chung WO, Werckenthin C, Schwarz S, Roberts MC (1999). Host range of the *ermF* rRNA methylase gene in human and animal bacteria. *J Antimicrob Chemother* 43:5–14.

Connell SR, Tracz DM, Nierhaus KH, Taylor DE (2003a). Ribosomal protection proteins and their mechanism of tetracycline resistance. *Antimicrob Agents Chemother* 47:3675–3681.

Connell SR, Trieber CA, Einfeldt E, Taylor DE, Nierhaus KH (2003b). Mechanism of Tet (O), perturbs the conformation of the ribosomal decoding center. *Mol Microbiol* 45:1463–1472.

Cousin SL Jr, Whittington WL, Roberts MR (2003). Acquired macrolide resistance genes in pathogenic *Neisseria* spp. isolated between 1940 and 1987. *Antimicrob Agents Chemother* 47:3877–3880.

DePaola A, Roberts MC (1995). Class D and E tetracycline resistance determinants in gram-negative catfish pond bacteria. *Molec Cell Probes* 9:311–313.

De Rossi E, Blokpoel MCJ, Cantoni R, Branzoni M, Riccardi G, Young DB, De Smet KAL, Ciferri O (1998). Molecular cloning and functional analysis of a novel tetracycline resistance determinant, tet(V), from *Mycobacterium smegmatis*. *Antimicrob Agents Chemother* 42:1931–1937.

Diaz-Torres ML, McNab R, Spratt DA, Villedieu A, Hunt N, Wilson M, Mullany P (2003). Novel tetracycline resistance determinate from the oral metagenome. *Antimicrob Agents Chemother* 47:1430–1432.

Di Luca MC, D'Ercole S, Petrelli D, Prenna M, Ripa S, Vitali LA (2010). Lysogenic transfer of *mef*(A) and*tet*(O) genes carried by Φm46.1 among group A streptococci. *Antimicrob Agents Chemother* 54(10):4464–4466.

Donato JJ, Moe LA, Converse BJ, Smart KD, Berklein FC, McManus PS, Handelsman J (2010). Metagenomic analysis of apple orchard soil reveals antibiotic resistance genes encoding predicted bifunctional proteins. *Appl Environ Microbiol* 76:4396–4401.

Dugan J, Rockey DD, Jones L, Andersen AA (2004). Tetracycline resistance in *Chlamydia suis* mediated by genomic isolated inserted into the chlamydial *inv*-like gene. *Antimicrob Agents Chemother* 48:3989–3995.

Facinelli B, Roberts MC, Giovanetti E, Casolari C, U. Fabio U, Varaldo PE (1993). Genetic basis of tetracycline resistance in food borne isolates of *Listeria innocua*. *Appl Environ Microbiol* 59:614–616.

Fournier P-E, Vallenet D, Barber V, Audic S, Ogata H, Poirel L, Richet H, Robert C, Mangenot S, Abergel C, Nordmann P, Weissenbach J, Raoult D, Claverie JM (2006). Comparative genomics of multidrug resistance in *Acinetobacter baumannii*. *PLoS Genet*, available: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1326220/pdf/pgen.0020007.pdf.

Furushita M, Shiba T, Maeda T, Yahata M, Kaneoka A, Takahashi Y, Torii K, Tadao Hasegawa T, Ohta M (2003). Similarity of tetracycline resistance genes isolated from fish farm bacteria to those from clinical isolates. *Appl Environ Microb* 69:5336–5342.

Garcillian-Barcia, MP, de la Cruz F (2008). Why is entry exclusion an essential feature of conjugative plasmids? *Plasmid* 60:1–18.

Ghosh S, LaPara TM (2007). The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. *ISME J* 1:191–203.

Ghosh S, Gralnick J, Roberts MC, Sadowsky M, LaPara T (2009). *Sphingobacterium* sp. strain PM2-P1–29 harbors a functional *tet*(X) gene encoding for the degradation of tetracycline. *J Appl Microbiol*106:1336–1342.

Gillespie MT, May JW, Skurray RA (1986). Detection of an integrated tetracycline resistance plasmid in the chromosome of methicillin-resistant *Staphylococcus aureus*. *J Gen Microbiol* 132:1723–1728.

Giovanetti E, Brenciani A, Lupidi R, Roberts MC, Varaldo PE(2003). The presence of the *tet* (O) gene in erythromycin and tetracycline-resistant strains of *Streptococcus pyogenes*. *Antimicrob Agents Chemother* 47:2844–2849.

Glad T, Fristiansen VF, Nielsen KM, Rusetti L, Wright A-D G, Sundset MA (2010). Ecological characterization of the colonic microbiota in arctic and sub-artic seals. *Microb Ecol* 60:320–330.

Gould LH, Limbago B (2010). *Clostridium difficile* in food and domestic animals: A new foodborne pathogen? *Clin Infect Dis* 51(5):577–582.

Grimdmann H, Aires-de-Sousa M, Boyce J, Tiemersma E (2006). Emergence and resurgence of methicillin-resistant *Staphylococcus aureus* as a public-health threat. *Lancet* 368:874–885.

Hughes VM, Datta N (1983). Conjugative plasmids in bacteria of the "pre-antibiotic" era. *Nature* 301:725–726.

Jacobs L, Chenia HY (2007). Characterization of integrons and tetracycline resistance determinants in *Aeromonas* spp. isolated from South African aquaculture systems. *Int J Food Microbiol* 114:295–306.

Johanesen PA, Lyras D, Bannam TL, Rood JI (2001). Transcriptional analysis of the *tet*(P) operon from *Clostridium perfringens*. *J Bacteriol* 183:7110–7119.

Kerry J, Hiney M, Coyne R, Nicgabhainn R, Gilroy D, Cabon D, Smith P (1995). Fish feed as a source of oxytetracycline-resistant bacteria in the sediments under fish farms. *Aquaculture* 240:89–100.

Kümmerer K (2004). Resistance in the environment. *J Antimicrob Chemother* 54:311–320.

L'Abee-Lund TM, Sorum H (2002). A global non-conjugative Tet C plasmid, pRAS3, from *Aeromonas salmonicida*. *Plasmid* 47:172–181.

Lancaster H, Roberts AP, Dedi R,Wilson M, Mullany P (2004). Characterization of Tn*916S*, a Tn*916*-like element containing the tetracycline resistance determinant *tet*(S). *J Bacteriol* 186:4395–4398.

Lancaster HP, Bedi R, Wilson M, Mullany P (2005). The maintenance in the oral cavity of children of tetracycline-resistant bacteria and the genes encoding such resistance. *J Antimicrob Chemother* 56:524–531.

Lawley TC, Burland V, Taylor DE (2000). Analysis of the complete nucleotide sequence of the tetracycline-resistant transposon Tn*10*. *Plasmid* 43:235–239.

Levy SB, McMurry LM, Barbosa TM, Burdett V, Courvalin P, Hillen W, Roberts MC, Rood JI, Taylor DE (1999). Nomenclature for new tetracycline resistance determinants. *Antimicrob Agents Chemother* 43:1523–1524.

Levy SB, McMurry LM, Roberts MC (2005). Tet protein hybrids. *Antimicrob Agents Chemother* 49:3099.

Leipe DD, Wolf YI, Koonin EV, Aravind L. (2002) Classification and evolution of P-loop GTPases and related ATPases. *J Mol Biol* 15:41–72.

Luo Z-Q, Farrand SK (1999). Cloning and characterization of a tetracycline resistance determinant present in *Agrobacterium tumefaciens* C58. *J Bacteriol* 181:618–626.

Marshall B, Roberts M, A. Smith A, Levy SB. (1984). Homogeneity of tetracycline-resistance determinants in *Haemophilus* species. *J Infect Dis* 149:1028–1029.

Melville CM, Scott KP, Mercer DK, Flint HJ (2001). Novel tetracycline resistance gene, *tet*(32), in the *Clostridium*-related human colonic anaerobe K10 and its transmission in vitro to the rumen anaerobe *Butyrivibrio fibrisolvens*. *Antimicrob Agents Chemother* 45:3246–3249.

Mendez B, Tachibana C, Levy SB (1980). Heterogeneity of tetracycline resistance determinants. *Plasmid* 3:99–108.

Miranda CD, Zemelman R (2002). Bacterial resistance to oxytetracycline in Chilean salmon farms. *Aquaculture* 212:31–47.

Miranda CD, Kehrenberg C, Ulep C, Schwarz S, Roberts MC (2003). Diversity of tetracycline resistance genes in bacteria from Chilean salmon farms. *Antimicrob Agents Chemother* 47:883–888.

Moore IF, Hughes DW, Wright GD (2005). Tigecycline is modified by the flavin-dependent monooxygenase TetX. *Biochemistry* 44:11829–11835.

Møller JK, Leth Bak A, Stenderup A, Zachariae H, Afzelius H (1977). Changing patterns of plasmid-mediated drug resistance during tetracycline therapy. *Antimicrob Agents Chemother* 11(3): 388–391.

Nawaz M, Khan AA, Khan S, Sung K, Steele R (2008). Isolation and characterization of tetracycline-resistant *Citrobacter* spp. from catfish. *Food Microbiol* 25:85–91.

Nonaka L, Suzuki S (2002). New Mg^{2+}-dependent oxytetracycline resistance determinant Tet 34 in *Vibrio* isolates from marine fish intestinal contents. *Antimicrob Agents Chemother* 476:1550–1552.

Nonaka L, Isshik Ti, Suzuki S (2000). The occurrence of the oxytetracycline resistant bacteria in the fish intestine and seawater environment. *Microb Environ* 15:223–228.

Norgren M, Scott JR (1991). The presence of conjugative transposon Tn*916* in the recipient strain does not impede transfer of a second copy of the element. *J Bacteriol* 173:319–324.

Palm GJ, Lederer T, Orth P, Saenger W, Takahashi M, Hilllen W, Hinrichs W (2008). Specific binding of divalent metal ions to tetracycline and to the Tet repressor/tetracycline complex. *J Biol Inorg Chem* 13:1097–1110.

Poeta P, Radhouani H, Pinto L, Martinho A, Rego V, Rodrigues R, Goncalves A, Rodrigues J, Estepa V, Torres C, Igrejas G (2009). Wild boars as reservoirs of extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* of different phylogenetic groups. *J Basic Microbiol* 49:584–588.

Rahman MH, Sakamoto KQ, Nonaka L, Suzuki S (2008). Occurrence and diversity of tetracycline *tet*(M) in enteric bacteria of Antarctic Adelie penguins. *J Antimicrob Chemother* 62:627–628.

Recchia GD, Hall RM (1995). Gene cassettes: A new class of mobile element. *Microbiology* 141:3015–3027.

Rice LB (2007). *Conjugative transposons*. In RA Bonomo and M Tolmasky (Eds.), *Enzyme-Mediated Resistance to Antibiotics: Mechanisms, Dissemination, and Prospects for Inhibition*. American Society for Microbiology, Washington, DC, pp. 271–284.

Roberts AP, Mullany P (2009). A modular master on the move: The Tn*916* family of mobile genetic elements. *Trends Microbiol* 17:251–258.

Roberts AP, Chandler M, Courvalin P, Guedon G, Mullany P, Pembroke T, Rood JI, Smith CJ, Summers AO, Tsuda M, Berg DE (2008). Revised nomenclature for transposable genetic elements. *Plasmid* 60:167–173.

Roberts MC (1989). Plasmids of *Neisseria gonorrhoeae* and other *Neisseria* species. *Rev Clin Microbiol* 2:S18–S23.

Roberts MC (1997). *Genetic mobility and distribution of tetracycline resistance determinants*. In *Antibiotic Resistance: Origins, Evolution, Selection and Spread*. Ciba Foundation Symposium 207. Wiley, Chichester, pp. 206–218.

Roberts MC (2005). MiniReview: Update on acquired tetracycline resistance genes. *FEMS Microbiol Lett* 245:195–203.

Roberts MC (2008). Update on macrolide–lincosamide–streptogramin, ketolide, and oxazolidinone (MLSKO) resistance genes. *FEMS Microbiol Lett* 282:147–159.

Roberts, MC, Knapp JS (1988a). Host range of the conjugative 25.2 Mdal tetracycline resistance plasmid from *Neisseria gonorrhoeae*. *Antimicrob Agents Chemother* 32:488–491.

Roberts MC, Knapp JS (1988b). Transfer of β-lactamase plasmids from *Neisseria gonorrhoeae* to *Neisseria meningitidis* and commensal *Neisseria* species by the 25.2-Megadalton conjugative plasmid. *Antimicrob Agents Chemother* 32:1430–1432.

Rolland RM, Hausfater G, Marshall B, Levy SB (1985). Antibiotic-resistant bacteria in wild primates: Increased prevalence in baboons feeding on human refuse. *Appl Environ Microbiol* 49:791–794.

Rowlinson M-C, Bruckner DA, Hinnebusch C, Nielsen K, Deville JG (2006). Clearance of *Cellulosimicrobium cellulans* bacteremia in a child without central venous catheter removal. *J Clin Microbiol* 44:2605–2654.

Shoemaker NB, Vlamakis H, Hayes K, Salyers AA (2001). Evidence for extensive resistance gene transfer among *Bacteroides* spp. and among *Bacteroides* and other genera in the human colon. *Appl Environ Microbiol* 67:561–568.

Silva N, Igrejas G, Figueiredo N, Goncalves A, Radhouani H, Rodrigues H, Poeta P (2010). Molecular characterization of antimicrobial resistance in enterococci and *Escherichia coli* isolates from European wild rabbit (*Oryctolagus cuniculus*). *Sci Total Environ* 408(20): 4871–4876.

Smith HO, Gwinn ML, Salzberg SL (1999). DNA uptake signal sequences in naturally transformable bacteria. *Res Microbiol* 150:603–616.

Soge OO, Beck N, White TM, Roberts MC (2008). A novel transposon, Tn6009, composed of a Tn*916*-like element linked to *Staphylococcus aureus*-like mer operon. *J Antimicrob Chemother* 62:674–680.

Soge OO, Tivoli L, Meschke JS, Roberts MC (2009). A conjugative macrolide resistance gene, *mef*(A), in environmental *Clostridium perfringens* carrying multiple macrolide and/or tetracycline resistance genes. *J Appl Microbiol* 106:34–40.

Sorum H, Roberts MC, Crosa JH (1992). Identification and cloning of a tetracycline resistance gene from the fish pathogen *Vibrio salmonicida*. *Antimicrob Agents Chemother* 36:611–615.

Speer B, Bedzyk S, Salyers AA (1991). Evidence that a novel tetracycline resistance gene found on two *Bacteroides* transposons encodes an NADP-requiring oxidoreductase. *J Bacteriol* 173:176–183.

Stanton TB, Humphrey SB (2003). Isolation of tetracycline-resistant *Megasphaera elsdenii* strains with novel mosaic gene combinations of *tet*(O) and *tet*(W) from swine. *Appl Environ Microbiol* 69:3874–3882.

Stanton TB, Humphrey SB, Scott KP, Flint HJ (2005). Hybrid *tet* genes and *tet* nomenclature: Request for opinions. *Antimicrob Agents Chemother* 49:1265–1266.

Suchland RJ, Sandoz KM, Jeffrey BM, Stamm WE, Rockey DD (2009). Horizontal transfer of tetracycline resistance among *Chlamydia* spp. in vitro. *Antimicrob Agents Chemother* 53:4604–4611.

Tauch A, Gotker S, Puhler A, Kalinowski J, Thierbach G (2002). The 27.8-kb R-plasmid pTET3 from *Corynebacterium glutamicum* encodes the aminoglycoside adenyltransferase gene cassette *aadA9* and the regulated tetracycline efflux system Tet 33 flanked by active copies of the widespread insertion sequence IS6100. *Plasmid* 48:117–129.

Teo JWP, Tan TMC, Poh CL (2002). Genetic determinants of tetracycline resistance in *Vibrio harveyi*. *Antimicrob Agents Chemother* 46:1038–1045.

Thaker M, Spanogiannopoulos P, Wright GD (2010). The tetracycline resistome. *Cell Mol Life Sci* 67:419–431.

Thompson SA, Maani EV, Lindell AH, King CJ, McArthur JC (2007). Novel tetracycline resistance determinant isolated form an environmental strain of *Serratia marcescens*. *Appl Environ Microbiol* 73:2199–2206.

Trujillo ME, Goodfellow M (2003). Numerical phonetic classification of clinically significant aerobic sporoactinomycetes and related organisms. *Antonie Van Leeuwenhoek* 84:39–68.

Van Hoek AHAM, Mayrhofer S, Doing KJ, Flórez AB, Mohammed S. Ammor MS, Mayo B, Aarts HJM (2008). Mosaic tetracycline resistance gene and their flanking regions in *Bifidobacterium thermophilum* and *Lactobacillus johnsonii*. *Antimicrob Agents Chemother* 52:248–252.

Verlicchi P, Galletti A, Masotti L (2010). Management of hospital wastewaters: the case of the effluent of a large hospital situated in a small town. *Water Sci Technol* 61:2507–2519.

Vo ATT, van Duijkeren E, Fluit AC, Gaastra W (2007). A novel *Salmonella* genomic island 1 and rare integron types in *Salmonella typhimurium* isolates from horses in The Netherlands. *J Antimicrob Chemother* 59:594–599.

Warburton, P, Roberts AP, Allan E Seville L, Lancaster H, Mullany P (2009). Characterization of *tet*(32) genes from the oral metagenome. *Antimicrob Agents Chemother* 53:273–276.

Watanabe T (1963). Infective heredity of multiple drug resistance in bacteria. *Bacteriol Rev* 27:87–115.

Waters VL (2001). Conjugation between bacterial and mammalian cells. *Nat Genet* 29: 375–376.

Weigel LM, Donlan M, Shin DH, Jensen B, Clark NC, McDougal LK, Zhu W, Musser KA, Thompson J, Kohlerschmidt D, Dumas N, Linberger RJ, Patel JB (2004). High-level vancomycin-resistant *Staphylococcus aureus* isolates associated with a polymicrobial biofilm. *Antimicrob Agents Chemother* 51:231–238.

Whittle G, Whitehead TR, Hamburger N, Nadja B. Shoemaker NB, Cotta MA, Salyers AA (2003). Identification of a new ribosomal protection type of tetracycline resistance gene, *tet* (36), from swine manure pits. *Appl Environ Microbiol* 69, 4151–4158.

Yang WR, Moore IF, Koteva KP, Bareich DC, Hughes DW, Wright GD (2004). TetX is a flavin-dependent monooxygenase conferring resistance to tetracycline antibiotics. *J Biol Chem* 279:52346–52352.

8

ENVIRONMENTAL ANTIBIOTIC RESISTOME: NEW INSIGHTS FROM CULTURE-INDEPENDENT APPROACHES

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8.1 ANTIBIOTICS, ENVIRONMENT, AND RESISTANCE

In our society, there are several settings where microorganisms need to be controlled: clinical units (for humans and animals), food production facilities, in industry, and in households. There are several ways to do so: by physical action (temperature, depletion of oxygen, depletion of nutrients, smooth surfaces), biological action (e.g., by vaccination or the use of beneficial microbes to out compete pathogens), and chemical action (the use of chemicals to impact on cell structures and processes). With respect to the latter, all such chemicals fall into the categories of antibiotics, antimicrobials, disinfectants, decontaminants, or biocides.

An antibiotic is a substance that affects (αντι) life (βιος), that is, organisms. Paracelsus' theorem and Julian Davies' hypothesis apply here too: All substances can be considered antibiotics; the right dose differentiates an antibiotic and a signaling molecule. The classification of a substance as an antibiotic always depends on the circumstances in which we made our observations: What community did we study, what concentration was present, how was the bioavailability, were other

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inhibitory substances present? A functional definition would serve our purposes best. An antibiotic can be functionally described as a molecule identified as having useful therapeutic activity in killing or inhibiting microbial growth (Yim et al., 2007). Bacteriocins or microcins are microbial products and inhibitors of the growth of many bacterial strains but without a pharmaceutically therapeutic use. They do not fit the functional definition of antibiotics, but since there is considerable interest in their development as therapeutic agents (Yim et al., 2007), they are on the short list.

Antibiotic resistance (AR) dissemination is one of the reasons bacterial infectious diseases are becoming more severe and require longer and more expensive treatments. In fact, infectious diseases that were formerly considered to be eradicated in developed countries, such as tuberculosis, are now a present and real threat. Bacteria developed several different mechanisms to render ineffective the antibiotics used against them. In the last decades, mechanisms conferring resistance to virtually all known substances have been detected and characterized from Gram-negative and Gram-positive isolates belonging to a wide range of genera. The dissemination of pathogenic strains resistant to multiple antibiotics presents an enormous concern, challenging clinical practices.

Resistance is a capacity that microorganisms may have that is based in traits (resistance elements) and expressed as (or measured by) population growth in the presence of an antibiotic at therapeutic concentrations. Extensive research has been conducted on this subject and several conclusions have been drawn. AR elements in pathogens have environmental origins and are not necessarily linked to exposure to antibiotics, although resistance mechanisms are most widespread in those settings (health units, countries) where antibiotic use is particularly intense (van de Sande-Bruinsma et al., 2008; Sabuncu et al., 2009). Large quantities of antibiotics are widely used in the treatment of infectious diseases in human medicine but also as therapeutic, prophylactic, and growth promotion agents in veterinary, agriculture, and aquaculture practices (Kümmerer, 2009).

Currently, only a fraction of the available resistance traits are proliferating in pathogens. Nevertheless, proliferation of new resistance traits is an ongoing process, as is the continuous emission of large volumes of antibiotic-resistant bacteria into the environment (the environment with immediate functions to the human populations, such as farmland and surface water) together with both nutrients (waste) and stressors (antibiotics, metals, disinfectants). Depending on the initial conditions and intensity and duration of exposure, a greater diversity of resistance elements is selected for and more of each individual element is produced. Spreading can occur because the resistant populations out compete others, taking up all space within the setting and cross boundaries, or by transfer of individuals (e.g., via air or rivers) across boundaries.

Most of the AR research has been and is still confined to the study of cultivable bacterial isolates. Given the fact that, independently of the environment being studied, the uncultivable fraction includes the majority of microbial community members, we can anticipate that the extent of the potential risk to human and ecological health of the so-called environmental resistance reservoirs is far from being determined and understood. Several aspects of the resistance thematic will potentially be further elucidated by using culture-independent molecular approaches. The aim of this chapter is to review culture-independent approaches potentially useful for AR assessment and to summarize novel findings that have already arisen

from studies based on such methodologies. The development of novel culture-independent methodologies and the awareness of their usefulness to study the molecular basis of microbial resistance will decidedly contribute to expand knowledge on this area.

8.2 ENVIRONMENTAL RESERVOIRS OF AR

The term *resistome* was coined by D'Costa et al. (2006) referring to "all resistance determinants present in soil" as "the soil resistome". Wright (2007) redefined resistome as the collection of all the AR genes in microorganisms: "the resistome is a collection of all the antibiotic resistance genes and their precursors in pathogenic and non-pathogenic bacteria. It includes resistance elements found in both pathogenic bacteria and antibiotic-producing bacteria, and cryptic resistance genes (which are not necessarily expressed) that are present in bacterial chromosomes. The resistome also includes precursor genes that encode proteins with modest antibiotic resistance activity, or affinity to antibiotics, that might evolve into effective resistance genes". Thaker et al. (2010) used the original concept: "the resistome concept refers to the aggregate of all antibiotic resistance mechanisms".

Given that resistomes are part of all environmental settings, they are potential reservoirs of resistance genes. In fact, the presence of antibiotic-resistant bacteria has been documented in a variety of natural environments from different geographical locations such as soil, groundwater, surface water, drinking water, rivers, lakes, oceans, and estuaries (Ash et al., 2002; Chee-Sanford et al., 2001; Goñi-Urriza et al., 2000; Henriques et al., 2006a; Schwartz et al., 2003). A few random examples are listed here that give some clues as to the origin and the conditions that select for AR. Jones et al. (1986) already described that the incidence of AR in aquatic bacteria (bacteria predominant in lake water excluding *Pseudomonas* spp., coliforms, and fecal streptococci) in Lake Windermere, receiving sewage effluents, was lower than that in *Pseudomonas* spp. and *Escherichia coli* from the same origin but higher than in other coliforms and fecal streptococci. Aquatic bacteria from two remote upland tarns, with hardly any anthropogenic influence, displayed comparative results to the lake bacteria. It was observed that nutrient poor conditions do not associate with reduced resistance levels (Jones et al., 1986). On another investigation, bacteria resistant to oxytetracycline (OTC) (counted on plates) were favored in marine sediments in the presence of high levels of unmedicated and sterilized fish feed; the data was obtained from facilities where OTC was absent and the sediment layer depth was 6 cm and the feed layer was 16 cm. Under feed layers of merely 1–2 cm, no relative increase in OTC-resistant bacteria was found. Here, nutrient-rich conditions were associated with increased resistance (Kapetanaki et al., 1995). Ample evidence for in situ cross resistance to metals and antibiotics in the terrestrial and aquatic environment had been documented (Berg et al., 2005; Stepanauskas et al., 2006).

If we look at any particular environment, we need to discern between a "natural" background of resistance (which is always present) and an "added" anthropogenic level (which may be absent in pristine areas or conserved habitats). The "natural background of resistance" is a concept that is still vague and difficult to handle but that deserves a good delineation of its contours for implementation in monitoring programs. Considering the large fraction of bacteria that are not amenable to culture,

culture-independent methods are needed to discern between determinants of resistance characteristic of a particular environment and those that, when present, are indicative of anthropogenic pressure; in parallel, quantitative approaches must be used to detect increase or decrease of molecular variants that may indicate perturbations of the system in consequence of human actions.

The origin of AR has been investigated in a Portuguese mesotrophic estuary under the influence of harbor facilities, industrial plants, domestic sewage inputs, and aquaculture. Henriques et al. (2006b) focused on the genes encoding for β-lactamases, the enzymes that deactivate β-lactam antibiotics. It was shown that various bacterial deoxyribonucleic acid (DNA) sequences extracted from the water samples were (almost) identical to β-lactamase gene sequences of enteric isolates. The patterns of molecular diversity of the bacterial DNA sequences, however, indicated that the origin of the β-lactam resistance in the estuary was diverse and that there were sequences of genes encoding for β-lactamases that were perhaps primordial of the resistance genes of enteric isolates; these relationships of ancestry therefore indicate that they are not of anthropogenic origin. This investigation showed that in situ AR had an environmental origin, in spite of the anthropogenic pollution with AR elements.

Considering antimicrobial resistance, what is a microorganism? Martiny et al. (2006) have defined a microorganism as having a mass of less than 10^{-5}g and a length of less than 500 μm. Microorganisms are found in the domains Bacteria and Archaea and also as microscopic members of the domain Eukarya (e.g., unicellular algae, some fungi, and protists). The abundance and diversity of resistance genes present in microorganisms are a result of a number of different factors. Bacteria have populated the Earth since about 3 billion years (Di Giulio, 2003), and viruses probably evolved simultaneously (Iyer et al., 2006). Fungi originated about 1 billion years ago (Lucking et al., 2009). This has given microbes plenty of time to develop mechanisms for survival: feeding, competition for nutrients, dealing with toxicants, and signaling. Where this involved chemicals, they produce and excrete; they also developed mechanisms to receive, tolerate, or resist such agents (Hall and Barlow, 2004; Garau et al., 2005; Yim et al., 2007). Such mechanisms are often also involved in primary metabolism or sporulation (Bibb, 2005; Nodwell, 2007). It has been demonstrated that environmental antibiotic producers have developed and optimized autoprotecting mechanisms to avoid the toxic activity of antibiotics (Cundliffe, 1992). Likewise, other microorganisms inhabiting the same ecological niches were forced to follow similar evolutionary routes. In other cases neighboring microorganisms acquired resistance through horizontal gene transfer of resistance genes from other microbial species. Horizontal gene transfer (HGT) is of main relevance in the plasticity and evolution of bacterial genes and genomes. Horizontal gene flow (HGF) is the phenomenon where a recipient bacterium acquires genetic material from a donor cell from a different strain or species. During the 1930s–1950s, the three essential mechanisms for gene transfer, transformation, transduction, and conjugation, were discovered (Fig. 8.1). Mobile genetic elements (MGEs) such as bacteriophages, plasmids, and transposons are involved in these processes (Burrus and Waldor, 2004; Frost et al., 2005; Thomas, 2000). Transformation is the transfer of extracellular DNA (e.g., after cell lysis) to a bacterium via inclusion. Transduction is the transfer of DNA to host bacteria by viral (bacteriophage) infection. Conjugation is the transfer of plasmids or other mobile genetic elements from one bacterium to another via a conjugation bridge, and it requires cell-to-cell contact.

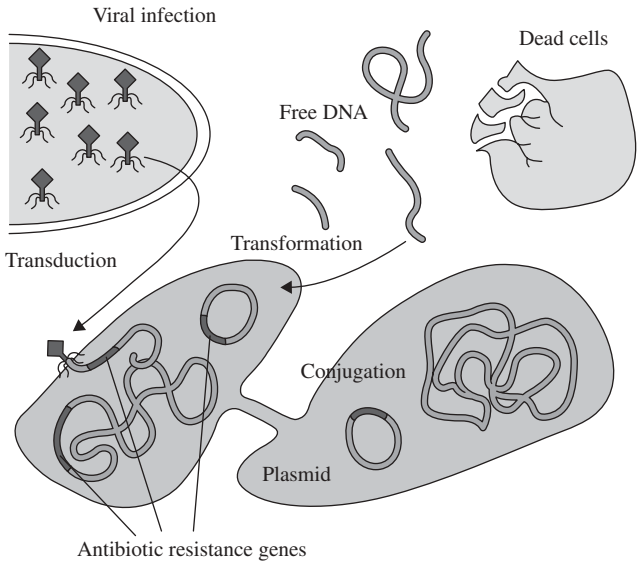

FIGURE 8.1 Transfer of AR genes.(By courtesy of Dr. T. Schwartz.)

Conjugation is generally thought to be the most important transfer mechanism. This can be mediated by plasmids or integrative conjugative elements that encode specific transfer genes (Thomas and Nielsen, 2005). Plasmids are double-stranded, usually circular, units of DNA that replicate within a cell, independently of the chromosomal DNA (Sorensen et al., 2005). Conjugative plasmids encode all the necessary proteins for mobilization. On the other hand, transference of mobilizable plasmids can be helped by a conjugative plasmid co-resident in the cell. Conjugative transposons are also able to promote their own intercellular conjugal transfer (Beuzon et al., 2004). Plasmids and conjugative transposons carrying AR genes have been frequently described (Bennett, 2008).

Gene transfer can also occur, driven by systems such as transposons and integrons, from one DNA molecule to another inside the same cell. Integrons are natural recombination systems that mediate the capture and expression of gene cassettes (Rowe-Magnus and Mazel, 2001). They can be found in bacterial chromosomes, plasmids, and transposons. Most of the gene cassettes so far described from bacterial isolates encode AR determinants (Moura et al., 2009).

The horizontal gene transfer of resistance genes between environmental bacteria and pathogens has been considered a main mechanism contributing to dissemination of AR (Baquero et al., 2009; Caratolli, 2001; Wright, 2007; Yim et al., 2007). Several molecular-based studies reported high incidence and diversity of mobile genetic elements carrying a variety of AR genes on isolates recovered from natural environments or anthropogenic-derived environments, such as wastewaters (Henriques et al., 2006a; Moura et al., 2007; Rosser and Young, 1999).

Considering that bacteria exchange genetic elements between species, we need to consider the level of organization of the biota with which we are dealing. The current description of approximately 10,000 validly named species of bacteria (http://www.bacterio.cict.fr/) constitutes an almost insignificant number when compared to the

estimated diversity of, for example, 6300 species per gram of soil (Curtis et al., 2002). This enormous phylogenetic diversity corresponds almost certainly to an equivalent or even higher unknown functional diversity. Microbial ecology examines the diversity and function of microorganisms and studies how these organisms interact with each other and with their environment. Microbial ecology provides a scientific frame to investigate the effects of AR on microbial communities taking their function and structure into account. Microorganisms live and function in assemblages of multiple species (McLandsborough et al., 2006). These microbial assemblages are often highly complex and structured, and these features are closely related to their relevance in the ecosystem. Specifically, microbial communities, such as biofilms, are able to maintain great stability in structure and function over time. They are capable of recovering from and adapting to radical habitat alterations by altering community physiology and composition. This plasticity is essential to the maintenance of the functional properties of the microbial communities. Many processes of pathogenesis are dependent of a structured community of microbial species rather than of one isolated strain (Anukam and Reid, 2007; Marsh, 2005). Resistant bacteria can mediate the survival of sensitive bacteria within biofilms of antibiotic producers, playing a major role in maintaining diversity in such environments (Narisawa et al., 2008).

Hot spots are physical locations where conditions favor relatively high resistance transfer rates. Such hot spots may be the guts of soil arthropods, manure, biofilms, plant rhizospheres, and upper aerobic sediment layers with high microbial densities (Ashelford et al., 2000; Hoffmann et al., 1998; Smalla et al., 2000; Schwartz et al., 2003; van Elsas and Bailey, 2002). Horizontal gene transfer is known to be enhanced by subminimal inhibition concentrations (MIC) of tetracycline (Celli and Trieu-Cuot, 1998), thus the presence of residues of antibiotics in the manure or sewage may add to the intensity of the HGT processes within these matrices and their receiving environments (surface waters, sediments, and soil).

Microbial communities are comprised of high numbers of very diverse and largely unknown organisms, and routinely it is only possible to study clear changes of the most abundant members of a microbial ecosystem. Laboratory studies on the effect on individual microbes can provide valuable background information. However, to investigate the effects on microbial ecosystems, populations must be studied in situ. Subtle community changes could be hard to identify and will be difficult to reliably attribute to the presence of resistance genes. Ultimately, since genetic resistance determinants can be transferred between individuals, the fate and effects of resistance genes on microbial communities is what interests us. There are several culture-independent techniques to shed light on some of the unexplained steps of the evolutionary processes of AR.

The origins of AR genes and their evolutionary routes have been greatly discussed in recent studies. It is now widely accepted that the main answers to these questions are probably hidden in the environment. Whether the AR gene pool, as we know it today, is a direct result of the use/misuse of antibiotics or alternatively it was simply gathered and mobilized from the gene pool of antibiotic producers remains one of the central themes of controversy. Aminov and Mackie (2007) reported extensive phylogenetic analysis of several groups of AR genetic determinants and found no evidence for transfer of such genes from the antibiotic-producing strains to pathogenic or commensal bacteria. During their careful considerations, the authors recognized

that some links were missing that prevented the analysis of evolutionary pathways of resistance to be conclusive. Another relevant topic that may be further elucidated by using culture-independent approaches concerns how AR and genetic exchange of resistance are shaping environment-resident bacterial communities and modulating their functional capabilities. Also, when confronted with molecular variants of resistance genes, the investigator has a dual question in his hands: Is that molecular variant associated with resident bacteria or did it appear as a consequence of human activities? Monitoring AR resistance in the natural environment can only be effective if the source of the resistance can be tracked. Culture-independent methodologies can be designed and applied for source-tracking purposes.

8.3 CULTURE-INDEPENDENT CHARACTERIZATION OF AR

8.3.1 The Unculturable Majority

The first reports highlighting the impossibility to cultivate the majority of the environmental microorganisms resulted from the so-called great plate count anomaly, meaning the evident discrepancy between numbers of microorganisms estimated by plating and by microscopy (Grimes et al., 1986; Torsvik and Ovreas, 2002). A number of studies estimated that, in aquatic environments, calculations of colony-forming units (CFUs) and microscope counts can differ by four to six orders of magnitude (Grimes et al., 1986) and in soil only 0.1–1% of bacteria were found to be readily cultivable under standard laboratory conditions (Torsvik and Ovreas, 2002). The unculturability of many microbial species/strains is not a characteristic exclusive of environmental microorganisms, but it has also been reported for commensal and pathogenic bacteria (Anukam and Reid, 2007; Marsh, 2005).

The reasons many microorganisms cannot be cultivated under laboratory conditions are not entirely understood, but in general this results from the impossibility to mimic the natural conditions, using instead highly artificial and restrictive growth conditions (Barer and Harwood, 1999). Clearly, the employment of culture-based methodologies introduces biases not only due to the unculturability of most microorganisms but also to the unpredictable different levels of cultivability of different phylogenetic groups. Even closely related microorganisms may show very different capacities to adapt to laboratory conditions (Kramme et al., 2004). Culture-based studies might also be biased due to the so-called viable but nonculturable state reported for some bacterial groups (Oliver, 1995).

8.3.2 Studying Resistance in Unculturable Bacteria

Culture-independent techniques have proven useful to unravel the phylogenetic diversity within microbial communities and to assess the molecular diversity of functional genes. Early studies targeted the 16S ribosomal ribonucleic acid (rRNA) gene of communities from environments such as the Sargasso Sea (Giovannoni et al., 1990) or hot springs (Ward et al., 1990). Latter other phylogenetic markers were used to study specific communities. Avrahami and co-workers (2003) identified temperature- or fertilizer-induced shifts in the community structure of ammonia oxidizers from an agricultural soil. In the case of this study, the target gene was the *amoA* gene coding for the α subunit of ammonia monooxygenase. Karr and

co-workers (2003) used a photosynthesis-specific gene, *pufM*, to study the composition of phototrophic purple bacteria in Lake Fryxell, Antarctica. In the last two decades, several culture-independent approaches were developed and the targets were also diversified, though the 16S RNA gene remains the marker of choice to study phylogenetic diversity. A number of studies have applied culture-independent approaches to unravel the molecular diversity of the resistome.

Methodologies are obviously chosen according to the aims of the study being conducted, but some technical constraints often are imposed by the nature of the samples. In Table 8.1, some advantages/limitations of culture-independent techniques previously applied to assess genetic determinants of bacterial resistance are summarized. Also, examples of the resistances and sources that have been targeted using each methodology are presented. When analyzing the results from culture-independent studies, we must be aware of some general biases introduced by such methodologies. First, it is very important to carefully design the sampling strategy. Particularly the incorrect collection, transportation, and storage of samples may introduce important biases into subsequent microbial community analysis. On the other hand, DNA extraction depends on lyses efficiency, which varies between microbial groups, and can be affected by environmental characteristics such as the presence of diverse contaminants. For example, deficient lyses could result in the preferential extraction of DNA from Gram-negative bacteria (Wintzingerode et al., 1997). Also, differential amplification of target genes can bias studies based on polymerase chain reaction (PCR) due to different affinities of primers to templates, different copy numbers of target genes, primer specificity, and DNA coextracted contaminants (Kirk et al., 2004). Amplified products may be artifacts or chimerical molecules or the result of contamination (Spiegelman et al., 2005). Additionally, PCR-based approaches target DNA fragments that may not always correspond to functional enzymes and consequently may have no impact on effective resistance. Detected genes may not be derived from intact cells but instead from free DNA or unviable cells; if that is the case, what is its contribution for gene transfer by transformation of naturally competent bacteria? And which phylogenetic lineages are more prone to natural transformation, and thus more likely to incorporate those "free" DNA sequences? Furthermore, generally the detected genes cannot be linked to their host bacterial species, and conclusions concerning their ability to transfer into recognized pathogens cannot be drawn.

8.3.2.1 Polymerase Chain Reaction Polymerase chain reaction (PCR) currently allows the production of millions of copies of a DNA fragment, with high reliability, within minutes. By using PCR-based approaches, a high number of samples can easily be processed. Also, small quantities of environmental DNA or RNA can be successful used. However, specific biases related to primers specificity and affinity can strongly affect results (Table 8.1). False-positive results are common due, for example, to the use of polymerases contaminated with DNA (Spangler et al., 2010). Accordingly, the adequate PCR controls should always be included in each experiment. It is also essential to determine the detection limits of primers, preferentially taking into account the effect of the contaminants in each specific sample (Volkmann et al., 2007).

Most of the culture-independent studies designed for AR assessment relied on PCR detection of resistance genes from environmental samples. Primers specific for

TABLE 8.1 Culture-Independent Molecular Techniques that have been Used to Detect/Characterize Antimicrobial Resistance Genetic Determinants Examples of Samples and Classes of Antibiotics Targeted in Previously Conducted Studies are Indicated

Technique	Advantages	Limitations	Target Samples	Target Resistance
PCR	Fast Easy to perform Reproducible High sensitivity Possibility to analyze a high number of samples Possibility to assess different genes in the same reaction	DNA extraction and PCR biases Primers based on known genes Not quantitative	Wastewater Drinking water Groundwater Estuarine water Seawater Rumina of cows Swine feed Feces Manure Sediment Soil	Sulfonamides Tetracyclines Aminoglycosides β-lactams
Quantitative PCR	Fast Easy to perform Reproducible High sensitivity Possibility to analyze a high number of samples Quantitative	DNA extraction and PCR biases Primers based on known genes	Wastewater Drinking water Groundwater Rumina of cows Swine feed Feces Manure Sediment Soil	Tetracyclines β-lactams Macrolides Lincosamides Sulfonamides Amphenicols
Hybridization	Fast Easy to perform Reproducible High sensitivity	DNA extraction biases Probes based on known genes	Estuarine water Wastewater Feces Soil	Tetracyclines Macrolides β-lactams Glycopeptides

(*Continued*)

TABLE 8.1. (Continued)

Technique	Advantages	Limitations	Target Samples	Target Resistance
	Analysis of a high number of samples Possibility to assess a high number of genes Avoid PCR biases Semiquantitative			lincosamides Streptogramins
PCR–DGGE	Fast Easy to perform Reproducible Possibility to analyze a high number of samples Possibility to detect molecular variants Allow to sequence bands in the gel	DNA extraction and PCR biases Primer design based on known genes Restricted to the analysis of small DNA fragments (< 700 bp)	Estuarine water Groundwater Rumina of cows Swine feed Feces Sediment Soil	Tetracyclines β-lactams
Gene libraries	Possibility to analyze molecular diversity Allow analysis of large DNA fragments	DNA extraction and PCR biases Primer on known genes Labor intensive and time consuming	Estuarine water Groundwater Swine feed Feces Soil	Tetracyclines β-lactams
Metagenomics	Possibility to analyze molecular diversity Potential to discover completely novel genes Avoid PCR biases	DNA extraction biases Cloning biases Needs high amounts of high-quality DNA Labor intensive and time consuming	Animal microbiome Soil Dental plaque Sediment Sludge Gut	Tetracyclines β-lactams Aminoglycosides Amphenicols Glycopeptides

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Pyrosequencing	Fast Cost effective Avoids cloning biases High throughput	DNA extraction and PCR biases Restricted to the analysis of small DNA fragments (<450 bp)	Clinical specimens	Rifampin Macrolides
Exogenous plasmid isolation	Easy to perform Isolation of only conjugative or mobilizable plasmids Potential to isolate plasmids independently of their abundance	Original bacterial host unknown Relies on plasmid encoded selectable markers Relies on conjugation/ mobilization Plasmids must be compatible with host	Sludge Pig manure Soil	Aminoglycosides Sulfonamides Amphenicols

several classes of resistance genes have already been designed and validated for culture-independent analysis. PCR-based methodologies were applied and found useful to confirm the occurrence of genes conferring resistance to sulfonamides, tetracyclines, aminoglycosides, and β-lactams in several different sample matrices such as wastewater, drinking water, coastal water, river sediments, soils, and manure (Agerso et al., 2004; Heuer et al., 2002; Pei et al., 2006; Schwartz et al., 2003; Storteboom et al., 2010).

8.3.2.2 Quantitative Polymerase Chain Reaction Conventional PCR is difficult to use in a quantitative manner due to reagent depletion and to loss of polymerase activity during amplification. To overcome this limitation quantitative real-time PCR (qPCR) is frequently used. This method detects amplicons during the early exponential phase of the amplification (Zhang and Fang, 2006). It involves the use of fluorescent markers, and the amount of fluorescence is directly related to the amount of product at the end of each cycle. The initial concentration of the target DNA can be estimated according to the change of product concentration with amplification cycles. qPCR allows us to rapidly quantify DNA, being also highly sensitive and accurate (Zhang and Fang, 2006). However, when used with DNA from a complex sample, the presence of PCR inhibitors may strongly influence the accuracy of the method. Serial dilutions of environmental DNA should be used for quantification in order to determine the presence and effect of contaminants (Harms et al., 2003).

Studies taking advantage of qPCR were conducted aiming at the detection and quantification of resistance genes in complex samples. Jernberg and co-workers (2007) used a qPCR-based approach to evaluate the incidence of genes conferring resistance to clindamycin and macrolides in DNA extracted from feces after clindamycin administration. The results suggested that long after the selection pressure had been removed, impacts on the human intestinal microbiota persisted and high levels of resistance could still be detected. Other studies used qPCR to investigate AR genes as emerging environmental contaminants. Levels of contamination by AR genes were higher in environments directly impacted by anthropogenic actions, and potential pathways for the spread of AR genes were identified (Pei et al., 2006; Pruden et al., 2006).

The abundance of AR genes can also be used as an indicator of water quality. The resistance genes *ermB*, *mecA*, $bla_{\text{SHV-5}}$, *ampC*, *tetO*, and *vanA* were used for that purpose by Böckelmann and co-workers (2009). Xi and co-workers (2009) found and quantified β-lactams, chloramphenicol, and sulfonamide resistance genes in drinking water. The authors observed that the water treatment processes might even increase AR prevalence. qPCR assays were also validated, optimized, and applied for quantification of genes conferring resistance to sulfonamides, vancomycin, methicillin, and β-lactams in wastewater samples and soil (Demanèche et al., 2008; McKinney et al., 2010; Lachmayr et al., 2009; Smith et al., 2004; Volkmann et al., 2004) and of genes conferring resistance to tetracyclines in wastewater, groundwater, and river sediments (McKinney et al., 2010; Koike et al., 2007; Peak et al., 2007; Pei et al., 2006). Optimized assays proved to be sensitive and allowed to obtain reliable and reproducible results from environmental samples.

8.3.2.3 Hybridization-Based Techniques Hybridization protocols rely on the use of specific probes to detect the presence of target sequences in a genome/community.

These are highly sensitive methods and can be used to analyze a high number of samples simultaneously. Probes can be designed based on multiple sequence alignments in order to target gene families. Also, the stringency conditions under which hybridization occurs can be manipulated to adjust specificity. Currently, several nonradiolabeled systems are commercially available such that it is possible to avoid radioactive labeling. In the case of complex samples, probes can be hybridized with environmental DNA or with previously obtained PCR fragments.

Hybridization-based methodologies were frequently used to detect AR genes in environmental samples using specific probes (Agerso et al., 2004; Henriques et al., 2006b). In fact, it was one of the first molecular techniques used for this purpose (Mendez et al., 1980). Malik and co-workers (2008) applied hybridization to detect *ampC, tetO*, *ermB*, *SHV-5*, *mecA*, and *vanA* genes in soil samples. The authors related the presence of *ampC* with irrigation with wastewater. The construction of macroarrays to screen for the presence of resistance genes in soils and animal fecal samples has been reported by Patterson and co-workers (2007). The incidence and distribution of genes conferring resistance to tetracyclines and erythromycin in such samples was assessed. The technique proved sensitive enough to detect genes present in $< 1\%$ of the bacterial population. The abundance of resistance genes was linked to the use of antibiotics and suggested that different classes of resistance genes are prevalent in animal gut environments compared with nongut environments such as soils.

Alternative hybridization-based methods can also be used, in a culture-independent manner, for AR assessment. For example, Zhou and colleagues (2009) developed a fluorescence in situ hybridization (FISH) protocol to determine the prevalence of a ribosomal modification responsible for macrolide, lincosamide, and streptogramin B resistance in communities from manure and soil (Zhou et al. 2009, 2010). In this case fixed samples were hybridized with oligonucleotide fluorescent probes that are small enough to penetrate the cells and hybridize specifically to rRNA. This is a quantitative method but obviously has a limited range of applications (resistance related to rRNA modifications).

8.3.2.4 Construction of Gene Libraries The construction and analysis of libraries of PCR amplicons obtained from environmental samples has been frequently reported, being the fragments most often from phylogenetic markers (Giovannoni et al., 1990; Ward et al., 1990). In fact cloning and sequencing of the 16S rRNA gene is still the most commonly used molecular technique in the field of microbial ecology. This methodology includes the extraction of nucleic acids, amplification and cloning of the target fragment, followed by sequencing and similarity searches against public databases. The analysis of gene libraries provides a comprehensive picture of the diversity of target genes within a community, but requires the scrutiny of a high number of clones from each sample, which is a time-consuming and laborious strategy.

This technique can provide useful information about the diversity of resistance genes in a complex sample. For this, primers targeting gene families, which include considerable molecular diversity, are especially useful. For example, more than 180 different genes encoding TEM-like β-lactamases have been reported (http://www.lahey.org/studies/) and many more are probably yet to be discovered. The corresponding enzymes often exhibit different activity profiles. Primers targeting gene families should be designed based on multiple alignments of sequences retrieved from

databases and identification of conserved regions. Large fragments can be amplified and cloned and thus the entire gene can frequently be analyzed.

Aminov and co-workers (2001) applied a strategy based on the PCR amplification of tetracycline efflux genes directly from total DNA, cloning of the resultant amplicons, and sequencing analysis of the constructed libraries. Samples were from swine feed and feces and from the lagoons and groundwater underlying swine production facilities known to be impacted by waste leakage. Extensive sequence analysis allowed the authors to conclude that the tetracycline resistance pool originates mainly from the gut of tetracycline-fed animals. This pool was probably mobile and persistent in the environment with the potential to enter the food chain.

An approach based on the construction and analysis of gene libraries was also applied to exploit the diversity of β-lactamase gene sequences in estuarine waters and to get information on the distinctive features of those sequences when compared to others previously reported from clinical environments (Henriques et al., 2006b). Most of the retrieved sequences were identical or very similar to β-lactamase gene sequences previously characterized from clinical isolates.

8.3.2.5 Denaturing Gradient Gel Electrophoresis (DGGE) A limited number of studies highlighted the usefulness of other culture-independent methodologies to investigate not only the presence but also the molecular diversity of resistance genetic determinants in complex samples. DGGE has been extensively applied in studies concerning the dynamics of microbial communities, which usually imply the analysis of a large number of samples. The technique separates different gene fragments based on their melting temperature, which is a function of their guanine–cytosine (G+C%) content (Muyzer et al., 1993). The basic methodology comprises extraction of environmental DNA, amplification using PCR with primers targeting part of the gene, and separation of the amplicons on a polyacrylamide gel with a gradient of increasing concentration of chemical denaturants (formamide and urea). Usually a 35 to 40-bp clamp is attached to the 5′ end of the forward primer to prevent complete denaturation of the DNA fragment (Muyzer et al., 1993). DGGE allows the rapid and simple analysis of a large number of samples and can be used in combination with band sequencing. Since the method implies DNA extraction and amplification, it suffers from the same biases as other PCR-based techniques. Also, the analysis is usually restricted to small DNA fragments (200–700 bp), thus losing much information about the diversity of molecular variants and, of course, preventing the deduction of whether they represent truly expressed genes.

In terms of AR, DGGE has been essentially used to screen gene libraries in order to detect molecular variants of a given resistance gene. Theoretically, DGGE can also be used to directly analyze a complex sample in order to determine the molecular diversity of resistance genes. In this case, the technique is limited to the ability to detect the dominant molecular variants. The intensity of each band depends on the abundance of each sequence but can be affected by PCR-related biases and so the method cannot be considered quantitative. Also the gel gradient can be optimized to assure the best separation of the amplicons, but the co-migration of fragments with distinct sequences cannot be excluded when analyzing a complex sample.

Aminov and co-workers (2001) developed primer sets targeting tetracycline resistance genes to detect and retrieve homologous sequences from environmental samples. The same primers were applied to assess the presence of such genes in waste

lagoons and groundwater (Chee-Sanford et al., 2001). In both cases, the authors applied a PCR–DGGE approach to study the molecular diversity, the identities, and the origin of the retrieved DNA fragments. Several classes of genes encoding resistance to tetracycline were found in total DNA extracted from the lagoons and from underlying groundwater. These studies demonstrated that tetracycline resistance genes occur in the environment as a direct result of agriculture and suggested that groundwater may be a potential source of AR in the food chain.

Kobayashi and co-workers (2007) showed that various tetracycline resistance genes encoding ribosomal protection proteins were widely distributed in the river and channel sediments of the Mekong Delta by applying a PCR–DGGE approach, using primers designed by Aminov et al. (2001). A PCR–DGGE-based approach was also applied to screen libraries of β-lactamase gene sequences from estuarine waters (Henriques et al., 2006b). This type of approach deserves to be tested for its performance in clinical specimens in order to evaluate its capability to discern the emergence of new molecular variants of already known and well-characterized genes.

8.3.2.6 Metagenomics Metagenomics includes methodologies that aim to assess the entire genomic information stored in DNA isolated from the microbial community. In fact, these methodologies have great potential for assessing all of the genetic competences of this community (Handelsman, 2004). The use of metagenomics to characterize new genes and gene products for various purposes has been reported in several studies (Handelsman, 2004; Gabor et al., 2007).

Metagenomic approaches do not rely on the design of DNA probes or primers derived from conserved regions of already known sequences, thus allowing identification of novel genes and avoiding the biases introduced by PCR. In most metagenome-based studies, total DNA is extracted from environmental samples, inserted into cloning vectors, and then propagated in *E. coli* strains (Stein et al., 1996). The resulting environmental libraries can be analyzed by conducting a sequence- or a function-based screening. When studying AR, a function-based screening is usually the method of choice as the phenotype conferred by resistance genes is easy to identify in culture plates. Since this screening is sequence independent, it has the potential to identify entirely novel genes. On the other hand, one drawback is the dependence on expression of the cloned genes by a foreign host.

A number of studies reported the usefulness of metagenomics to evaluate the potential risk of bacterial communities to human health, namely by the analysis of molecular diversity of AR genetic determinants. Riesenfeld et al. (2004) constructed four metagenomic libraries containing a total of 4.1 gigabases of cloned soil DNA. The authors detected nine clones expressing resistance to aminoglycosides and one expressing tetracycline resistance. In general, retrieved sequences were significantly different from previously reported sequences. A similar approach allowed cloning novel tetracycline, amoxicillin, and gentamicin resistance determinants from the oral metagenome (Diaz-Torres et al., 2003, 2006).

Molecular analysis of a metagenomic library from the cold-seep sediments was performed to determine the prevalence and genotypes of β-lactamase genes (Song et al., 2005). The resistance levels conferred by the β-lactamases identified were similar to resistance levels conferred by the enzymes isolated from clinical strains. Extended spectrum β-lactamases were identified. The authors demonstrated that most of the diversity of these enzymes is not the result of recent evolution.

More recently, other environments were assessed including the human and animal microbiome (Kazimierczak et al., 2009; Sommer et al., 2009), soils (Allen et al., 2009; Donato et al., 2010) and sludge (Mori et al., 2008; Parsley et al., 2010). These studies allowed the identification of genetic determinants conferring resistance to β-lactams (Allen et al., 2009; Donato et al., 2010; Parsley et al., 2010; Sommer et al., 2009), tetracyclines (Kazimierczak et al. 2009; Sommer et al. 2009), amphenicols (Parsley et al., 2010; Sommer et al., 2009), aminoglycosides (Donato et al., 2010; Parsley et al., 2010; Sommer et al., 2009), and glycopeptides (Mori et al., 2008). Most of the characterized genes have not been previously identified and were evolutionarily distant from known resistance genes. However, in some cases, especially when analyzing human or animal associated communities, known resistance genes were also identified (Diaz-Torres et al., 2003, 2006; Kazimierczak et al., 2009; Sommer et al., 2009). Using a metagenomic approach Kazimierczak and co-workers (2009) were also able to verify that the majority of resistance genes identified from the pig gut were associated with putative mobile genetic elements.

8.3.2.7 Pyrosequencing Pyrosequencing is a novel and successful sequencing strategy (Ronaghi, 2001). The procedure generally consists on generating 5′-biotinylated PCR products (e.g., from environmental DNA), processing the PCR products to obtain single-stranded DNA, annealing of the primer, and sequencing. The sequencing method is based on the detection of the pyrophosphate released when DNA polymerase incorporates nucleotides into a growing DNA chain. The pyrophosphate is used to convert adenosine monophosphate (AMP) into adenosine triphosphate (ATP), which is then used to produce visible light. The light signal intensity is proportional to the number of nucleotides incorporated. This method is faster and more cost effective than sequencing methods previously described, and it does not require cloning of the DNA, avoiding biases associated with this step in metagenomics. However, it can only be used to analyze small DNA fragments (<450 bp) and if, based on PCR, it suffers from the biases associated with this technology.

Especially for the detection of mutations, pyrosequencing offers high accuracy, speed, and high throughput. This method has already been used to detect mutations in genes conferring resistance to antibiotics (Moder et al., 2007; Halse et al., 2010). In a few cases the method was validated to study resistance in a culture-independent manner. Moder and colleagues (2007) developed a pyrosequencing assay to determine macrolide resistance, associated with 23S rRNA point mutations, in *Helicobacter pylori*. Macrolide resistance was also determined directly out of gastric biopsies, without culturing *H. pylori*. Culture-independent and culture-dependent strategies gave identical results, and pyrosequencing was a fast and reliable method for determining macrolide resistance in *H. pylori*.

A pyrosequencing assay was also developed for detection in clinical specimens of both the presence of *Mycobacterium tuberculosis* and mutations within the *rpoB* gene that are associated with rifampin resistance (Halse et al., 2010). Authors reported high concordance between results obtained with this assay and results from conventional methods. However, the pyrosequencing method was faster and less expensive.

8.3.3 Assessing Mobile Resistance among Unculturable Microorganisms

Mobile genetic elements contribute to bacterial genomic plasticity, allowing gene flow and favoring genetic variability. Frequently, genes encoding AR are located on

mobile genetic elements that can be exchanged among bacteria of different taxonomic affiliation (Caratolli, 2001). Plasmids, transposons, integrons, and other integrative elements contribute to the spread of AR, playing a major role in the dissemination of resistance between environmental bacteria and from those to pathogens. Culture-independent approaches designed to focus on mobile genetic elements have the potential to characterize not only the occurrence and molecular diversity of resistance genes but also to assess their potential for mobility between species and strains.

Theoretically, PCR amplification of gene cassettes from community DNA using primers specific for integron-conserved regions is a straightforward method to analyze many samples for the presence of mobile AR genes. That strategy has been assayed in a number of studies (Elsaied et al., 2007; Moura et al., 2010; Nemergut et al., 2004). However, the detection of open reading frames with noticeable homology with AR genes occurred rarely, suggesting that integrons may contribute to gene transfer in response to selective pressures other than the presence of antibiotics. Results can also be biased by a prime's limited specificity and different affinity.

The isolation of plasmids from the environment can be achieved by exogenous isolation in either a bi- or triparental mating or transformation of recipient strains with plasmid DNA extracted directly from environmental samples. These methodologies are dependent on the ability to cultivate the recipient strains but can be used to capture plasmids from unculturable bacteria (Hill et al., 1996). A number of plasmids have been captured from natural or anthropogenic-derived environments using such techniques, and their nucleotide sequence has been completely determined. A variety of AR genes were identified. Additionally, the capture of plasmids into selected bacterial hosts, either by exogenous isolation or by transformation, allowed the characterization of the resistance phenotypes. However, the recipient strains used to capture plasmids obviously select for certain types of plasmids and the use of different hosts is advisable.

Dröge et al. (2000) isolated several plasmids from activated sludge by using an exogenous isolation procedure. The presence of open reading frames with significant similarity with AR genes was reported by the authors. High incidences of broad host range plasmids, conferring resistance to several antibiotics, were detected in activated sludge, with obvious repercussions on the dissemination of AR on environmental bacterial populations. Subsequent studies reported the complete nucleotide sequence of 4 plasmids out of the 12 isolated by Dröge and co-workers (Heuer et al., 2004; Schlüter et al., 2003, 2005; Tauch et al., 2003).

Self-transferable plasmids were isolated in *E. coli* CV60 and *Pseudomonas putida* UWC1 recipients from bacterial communities of pig manure (Smalla et al., 2000). Genes conferring resistance to several antibiotics such as streptomycin, sulfonamide, streptothricin, kanamycin, and chloramphenicol were found. The nucleotide sequences of plasmids pIE115 and pIE1130, isolated during this study, were completely determined.

Szczepanowski and co-workers (2004) analyzed the bacterial populations residing in the activated sludge compartment of a wastewater treatment plant for the presence of plasmids conferring erythromycin resistance to the host bacterium. Ten different AR plasmids were isolated by a transformation-based approach into competent *E. coli* DH5 or *Pseudomonas* sp. GFP1. The selected plasmids conferred resistance to up to eight different classes of antibiotics. From this study, pRSB101, pRSB105, and

pRSB107 were completely sequenced (Schlüter et al., 2007; Szczepanowski et al., 2004, 2005).

Metagenomic approaches for studying the mobile metagenome aim to assess all mobile genetic elements resident in a bacterial community, regardless of the capability to culture the host bacteria. Recently, a novel culture-independent method to capture plasmids from complex microbial communities was described and designated TRACA (Jones and Marchesi, 2007). This method is based on the metagenomic DNA and is independent of plasmid-selectable markers or a plasmid's ability to mobilize and replicate in recipient strains. Instead, plasmids in the target community are provided with an origin of replication and a selectable marker, by tagging plasmids with a transposon encoding these functions. This method has been applied to the study of the human gut mobile metagenome (Jones and Marchesi, 2007).

Aiming to detect plasmid-borne AR genes in wastewater, Szczepanowski and colleagues used a strategy based on the amplification of resistance genes from total plasmid DNA directly extracted from wastewater samples (Szczepanowski et al., 2009). The authors designed and applied 192 resistance-gene-specific PCR primer sets and detected genes conferring resistance to a wide variety of antibiotics.

The analysis of multiple samples along the wastewater treatment plant allowed the authors to suggest that resistance determinants might be disseminated in habitats downstream of the sewage plant.

8.4 FINAL CONSIDERATIONS

Evidence has been gathered confirming that environmental microbial communities comprise a flexible and wide pool of resistance genes. The implications of such a pool to human and ecological health have been extensively discussed in recent years, and concern increases due to the ability of AR genes to rapidly evolve and to be mobilized into other hosts. However, the characterization of the environmental reservoirs of AR is far from being satisfactory and clearly our understanding of the microbial ecology of resistance is incomplete.

A precise definition of what is a reservoir of AR genes is needed that applies to both qualitative and quantitative terms. For that, several different ecological niches must be exploited for their content on AR genetic determinants in order to evaluate the extent of the molecular diversity; in this context molecular approaches that allow the determination of quantitative data are needed. Information on the extent of the molecular diversity and the amount of each molecular variant present in a particular environment will constitute a valuable tool to objectively assess the risk represented by AR genes. Mapping niches for their content in AR genetic determinants will allow comparisons between settings under different influences, thus contributing to the unveiling of the forces that favor the emergence of new variants and promote their transmission to pathogens. The fact that the majority of microorganisms cannot be cultivated under laboratory conditions raises the complexity of the already difficult task of characterizing resistance among complex microbial communities.

Large sets of data, like the one obtained in 2004 by the Craig Venter team (Venter et al., 2004), must be intensively investigated using bioinformatics to discern what sequences might represent AR genes, in what amounts are each sequence represented, and to infer whether or not they correspond to truly expressed genes.

The same type of data sets are also informative about the genetic environment of each resistance gene, thus allowing inferring about their association with mobile genetic elements and their capability to move between bacterial strains.

Culture-independent approaches increase and broaden the spectrum of molecular variants already detected on isolated bacteria. The large diversity revealed by methods that directly capture molecules is of great value to investigate the evolution of AR genes, their routes of dispersion, and the factors that contribute to their amplification in a certain environment. The detection of new molecular variants without cultivation of the organisms that host those molecules poses a new challenge: Methodologies to investigate the expression of those putative genes must be put in practice and the kinetics of their products must be better understood.

This mini review discussed several topics of the AR thematic, with emphasis on environmental AR, which can potentially be clarified using culture-independent approaches. A number of culture-independent studies, which have already been reported, and contributed with significant data to clarify these aspects, were referred. Relevance was also given to methodological considerations, namely the specificities, advantages, and limitations of different culture-independent approaches with potential to be applied to the study of AR.

Despite the acknowledged limitations, several studies demonstrated the applicability of culture-independent approaches to estimate the resistance gene pool and to follow the flux of such genes, independently of the ability to culture their original hosts. Novel perspectives on the ecology of AR genes have been achieved using culture-independent technologies. Potential sources for resistance dissemination have been identified. The flow of resistance genes between environmental and clinical compartments has been assessed. Novel and useful culture-independent tools have been designed and optimized to evaluate and discriminate the impact of antibiotic use on microbial communities.

The results gathered from the environment may provide an early advice system for future clinically relevant AR mechanisms. Aiming toward this, further studies are needed to provide conclusive answers to remaining key questions concerning the incidence, fate, and transport of antibiotic-resistant bacteria in natural environments, the major sources contributing to the input of AR into the environment and the magnitude and extent of AR gene migration from the environmental bacterial communities into specific bacterial species that are pathogenic to humans or animals. Data already obtained should be carefully analyzed so that efficient mitigation policies can be implemented to reduce dissemination of AR. The hypothesis that AR genes should be considered as potential environmental contaminants, representing per se an ecological threat, is also worthy of investigation. We trust that to achieve these objectives, several different approaches should be applied. For this purpose, a priority should be to compose more detailed estimations of the differences between the results obtained using culture-dependent and culture-independent methods.

Joining qualitative and quantitative data about the repertoire of molecular determinants of resistance, information about their phylogenetic context, and the genetic environments where they evolve will allow us to implement monitoring policies and to make qualified decisions about where and when mitigation measures are needed. The availability of quantitative data on the presence of antibiotic determinants and their hosts in specific niches will greatly help to establish monitoring strategies and to design monitoring strategies in a more rational basis.

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REFERENCES

Agerso Y; Sengelov G, Jensen LB (2004). Development of a rapid method for direct detection of *tet*(M) genes in soil from Danish farmland. *Environ Int* 30:117–122.

Allen HK, Moe LA, Rodbumrer J, Gaarder A, Handelsman J (2009). Functional metagenomics reveals diverse β-lactamases in a remote Alaskan soil. *ISME J* 3:243–251.

Aminov RI, Mackie RI (2007). Evolution and ecology of antibiotic resistance genes. *FEMS Microbiol Lett* 271:147–161.

Aminov RI, Garrigues-JeanJean N, Mackie RI (2001). Molecular ecology of tetracycline resistance: Development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. *Appl Environ Microbiol* 67:22–32.

Anukam KC, Reid G (2007). Organisms associated with bacterial vaginosis in Nigerian women as determined by PCR-DGGE and 16S rRNA gene sequence. *Afr Health Sci* 7:68–72.

Ash RJ, Mauck B, Morgan M (2002). Antibiotic resistance of Gram-negative bacteria in rivers, United States. *Emerg Infect Dis* 8:713–716.

Ashelford KE, Norris SJ, Fry JC, Bailey MJ, Day MJ (2000). Seasonal population dynamics and interactions of competing bacteriophages and their host in the rhizosphere. *Appl Environ Microbiol* 66:4193–4199.

Avrahami S, Liesack W, Conrad R (2003). Effects of temperature and fertilizer on activity and community structure of soil ammonia oxidizers. *Environ Microbiol* 5:691–705.

Baquero F, Alvarez-Ortega C, Martínez JL (2009). Ecology and evolution of antibiotic resistance. *Environ Microbiol Rep* 1(6):469–476.

Barer MR, Harwood CR (1999). Bacterial viability and culturability. *Adv Microbiol Physiol* 41:93–137.

Bennett PM (2008). Plasmid encoded antibiotic resistance: Acquisition and transfer of antibiotic resistance genes in bacteria. *Br J Pharmacol* 153:S347–357.

Berg J, Tom-Petersen A, Nybroe O (2005). Copper amendment of agricultural soil selects for bacterial antibiotic resistance in the field. *Lett Appl Microbiol* 40:146–151.

Beuzon CR, Chessa D, Casadesus J (2004). IS200: An old and still bacterial transposon. *Int Microbiol* 7:3–12.

Bibb MJ (2005). Regulation of secondary metabolism in *Streptomycetes*. *Curr Opin Microbiol* 8:208–215.

Böckelmann U, Dörries H-H, Ayuso-Gabella MN, de Marçay MS, Tandoi V, Levantesi C, Masciopinto C, van Houtte E, Szewzyk U, Wintgens T, Grohmann E (2009). Quantitative PCR monitoring of antibiotic resistance genes and bacterial pathogens in three European artificial groundwater recharge systems. *Appl Environ Microbiol* 75:154–163.

Burrus V, Waldor MK (2004). Shaping bacterial genomes with integrative and conjugative elements. *Res Microbiol* 155:376–386.

Caratolli A (2001). Importance of integrons in the diffusion of resistance. *Vet Res* 32:243–259.

Celli J, Trieu-Cuot P (1998). Circularization of Tn916 is required for expression of the transposon-encoded transfer functions: Characterization of long tetracycline-inducible transcripts reading through the attachment site. *Mol Microbiol* 28:103–117.

Chee-Sanford JC, Aminov RI, Krapac IJ, Garrigues-Jeanjean N, Mackie RI (2001). Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Appl Environ Microbiol* 67:1494–1502.

Cundliffe E (1992). Self-protection mechanisms in antibiotic producers. *Ciba Found Symp* 171:199–208.

Curtis TP, Sloan W, Scannell J (2002). Estimating prokaryotic diversity and its limits. *PNAS USA* 99:10494–10499.

D'Costa VM, McGrann KM, Hughes DW, Wright GD (2006). Sampling the antibiotic resistome. *Science* 311:374–377.

Demanèche S, Sanguin H, Poté J, Navarro E, Bernillon D, Mavingui P, Wildi W, Vogel TM, Simonet P (2008). Antibiotic-resistant soil bacteria in transgenic plant fields. *PNAS USA* 105:3957–3962.

Diaz-Torres ML, McNab R, Spratt DA, Villedieu A, Hunt N, Wilson M, Mullany P (2003). Novel tetracycline resistance determinant from the oral metagenome. *Antimicrob Agents Chemother* 47:1430–1432.

Diaz-Torres ML, Villedieu A, Hunt N, McNab R, Spratt DA, Allan E, Mullany P, Wilson M (2006). Determining the antibiotic resistance potential of the indigenous oral microbiota of humans using a metagenomic approach. *FEMS Microbiol Lett* 258:257–262.

Di Giulio M (2003). The universal ancestor and the ancestor of bacteria were hyperthermophiles. *J Mol Evol* 57(6):721–730.

Donato JJ, Moe LA, Converse BJ, Smart KD, Berklein FC, McManus PS, Handelsman J (2010). Metagenomic analysis of apple orchard soil reveals antibiotic resistance genes encoding predicted bifunctional proteins. *Appl Environ Microbiol* 76:4396–4401.

Dröge M, Pühler A, Selbitschka W (2000). Phenotypic and molecular characterization of conjugative antibiotic resistance plasmids isolated from bacterial communities of activated sludge. *Mol Genomics Genet* 263:471–482.

Elsaied H, Stokes HW, Nakamura T, Kitamura K, Fuse H, Maruyama A (2007). Novel and diverse integron integrase genes and integron-like gene cassettes are prevalent in deep-sea hydrothermal vents. *Environ Microbiol* 9:2298–2312.

Frost LS, Leplae R, Summers AO, Toussaint A. (2005). Mobile genetic elements: The agents of open source evolution. *Nat Rev Microbiol* 3:722–732.

Gabor E, Liebeton K, Niehaus F, Eck J, Lorenz P (2007). Updating the metagenomics toolbox. *Biotechnol J* 2:201–206.

Garau G, Di Guilmi AM, Hall BG (2005). Structure-based phylogeny of the metallo-β-lactamases. *Antimicrob Agents Chemother* 49:2778–2784.

Giovannoni SJ, Britschgi TB, Moyer CL, Field KG (1990). Genetic diversity in Sargasso Sea bacterioplankton. *Nature* 345:60–63.

Goñi-Urriza M, Capdepuy M, Arpin C, Roques C, Caumette P, Quentin C (2000). Impact of an urban effluent on antibiotic resistance of riverine *Enterobacteriaceae* and *Aeromonas* spp. *Appl Environ Microbiol* 66:125–132.

Grimes DJ, Atwell RW, Brayton PR, Palmer LM, Rollins DM, Roszak DB, Singleton FL, Tamplin ML, Colwell RR (1986). The fate of enteric pathogenic bacteria in estuarine and marine environments. *Microbiol Sci* 3:324–329.

Hall BG, Barlow M (2004). Evolution of the serine β-lactamases: Past, present and future. *Drug Resist Updat* 7:111–123.

Halse TA, Edwards J, Cunningham PL, Wolfgang WJ, Dumas NB, Escuyer VE, Musser KA (2010). Combined real-time PCR and *rpoB* gene pyrosequencing for rapid identification of *Mycobacterium tuberculosis* and determination of rifampin resistance directly in clinical specimens *J Clin Microbiol* 48:1182–1188.

Handelsman J (2004). Metagenomics: Application of genomics to uncultured microorganisms. *Microbiol Mol Biol Rev* 68:669–685.

Harms G, Layton AC, Dionisi HM, Gregory IM, Garrett VM, Hawkins SA, Robinson KG, Sayler GS (2003). Real-time PCR quantification of nitrifying bacteria in a municipal wastewater treatment plant. *Environ Sci Technol* 37:343–351.

Henriques I, Fonseca F, Alves A, Saavedra MJ, Correia A (2006a). Occurrence and diversity of integrons and β-lactamase genes among ampicillin-resistant isolates from estuarine waters. *Res Microbiol* 157:938–947.

Henriques I, Moura A, Alves A, Saavedra MJ, Correia A (2006b). Analyzing diversity among β-lactamase encoding genes in aquatic environments. *FEMS Microbiol Ecol* 56:418–429.

Heuer H, Krögerrecklenfort E, Wellington EMH, Egan S, van Elsas JD, van Overbeek L, Collard JM, Guillaume G, Karagouni AD, Nikolakopoolou TL, Smalla K (2002). Gentamicin resistance genes in environmental bateria: Prevalence and transfer. *FEMS Microbiol Ecolo* 42:289–302.

Heuer H, Szczepanowski R, Schneiker S, Puhler A, Top EM, Schlüter A (2004). The complete sequences of plasmids pB2 and pB3 provide evidence for a recent ancestor of the IncP-1beta group without any accessory genes. *Microbiology* 150:3591–3599.

Hill KE, Marchesi JR, Fry JC (1996). Conjugation and mobilization in the epilithon. In ADL Akkermans, JD van Elsas, and FJ de Bruijn (Eds.), *Molecular Microbial Ecology Manual 5.2.2*, Kluwer Academic, Dordrecht, The Netherlands, pp. 1–28.

Hoffmann A, Thimm T, Dröge M, Moore ERB, Munch JC, Tebbe CC (1998). Intergenic transfer of conjugative and mobilizable plasmids harbored by *Escherichia coli* in the gut of the soil microarthropod *Folsomia candida*. *Appl Environ Microbiol* 64:2652–2659.

Iyer LM, Balaji S, Koonin EV, Aravind L (2006). Evolutionary genomics of nucleo-cytoplasmic large DNA viruses. *Virus Res* 117(1):156–184.

Jernberg C, Löfmark S, Edlund C, Jansson JK (2007). Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. *ISME J* 1:56–66.

Jones BV, Marchesi JR (2007). Transposon-aided capture (TRACA) of plasmids resident in the human gut mobile metagenome. *Nat Methods* 4:55–61.

Jones JG, Gardener S, Simon BM, Pickup RW (1986). Antibiotic resistant bacteria in Windermere and two remote upland tarns in the English Lake District. *J Appl Microbiol* 60:443–453.

Kapetanaki M, Kerry J, Hiney M, O'Brian C, Coyne R, Smith P (1995). Emergence, in oxytetracycline-free marine mesocosms, of microorganisms capable of colony formation on oxytetracycline-containing media. *Aquaculture* 134:227–236.

Karr EA, Sattley WM, Jung DO, Madigan MT, Achenbach LA (2003). Remarkable diversity of phototrophic purple bacteria in a permanently frozen Antarctic lake. *Appl Environ Microbiol* 69:4910–4914.

Kazimierczak KA, Scott KP, Kelly D, Aminov RI (2009). Tetracycline resistome of the organic pig gut. *Appl Environ Microbiol* 75(6):1717–1722.

Kirk JL, Beaudette LA, Hart M, Moutoglis P, Klironomos JN, Lee H, Trevors JT (2004). Methods of studying soil microbial diversity. *J Microbiol Methods* 58:169–188.

Kobayashi T, Suehiro F, Tuyen BC, Suzuki S (2007). Distribution and diversity of tetracycline resistance genes encoding ribosomal protection proteins in Mekong river sediments in Vietnam. *FEMS Microbiol Ecol* 59:729–737.

Koike S, Krapac IG, Oliver HD, Yannarell AC, Chee-Sanford JC, Aminov RI, Mackie RI (2007). Monitoring and source tracking of tetracycline resistance genes in lagoons and groundwater adjacent to swine production facilities over a 3-year period. *Appl Environ Microbiol* 73:4813–4823.

Kramme S, Bretzel G, Panning M, Kawuma J, Drosten C (2004). Detection and quantification of *Mycobacterium leprae* in tissue samples by real-time PCR. *Med Microbiol Immunol* 193:189–193.

Kümmerer K (2009). Antibiotics in the aquatic environment—A review—Part II. *Chemosphere* 75:435–441.

Lachmayr KL, Kerkhof LJ, DiRienzo AG, Cavanaugh CM, Ford TE (2009). Quantifying nonspecific TEM β-lactamase (blaTEM) genes in a wastewater stream. *Appl Environ Microbiol* 75:203–211.

Lucking R, Huhndorf S, Pfister D, Rivas Plata E, Lumbsch H (2009). Fungi evolved right on track. *Mycologia* 101(6):810–822.

Malik A, Çelik EK, Bohn C, Böckelmann U, Knobel K, Grohmann E (2008). Detection of conjugative plasmids and antibiotic resistance genes in anthropogenic soils from Germany and India. *FEMS Microbiol Lett* 279:207–216.

Marsh PD (2005). Dental plaque: biological significance of a biofilm and community life-style. *J Clini Periodontol* 32:7–15.

Martiny JBH, Bohannan BJM, Brown JH, Colwell RK, Fuhrman JA, Green JL, Horner-Devine MC, Kane M, Krumins JA, Kuske CR, Morin PJ, Naeem S, Øvreås L, Reysenbach A-L, Smith VH, Staley JT (2006). Microbial biogeography: Putting microorganisms on the map. *Nat Rev Microbiol* 4:102–112.

McKinney C, Loftin K, Meyer MT, Davis JG, Pruden A (2010). *tet* and *sul* antibiotic resistance genes in livestock lagoons of various operation type, configuration, and antibiotic occurrence. *Environ Sci Technol* 44:6102–6109.

McLandsborough L, Rodriguez A, Pérez-Conesa D, Weiss J (2006). Biofilms: At the interface between biophysics and microbiology. *Food Biophys* 1:94–114.

Mendez B, Tachibana C, Levy SB (1980). Heterogeneity of tetracycline resistance determinants. *Plasmid* 3:99–108.

Moder K-A, Layer F, Konig W, Konig B (2007). Rapid screening of clarithromycin resistance in *Helicobacter pylori* by pyrosequencing. *J Med Microbiol* 56:1370–1376.

Mori T, Mizuta S, Suenaga H, Miyazaki K (2008). Metagenomic screening for bleomycin resistance genes. *Appl Environ Microbiol* 74:6803–6805.

Moura A, Henriques I, Ribeiro R, Correia A (2007). Prevalence and characterization of integrons from bacteria isolated from a slaughterhouse wastewater treatment plant. *J Antimicrob Chemother* 60:1243–1250.

Moura A, Soares M, Pereira C, Leitão N, Henriques I, Correia A (2009). INTEGRALL: A database and search engine for integrons, integrases and gene cassettes. *Bioinformatics* 25:1096–1098.

Moura A, Henriques I, Smalla K, Correia A (2010). Wastewater bacterial communities bring together broad-host range plasmids, integrons and a wide diversity of uncharacterized gene cassettes. *Res Microbiol* 161:58–66.

Muyzer G, de Waal EC, Uitterlinden AG (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction amplified genes coding for 16S rRNA. *Appl Environ Microbiol* 59:695–700.

Narisawa N, Haruta S, Arai H, Ishii M, Igarashi Y (2008). Coexistence of antibiotic-producing and antibiotic-sensitive bacteria in biofilms is mediated by resistant bacteria. *Appl Environ Microbiol* 74:3887–3894.

Nemergut DR, Martin AP, Schmidt SK (2004). Integron diversity in heavy-metal- contaminated mine tailings and inferences about integron evolution. *Appl Environ Microbiol* 70:1160–1168.

Nodwell JR (2007). Novel Links between antibiotic resistance and antibiotic production. *J Bacteriol* 189(10):3683–3685.

Oliver JD (1995). The viable but non-culturable state in the human pathogen *Vibrio vulnificus*. *FEMS Microbiol Lett* 133:203–208.

Parsley LC, Consuegra EJ, Kakirde KS, Land AM, Harper WF Jr, Liles MR (2010). Identification of diverse antimicrobial resistance determinants carried on bacterial, plasmid, or viral metagenomes from an activated sludge microbial assemblage. *Appl Environ Microbiol* 76(11):3753–3757.

Patterson AJ, Colangeli R, Spigaglia P, Scott KP (2007). Distribution of specific tetracycline and erythromycin resistance genes in environmental samples assessed by macroarray detection. *Environ Microbiol* 9:703–715.

Peak N, Knapp CW, Yang RK, Hanfelt MM, Smith MS Aga DS, Graham DW (2007). Abundance of six tetracycline resistance genes in wastewater lagoons at cattle feedlots with different antibiotic use strategies. *Environ Microbiol* 9:143–151.

Pei R, Kim SC, Carlson KH, Pruden A (2006). Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Rese* 40:2427–2435.

Pruden A, Pei R, Storteboom H, Carlson KH (2006). Antibiotic resistance genes as emerging contaminants: Studies in Northern Colorado. *Environ Sci Technol* 40:7445–7450.

Riesenfeld CS, Goodman RM, Handelsman J (2004). Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. *Environ Microbiol* 6(9):981–989.

Ronaghi M (2001). Pyrosequencing sheds light on DNA sequencing. *Genome res* 11:3–11.

Rosser SJ, Young H-K (1999). Identification and characterization of class 1 integrons in bacteria from an aquatic environment. *J Antimicrob Chemother* 44:11–18.

Rowe-Magnus DA, Mazel D (2001). Integrons: Natural tools for bacterial genome evolution. *Curr Opin Microbiol* 4:565–569.

Sabuncu E, David J, Bernède-Bauduin C, Pépin S, Leroy M, Boëlle P-Y, Watier L, Guillemot D (2009). Significant reduction of antibiotic use in the community after a nationwide campaign in France, 2002–2007. *PLoS Med* 6(6):e1000084.

Schlüter A, Heuer H, Szczepanowski R, Forney LJ, Thomas CM, Puhler A, Top EM (2003). The 64 508 bp IncP-1beta antibiotic multiresistance plasmid pB10 isolated from a wastewater treatment plant provides evidence for recombination between members of different branches of the IncP-1beta group. *Microbiology* 149:3139–3153.

Schlüter A, Heuer H, Szczepanowski R, Poler SM, Schneiker S, Puhler A, Top EM (2005). Plasmid pB8 is closely related to the prototype IncP-1beta plasmid R751 but transfers poorly to *Escherichia coli* and carries a new transposon encoding a small multidrug resistance efflux protein. *Plasmid* 54:135–148.

Schlüter A, Szczepanowski R, Kurz N, Schneiker S, Krahn I, Puhler A (2007). Erythromycin resistance-conferring plasmid pRSB105, isolated from a sewage treatment plant, harbors a new macrolide resistance determinant, an integron-containing Tn402-like element, and a large region of unknown function. *Appl Environ Microbiol* 73:1952–1960.

Schwartz T, Kohnen W, Jansen B, Obst U (2003). Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS Microbiol Ecol* 43:325–336.

Smalla K, Heuer H, Götz A, Niemeyer D, Krögerrecklenfort E, Tietze E (2000). Exogenous isolation of antibiotic resistance plasmids from piggery manure slurries reveals a high prevalence and diversity of IncQ-like plasmids. *Appl Environ Microbiol* 66:4854–4862.

Smith MS, Yang RK, Knapp CW, Niu Y, Peak N, Hanfelt MM, Galland JC, Graham DW (2004). Quantification of tetracycline resistance genes in feedlot lagoons by real-time PCR. *Appl Environ Microbiol* 70:7372–7377.

Sommer MO, Dantas G, Church GM (2009). Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science* 325:1128–31.

Song JS, Jeon JH, Lee JH, Jeong SH, Jeong BC, Kim SJ, Lee JH, Lee SH (2005). Molecular characterisation of TEM-type β-lactamases identified in cold-seep sediments of Edison Seamount (south of Lihir Island, Papua New Guinea). *J Microbiol* 43:172–178.

Sorensen SJ, Bailey M, Hansen LH, Kroer N, Wuertz S (2005). Studying plasmid horizontal transfer in situ: A critical review. *Nat Rev Microbiol* 3:700–710.

Spangler S, Goddard NL, Thaler DS (2010). Optimizing *Taq* polymerase concentration for improved signal-to-noise in the broad range detection of low abundance bacteria. *PLoS ONE* 4(9):e7010.

Spiegelman D, Whissell G, Greer CW (2005). A survey of the methods for the characterization of microbial consortia and communities. *Can J Microbiol* 51:355–386.

Stein JL, Marsh TL, Wu KY, Shizuya H, DeLong EF (1996). Characterization of uncultivated prokaryotes: Isolation and analysis of a 40-kilobase-pair genome fragment from a planktonic marine archaeon. *J Bacteriol* 178:591–599.

Stepanauskas R, Glenn TC, Jagoe CH, Tuckfield RC, Lindell AH, King CJ, McArthur JV (2006). Coselection for microbial resistance to metals and antibiotics in freshwater microcosms. *Environ Microbiol* 8:1510–1514.

Storteboom H, Arabi M, Davis JG, Crimi B, Pruden A (2010). Identification of antibiotic-resistance-gene molecular signatures suitable as tracers of pristine river, urban, and agricultural sources. *Environ Sci Technol* 44:1947–1953.

Szczepanowski R, Krahn I, Linke B, Goesmann A, Puhler A, Schlüter A (2004). Antibiotic multiresistance plasmid pRSB101 isolated from a wastewater treatment plant is related to plasmids residing in phytopathogenic bacteria and carries eight different resistance determinants including a multidrug transport system. *Microbiology* 150:3613–3630.

Szczepanowski R, Braun S, Riedel V, Schneiker S, Krahn I, Puhler A, Schlüter A (2005). The 120 592 bp IncF plasmid pRSB107 isolated from a sewage-treatment plant encodes nine different antibiotic-resistance determinants, two iron-acquisition systems and other putative virulence-associated function. *Microbiology* 151:1095–1111.

Szczepanowski R, Linke B, Krahn I, Gartemann KH, Gutzkow T, Eichler W, Puhler A, Schlüter A (2009). Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. *Microbiology* 155:2306–2319.

Tauch A, Schlüter A, Bischoff N, Goesmann A, Meyer F, Puhler A (2003). The 79,370-bp conjugative plasmid pB4 consists of an IncP-1beta backbone loaded with a chromate resistance transposon, the *str*A-*str*B streptomycin resistance gene pair, the oxacillinase gene $bla_{(NPS-1)}$, and a tripartite antibiotic efflux system of the resistance-nodulation-division family. *Mol Genet Genomics* 268:570–584.

Thaker M, Spanogiannopoulos P, Wright GD (2010). The tetracycline resistome. *Cell Mol Life Sci* 67:419–431.

Thomas CM (2000). *The Horizontal Gene Pool; Bacterial Plasmids and Gene Spread*. Harwood Scientific, Amsterdam.

Thomas CM, Nielsen KM (2005). Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nat Rev Microbiol* 3:711–721.

Torsvik V, Ovreas L (2002). Microbial diversity and function in soil: From genes to ecosystems. *Curr Opin Microbiol* 5:240–245.

van de Sande-Bruinsma N, Grundmann H, Verloo D, Tiemersma E, Monen J, Goossens H, Ferech M, European Antimicrobial Resistance Surveillance System Group (2008). European Surveillance of Antimicrobial Consumption Project Group. Antimicrobial drug use and resistance in Europe. *Emerg Infect Dis* 14(11):1722–1730.

van Elsas JD, Bailey MJ (2002). The ecology of transfer of mobile genetic elements. *FEMS Microbiol Ecol* 42:187–197.

Venter JC, Remington K, Heidelberg JF, Halpern AL, Rusch D, Eisen JA, Wu D, Paulsen I, Nelson KE, Nelson W, Fouts DE, Levy S, Knap AH, Lomas MW, Nealson K, White O, Peterson J, Hoffman J, Parsons R, Baden-Tillson H, Pfannkoch C, Rogers YH, Smith HO (2004). Environmental genome shotgun sequencing of the Sargasso Sea. *Science* 304:66–74.

Volkmann H, Schwartz T, Bischoff P, Kirchen S, Obst U (2004). Detection of clinically relevant antibiotic-resistance genes in municipal wastewater using real-time PCR (TaqMan). *J Microbiol Methods* 56:277–286.

Volkmann H, Shwartz T, Kirshen S, Stofer S, Obst U (2007). Evaluation of inhibition and cross-reaction effects on real-time PCR applied to the total DNA of wastewater samples for the quantification of bacterial antibiotic resistance genes and taxon-specific targets. *Mol Cell Probes* 21:125–132.

Ward DM, Weller R, Bateson MM (1990). 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature* 345:63–65.

Wintzingerode FV, Gobel UB, Stackebrandt E (1997). Determination of microbial diversity in environmental samples: Pitfalls of PCR-based rRNA analysis. *FEMS Microbiol Rev* 21:213–229.

Wright GD (2007). The antibiotic resistome: The nexus of chemical and genetic diversity. *Nat Rev Microbiol* 5:175–186.

Xi C, Zhang Y, Marrs CF, Ye W, Simon C, Foxman B, Nriagu J (2009). Prevalence of antibiotic resistance in drinking water treatment and distribution systems. *Appl Environ Microbiol* 75:5714–5718.

Yim G, Wang HH, Davies JE (2007). Antibiotics as signalling molecules. *Philos Trans R Soc B* 362:1195–1200.

Zhang T, Fang HHP (2006). Applications of real-time polymerase chain reaction for quantification of microorganisms in environmental samples. *Appl Microbiol Biotechnol* 70:281–289.

Zhou Z, Raskin L, Zilles JL (2009). Identification of macrolideresistant microorganisms on antimicrobial-free swine farms. *Appl Environ Microbiol* 75:5814–5820.

Zhou Z, Raskin L, Zilles JL (2010). Effects of swine manure on macrolide, lincosamide, and streptogramin B antimicrobial resistance in soils. *Appl Environ Microbiol* 76:2218–2224.

PART II

FATE

9

ENVIRONMENTAL POLLUTION BY ANTIBIOTIC RESISTANCE GENES

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9.1 INTRODUCTION

Since World War II, antibiotics have been widely used for the treatment of infectious diseases, likely being the most successful group of drugs developed by humankind. Nevertheless, the use of these chemicals is not limited to treating human infections. They have been used as well in farming, aquaculture, and agriculture both for treating (or preventing) infections and as growth promoters in animals (Cabello, 2006; McManus et al., 2002; Singer et al., 2003; Smith et al., 2002). All of these practices imply that large amounts of antibiotics have been (and are being) released in many natural ecosystems (Hu et al., 2010; Kümmerer, 2009a; 2009b; Lindberg et al., 2007; Martinez, 2009a; McArthur and Tuckfield, 2000; Yiruhan et al., 2010). Although it has been described that pollution by antibiotics might have some effects on the development of plants (Xie et al., 2010), the major effect of this contamination will be on the overall composition of the environmental microbiota (Grimes et al., 1984; Naslund et al., 2008), mainly in terms of selecting antibiotic-resistant bacteria (Baya et al., 1986; Blanco et al., 2009; Brandt et al., 2009; Martinez, 2008, 2009a). Despite this situation, and although the effect of the use of antibiotics on the development of resistance by human and animal bacterial pathogens has been studied in detail (Aarestrup, 2005; Alekshun and Levy, 2007; Ferber, 2003; Levy, 1998; Levy and Marshall, 2004; Levy and O'Brien, 2005; Pereira-Leal et al., 2006; Singer et al., 2003), information on the effect of pollution by antibiotics on the population dynamics of the environmental microbiosphere is still scarce. One important aspect of this type of pollution is that the release of antibiotics present in wastewaters from farms, hospitals, or cities is usually accompanied by the release of resistance genes currently

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disseminated among human and animal-linked bacteria (Baquero et al., 2008; Kümmerer, 2004; Martinez, 2008, 2009a). Agricultural fertilization using organic wastes, mainly from livestock and sewage sludge from urban effluents, contributes as well to the release of antibiotic resistance genes in natural ecosystems (Blanco et al., 2009; Gerba and Smith, 2005; O'Connor et al., 2005).

Several studies have addressed the prevalence of antibiotic resistance genes in natural ecosystems, and, occasionally, the finding of antibiotic-resistant bacteria has been considered as evidence of contamination with an anthropogenic origin. As we will see later on, this might occur when the resistance determinants found are the same as those already disseminated among human pathogens (Baquero et al., 2009; Martinez, 2008, 2009a). However, natural ecosystems (and not clinical ones) are the origin of the resistance elements (Aminov, 2009; Baquero et al., 2009; Davies, 1994, 1997; Martinez, 2008, 2009b) acquired by human pathogens by horizontal gene transfer (HGT) in such a way that the finding of an unknown resistance gene in a natural (not clinical) ecosystem should not be considered as a surprise. In this regard, it is important to mention here that antibiotic resistance genes are ubiquitous in all ecosystems, either contaminated or pristine, or even in the commensal microbiota of wild animals (Allen et al., 2009; D'Acosta et al., 2006; Wright, 2007).

Nevertheless, among all these determinants capable of conferring resistance upon their transfer to a new host, only some of them have been acquired by human pathogens (Alekshun and Levy, 2007; Baquero et al., 2009; Fajardo et al., 2009; Martinez et al., 2007, 2009b). When talking about pollution by resistance genes (Pruden et al., 2006), we thus refer to these specific determinants that have been acquired by human-linked bacteria after the introduction of antibiotics for therapy. Indeed, the analysis of bacterial isolates from the preantibiotic era demonstrated that the types and amounts of plasmids carried by pathogenic bacteria were similar to what can be found today, the only difference being that the plasmids from the preantibiotic era did not contain resistance determinants, whereas currently they harbor resistance genes acquired through HGT, acquisition and spread being direct consequences of the strong selective pressure exerted during the treatment of the infections (Datta and Hughes, 1983).

It is thus clear that one of the consequences of the use of antibiotics for therapy has been the spread of a number of specific antibiotic resistance genes among human pathogens (and commensals). In other words, over the course of the last 60 years, there has been a strong enrichment and a wide spread among different hosts of a small subset of resistance genes [as compared with the large number of different resistance determinants present in natural ecosystems; see (D'Acosta et al., 2006)]. For instance, the plasmid-encoded TEM1 β-lactamase (and its derivatives) is currently widely disseminated (Bradford, 2001; Paterson and Bonomo, 2005), but its origin in the chromosome of an unknown microorganism is still ignored. This supports the notion that, before the antibiotic era, the bla_{TEM1} gene was present just in the chromosome of a specific environmental bacterial species, and the use of β-lactams (and further selection) allowed its integration into successful gene transfer units with the consequence of its dissemination and fast evolution. Whereas it is clear that the use of antibiotics has led to the enrichment in clinical isolates of some antibiotic resistance genes, the effect of this utilization in the environmental microbiota has been studied in less detail.

At first sight, and although the antibiotic selective pressure is lower in natural ecosystems than in clinical settings, we have to take into consideration that from the

time that antibiotics were introduced for therapy, they have been common environmental pollutants (de Souza et al., 2009; Hu et al., 2010; Karci and Balcioglu, 2009; Venglovsky et al., 2009; Yiruhan et al., 2010), constantly introduced in natural ecosystems from human, animal and, industrial (e.g., antibiotics–producing companies) waste disposals. Human activities, including the use of antibiotics for human treatment, but also in farms and fisheries, as well as the use of organic wastes for agricultural fertilization, might have an impact the environmental microbiota in three aspects that are relevant for the evolution and spread of resistance with clinical relevance: (i) Selection of antibiotic resistant microorganisms in natural ecosystems, including intrinsically resistant bacteria. Given that some relevant opportunistic pathogens such as *Pseudomonas aeruginosa* (Lister et al., 2009), different species of the genus *Burkholderia* (McGowan, 2006; Spicuzza et al., 2008), *Acinetobacter baumanii* (Navon-Venezia et al., 2005; Sader and Jones, 2005), or *Stenotrophomonas maltophilia* (Looney et al., 2009; Sanchez et al., 2009), which are characterized by presenting low susceptibility to antibiotics, have an environmental origin, the enrichment of these populations by the indiscriminate release of antibiotics might impact human health; (ii) spreading of antibiotic resistance determinants by gene transfer elements (Baquero, 2004). Since gene transfer units confer a cost to the new host (fitness costs), gene transfer is a rewarding evolutionary strategy (Baquero, 2004; Baquero et al., 2009; Martinez et al., 2007) only when the element present in the gene transfer unit confers an adaptive advantage to the new host (Fig. 9.1). In the case of resistance genes, this adaptive advantage occurs upon pollution by antibiotics (Martinez, 2009a), in such a way that antibiotic resistance can be considered as a colonization factor in environments with high antibiotic load (Martinez and Baquero, 2002). (iii) The maintenance and difficult eradication of the antibiotic resistance genes from natural ecosystems. One important aspect in the pollution by antibiotic resistance genes is that the wastes from hospitals and farms contain not only resistance genes but their selectors as well—the antibiotics. The constant release of antibiotics into natural ecosystems, together with resistance genes, will thus favor the maintenance and dissemination of resistance among environmental microbiota (Martinez, 2009a).

Environmental pollution with antibiotic resistance genes might be a relevant problem for human health since their release upon integration in gene transfer units might allow their successful spread into natural bacterial populations, creating a relevant reservoir of these elements (Simoes et al., 2010) that, in turn, might be difficult to eradicate (Martinez et al., 2007). In this chapter, we will discuss the impact of the contamination of natural ecosystems by antibiotic resistance determinants for these ecosystems and for human health as well.

9.2 ANTIBIOTICS AND ANTIBIOTIC RESISTANCE GENES AS REGULAR COMPONENTS OF NATURAL ECOSYSTEMS

The first antimicrobial agents were derivatives of heavy metals (MacLeod, 1912) or arsenic (Thorburn, 1983) or compounds obtained by chemical synthesis such as sulfonamides (Yates, 1939). However, after the finding by Fleming of the naturally produced antibiotic penicillin (Fleming, 1944), it was evident that environmental microorganisms should be able to produce antimicrobials. In a seminal paper,

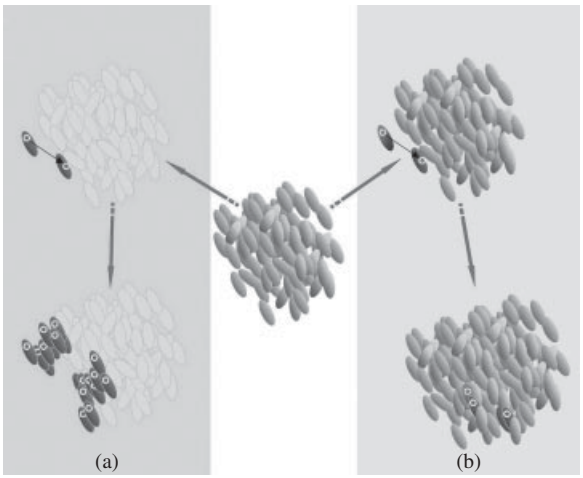

FIGURE 9.1 Second-order selection of transfer of antibiotic resistance genes in the presence of antibiotics. Once an antibiotic resistance gene is present in a gene transfer element, it can be spread among different hosts. (a) Nevertheless, carrying this element will produce a fitness cost, so that in the absence of antibiotics' selective pressure, resistant bacteria, including those that have received the resistance gene by horizontal gene transfer from the original host, will be out competed by the susceptible ones (b). Under circumstances of no selection, acquisition of novel genetic elements is not adaptive. In the presence of antibiotics, however, the growth of susceptible bacteria will be inhibited, and only those containing resistance genes (either the original ones or those that have received the resistance determinant by gene transfer) will be able to grow (a), thus favoring the dissemination of these resistance determinants. *(See color insert.)*

Waksman and Woodruff suggested searching for inhibitors of human pathogens in soil microorganisms based on an ecological hypothesis (Waksman and Woodruff, 1940). Given that human and animals had been releasing large numbers of bacterial pathogens in natural ecosystems for several years and that, in spite of this constant contamination, natural ecosystems do not harbor large numbers of human pathogens, it should be a valid interpretation that the microorganisms in these ecosystems might produce inhibitors of pathogens. The fruitful finding of these compounds, mainly produced by *Actinomyces*, led to the conclusion that the role of antibiotics in natural ecosystems would be indeed fighting against competitors. This can be true on occasions, however, the fact that the concentration of antibiotics in natural ecosystems is likely much lower than the one used for therapy cast doubts on the universal validity of this statement. In fact, some recent articles propose that antibiotics might be signaling molecules at the low concentrations at which they can be found in natural ecosystems (Davies, 2006; Davies et al., 2006; Fajardo and Martinez, 2008; Linares et al., 2006; Yim et al., 2006a; 2006b, 2007).

In contrast to the notion that antibiotics might be weapons in intermicrobial warfare, the most common idea concerning resistance genes is that they are shields to avoid antibiotics' activity, creating the scenario of a "Red Queen" evolution trend in which each partner (the antibiotic producer or the resistant bacteria) must run (evolve) as fast as possible to maintain the homeostasis of the system (Benton, 2010; Clay and Kover, 1996; Laskaris et al., 2010). Under this view, resistance genes should

have originated in the same ecosystem as antibiotics; in other words in natural (nonclinical) environments (Baquero et al., 2009; Fajardo et al., 2009; Martinez, 2008). This idea is in agreement with the fact that the plasmids present in human-linked bacteria isolated from the preantibiotic era did not carry resistance genes (Datta and Hughes, 1983). Among the most suitable organisms that can be the origin of the resistance determinants currently disseminated in bacterial pathogens, we have to take into consideration the antibiotic producers because these organisms require elements capable to protect themselves from the antibiotics that they produce (Benveniste and Davies, 1973; Cundliffe, 1992; Marshall et al., 1998). Several of the resistance determinants acquired by human pathogens through HGT are enzymes that modify the antibiotic: chloramphenicol acetyl transferases acetylate chloramphenicol (Shaw, 1983); β-lactamases hydrolyze β-lactams (Bush and Jacoby, 2010), and several enzymes that acetylate, phosphorylate, or adenylate the aminoglycoside antibiotics (Shaw et al., 1993). The presence of these enzymes in the microorganisms that produce those antibiotics has been demonstrated (Benveniste and Davies, 1973). For example, many species of *Streptomyces*, a natural producer of aminoglycosides, possess aminoglycoside-modifying enzymes capable of inactivating members of this family of antibiotics (Argoudelis and Coats, 1969). Similarly, acetylation of cloramphenicol is commonly found in chloramphenicol producers (Lietman, 1972), as well as tetracycline-resistant determinants such as *otr*A and *otr*B (Pang et al., 1994), which have been found in the tetracycline producers and in members of pathogenic Mycobacteria.

Similar to antibiotics, which may have other functional roles in addition to the inhibition of competitors in natural ecosystems (Davies, 2006; Davies et al., 2006; Fajardo and Martinez, 2008; Linares et al., 2006; Yim et al., 2006a), resistance genes may have other functions in their original hosts than resistance to bacteria (Baquero et al., 2009; Martinez, 2008; Martinez et al., 2009b). Moreover, in the case of producers, it has been stated that on occasion, an antibiotic-modifying enzyme might be a part of a biosynthetic pathway more than an element involved in the autoprotection from antibiotic action (Benveniste and Davies, 1973). In fact, in a few specific cases, the resistance genes present in pathogens have been accurately tracked and their original hosts were not antibiotic producers. For example, the plasmid-encoded *qnr* genes have originated from the chromosomes of aquatic bacteria such as *Shewanella algae* (Poirel et al., 2005a, 2005b; Sanchez et al., 2008) and the CTXM β-lactamases are originally present in the chromosome of *Kluyvera ascorbata* (Canton and Coque, 2006; Humeniuk et al., 2002). Neither of these microorganisms produce antibiotics, and it is unlikely that they are under constant antibiotic selective pressure, mainly in the case of the *qnr* determinants, since they confer resistance to the quinolone family of synthetic antibiotics.

Examples of antibiotic resistance determinants for which a role other than resistance has been proposed include multidrug (MDR) efflux pumps, aminoglycoside-inactivating enzymes, and β-lactamases. MDR efflux pumps are the more versatile group of genes with a physiological function other than antibiotic resistance (Saier and Paulsen, 2001). These resistance genes can be found in all prokaryotic and eukaryotic organisms (Nikaido, 1998a, 1998b; Saier and Paulsen, 2001), contributing to the intrinsic resistance to antibiotics (Nikaido, 1994) and anticancer drugs (Twentyman, 1997), respectively. It is possible to find as many as 20 different MDR determinants in a genome of a single bacterium. But the most important

feature of these determinants is their capability to extrude a great variety of molecules such as solvents, detergents, aromatic compounds (Isken and de Bont, 1996; Li et al., 1998; Ramos et al., 2002), heavy metals (Silver and Phung, 1996), or quorum-sensing (QS) signal molecules (Aendekerk et al., 2005; Evans et al., 1998; Kohler et al., 2001). Heavy metals and aromatic compounds derived from degradation processes are natural products frequently found in several ecosystems. It is thus possible that MDR determinants from environmental bacteria could be selected for the protection of these organisms against those toxic compounds or for signal trafficking in the case of those pumps capable of extruding QS signals (Martinez et al., 2009a).

Strong arguments support the notion that MDR pumps are evolutionary ancient elements. Phylogenetic studies have shown that the type of transporters and their substrates maintain a strong correlation with the physiology of the respective organism (Ren and Paulsen, 2005). Besides, and although chromosomally encoded transporters could be variable in the same species, depending on the particular ecotype, in many cases bacterial species belonging to the same genus share most of these MDR efflux pumps (Aarestrup, 2005). Finally, the genes coding for these elements usually belong to the bacterial core genome (Alonso et al., 2004). Another good example of resistance genes with an original role different from resistance concerns are aminoglycoside-modifying enzymes. Many bacterial species such as *Providencia stuartii* (Macinga and Rather, 1999), *Stenotrophomonas maltophilia* (Lambert et al., 1999), *Serratia* sp. (Shaw et al., 1992), or Mycobacteria (Ainsa et al., 1997) present these genes in the chromosome of all isolates, indicating that these resistance elements have not been recently acquired as the consequence of antibiotic selective pressure. It has been stated that they may have evolved from sugar kinases and acetyltransferases (Macinga and Rather, 1999) and might play important roles in bacterial metabolism. An example of this situation is the chromosomally encoded acetyltransferase [AAC(2′)-Ia] from *P. stuartii*. This enzyme shows at least one clear physiological function, which is the acetylation of peptidoglycan (Payie et al., 1995). It is important to note that *P. stuartii* is not always in contact with aminoglycosides. Thus, the most suitable function for this enzyme is cell wall metabolism and not antibiotic resistance. A final example concerns β-lactamases. It is worth mentioning that several *Enterobacteriaceae* harbor chromosomally encoded β-lactamases (Lindberg and Normark, 1986; Livermore and Woodford, 2006), despite the fact that the gut is not known to contain β-lactam producers. It has been stated that the most suitable function of these determinants might deal with the cell wall synthesis, evolving from carboxypeptidases or transpeptidases (Adachi et al., 1992; Knox et al., 1996), and the activity against antibiotics is an emergent property of the system only in the presence of the β-lactam.

The analysis of comprehensive libraries of mutants from different bacterial species (Breidenstein et al., 2008; Dotsch et al., 2009; Fajardo et al., 2008; Tamae et al., 2008) demonstrates that several genes participate in the intrinsic resistance phenotype of these microorganisms, further supporting the observation that genes not originally selected for conferring resistance can contribute to this phenotype.

From the information discussed above, it is clear that a natural background of antibiotics and of resistance determinants exists in environmental microbiota. The variability of these elements is much higher than that found in clinics in which only a few antibiotics are used in therapy and the same resistance genes are found in

different pathogens and at different geographical locations. What can be thus observed is that the utilization of antibiotics has produced the spread of few specific resistance genes (Baquero et al., 2009; Martinez, 2008). When compared to what is found in natural ecosystems (D'Acosta et al., 2006; Wright 2007), this means that antibiotics reduce the diversity of the potential resistance genes, increasing the concentration of those that are being selected (Baquero et al., 2009; Martinez, 2008; Martinez, 2009a).

Since, as discussed, natural ecosystems constitute both the origin and the reservoir of resistance determinants, it is important to know whether the contamination by antibiotics and resistance determinants might produce a similar effect in the population dynamics of the microbiota present in these natural habitats.

9.3 ANTIBIOTIC RESISTANCE GENES AS POLLUTANTS

Given that resistance genes originate in, and are regular inhabitants of, natural ecosystems, it is important to distinguish when resistance can be considered as a result of pollution and when it represents the normal state of a given ecosystem (Baquero et al., 2009; Martinez, 2008, 2009a, 2009b). An important index of pollution by antimicrobials is the increase of resistant organisms above the normal value. Nevertheless, this increase does not necessarily imply contamination by resistance genes, since antibiotic pollution might enrich the population of intrinsically resistant bacteria as well (see above). Moreover, it has been described that contamination by biocides can enrich the population of bacteria containing some biocide resistance determinants that are frequently associated with resistance genes in gene capture units such as integrons (Gaze et al., 2005; Wright et al., 2008). When defining pollution by resistance genes, we must thus distinguish between the enrichment of naturally resistant bacteria, which is a consequence of pollution with antibiotics (or other toxics, see above) and the release of those genes that have been already acquired by human pathogens and are present in the wastes discharged in nature. For example, it has been described that natural antibiotic-resistant bacterial populations can be found in regions where contact with humans is unlikely, such as the deep terrestrial subsurface (Brown and Balkwill, 2009), the deep Greenland ice core (Miteva et al., 2004), or the waters of the Antarctic Ocean (De Souza et al., 2006). The resistance genes present in these bacteria most likely constitute their intrinsic resistome, understood as all the genes that confer resistance to a specific bacterial species (Breidenstein et al., 2008; Fajardo et al., 2008; Mori et al., 2008; Tamae et al., 2008), and are not the result of any contamination by antibiotic resistance genes. Throughout this chapter, only those genes that have already been acquired by human/animal-linked microbiotas (pathogens or commensals) will be considered as pollutant resistance genes.

The main cause of environmental pollution by antibiotic resistance genes results from the release of residues from houses, hospitals, and farms, together with the use of wastes containing these compounds as fertilizers in agriculture. It is important to note that these residues usually contain both antibiotics and resistant bacteria (Kümmerer and Henninger, 2003). In other words, they contain the genes for adaptation (resistance genes) and the selecting agent (the antibiotic), in such a way that the maintenance and eventual spread of resistance by the contact of these wastes

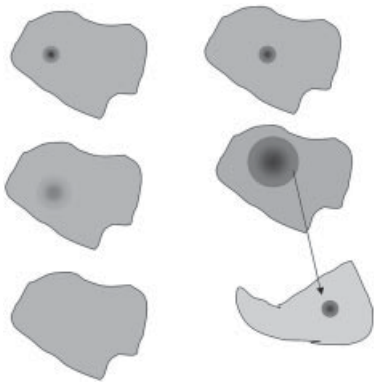

FIGURE 9.2 Different fate of contamination by antibiotics and by antibiotic resistance genes. Antibiotics are chemical compounds that are degraded by time and diluted across space. Their diffusion in natural ecosystems produce a reduction in their concentration and, if there are not more pollution events, the contaminant will disappear sooner or later (a). Contrary to this situation, antibiotic resistance genes are autoreplicative elements, so that their concentration can increase in the presence of antibiotics and eventually can travel across long distances to occupy environments were they were not originally released using wild animals as vectors (b). Antibiotic contamination are shown in orange, and pollution by antibiotic resistance determinants are shown in red. *(See color insert.)*

with natural populations of environmental bacteria is enhanced. Studies in sediments located nearby cities and sewage tributaries have shown that the presence of contaminant resistance genes is ubiquitous and the amount of these genes is much higher than that found in pristine environments (Pei et al., 2006). Nevertheless, as stated above, pristine environments without any historical record of contamination by antibiotics also harbor antibiotic resistance genes. This suggests that contamination could increase resistance, but resistance can persist and eventually spread even in the absence of pollution with antibiotics.

To understand this apparent paradox, we must take into consideration that the behavior in natural ecosystems of contaminating antibiotic resistance genes is very different from the situation observed for any other pollutant (Fig. 9.2). The release of a chemical pollutant in any given environment produces a gradient of contamination in time and space. The gradient in the space depends on the environment where pollution occurs (soil or water) or the intrinsic diffusion properties of the pollutant (mainly, its solubility). Although the concentration of the pollutant might be locally higher than at the point of contamination [e.g., active adsorption at sediments (Xu and Li, 2010)], the total amount of pollutant cannot be higher than released. The concentration gradient along time depends on the degradation of the pollutant in natural ecosystems, including physicochemical and biological degradation processes. It is thus expected that, for any type of pollution, if the release of the contaminant ceases, pollution will decrease and eventually disappear (although some chemical compounds are extremely resilient to degradation).

In sharp contrast with this situation, contaminant-resistant genes are autoreplicative pollutants, such that their concentration can increase if there exists an appropriate selective force. In addition, they can travel for long distances

(e.g., in the microbiota of birds; see below) without reduction in their concentration. In other words, unlike contamination by chemical compounds (including antibiotics), the maintenance of pollutant resistance genes in natural ecosystems does not depend on their constant release, since the host bacteria can grow and the genes, which are present in gene transfer units, can spread among several bacterial species in different habitats. In this regard, it has been described that identical antibiotic resistance genes have been found in disconnected aquatic systems (Picao et al., 2008). The finding of the same resistance elements, in different geographical allocations and in different microorganisms, suggests that human interventions, not necessarily involving contamination, may contribute to accelerate their spread. For instance, it is known that the traffic of ships around the world favors the dissemination of bacteria even between different oceans and continents (Ruiz et al., 2000). In a global world, with constant interchange of people and goods, resistance that originated in areas with high use of antibiotics will sooner or later invade comparatively clean environments. Other elements unlinked to human activities can also favor the dissemination of resistance genes. This is the case with the migration of wild animals harboring resistant bacteria, which allows this dissemination across long distances (Poeta et al., 2008, 2009; Sjolund et al., 2008) and enables the presence of antibiotic resistance genes in places with very low or even no human activities.

One important issue concerning pollutant resistance genes is that they are encoded in gene transfer units (Baquero, 2004). Chromosomally encoded antibiotic resistance genes can be enriched in the presence of antibiotics because of the enrichment of the bacterial species harboring them. Since any given bacterial species can colonize a limited number of ecosystems, this can lead to the expansion of those genes just at those specific ecosystems. If the human body is not one of these habitats, the enrichment of these intrinsically resistant microorganisms in natural ecosystems will not have a direct influence on human health. As an example, the potential selection of antibiotic producers such as *Streptomyces* as the consequence of the contamination with antibiotics will not directly impact human heath since these organisms are not pathogens. An indirect effect might occur, however, if this enrichment favors the transfer of the resistance genes from the producers to a human pathogen, an issue that has not yet been analyzed in detail.

As discussed above, the enrichment of intrinsically resistant bacteria might be relevant in the case of opportunistic pathogens with an environmental origin (Alonso et al., 2001). However, these pathogens rarely infect healthy people, and the most frequent source of infection is the hospital or the patient's home, such that the impact of antibiotic pollution on the emergence of infections due to intrinsically resistant bacteria will likely be low. A different situation may occur for plasmid-encoded resistance determinants since their presence in gene transfer units allows their flow among different bacterial species, so that they can more easily colonize different ecosystems, including the human host. In this regard, we must consider the integration of a chromosomally encoded resistance determinant into a gene transfer unit as the hallmark that allows the dissemination of this determinant among human pathogens. One example of this situation has been described for *qnr* genes. These quinolone resistance determinants are present in plasmids in *Enterobacteriaceae* (Robicsek et al., 2006; Strahilevitz et al., 2009). Their origin is found in some species of water-dwelling microorganisms in which they are chromosomally encoded (Poirel et al., 2005a; Sanchez et al., 2008). The presence of a *qnr*-encoded plasmid in the

environmental nonpathogenic bacteria *Aeromonas* (Cattoir et al., 2008) has recently been described. The finding of the same plasmid in another isolate of *Aeromonas allosaccharophila* (Picao et al., 2008) from a lake geographically distant from the place where the first isolate was recovered indicates that the integration of resistance genes in gene transfer units allows their efficient spread in natural ecosystems, an issue already established for several other resistance determinants (Martinez et al., 2007).

Contrasting the situation with chemical pollutants, in which the evolution of the contamination depends on the physicochemical characteristics of the compound and its degradation (either by physicochemical or biological processes) in natural ecosystems, the evolution of pollution by resistance genes depends on the capability for colonizing natural environments of the bacteria that host them and on their transfer among different bacterial species. This situation is analogous to the introduction of an alien species into a new habitat in which case the spread depends on the adaptability of this species to the novel environment and on the presence of competitors. An example of this situation has been the introduction of rabbits in Australia, which rendered an explosive expansion of the lagomorphs on that continent (Cowan and Tyndale-Biscoe, 1997). Introduction of pollutant antibiotic resistance genes does not produce such a drastic effect. However, it is clear that these autoreplicative pollutants can be maintained and are able to expand their presence in noncontaminated environments and even to environments without a high load of antibiotics. In agreement with this issue, the analysis of historical soils has demonstrated that there is an increasing prevalence of antibiotic resistance elements in these ecosystems since the antibiotics were introduced for therapy (Knapp et al., 2010). As stated above, this includes pristine environments such as the Antarctic Ocean (De Souza et al., 2006), the gut microbiota from isolated human populations that have not been in contact with antibiotics (Bartoloni et al., 2009), and microbiotas from wild animals (Livermore et al., 2001; Rolland et al., 1985). As rightly stated by Blanco et al. (2009), the transfer of bacterial resistance to wildlife is an important risk for environmental health. The chances for this transfer increase when agricultural manuring includes fecal material from livestock operations and urban areas that contain multiresistant enteric bacteria (Blanco et al., 2009). The finding in seagull feces of a high number of multidrug resistant *Escherichia coli* strains harboring well-known β-lactamase genes (Simoes et al., 2010) indicates that, once resistance genes are released in natural ecosystems, several vectors contribute to their dissemination, even between different continents without the need of a geographical connectivity with the original contamination source. The presence of antibiotic-resistant bacteria on the microbiota of avian scavengers, such as vultures, which consume stabled livestock carrion, likely containing antibiotics and resistance elements, further support the role that birds may have on the dissemination of resistance at long distances (Blanco et al., 2007).

9.4 MECHANISMS OF MAINTENANCE AND SPREAD OF RESISTANCE GENES IN NATURAL ECOSYSTEMS

As discussed above, the simultaneous pollution by antibiotics and resistance genes leads to the local enrichment of pollutant resistance genes, enhanced by the presence

of the antibiotics that both select the resistance organisms and favor their dissemination by a second-order selection process (Fig. 9.1) triggered by the presence of the selector (Salyers and Amabile-Cuevas, 1997; Thomas and Nielsen, 2005). In the absence of antibiotics, the concentration of pollutant resistance genes harbored in gene transfer units diminishes (Gonzalo et al., 1989). One good example of this situation is the treatment of some fish diseases in aquaculture. When an important bacterial outbreak occurs, large amounts of antibiotics are used, leading to an increase in the prevalence of resistance genes in the water of the vicinity of the containment enclosure. However, when the outbreak is over and the use of antibiotic is restored to normal levels, the amount of bacteria carrying antibiotic resistance plasmids diminishes considerably. The analysis of resistance in animals living in areas with dense human populations in comparison with those living in nonpopulated areas demonstrate this trend (Allen et al., 2010). For example, 90% bacterial isolates from mice and voles analyzed in a densely populated region of rural England were resistant to β-lactams (Gilliver et al., 1999), whereas fecal *Enterobacteriaceae* obtained in Finland (a much less populated area) from wild elk, deer, and voles were mostly susceptible to antibiotics (Osterblad et al., 2001). Likewise, it has been found that baboons and apes living in contact with humans and having daily contact with unprocessed human refuse harbor more antibiotic-resistant bacteria than those living in regions far away from human activities (Rolland et al., 1985; Rwego et al., 2008). All these observations fit with the idea that the major source for the pollution by resistance genes is human activity and that in the absence of this activity resistance might eventually disappears. This concept has been applied to the clinical world in order to replace resistant populations by susceptible ones by changing the antibiotics used in therapy (Austin et al. 1999). Although the strategy of drug removal seems to have some success on occasions (Guillemot et al., 2005; Seppala et al., 1997), in most cases, resistance diminishes but does not disappear. In some other cases, removing the antibiotics does not drastically alter the incidence of resistance. For instance, it has been recently shown that a 2-year discontinuation in the use of trimethoprim had not reduced the *E. coli* resistance rates to this compound in Sweden (Sundqvist et al., 2010). This stability of resistance in the absence of direct selection pressure is likely due to the spread of trimethoprim resistance genes in several different bacteria and mobile elements, which frequently harbor other resistance determinants that might serve for co-selecting resistance to trimethoprim (see below).

Similarly, and although it is clear that pristine environments contain lower amounts of resistance genes, it has been demonstrated that these ecosystems might contain the same resistance elements currently present in human pathogens. In other words, they can harbor pollutant resistance genes in the absence of both direct contamination and the selective force exerted by the antibiotics (Pallecchi et al., 2008). Examples of this situation are the finding of resistance genes in bacteria obtained from remote human populations (Bartoloni et al., 2009; Grenet et al., 2004) or in wild animals despite the fact that they do not have any known contact with antibiotics (Gilliver et al., 1999; Livermore et al., 2001), indicating that total eradication of resistance genes is difficult if not impossible.

As stated above, resistance genes are replicative elements, and they will disappear only if the bacterial populations harboring them are out competed by susceptible partners. Indeed, it has been widely accepted that acquisition of resistance confers a fitness cost (Andersson and Levin, 1999; Bouma and Lenski, 1988; Dahlberg and

Chao, 2003) and in the absence of selection, their susceptible partners might displace resistant bacteria. However, this statement presents some caveats because available data obtained mainly from the study of human and animal bacterial pathogens indicates that the rate of loss of resistance at the community level is low (Andersson and Hughes, 2010). The above-mentioned finding of the same resistance genes in bacterial pathogens and in environments without any history of antibiotic contamination indicates that, like in human-linked habitats, the resistance determinants are resilient for their elimination in natural ecosystems, even in the absence the selective pressure of antibiotics (Pallecchi et al., 2008).

To understand the reasons for this persistence, some issues must be taken into consideration. The first element that may contribute to the persistence of pollutant resistance genes in natural ecosystems is their presence in mobile genetic elements, which are "easy to get" and "hard to lose." The main cause of this phenomenon is that lateral transfer of resistance genes between bacteria of different species, genera, or even families occurs easily and frequently in nature. Many examples of identical copies of the same resistance gene distributed among distantly related bacteria have been described. For example, the gene *tetM* can be found in a variety of Gram-positive and Gram-negative bacteria (Roberts et al., 1996; Salyers and Shoemaker, 1996; Salyers et al., 1995), indicating the promiscuity of the elements carrying the resistance genes among a broad range of hosts. It is important to mention that broad-host-range plasmids are ubiquitous in environmental bacteria (Götz et al., 1996), indicating that their presence does not necessarily compromise the fitness of their hosts.

One of the reasons for the persistence of gene transfer units, even in the absence of selective pressure, is the structure of these elements themselves. For example, many plasmids, including those carrying resistance genes, encode toxin–antitoxin systems (Hayes, 2003). These systems are formed by a bacterial killer (the toxin) and an antitoxin that binds the toxin, thereby precluding its activity. The antitoxin is less stable than the toxin, such that its constant production is needed to avoid cell death. If the bacterial cell loses the plasmid, the antitoxin is rapidly degraded, but the toxin remains for sufficient time to kill the bacterium. Because of this, toxin–antitoxin systems are very efficient elements for the stabilization of plasmids in their host cells. Another relevant factor for the stabilization of resistant plasmid is co-selection (Dang et al., 2006; Stepanauskas et al., 2006). Resistance plasmids frequently harbor several different resistance genes such that selection for one of these genes will co-select the others. This is not necessarily relevant in the case of noncontaminated environments. However, resistance plasmids can also harbor determinants with an adaptive value for colonizing natural ecosystems such as siderophores, toxins, colonization factors, microcins, or resistance to biocides or heavy metals (De Souza et al., 2006; Dhakephalkar and Chopade, 1994; Gonzalo et al., 1989; Hermansson et al., 1987; Martinez et al., 1989; Martinez and Perez-Diaz, 1990; Martinez-Suarez et al., 1987; Stepanauskas et al., 2006), which might favor co-selection of resistance in natural ecosystems. Antibiotic resistance genes are frequently found forming clusters in integrons. Integrons are gene capture units capable of recruiting several different gene cassettes (Mazel, 2006). The backbone of type I integrons contains a determinant that confers resistance to quaternary ammonium compounds, a commonly used industrial biocide and thus industrial pollution is one important issue for co-selection of resistance genes in natural ecosystems (Biyela et al., 2004; Gaze et al., 2005; Wright et al., 2008).

A final consideration concerns the effect of resistance on bacterial fitness. Although in several occasions acquisition of resistance produces a metabolic burden, which diminishes the competitivity of resistant bacteria, the fitness costs of antibiotic resistance is not always the same. Indeed, it has been described that some resistance mechanisms have no cost for bacteria (Balsalobre and de la Campa, 2008). In this case the resistant microorganisms should not be out competed by their susceptible counterparts. Furthermore, the effect of resistance on bacterial fitness can be specific for the genetic background and for the host environment. As an example, the same *gyrA* mutation that leads to quinolone resistance reduces the virulence of one strain of *Campylobacter jejuni* but has the opposite effect on another strain (Luo et al., 2005), indicating that, in some instances, resistance might increase bacterial fitness, depending on the genetic background of the host. The analysis of rifampin mutants of *Bacillus subtilis* has demonstrated as well that some mutations produce a metabolic rewiring that allows bacteria to more efficiently use some substrates than the susceptible strains (Maughan et al., 2004), further supporting the notion that resistance might increase fitness in some specific habitats. Even in the cases in which resistance renders strong fitness costs that diminish the ecological competitivity of resistant bacteria, stabilization is allowed by the acquisition of secondary mutations (compensatory mutations) that compensate the costs associated to resistance (Bjorkman et al., 2000; Lofmark et al., 2008; Paulander et al., 2007).

Altogether, these factors allow the stabilization of pollutant resistance genes both in contaminated and in pristine environments, indicating that the release of these elements into natural ecosystems is likely a one-way road. Studies on the effects on antibiotic resistance of the ban in the use avoparcin for farming purposes demonstrates a decline in the prevalence of resistance organisms in the animals. However, a reversal to a situation of full susceptibility in all isolates has not been observed (Aarestrup, 2005; Aarestrup et al., 2001). We can predict that a similar pattern will likely be observed in natural ecosystems.

The effects of pollution by antibiotics and by resistance genes harbored by gene transfer units on the genetic structure of bacterial populations is unknown, but it is likely to be irreversible and may impact the future evolution of environmental microbiota with consequences for human health that are difficult to predict.

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REFERENCES

Aarestrup FM (2005). Veterinary drug usage and antimicrobial resistance in bacteria of animal origin. *Basic Clin Pharmacol Toxicol* 96:271–281.

Aarestrup FM, Seyfarth AM, Emborg HD, Pedersen K, Hendriksen RS, Bager F (2001). Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark. *Antimicrob Agents Chemother* 45:2054–2059.

Adachi H, Ishiguro M, Imajoh S, Ohta T, Matsuzawa H (1992). Active-site residues of the transpeptidase domain of penicillin-binding protein 2 from *Escherichia coli*: Similarity in catalytic mechanism to class A beta-lactamases. *Biochemistry* 31:430–437.

Aendekerk S, Diggle SP, Song Z, Hoiby N, Cornelis P, Williams P, Camara M (2005). The MexGHI-OpmD multidrug efflux pump controls growth, antibiotic susceptibility and virulence in *Pseudomonas aeruginosa* via 4-quinolone-dependent cell-to-cell communication. *Microbiology* 151:1113–1125.

Ainsa JA, Perez E, Pelicic V, Berthet FX, Gicquel B, Martin C (1997). Aminoglycoside 2′-*N*-acetyltransferase genes are universally present in mycobacteria: Characterization of the aac(2′)-Ic gene from *Mycobacterium tuberculosis* and the aac(2′)-Id gene from *Mycobacterium smegmatis*. *Mol Microbiol* 24:431–441.

Alekshun MN, Levy SB (2007). Molecular mechanisms of antibacterial multidrug resistance. *Cell* 128:1037–1050.

Allen HK, Cloud-Hansen KA, Wolinski JM, Guan C, Greene S, Lu S, Boeyink M, Broderick NA, Raffa KF, Handelsman J (2009) Resident microbiota of the Gypsy moth midgut harbors antibiotic resistance determinants. *DNA and Cell Biology*, 28(3): 109–117

Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J (2010). Call of the wild: Antibiotic resistance genes in natural environments. *Nat Rev Microbiol* 8:251–259.

Alonso A, Sanchez P, Martinez JL (2001). Environmental selection of antibiotic resistance genes. *Environ Microbiol* 3:1–9.

Alonso A, Morales G, Escalante R, Campanario E, Sastre L, Martinez JL (2004). Overexpression of the multidrug efflux pump SmeDEF impairs *Stenotrophomonas maltophilia* physiology. *J Antimicrob Chemother* 53:432–434.

Aminov RI (2009). The role of antibiotics and antibiotic resistance in nature. *Environ Microbiol* 11:2970–2988.

Andersson DI, Levin BR (1999). The biological cost of antibiotic resistance. *Curr Opin Microbiol* 2:489–493.

Andersson DI, Hughes D (2010). Antibiotic resistance and its cost: Is it possible to reverse resistance? *Nat Rev Microbiol* 8:260–271.

Argoudelis AD, Coats JH (1969). Microbial transformation of antibiotics. II. Phosphorylation of lincomycin by *Streptomyces* species. *J Antibiot (Tokyo)* 22:341–343.

Austin DJ, Kristinsson KG, Anderson RM (1999). The relationship between the volume of antimicrobial consumption in human communities and the frequency of resistance. *Proc Natl Acad Sci USA* 96:1152–1156.

Balsalobre L, de la Campa AG (2008). Fitness of *Streptococcus pneumoniae* fluoroquinolone-resistant strains with topoisomerase IV recombinant genes. *Antimicrob Agents Chemother* 52:822–830.

Baquero F (2004). From pieces to patterns: Evolutionary engineering in bacterial pathogens. *Nat Rev Microbiol* 2:510–518.

Baquero F, Martinez JL, Canton R (2008). Antibiotics and antibiotic resistance in water environments. *Curr Opin Biotechnol* 19:260–265.

Baquero F, Alvarez-Ortega C, Martinez JL (2009). Ecology and evolution of antibiotic resistance. *Environ Microbiol Rep* 1:469–476.

Bartoloni A, et al. (2009). Antibiotic resistance in a very remote Amazonas community. *Int J Antimicrob Agents* 33:125–129.

Baya AM, Brayton PR, Brown VL, Grimes DJ, Russek-Cohen E, Colwell RR (1986). Coincident plasmids and antimicrobial resistance in marine bacteria isolated from polluted and unpolluted Atlantic Ocean samples. *Appl Environ Microbiol* 51:1285–1292.

Benton MJ (2010). Evolutionary biology: New take on the Red Queen. *Nature* 463:306–307.

Benveniste R, Davies J (1973). Aminoglycoside antibiotic-inactivating enzymes in actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria. *Proc Natl Acad Sci USA* 70:2276–2280.

Biyela PT, Lin J, Bezuidenhout CC (2004). The role of aquatic ecosystems as reservoirs of antibiotic resistant bacteria and antibiotic resistance genes. *Water Sci Technol* 50:45–50.

Bjorkman J, Nagaev I, Berg OG, Hughes D, Andersson DI (2000). Effects of environment on compensatory mutations to ameliorate costs of antibiotic resistance. *Science* 287:1479–1482.

Blanco G, et al. (2007). Geographical variation in cloacal microflora and bacterial antibiotic resistance in a threatened avian scavenger in relation to diet and livestock farming practices. *Environ Microbiol* 9:1738–1749.

Blanco G, Lemus JA, Grande J (2009). Microbial pollution in wildlife: Linking agricultural manuring and bacterial antibiotic resistance in red-billed choughs. *Environ Res* 109:405–412.

Bouma JE, Lenski RE (1988). Evolution of a bacteria/plasmid association. *Nature* 335:351–352.

Bradford PA (2001). Extended-spectrum beta-lactamases in the 21st century: Characterization, epidemiology, and detection of this important resistance threat. *Clin Microbiol Rev* 14:933–951.

Brandt KK, Sjoholm OR, Krogh KA, Halling-Sorensen B, Nybroe O (2009). Increased pollution-induced bacterial community tolerance to sulfadiazine in soil hotspots amended with artificial root exudates. *Environ Sci Technol* 43:2963–2968.

Breidenstein EB, Khaira BK, Wiegand I, Overhage J, Hancock RE (2008). Complex ciprofloxacin resistome revealed by screening a *Pseudomonas aeruginosa* mutant library for altered susceptibility. *Antimicrob Agents Chemother* 52:4486–4491.

Brown MG, Balkwill DL (2009). Antibiotic resistance in bacteria isolated from the deep terrestrial subsurface. *Microb Ecol* 57:484–493.

Bush K, Jacoby GA (2010). Updated functional classification of beta-lactamases. *Antimicrob Agents Chemother* 54:969–976.

Cabello FC (2006). Heavy use of prophylactic antibiotics in aquaculture: A growing problem for human and animal health and for the environment. *Environ Microbiol* 8:1137–1144.

Canton R, Coque TM (2006). The CTX-M beta-lactamase pandemic. *Curr Opin Microbiol* 9:466–475.

Cattoir V, Poirel L, Aubert C, Soussy CJ, Nordmann P (2008). Unexpected occurrence of plasmid-mediated quinolone resistance determinants in environmental *Aeromonas* spp. *Emerg Infect Dis* 14:231–237.

Clay K, Kover PX (1996). The Red Queen Hypothesis and plant/pathogen interactions. *Annu Rev Phytopathol* 34:29–50.

Cowan PE, Tyndale-Biscoe CH (1997). Australian and New Zealand mammal species considered to be pests or problems. *Reprod Fertil Dev* 9:27–36.

Cundliffe E (1992). Self-protection mechanisms in antibiotic producers. *Ciba Found Symp* 171:199–208.

D'Acosta VM, McGrann KM, Hughes DW, Wright GD (2006). Sampling the antibiotic resistome. *Science* 311:374–377.

Dahlberg C, Chao L (2003). Amelioration of the cost of conjugative plasmid carriage in *Eschericha coli* K12. *Genetics* 165:1641–1649.

Dang H, Song L, Chen M, Chang Y (2006). Concurrence of *cat* and *tet* genes in multiple antibiotic-resistant bacteria isolated from a sea cucumber and sea urchin mariculture farm in China. *Microb Ecol* 52:634–643.

Datta N, Hughes VM (1983). Plasmids of the same Inc groups in Enterobacteria before and after the medical use of antibiotics. *Nature* 306:616–617.

Davies J (1994). Inactivation of antibiotics and the dissemination of resistance genes. *Science* 264:375–382.

Davies JE (1997). Origins, acquisition and dissemination of antibiotic resistance determinants. *Ciba Found Symp* 207:15–27.

Davies J (2006). Are antibiotics naturally antibiotics? *J Ind Microbiol Biotechnol* 33:496–499.

Davies J, Spiegelman GB, Yim G (2006). The world of subinhibitory antibiotic concentrations. *Curr Opin Microbiol* 9:445–453.

De Souza MJ, Nair S, Loka Bharathi PA, Chandramohan D (2006). Metal and antibiotic-resistance in psychrotrophic bacteria from Antarctic Marine waters. *Ecotoxicology* 15:379–384.

de Souza SM, Vasconcelos EC, Dziedzic M, de Oliveira CM (2009). Environmental risk assessment of antibiotics: An intensive care unit analysis. *Chemosphere* 77:962–967.

Dhakephalkar PK, Chopade BA (1994). High levels of multiple metal resistance and its correlation to antibiotic resistance in environmental isolates of *Acinetobacter*. *Biometals* 7:67–74.

Dotsch A, Becker T, Pommerenke C, Magnowska Z, Jansch L, Haussler S (2009). Genome-wide identification of genetic determinants of antimicrobial drug resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 53:2522–2531.

Evans K, Passador L, Srikumar R, Tsang E, Nezezon J, Poole K (1998). Influence of the MexAB-OprM multidrug efflux system on quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* 180:5443–5447.

Fajardo A, Martinez JL (2008). Antibiotics as signals that trigger specific bacterial responses. *Curr Opin Microbiol* 11:161–167.

Fajardo A, et al. (2008). The neglected intrinsic resistome of bacterial pathogens. *PLoS ONE* 3:e1619.

Fajardo A, Linares JF, Martinez JL (2009). Towards an ecological approach to antibiotics and antibiotic resistance genes. *Clin Microbiol Infect* 15:14–16.

Ferber D (2003). Antibiotic resistance. WHO advises kicking the livestock antibiotic habit. *Science* 301:1027.

Fleming A (1944). Penicillin: The Robert Campbell Oration. *Ulster Med J* 13:95–122.

Gaze WH, Abdouslam N, Hawkey PM, Wellington EM (2005). Incidence of class 1 integrons in a quaternary ammonium compound-polluted environment. *Antimicrob Agents Chemother* 49:1802–1807.

Gerba CP, Smith JE, Jr. (2005). Sources of pathogenic microorganisms and their fate during land application of wastes. *J Environ Qual* 34:42–48.

Gilliver MA, Bennett M, Begon M, Hazel SM, Hart CA (1999). Antibiotic resistance found in wild rodents. *Nature* 401:233–234.

Gonzalo MP, Arribas RM, Latorre E, Baquero F, Martinez JL (1989). Sewage dilution and loss of antibiotic resistance and virulence determinants in *E. coli*. *FEMS Microbiol Lett* 50:93–96.

Götz A, et al. (1996). Detection and characterization of broad-host-range plasmids in environmental bacteria by PCR. *Appl Environ Microbiol* 62:2621–2628.

Grenet K, et al. (2004). Antibacterial resistance, Wayampis Amerindians, French Guyana. *Emerg Infect Dis* 10:1150–1153.

Grimes DJ, Singleton FL, Colwell RR (1984). Allogenic succession of marine bacterial communities in response to pharmaceutical waste. *J Appl Bacteriol* 57:247–261.

Guillemot D, et al. (2005). Reduction of antibiotic use in the community reduces the rate of colonization with penicillin G-nonsusceptible *Streptococcus pneumoniae*. *Clin Infect Dis* 41:930–938.

Hayes F (2003). Toxins-antitoxins: Plasmid maintenance, programmed cell death, and cell cycle arrest. *Science* 301:1496–1499.

Hermansson M, Jones GW, Kjelleberg S (1987). Frequency of antibiotic and heavy metal resistance, pigmentation, and plasmids in bacteria of the marine air-water interface. *Appl Environ Microbiol* 53:2338–2342.

Hu X, Zhou Q, Luo Y (2010). Occurrence and source analysis of typical veterinary antibiotics in manure, soil, vegetables and groundwater from organic vegetable bases, northern China. *Environ Pollut* 158:2992–2998.

Humeniuk C, Arlet G, Gautier V, Grimont P, Labia R, Philippon A (2002). Beta-lactamases of *Kluyvera ascorbata*, probable progenitors of some plasmid-encoded CTX-M types. *Antimicrob Agents Chemother* 46:3045–3049.

Isken S, de Bont JA (1996). Active efflux of toluene in a solvent-resistant bacterium. *J Bacteriol* 178:6056–6058.

Karci A, Balcioglu IA (2009). Investigation of the tetracycline, sulfonamide, and fluoroquinolone antimicrobial compounds in animal manure and agricultural soils in Turkey. *Sci Total Environ* 407:4652–4664.

Knapp CW, Dolfing J, Ehlert PA, Graham DW (2010). Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. *Environ Sci Technol* 44:580–587.

Knox JR, Moews PC, Frere JM (1996). Molecular evolution of bacterial beta-lactam resistance. *Chem Biol* 3:937–947.

Kohler T, van Delden C, Curty LK, Hamzehpour MM, Pechere JC (2001). Overexpression of the MexEF-OprN multidrug efflux system affects cell-to-cell signaling in *Pseudomonas aeruginosa*. *J Bacteriol* 183:5213–5222.

Kümmerer K (2004). Resistance in the environment. *J Antimicrob Chemother* 54:311–320.

Kümmerer K (2009a). Antibiotics in the aquatic environment—A review—Part II. *Chemosphere* 75:435–441.

Kümmerer K (2009b). Antibiotics in the aquatic environment—A review—Part I. *Chemosphere* 75:417–434.

Kümmerer K, Henninger A (2003). Promoting resistance by the emission of antibiotics from hospitals and households into effluent. *Clin Microbiol Infect* 9:1203–1214.

Lambert T, Ploy MC, Denis F, Courvalin P (1999). Characterization of the chromosomal aac(6′)-Iz gene of *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* 43:2366–2371.

Laskaris P, Tolba S, Calvo-Bado L, Wellington L (2010). Coevolution of antibiotic production and counter-resistance in soil bacteria. *Environ Microbiol* 12:783–796.

Levy SB (1998). Multidrug resistance—A sign of the times. *N Engl J Med* 338:1376–1378.

Levy SB, Marshall B (2004). Antibacterial resistance worldwide: Causes, challenges and responses. *Nat Med* 10:S122–129.

Levy SB, O'Brien TF (2005). Global antimicrobial resistance alerts and implications. *Clin Infect Dis* 41:S219–220.

Li XZ, Zhang L, Poole K (1998). Role of the multidrug efflux systems of *Pseudomonas aeruginosa* in organic solvent tolerance. *J Bacteriol* 180:2987–2991.

Lietman PS (1972). Pharmacologic effects on developing enzyme systems. *Fed Proc* 31:62–64.

Linares JF, Gustafsson I, Baquero F, Martinez JL (2006). Antibiotics as intermicrobial signaling agents instead of weapons. *Proc Natl Acad Sci USA* 103:19484–19489.

Lindberg F, Normark S (1986). Contribution of chromosomal beta-lactamases to beta-lactam resistance in enterobacteria. *Rev Infect Dis* 8:S292–304.

Lindberg RH, Bjorklund K, Rendahl P, Johansson MI, Tysklind M, Andersson BA (2007). Environmental risk assessment of antibiotics in the Swedish environment with emphasis on sewage treatment plants. *Water Res* 41:613–619.

Lister PD, Wolter DJ, Hanson ND (2009). Antibacterial-resistant *Pseudomonas aeruginosa*: Clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clin Microbiol Rev* 22:582–610.

Livermore DM, Woodford N (2006). The beta-lactamase threat in Enterobacteriaceae, Pseudomonas and Acinetobacter. *Trends Microbiol* 14:413–420.

Livermore DM, et al. (2001). Antibiotic resistance in bacteria from magpies (*Pica pica*) and rabbits (*Oryctolagus cuniculus*) from west Wales. *Environ Microbiol* 3:658–661.

Lofmark S, Jernberg C, Billstrom H, Andersson DI, Edlund C (2008). Restored fitness leads to long-term persistence of resistant *Bacteroides* strains in the human intestine. *Anaerobe* 14:157–160.

Looney WJ, Narita M, Muhlemann K (2009). *Stenotrophomonas maltophilia*: An emerging opportunist human pathogen. *Lancet Infect Dis* 9:312–323.

Luo N, Pereira S, Sahin O, Lin J, Huang S, Michel L, Zhang Q (2005). Enhanced in vivo fitness of fluoroquinolone-resistant *Campylobacter jejuni* in the absence of antibiotic selection pressure. *Proc Natl Acad Sci USA* 102:541–546.

Macinga DR, Rather PN (1999). The chromosomal 2′-*N*-acetyltransferase of *Providencia stuartii*: Physiological functions and genetic regulation. *Front Biosci* 4:D132–140.

MacLeod C (1912). Electric metallic colloids and their therapeutical applications. *Lancet* 179:322.

Marshall CG, Lessard IA, Park I, Wright GD (1998). Glycopeptide antibiotic resistance genes in glycopeptide-producing organisms. *Antimicrob Agents Chemother* 42:2215–2220.

Martinez JL (2008). Antibiotics and antibiotic resistance genes in natural environments. *Science* 321:365–367.

Martinez JL (2009a). Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environ Pollut* 157:2893–2902.

Martinez JL (2009b). The role of natural environments in the evolution of resistance traits in pathogenic bacteria. *Proc Biol Sci* 276:2521–2530.

Martinez JL, Baquero F (2002). Interactions among strategies associated with bacterial infection: pathogenicity, epidemicity, and antibiotic resistance. *Clin Microbiol Rev* 15:647–679.

Martinez JL, Perez-Diaz JC (1990). Cloning of the determinants for microcin D93 production and analysis of three different D-type microcin plasmids. *Plasmid* 23:216–225.

Martinez JL, Cercenado E, Baquero F (1989). Aerobactin production and plasmid distribution in *Escherichia coli* clinical isolates. *FEMS Microbiol Lett* 51:41–44.

Martinez JL, Baquero F, Andersson DI (2007). Predicting antibiotic resistance. *Nat Rev Microbiol* 5:958–965.

Martinez JL, Sanchez MB, Martinez-Solano L, Hernandez A, Garmendia L, Fajardo A, Alvarez-Ortega C (2009a). Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. *FEMS Microbiol Rev* 33:430–449.

Martinez JL, Fajardo A, Garmendia L, Hernandez A, Linares JF, Martinez-Solano L, Sanchez MB (2009b). A global view of antibiotic resistance. *FEMS Microbiol Rev* 33:44–65.

Martinez-Suarez JV, Martinez JL, Lopez de Goicoechea MJ, Perez-Diaz JC, Baquero F, Meseguer M, Linares J (1987). Acquisition of antibiotic resistance plasmids in vivo by extraintestinal *Salmonella* spp. *J Antimicrob Chemother* 20:452–453.

Maughan H, Galeano B, Nicholson WL (2004). Novel rpoB mutations conferring rifampin resistance on *Bacillus subtilis*: global effects on growth, competence, sporulation, and germination. *J Bacteriol* 186:2481–2486.

Mazel D (2006). Integrons: Agents of bacterial evolution. *Nat Rev Microbiol* 4:608–620.

McArthur JV, Tuckfield RC (2000). Spatial patterns in antibiotic resistance among stream bacteria: Effects of industrial pollution. *Appl Environ Microbiol* 66:3722–3726.

McGowan JE Jr (2006). Resistance in nonfermenting gram-negative bacteria: Multidrug resistance to the maximum. *Am J Infect Control* 34:S29–37.

McManus PS, Stockwell VO, Sundin GW, Jones AL (2002). Antibiotic use in plant agriculture. *Annu Rev Phytopathol* 40:443–465.

Miteva VI, Sheridan PP, Brenchley JE (2004). Phylogenetic and physiological diversity of microorganisms isolated from a deep greenland glacier ice core. *Appl Environ Microbiol* 70:202–213.

Mori T, Mizuta S, Suenaga H, Miyazaki K (2008). Metagenomic screening for bleomycin resistance genes. *Appl Environ Microbiol* 74:6803–6805.

Naslund J, Hedman JE, Agestrand C (2008). Effects of the antibiotic ciprofloxacin on the bacterial community structure and degradation of pyrene in marine sediment. *Aquat Toxicol* 90:223–227.

Navon-Venezia S, Ben-Ami R, Carmeli Y (2005). Update on *Pseudomonas aeruginosa* and *Acinetobacter baumannii* infections in the healthcare setting. *Curr Opin Infect Dis* 18:306–313.

Nikaido H (1994). Prevention of drug access to bacterial targets: Permeability barriers and active efflux. *Science* 264:382–388.

Nikaido H (1998a). Antibiotic resistance caused by gram-negative multidrug efflux pumps. *Clin Infect Dis* 27:S32–41.

Nikaido H (1998b). Multiple antibiotic resistance and efflux. *Curr Opin Microbiol* 1:516–523.

O'Connor GA, Elliott HA, Basta NT, Bastian RK, Pierzynski GM, Sims RC, Smith JE Jr (2005). Sustainable land application: An overview. *J Environ Qual* 34:7–17.

Osterblad M, Norrdahl K, Korpimaki E, Huovinen P (2001). Antibiotic resistance. How wild are wild mammals? *Nature* 409:37–38.

Pallecchi L, Bartoloni A, Paradisi F, Rossolini GM (2008). Antibiotic resistance in the absence of antimicrobial use: Mechanisms and implications. *Expert Rev Anti Infect Ther* 6:725–732.

Pang Y, Brown BA, Steingrube VA, Wallace RJ Jr, Roberts MC (1994). Tetracycline resistance determinants in *Mycobacterium* and *Streptomyces* species. *Antimicrob Agents Chemother* 38:1408–1412.

Paterson DL, Bonomo RA (2005). Extended-spectrum beta-lactamases: A clinical update. *Clin Microbiol Rev* 18:657–686.

Paulander W, Maisnier-Patin S, Andersson DI (2007). Multiple mechanisms to ameliorate the fitness burden of mupirocin resistance in *Salmonella typhimurium*. *Mol Microbiol* 64:1038–1048.

Payie KG, Rather PN, Clarke AJ (1995). Contribution of gentamicin 2′-*N*-acetyltransferase to the O acetylation of peptidoglycan in *Providencia stuartii*. *J Bacteriol* 177:4303–4310.

Pei R, Kim SC, Carlson KH, Pruden A (2006). Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Res* 40:2427–2435.

Pereira-Leal JB, Levy ED, Teichmann SA (2006). The origins and evolution of functional modules: Lessons from protein complexes. *Philos Trans R Soc B* 361:507–517.

Picao RC, Poirel L, Demarta A, Silva CS, Corvaglia AR, Petrini O, Nordmann P (2008). Plasmid-mediated quinolone resistance in *Aeromonas allosaccharophila* recovered from a Swiss lake. *J Antimicrob Chemother* 62:948–950.

Poeta P, et al. (2008). Seagulls of the Berlengas natural reserve of Portugal as carriers of fecal *Escherichia coli* harboring CTX-M and TEM extended-spectrum beta-lactamases. *Appl Environ Microbiol* 74:7439–7441.

Poeta P, et al. (2009). Wild boars as reservoirs of extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* of different phylogenetic groups. *J Basic Microbiol* 49:584–588.

Poirel L, Liard A, Rodriguez-Martinez JM, Nordmann P (2005a). Vibrionaceae as a possible source of Qnr-like quinolone resistance determinants. *J Antimicrob Chemother* 56:1118–1121.

Poirel L, Rodriguez-Martinez JM, Mammeri H, Liard A, Nordmann P (2005b). Origin of plasmid-mediated quinolone resistance determinant QnrA. *Antimicrob Agents Chemother* 49:3523–3525.

Pruden A, Pei R, Storteboom H, Carlson KH (2006). Antibiotic resistance genes as emerging contaminants: Studies in northern Colorado. *Environ Sci Technol* 40:7445–7450.

Ramos JL, et al. (2002). Mechanisms of solvent tolerance in gram-negative bacteria. *Annu Rev Microbiol* 56:743–768.

Ren Q, Paulsen IT (2005). Comparative analyses of fundamental differences in membrane transport capabilities in prokaryotes and eukaryotes. *PLoS Comput Biol* 1:e27.

Roberts MC, Chung WO, Roe DE (1996). Characterization of tetracycline and erythromycin resistance determinants in *Treponema denticola*. *Antimicrob Agents Chemother* 40:1690–1694.

Robicsek A, Jacoby GA, Hooper DC (2006). The worldwide emergence of plasmid-mediated quinolone resistance. *Lancet Infect Dis* 6:629–640.

Rolland RM, Hausfater G, Marshall B, Levy SB (1985). Antibiotic-resistant bacteria in wild primates: Increased prevalence in baboons feeding on human refuse. *Appl Environ Microbiol* 49:791–794.

Ruiz GM, Rawlings TK, Dobbs FC, Drake LA, Mullady T, Huq A, Colwell RR (2000). Global spread of microorganisms by ships. *Nature* 408:49–50.

Rwego IB, Isabirye-Basuta G, Gillespie TR, Goldberg TL (2008). Gastrointestinal bacterial transmission among humans, mountain gorillas, and livestock in Bwindi Impenetrable National Park, Uganda. *Conserv Biol* 22:1600–1607.

Sader HS, Jones RN (2005). Antimicrobial susceptibility of uncommonly isolated non-enteric Gram-negative bacilli. *Int J Antimicrob Agents* 25:95–109.

Saier MH Jr, Paulsen IT (2001). Phylogeny of multidrug transporters. *Semin Cell Dev Biol* 12:205–213.

Salyers AA, Amabile-Cuevas CF (1997). Why are antibiotic resistance genes so resistant to elimination? *Antimicrob Agents Chemother* 41:2321–2325.

Salyers AA, Shoemaker NB (1996). Resistance gene transfer in anaerobes: New insights, new problems. *Clin Infect Dis* 23:S36–43.

Salyers AA, Shoemaker NB, Stevens AM, Li LY (1995). Conjugative transposons: An unusual and diverse set of integrated gene transfer elements. *Microbiol Rev* 59:579–590.

Sanchez MB, Hernandez A, Rodriguez-Martinez JM, Martinez-Martinez L, Martinez JL (2008). Predictive analysis of transmissible quinolone resistance indicates *Stenotrophomonas maltophilia* as a potential source of a novel family of Qnr determinants. *BMC Microbiol* 8:148.

Sanchez MB, Hernandez A, Martinez JL (2009). Stenotrophomonas maltophilia drug resistance. *Future Microbiol* 4:655–660.

Seppala H, Klaukka T, Vuopio-Varkila J, Muotiala A, Helenius H, Lager K, Huovinen P (1997). The effect of changes in the consumption of macrolide antibiotics on erythromycin resistance in group A streptococci in Finland. Finnish Study Group for Antimicrobial Resistance. *N Engl J Med* 337:441–446.

Shaw WV (1983). Chloramphenicol acetyltransferase: Enzymology and molecular biology. *CRC Crit Rev Biochem* 14:1–46.

Shaw KJ, et al. (1992). Characterization of the chromosomal aac(6′)-Ic gene from *Serratia marcescens*. *Antimicrob Agents Chemother* 36:1447–1455.

Shaw KJ, Rather PN, Hare RS, Miller GH (1993). Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiol Rev* 57:138–163.

Silver S, Phung LT (1996). Bacterial heavy metal resistance: New surprises. *Annu Rev Microbiol* 50:753–789.

Simoes RR, Poirel L, Da Costa PM, Nordmann P (2010). Seagulls and beaches as reservoirs for multidrug-resistant *Escherichia coli*. *Emerg Infect Dis* 16:110–112.

Singer RS, Finch R, Wegener HC, Bywater R, Walters J, Lipsitch M (2003). Antibiotic resistance—The interplay between antibiotic use in animals and human beings. *Lancet Infect Dis*, 3:47–51.

Sjolund M, et al. (2008). Dissemination of multidrug-resistant bacteria into the Arctic. *Emerg Infect Dis* 14:70–72.

Smith DL, Harris AD, Johnson JA, Silbergeld EK, Morris JG Jr (2002). Animal antibiotic use has an early but important impact on the emergence of antibiotic resistance in human commensal bacteria. *Proc Natl Acad Sci USA* 99:6434–6439.

Spicuzza L, Sciuto C, Vitaliti G, Di Dio G, Leonardi S, La Rosa M (2008). Emerging pathogens in cystic fibrosis: Ten years of follow-up in a cohort of patients. *Eur J Clin Microbiol Infect Dis* 28:191–195.

Stepanauskas R, Glenn TC, Jagoe CH, Tuckfield RC, Lindell AH, King CJ, McArthur JV (2006). Coselection for microbial resistance to metals and antibiotics in freshwater microcosms. *Environ Microbiol* 8:1510–1514.

Strahilevitz J, Jacoby GA, Hooper DC, Robicsek A (2009). Plasmid-mediated quinolone resistance: A multifaceted threat. *Clin Microbiol Rev* 22:664–689.

Sundqvist M, et al. (2010). Little evidence for reversibility of trimethoprim resistance after a drastic reduction in trimethoprim use. *J Antimicrob Chemother* 65:350–360.

Tamae C, et al. (2008). Determination of antibiotic hypersensitivity among 4,000 single-gene-knockout mutants of *Escherichia coli*. *J Bacteriol* 190:5981–5988.

Thomas CM, Nielsen KM (2005). Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nat Rev Microbiol* 3:711–721.

Thorburn AL (1983). Paul Ehrlich: Pioneer of chemotherapy and cure by arsenic (1854–1915). *Br J Vener Dis* 59:404–405.

Twentyman PR (1997). Transport proteins in drug resistance: Biology and approaches to circumvention. *J Intern Med Suppl* 740:133–137.

Venglovsky J, Sasakova N, Placha I (2009). Pathogens and antibiotic residues in animal manures and hygienic and ecological risks related to subsequent land application. *Bioresour Technol* 100:5386–5391.

Waksman SA, Woodruff HB (1940). The soil as a source of microorganisms antagonistic to disease-producing bacteria. *J Bacteriol* 40:581–600.

Wright GD (2007). The antibiotic resistome: The nexus of chemical and genetic diversity. *Nat Rev Microbiol* 5:175–186.

Wright MS, Baker-Austin C, Lindell AH, Stepanauskas R, Stokes HW, McArthur JV (2008). Influence of industrial contamination on mobile genetic elements: Class 1 integron abundance and gene cassette structure in aquatic bacterial communities. *ISME J* 2:417–428.

Xie X, Zhou Q, Bao Q, He Z, Bao Y (2011). Genotoxicity of tetracycline as an emerging pollutant on root meristem cells of wheat (*Triticum aestivum* L.). *Environ Toxicol* 26(4):417–423.

Xu XR, Li XY (2010). Sorption and desorption of antibiotic tetracycline on marine sediments. *Chemosphere* 78:430–436.

Yates AL (1939). The action of sulphonamide in infective colds. *Can Med Assoc J* 41:275–278.

Yim G, Wang HH, Davies J (2006a). The truth about antibiotics. *Int J Med Microbiol* 296:163–170.

Yim G, de La Cruz F, Spiegelman GB, Davies J (2006b). Transcription modulation of *Salmonella enterica* serovar Typhimurium promoters by sub-MIC levels of rifampin. *J Bacteriol* 188:7988–7991.

Yim G, Wang HH, Davies J (2007). Antibiotics as signalling molecules. *Philos Trans R Soc B* 362:1195–1200.

Yiruhan, et al. (2010). Determination of four fluoroquinolone antibiotics in tap water in Guangzhou and Macao. *Environ Pollut* 158:2350–2358.

10

QUANTIFYING ANTHROPOGENIC IMPACTS ON ENVIRONMENTAL RESERVOIRS OF ANTIBIOTIC RESISTANCE

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10.1 OVERVIEW

Antibiotic resistance remains a particularly challenging "contaminant" to track in the environment given its natural background occurrence and ability to be horizontally transferred among bacteria. This chapter will explore the impact of human activities on environmental reservoirs of resistance and define the unique nature of *antibiotic resistance genes (ARGs)* as contaminants. Insight will be provided into the potential to track the fate of ARGs in the watershed using a recently developed molecular signature approach. Of particular interest are the dominant processes driving ARG proliferation in anthropogenically impacted environments. For example, are *antibiotic-resistant bacteria (ARB)* and ARGs primarily transported to surface waters from *wastewater treatment plants (WWTPs)* and *animal feeding operations (AFOs)* or are they selected among the native bacterial flora by antibiotic waste streams and other selective pressures? The answers to these questions have critical implications for potential antibiotic resistance management strategies.

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10.2 ANTIBIOTIC RESISTANCE GENES AS CONTAMINANTS

Contaminants are defined as undesired constituents in the environment, typically resulting from human activities. Clearly, antibiotic resistance represents an undesired property among pathogenic bacteria. It has been documented that the rate of antibiotic resistance among many disease-causing bacteria has been steadily increasing, thus undermining the reliability of antibiotics in fighting disease. In the United States it is estimated that about 98,000 patients die each year from hospital-acquired infections (Klevens et al., 2007), which is a notable increase from 13,300 deaths in 1992 (Cardo et al., 2004). According to most recent estimates, 70% of all hospital-acquired infections are resistant to at least one class of antibiotic (Leeb, 2004). Although a large body of research exists characterizing the clinical aspect of antibiotic resistance, the problem continues to worsen with no clear solutions in sight. Consideration of ARGs as environmental contaminants may offer fresh perspective in combating antibiotic resistance.

Numerous studies have revealed that human activities elevate antibiotic resistance levels in the environment. For example, examination of the Cache La Poudre (Poudre) River sediments in northern Colorado along a gradient of adjacent urban and agricultural land-use activities revealed concentrations of tetracycline ARGs (*tet*O and *tet*W) and sulfonamide ARGs (*sul*I and *sul*II) 2–3 orders of magnitude higher compared to the pristine upstream portion of the river (Pei et al., 2006; Pruden et al., 2006). In the extreme case of the heavily polluted River Ganga in India, 25% of *Escherichia coli* isolated were resistant to three or more antibiotics and also carried high loads of virulence genes (Ram et al., 2007). For comparison, resistance of *E. coli* to four or more antibiotics ranged from approximately 17 to 40% (for antibiotic concentrations of 20–100 mg/L) in treated wastewater in the United States, which was more than double the resistance rate observed in the influent (Lefkowitz and Duran, 2009). Particularly compelling is a recent analysis of archived soils from the Netherlands spanning the antibiotic era, indicating marked increases in abundance of tetracycline, erythromycin, and β-lactam ARGs from 1940 to 2008 (Knapp et al., 2010). Thus, though the mechanisms are not entirely clear, human activities, especially those during the antibiotic era, have had a profound influence on the magnitude of antibiotic reservoirs of resistance.

Rising levels of antibiotic resistance in environmental reservoirs would not be of concern if these are inherently disconnected from the resistance elements observed in human pathogens; however, the opposite appears to be the situation. A very direct link has been noted in the case of the observed exchange of tetracycline resistance-encoding plasmids between the fish pathogen *Aeromonas* isolated from aquaculture facilities and pathogenic *E. coli* isolated from hospital effluent (Rhodes et al., 2000). Similarly, the origins of clinically relevant quinolone ARGs, *qnr*A and *qnr*S, have both been traced to waterborne bacteria, *Shewanella algae* and *Vibrio splendidus* (Poirel et al., 2005; Cattoir et al., 2007). ARGs observed in the clinic have also been observed to originate from antibiotic-producing bacteria present in soil; for example, tetracycline ARG *otrA* and *otrB* are found in mycobacteria and originated in *Streptomyces rimosus* (Pang et al., 1994). In terms of the broader antibiotic resistome, the vast majority of antibiotics in use today are derived from soil organisms, thus clinical antibiotic resistance is also likely to have emerged from the same nonclinical habitats (Alonso et al., 2001). Interestingly, even in remote Alaskan soil the diverse

β-lactamases that were discovered there were readily transformed and functional within recipient *E. coli* cells (Allen et al., 2009).

Fundamental to the definition of ARGs as contaminants is their tendency to be associated with mobile genetic elements, such as plasmids, transposons, and integrons. Such mobile genetic elements drive horizontal gene transfer between even unrelated bacteria (Shoemaker et al., 2001) and thus can be described as transcending their bacterial hosts. Interestingly, phylogenetic analysis of a diverse array of ARGs spanning various classes of antibiotics revealed that the dominance of horizontal gene transfer events represents a fundamental feature of the postantibiotic era relative to the preantibiotic era (Aminov and Mackie, 2007). Thus, horizontal gene transfer is of primary concern in combating the present phenomenon of elevated antibiotic resistance associated with widespread antibiotic use. A most recent case in point is that of the emergence of the New Delhi metallo- β-lactamase gene (bla_{NDM}-1), which is housed by a readily transferable 180-kb region that confers resistance to all known antibiotics, except polymyxin and colistin (Yong et al., 2009). Of note is that it is the genetic element that is of concern, not necessarily a particular pathogenic bacterium, as this element has already been found in three major pathogens: *E. coli*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii* (Kumarasamy et al., 2010). Thus, as the antibiotic era takes its course, we see a paradigm shift away from the traditional "pathogen" and a focus of attention toward the deoxyribonucleic acid (DNA) that imparts resistance and its transfer.

A focus on ARGs as contaminants further offers several methodological advantages. Most importantly, molecular techniques provide a means to directly examine resistance elements present in environmental samples and thus circumvent limitations imposed by culture bias. Recent metagenomic studies have provided groundbreaking insight into the true nature of the antibiotic resistome, revealing that the diversity of resistance elements is immense, more so than anyone could have imagined. For example, on average, only 69.5% similarity to known ARGs was observed in the nucleotide sequences among the 95 distinct ARGs isolated from the guts of healthy humans (Sommer et al., 2009). Similarly, phylogenetic analysis of β-lactamases recovered from remote Alaskan soil indicated that they diverged deeply from previously described β-lactamases (Allen et al., 2009). Furthermore, the diversity was remarkable, such that no two β-lactamases isolated were found to have the same phenotype. Thus, while culture-based studies are certainly of value in providing antibiotic resistance information related to well-studied models, such as *E. coli*, they likely only scratch the surface of the underlying phenomena truly driving observed resistance patterns. Furthermore, techniques such as quantitative polymerase chain reaction (qPCR) provide a convenient means to directly quantify the "contaminant" of interest, a critical asset in developing any "pollution" control measure. Already qPCR is gaining wide acceptance as a method of choice for quantifying ARGs in various environmental matrices, including rivers (Luo et al., 2010; Pruden et al., 2006; Pei et al., 2006), lakes (Zhang et al., 2009a; Auerbach et al., 2007), livestock lagoons (McKinney et al., 2010; Peak et al., 2007; Smith et al., 2004), WWTP-activated sludge (Auerbach et al., 2007; Volkmann et al., 2004), anaerobic digesters (Ghosh et al., 2009), soil (Heuer et al., 2009), groundwater (Böckelmann et al., 2009; Mackie et al., 2006), and drinking water systems (Xi et al., 2009).

Thus, efforts to contain antibiotic resistance have so far been of limited effectiveness and may benefit from greater attention on ARGs and mobile genetic elements as

TABLE 10.1 Why Are ARGs Contaminants?

- ARGs are undesired constituents with negative impacts on human health.
- Anthropogenic sources/influences amplify ARG occurrence in the environment.
- Links have been identified between environmental sources of ARGs and human disease.
- ARGs exist and are transferred across bacterial species boundaries, thus transcending the host cell.
- Molecular methods allow ARGs to be directly quantified and characterized without culture bias.

the contaminants of concern, beyond focus on individual strains and cultures. Appropriate definition of the contaminant is a critical step in developing effective strategies for controlling any pollutant of interest (Table 10.1).

10.3 ENVIRONMENTAL SCIENCE AND ENGINEERING PERSPECTIVE ON CONTROL OF ANTIBIOTIC RESISTANCE

10.3.1 Mass Balance Approach

The vast majority of antibiotic resistance studies have been conducted in the clinical realm, while most recently appreciation has been growing for the importance of environmental reservoirs of resistance (Allen et al., 2010; Baquero et al., 2008; Martinez, 2008). This has opened the door to new perspective on the problem of antibiotic resistance. In particular, the discipline of environmental science and engineering provides a framework for defining the processes that govern the movement of contaminants in the environment as well as approaches for effectively controlling such movement when it is undesired.

At the core of determining the fate and transport of any contaminant is the principle of mass balance. First, a control volume of interest is defined, which may consist of any environmental matrix, such as sediment, water, soil, or air. Second, flows into and out of the control volume are defined, and these may either be enriched in or devoid of the contaminant of concern. Third, any processes that may be contributing either to the generation or degradation of the contaminant of interest within the control volume are considered. Thus, the generalized mass balance equation is

$$\text{Accumulation} = \text{In} - \text{Out} + \text{Generation} - \text{Degradation} \tag{10.1}$$

Accumulation is zero at steady state. At steady state in a closed system (batch) there is no net change in concentration of the contaminant with time, whereas in a system with flow (continuously mixed flow reactor or plug flow) steady state is defined as a stable concentration of the contaminant exiting the control volume.

With the mass balance framework in place, focus is then on the processes of interest occurring within the control volume, which may contribute to generation, degradation, or otherwise cause the contaminant to remain within the system (e.g., sorption). In the case of ARGs, this is where the main challenge lies. Their behavior in the environment is only beginning to be understood and what we do know indicates that they are fraught with complexity. The following paragraphs discuss

key phenomena that will contribute to amplification, attenuation, or persistence within a given control volume.

10.3.2 Extracellular ARGs

First of all, ARGs exist not only intracellularly but their extracellular form is also deserving of attention. Using differential extraction methods, the percent extracellular DNA in various soil horizons was estimated to range from 10.4 to 60.1% of the total DNA (Agnelli et al., 2004). Significant downward movement of extracellular DNA has been observed both in soil columns (Pote et al., 2003) and in the field (Agnelli et al., 2004). Furthermore, soil components, such as clay, have been demonstrated to bind to DNA and protect it from DNase (Demanèche et al., 2001; Crecchio et al., 2005) while still maintaining integrity to transform bacterial cells (Crecchio et al., 2005; Crecchio and Stotzky, 1998). Thus, sorption behavior, advection, dispersion, rates of decay by DNases, and rates of transformation (uptake) into bacteria represent potentially important factors governing the fate and transport of extracellular ARGs in the environment. First-order degradation kinetics are typically applied as a first approximation for the disappearance of a contaminant of interest and appear to be a reasonable first step for extracellular ARGs. Though the extracellular distinction was not made, Engemann et al. (2008) were able to successfully apply first-order models and calculate corresponding rate constants corresponding to the disappearance of tetracycline ARGs in mesocosms receiving cattle feedlot wastewater (Engemann et al., 2008).

10.3.3 Intracellular ARGs

Of course, the dominant form of ARGs in the environment is intracellular, where they are harbored within bacterial hosts. Like DNA, bacterial cells may also attach to soil and sediment material, which may then enhance their persistence. Based on centrifugation methods, an average of 40% of fecal indicator bacteria have been observed to attach to soil particles (Krometis et al., 2007). Centrifugation methods are considered to provide relevant information regarding the portion of bacteria that are removable by settling following a storm event; however, they generally underestimate the total portion of bacteria interacting with soil particles. For example, standard filtration-based methods employing pure cultures of *E. coli* have estimated attachment to be as high as 90% (Krometis et al., 2009). Biofilms represent a special case of bacterial adhesion to soil and sediment particles and are generally the preferred mode of bacterial growth relative to suspended growth in the bulk solution, provided that time is sufficient to establish. Interestingly, it was observed that certain tetracycline ARGs (especially *tet*W) readily migrated to biofilms in an aquatic mesocosm study receiving feedlot wastewater (Engemann et al., 2008). While the transport of suspended bacteria carrying ARGs would be expected to be dominated by advection, the sorbed bacterial cells will be governed by more complex transport processes typifying soil erosion and sediment transport.

10.3.4 Proliferation of Intracellular ARGs by Growth and Selection Pressure

Unlike the free-DNA form, however, intracellular ARGs are subject to the fundamental laws of biology. Each bacterial host is unique in its response to

environmental stimuli and will grow on a different range of carbon substrates. Classic Monod kinetics defines bacterial growth rates as a function of substrate concentration, with a distinct array of corresponding kinetic coefficients (μ_{max}, K_s, Y, k_d, etc.) for each bacteria–substrate pair. Thus, increased substrate concentration is expected to result in a net increase in ARGs as bacterial hosts replicate.

Monod kinetics may be modified to consider the presence of inhibitory substances, which is of particular relevance to antibiotic resistance. The *minimum inhibitory concentration (MIC)* is defined as the minimum concentration of antibiotic that inhibits bacterial growth (Andrews, 2001). Of course, bacteria possessing ARGs are characterized by higher MICs, resulting in selective enrichment of resistant bacteria: *this is conventionally considered to be the fundamental phenomenon driving the problem of antibiotic resistance*. Environmental surveys indicate that antibiotic concentrations observed in the environment are typically well below known MIC thresholds (Kolpin et al., 2002), except in extreme cases of uncontrolled antibiotic release such as has been observed in India (Fick et al., 2009). However, it must be recognized that antibiotics play a much broader role in nature than merely as bactericidal agents (Allen et al., 2010). Antibiotics represent a subset of small bioactive compounds that have existed for millennia before the antibiotic era. At the small concentrations that typically occur in nature, antibiotics appear to serve as signaling molecules and thus may actually enhance bacterial growth under some conditions (Allen et al., 2010). Furthermore, antibiotic concentrations well below MICs have been observed to induce transcription of ARGs (Davies et al., 2006) and therefore may also still play a role in their selection in the environment. Even at high concentrations antibiotics have been observed to serve as carbon and energy sources for certain bacteria (Dantas et al., 2008). For these reasons, when including antibiotic concentrations in models, it may be wise to consider the full range of antibiotic concentrations, from low to high, as potential factors enhancing selection of strains carrying ARGs.

At the same time, one-antibiotic one-ARG models of selection of antibiotic resistance are not likely to be representative of actual phenomena occurring in the environment. Co-selection occurs when resistance elements are physically connected to each other, for example, existing on the same plasmid or transposon. Thus, use of one antibiotic does not necessarily impose selection pressure on just one ARG, or even one class of ARGs. In some instances, more than 100 ARG cassettes have been found to be physically connected on the same *multiple antibiotic resistance (MAR)* superintegron (Rowe-Magnus et al., 2001). The phenomenon of co-selection was well illustrated in the case of the ban of avoparcin in Europe. Notably, it was not until an antibiotic of a different class, tylosin (a macrolide), was also discontinued that vancomycin-resistantrom Enterococci prevalence finally began to diminish (Aarestrup et al., 2001). This was found to be a result of the physical linkage of the ARGs, leading to their co-selection.

Also critical to consider is that antibiotics are not the only selective agents of ARGs in the environment. In particular, bacterial mechanisms for fighting other chemicals, such as heavy metals and disinfectants, have been demonstrated to indirectly select for antibiotic resistance (Stepanauskas et al., 2005; Fricke et al., 2008). The ability of heavy metals to co-select ARGs has been well documented (Baker-Austin et al., 2006). Certain organic contaminants may also select for ARGs, for example, a recent study demonstrated that genes coding for multidrug efflux pumps (such as MexAB-OprM) are strongly upregulated in soil bacteria upon

exposure to the environmental contaminant pentachlorophenol (Muller et al., 2007). Therefore, antibiotics are not the only constituent to consider in incorporating factors that lead to an increase in antibiotic resistance into models. In the era of "emerging contaminants," the list of human-derived pharmaceuticals and personal care products detected in the environment continues to grow (Kolpin et al., 2002) and potential impacts on antibiotic resistance remain undefined. One potentially effective approach may be to lump together various selective agents potentially present in an environment into an "anthropogenic selection index factor." The gradient of ARG concentrations observed corresponding to the level of human impact in the Poudre River suggests that this approach may effectively describe antibiotic resistance levels (Pruden et al., 2006). Furthermore, though environments truly untouched by humans are few and far between, a near absence of antibiotic resistance in some "pristine" environments have been revealed, for example, in the commensal enterobacteria of Galapagos iguanas (Thaller et al., 2010).

10.3.5 Horizontal Gene Transfer of ARGs

Intimately related to the phenomenon of co-selection is horizontal gene transfer. MAR gene cassettes and ARG are notorious for being associated with plasmids and/or transposons that facilitate horizontal gene transfer (Rowe-Magnus et al., 2001) and also virulence factors that cause resistant pathogens to be especially aggressive (Aendekerk et al., 2005; Gill et al., 2005). As noted above, horizontal gene transfer appears to be the dominant process of ARG evolution following the onset of the antibiotic era (Aminov and Mackie, 2007) and the driver of antibiotic resistance among pathogenic bacteria (Davies, 1994, 1997). Interestingly, horizontal gene transfer has been noted to be stimulated in response to stress events, such as the generalized SOS-response (Beaber et al., 2004), further supporting the notion that antibiotic resistance is not propagated solely by antibiotic pressures. Thus, horizontal gene transfer is perhaps the most fundamental aspect of the challenge of combating antibiotic resistance.

In terms of modeling, horizontal gene transfer will result in a net increase in ARGs, with subsequent amplification both within the original and new host bacterial cells. If horizontal gene transfer rates can be measured, then the next step will be to consider amplification within the host cells. Assuming transfer from nonnative to native bacteria, amplification is likely to be enhanced in the new host cell as a result of its superior fitness in the corresponding environment. However, from a practical perspective, it may be equally effective to consider simpler models. For example, ARGs could be treated as independent entities responding to antibiotic, metals, or other stress events in the environment, regardless of their presence within host cells. Thus, it would be of interest to compare detailed mechanistic models with simplified ones for better estimating the quantitative role of horizontal gene transfer in propagating ARGs in the environment.

10.3.6 Attenuation versus Persistence of ARGs in Absence of Antibiotic Selection Pressure

In terms of attenuation mechanisms of intracellular ARGs, conventional wisdom dictates that as antibiotic selection pressure is removed, ARGs will attenuate.

The ban of subtherapeutic antibiotic use in agriculture first in Denmark in 1999 and 6 years later across the entire European Union provides the opportunity to observe if this is truly the case. As expected, a sharp decline in bacterial resistance was observed in animal fecal material (Aarestrup et al., 2001). Although a corresponding reduction in the carriage of vancomycin-resistant enterococci (VRE) among healthy humans has been observed, a corresponding decrease in actual VRE infections has not (Klare et al., 1999; Van den Bogaard et al., 2000). Attenuation of antibiotic resistance following the removal of the antibiotic is considered to be a result of selective disadvantage at play by investing energy in maintaining genes not necessary for survival, a phenomenon referred to as the "cost of resistance."

However, cost of resistance does not tell the entire story, and it is apparent that early experiments likely overestimated fitness costs (Summers, 2002). Several ARGs have been observed to carry no measurable fitness cost (Andersson and Levin, 1999). Further, fitness costs can be eliminated by various genetic modifications. For example, inducible expression allows the ARG only to be turned on when it is needed, such as has been observed in the case of vancomycin-resistant enterococci (Foucault et al., 2010). In any case, persistence of ARGs long after the antibiotic selection pressure has been removed is a commonly observed phenomenon (Lipsitch et al., 2000; Ghosh and La Para, 2007; Pei et al., 2007). In parallel to the misconceptions noted above regarding antibiotics, ARGs themselves appear to possess broad function beyond resisting antibiotics, a role that has largely been thrust upon them as a result of the antibiotic era (Allen et al., 2010; Martinez, 2008). For this reason ARGs have recently been referred to as "accidental resistance genes" (Allen et al., 2010). ARGs and their prototypes are abundant in nature, even in pristine environments (Allen et al., 2009), and existed well before the antibiotic era (Aminov, 2009). ARGs have been noted as being remarkable not only in their ability to disseminate but also to persist. This is exemplified by the high similarity noted between ARGs present in an isolated population in Bolivia with minimal access to health care and thus an absence of selection pressure and ARGs from antibiotic-exposed environments (Bartoloni et al., 2004; Pallecchi et al., 2007). Thus, clearly, the phenomena driving ARG attenuation are more complex than simply removing the antibiotic.

10.3.7 Considerations for Modeling the Fate and Transport of ARGs in the Environment

Mechanistic models are required to provide a comprehensive understanding of the phenomena at play in the proliferation of ARGs in the environment and thus to identify the strategic points of attack by which to guide successful management strategies. Clearly, the processes governing the amplification and attenuation of ARGs as environmental contaminants are complex and will be challenging to model. Yet the mass balance approach provides a basic framework by which ARGs may be conceptualized as contaminants and the problem may be tackled in a step-by-step fashion (Table 10.2). Appropriate assumptions and approximations will be necessary, but may be validated by real-world data. A successful parallel exists in the medical realm in which it was determined through modeling that cycling of antibiotic use in hospitals was an ineffective management strategy, though the practice intuitively seemed justified and was widely implemented (Bergstrom et al., 2004).

TABLE 10.2 ARG Proliferation (+), Attenuation (−), and Persistence (0)

	Extracellular	Intracellular
+	• Transformation	• Growth • Direct selection pressure • Co-selection • Horizontal gene transfer
−	• Decay by DNase or hydrolysis	• Death, decay • Fitness cost of resistance
0	• Sorption	• Sorption • Biofilms

Numerous baseline models for contaminant fate and transport exist in the environmental science and engineering literature but will need to be modified to be considerate of processes such as selection, maintenance, attenuation, and horizontal gene transfer.

10.4 MOLECULAR SIGNATURES APPROACH TO TRACKING ARGs AT THE WATERSHED SCALE

Approaching the problem from the opposite side, empirical observation of ARG occurrence in the environment provides critical information for guiding which factors deserve the keenest attention in the development of mechanistic models of fate and transport. The following criteria have been identified as essential for linking ARGs in the environment to their sources:

- *An idealized and well-characterized environmental system* is required to assist in accounting for the inherent heterogeneity in background antibiotic resistance patterns.
- *Suitable tracer ARGs* are needed that are capable of distinguishing different natural or anthropogenic sources in the watershed and that are sufficiently abundant to avoid methodological difficulties.
- *Multiple lines of evidence* are also ideal given the empirical nature of the approach and the numerous factors outlined in the previous sections that can lead to the presence and persistence of ARGs in a given environmental reservoir.

Ideally, key patterns of ARG occurrence and persistence may then be applied to explore hypotheses related to the fundamental phenomena driving their proliferation in various environments. This information may then provide direct guidance to the improvement of mechanistic models, and vice versa.

10.4.1 Poudre River as a Model System for Tracking Antibiotic Resistance in the Environment

As noted by Singer et al. (2006), linking the causes with actual resistance patterns requires incorporation of suitable controls as well as examination over appropriate

spatial and temporal scales. The South Platte River Basin, and in particular the Cache La Poudre (Poudre) River watershed, in northern Colorado possess many such ideal qualities for quantifying the effects of human impacts on antibiotic resistance patterns observed in surface water (Yang and Carlson, 2003; Pruden et al., 2006; Pei et al., 2006).

Perhaps the greatest virtue of the Poudre River as a model system is its pristine origin, which arises from snow melt emanating from the Rocky Mountains. The river travels about 82 km downstream from the most upstream monitoring point and through the mouth of the Poudre Canyon before encountering a lightly impacted zone just upstream from the city of Fort Collins. As it passes through Fort Collins, which has a population of approximately 140,000, there is one wastewater treatment plant with a discharge flow of 6 million gallons (23,000 m^3) per day (6 MGD) that directly discharges to the river, as required by Colorado Water Law, and a second 20-MGD WWTP that irrigates in the vicinity. At the point before its confluence with the South Platte River at Greeley, 43 MGD of WWTP effluent and 1461 acres (591 ha) of animal feeding operations (AFOs) have been identified upgradient in the watershed (Storteboom et al., 2010b). Thus, the Poudre River offers a gradient of anthropogenic influence, with a pristine origin available for contrast.

In more recent studies, sampling has been expanded to include the South Platte River, of which the Poudre River is a tributary. In contrast to the Poudre River, the South Platte River is heavily impacted, receiving major influences from wastewater discharges as it flows through the Denver metropolitan area. During low flow, the wastewater effluent component of the river can approach 90%. As the South Platte River flows northeast away from Denver, the land use of the watershed becomes dominated by concentrated AFOs and cropland.

Figure 10.1 provides an overview of the land use and the distribution of WWTPs and AFOs in the South Platte River Basin, including the South Platte and Poudre rivers. The sampling points that have been monitored in past studies are also indicated in Figure 10.1.

10.4.2 Antibiotics and ARGs in the Poudre River

Also an immense asset of the Poudre River as a model system is that it has been well characterized in terms of occurrence of antibiotics (Kim and Carlson, 2006, 2007; Yang and Carlson, 2003). In general, the antibiotic analyses of the Poudre River sediments and water have supported the classification of site 1 as pristine, site 2 as light agriculture influenced, site 3b as predominantly urban influenced, and sites 4 and 5 as heavily agricultural/mixed-urban influenced. Of the various tetracycline, β-lactam, macrolide, sulfonamide, and ionophore antibiotics that have been tested, none have ever been detected in the pristine region. The remaining sites are generally characterized by increasing concentration and frequency of detection as the river flows downstream. Notably, sulfamethoxazole, which is only used in humans, has primarily been detected at site 3 (up to 0.32 μg/L in the water), immediately downstream from the Fort Collins WWTPs (Kim and Carlson, 2007; Yang and Carlson, 2003). By contrast, ionophores, which are only used in agriculture, greatly increase in detection frequency and concentration at sites 4 and 5 (ranging from about 5 to 30 μg/kg sediment) (Kim and Carlson, 2006). Similarly, chlortetracycline, which is also only used in agriculture, was found to be greatest in concentration at

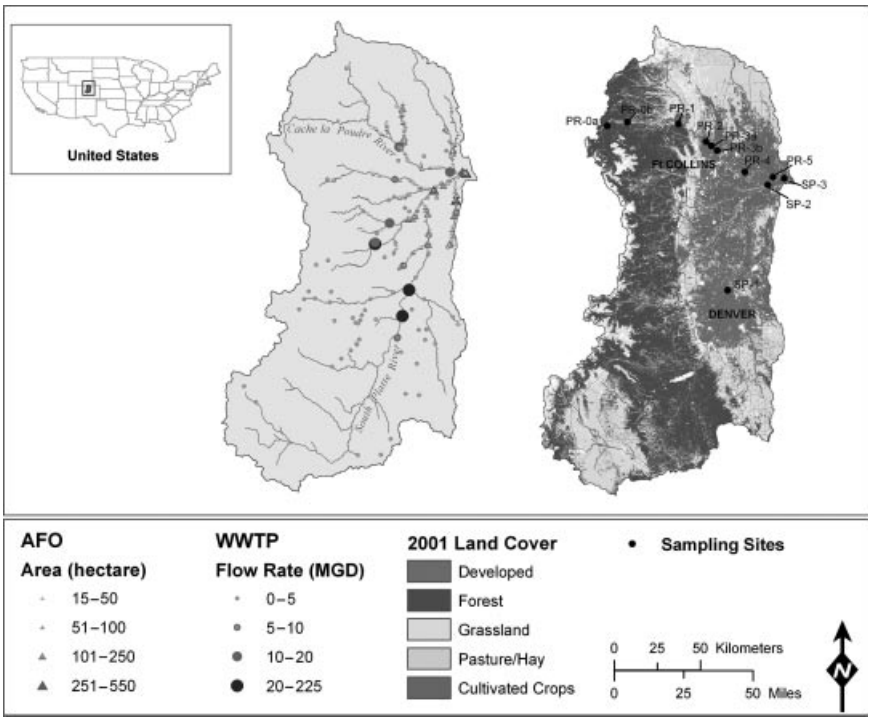

FIGURE 10.1 Land cover classification of South Platte River Watershed, including the South Platte River, the Cache La Poudre River, and their confluence. Sampling sites are indicated in the left panel and wastewater treatment plants (WWTPs) and animal feeding operations (AFOs) are indicated in the right panel. *(See color insert.)*

site 4 (up to 30.8 μg/kg sediment) (Kim and Carlson, 2007). Notably, all aqueous antibiotic concentrations detected to date are well below known MIC thresholds (Andrews, 2001).

An initial survey of tetracycline (*tet*O, *tet*W) and sulfonamide (*sul*I and *sul*II) ARGs along the Poudre River by qPCR revealed a striking pattern (Pruden et al., 2006; Pei et al., 2007). While three of the four ARGs were detected sporadically and at low concentrations at the pristine PR-1 over the five sampling dates, the detection frequency and the overall concentrations of all four ARGs increased dramatically downstream (Pruden et al., 2006). For example, ~2–3 orders of magnitude difference in *tet*W concentration was observed between PR-1 and PR-3 or PR-4 within each individual sampling event (Fig.10.2). These observations clearly demonstrated an impact of human activities on the levels of ARGs in the river sediments. However, the immediate follow-up question could not be answered based on this study: From where did the ARGs come?

10.4.3 Molecular Signatures as Tracers of Sources of Antibiotic Resistance Genes

Antibiotics generally have known use patterns in humans and livestock, which facilitate the determination of their sources when they are found in the environment. However, the distinction between human and animal sources of ARGs is not as

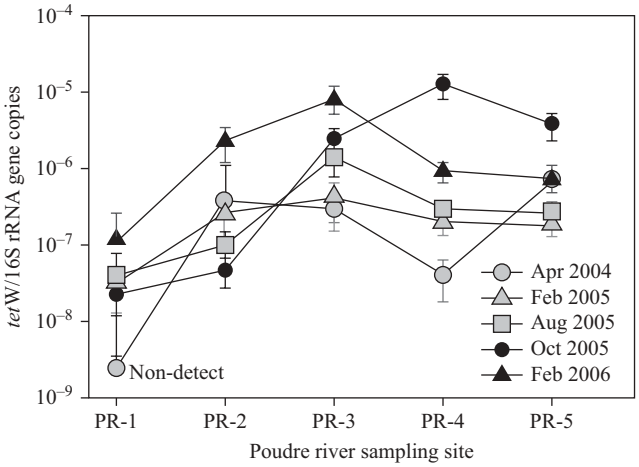

FIGURE 10.2 Average *tet*W measured by qPCR at five Poudre River sampling sites (Fig. 10.1) over five sampling events. Values are normalized to 16S ribosomal ribonucleic acid (rRNA) gene copies to account for variation in total bacterial biomass at various sampling sites.

precise. As noted above, clinical and pristine ARGs can generally be discerned by phylogenetic analysis (Aminov and Mackie, 2007). Thus, one strategy may be to survey ARGs known to be relatively abundant in the clinical versus agricultural realms. Particularly notorious ARGs that threaten human health include *van*A, which imparts resistance to vancomycin (a last-resort antibiotic), and *mec*A, which encodes methicillin resistance, a characteristic feature of methicillin-resistant *Staphylococcus aureus* (MRSA). By contrast, florfenicol is an antibiotic that has solely been approved for use in livestock, both the United States and the European Union, thus corresponding ARG, such as *flo*R or *fex*A, are of interest as markers of agricultural influence on antibiotic resistance patterns (Schwarz et al., 2004). However, attempts to detect *van*A, *mec*A, or *flo*R by qPCR or traditional PCR within the South Platte River Basin were unsuccessful. This is likely because these ARGs are not present in sufficient abundance in the environment, or the available primers are not sufficiently broad to capture the diversity present within environmental reservoirs. Similarly, only limited detection of *van*A (Böckelmann et al., 2009; Volkmann et al., 2004), *mec*A (Böckelmann et al., 2009; Volkmann et al., 2004; Schwartz et al., 2003), and *flo*R (Lang et al., 2010) has been reported in environmental matrices.

10.4.3.1 Why Tetracycline and Sulfonamide ARGs? By contrast, sulfonamide and tetracycline ARGs are relatively abundant in human-impacted environments and have been the focus of numerous studies documenting WWTP and livestock impacts to the soil, water, and sediment environment (Heuer et al., 2009; Heuer and Smalla, 2007; Koike et al., 2007; Chee-Sanford et al., 2001; Auerbach et al., 2007; Pruden et al., 2006; Pei et al., 2006). Tetracycline ARGs provide an advantage in that they are diverse, yet well characterized, with 11 efflux (Aminov et al., 2002) and 8 ribosomal protection factor (Aminov et al., 2001) variants described to date. The *tet*X gene, which encodes enzymatic degradation of tetracyclines, has also been described (Speer and Salyers, 1989). Given the noted complexities governing the behavior of ARGs in

TABLE 10.3 Why tet and sul ARGs?

- Widespread in the environment, which simplifies their detection.
- Well characterized in the literature, which facilitates methodology and comparative studies.
- Harbor sufficient diversity for phylogenetic comparison.
- *tet* and *sul* ARGs together represent diverse resistance mechanisms.
- *tet* and *sul* ARGs often display distinct responses to stimuli.
- Diminished utility of corresponding antibiotics in human medicine: May provide insight into means to prevent spread of more critical ARGs.

the environment, it is wise to examine more than one class of ARGs, and sulfonamide ARGs provide a highly suitable complement to the information obtained from tetracycline ARGs. Sulfonamides target dihydropteroate synthase (DHPS) in the folic acid pathway and corresponding ARGs encode variant DHPS enzymes (Sköld, 2000). Sulfonamide antibiotics represent one of the few classes of truly synthetic antibiotics, thus their occurrence is more likely to be associated with human impacts. Also, the *sul*I ARG has been observed to be physically linked to class I integrons in some cases (Antunes et al., 2005) and thus may provide an indicator of horizontal gene transfer potential. Furthermore, *tet* and *sul* ARG have been observed to exhibit opposite responses to environmental stimuli, such as biological treatment, temperature, and redox state, and *sul* ARGs appear to be particularly recalcitrant (Luo et al., 2010; McKinney et al., 2010; Pei et al., 2007). Though tetracyclines and sulfonamides have largely lost their utility in human medicine due to widespread resistance, this same feature makes their corresponding ARGs attractive models for characterizing the behavior of resistance elements in the environment. Furthermore, understanding how tetracycline and sulfonamide ARGs respond and move through the environment may help to predict how resistance to future antibiotics that are of human health value will behave, if left unchecked. The various advantages of targeting *tet* and *sul* ARGs for delineating human impacts to the environment are summarized in Table 10.3.

10.4.3.2 Development of the ARG Molecular Signatures Approach Of course, the disadvantage of *tet* and *sul* ARGs as tracers of anthropogenic influence is that, until recently, unique signatures linking them to their sources were not available. This feat was recently achieved by Storteboom and colleagues through their extensive characterization of the South Platte River Basin and the development of the "molecular signatures" approach to tracking the sources of ARGs (Storteboom et al., 2010a) (Table 10.4). The molecular signature consists of two components: (1) the frequency of detection (FOD) of *tet* and *sul* ARGs and (2) phylogenetic/phylotypic analysis of the *tet*W ARG.

To identify FOD patterns characteristic of putative sources, the FOD of 11 tetracycline and 2 sulfonamide ARGs was investigated by PCR in several AFOs and WWTPs located in the watershed, along with 3 sites located in the pristine upstream portion of the Poudre River (sites PR-0a, PR-0b, and PR-1) (Storteboom et al., 2010a). Using correspondence analyses, it was found that *tet*C, *tet*E, and *tet*O (group *tet* CEO) were loaded in the direction of the WWTPs, while *tet*H, *tet*Q, *tet*S, and *tet*T (group *tet* HQST) were loaded in the direction of the AFOs investigated.

TABLE 10.4 The ARG Molecular Signature Approach

- Signature consists of two main components:
  - Frequency of detection of *tet* and *sul* ARGs
  - Analysis of *tet*W (ubiquitous ARG)
  - *tet*W RFLP
  - *tet*W sequencing
- Analysis of components:
  - Correspondence analysis to determine which ARGs and *tet*W RFLP patterns are associated with pristine, WWTP, or AFO sources
  - Discriminant analysis applied to patterns to classify impacted river sites relative to putative sources
  - Phylogenetic analysis of *tet*W sequences and determination of significant differences between impacted river sites and putative sources

Discriminant analysis applied to *tet* CEO versus *tet* HQST resulted in unambiguous separation of the WWTPs, the AFOs, and the pristine sampling environments. Interestingly, the *sul*I was detected in 100% of sources and only in 4% of pristine samples, which is consistent with this ARG corresponding to a synthetic antibiotic of human origin and with the general recalcitrance of this ARG.

Notably, *tet*W was the most frequently detected ARG at the pristine sites (33% detection rate) and was also detected in 96%–100% of AFO and WWTP samples. This made *tet*W a prime candidate for comparison by phylogenetic analysis, as this would not be possible for an ARG that is absent at the pristine sites. Similarly, *tet*W phylogenetics has been described as a useful tool for tracking the sources of ARGs in groundwater adjacent to livestock lagoons (Koike et al., 2007). As predicted, phylogenetics of the *tet*W ARG provided clear branching between WWTP, AFO, and pristine sampling environments (Storteboom et al., 2010a). As a secondary method of characterizing the *tet*W ARGs, cloning and *restriction fragment length polymorphism (RFLP)* analysis was performed to distinguish the phylotypes. Four dominant *tet*W RFLP patterns emerged, which were designated as c1, c2, c3, and c4. Correspondence analysis was performed on the RFLP patterns, and it was found that pattern c1 was loaded in the direction of the AFOs, while c2 was loaded in the direction of the WWTPs. Discriminant analysis applied to pattern c1 versus c2 was found to effectively classify the AFO and WWTP environments. This is encouraging because RFLP analysis is less costly than DNA sequencing and thus may make the approach more accessible.

10.5 TRACKING ARGs IN THE POUDRE RIVER AND THE SOUTH PLATTE RIVER BASIN

10.5.1 Applying the Molecular Signatures Approach to Reveal the Sources of ARGs

The molecular signatures approach defined by Storteboom and colleagues (2010a) (Table 10.4) was applied to track the sources of ARGs in the South Plate River Basin

(Storteboom et al., 2010b). River water and bed sediment samples were collected at the eight Poudre River and three South Platte River sampling sites (Fig. 10.1) four times over a one-year period, including both low-flow and high-flow events. This comprehensive sampling approach was intended to capture seasonal and sampling matrix variability. Because the total FODs vary among different sample matrices (e.g., the total number of ARG detections in river sediments will be lower than in AFO lagoons), the FODs of the *tet* CEO and *tet* HQST ARGs were normalized to the total FOD for each sample type. This approach made it possible to compare the likely sources of ARGs to the river sites on a more even playing field and not have the result dominated by the total FOD.

Application of the ARG molecular signatures approach to the river sediment and water samples yielded a very interesting result: All of the impacted river sites were classified by all lines of evidence employed as predominantly WWTP influenced, except for SP-3 (Storteboom et al., 2010b) (see Fig. 10.1). The fact that SP-1, which is immediately downstream of a major WWTP, was most strongly classified as WWTP influenced provided a good indication of the validity of the method. Development of a *Geographical Information Systems (GIS)* database of known locations of WWTPs and AFOs in the watershed aided in validating the molecular signatures approach and also provided insight into the relative contribution of WWTP loading versus upgradient AFO acreage resulting in a site classification as WWTP versus AFO influenced. SP-3 was perhaps the most difficult to classify based on land use alone, as it is just downstream from the confluence of both rivers and thus receives heavy influence both from WWTPs (455 MGD) and AFOs (6553 acres). For this reason it is not surprising that SP-3 was classified as AFO influenced by the FOD method, and as not significantly different from either source based on Unifrac significance testing of the *tet*W phylogenetic trees. Also of interest is that PR-2 shifted the closest to the pristine portion of the river when normalized to *sul*I, the ARG that appeared to be the most indicative of human-derived sources. In addition to the high level of consistency observed between the three pristine sampling sites spanning over 54 km, their similarity to the least impacted PR-2 (according the GIS analysis) 83 km downstream instills confidence that the native ARGs would have maintained relative consistency in the background along the length of the Poudre in the absence of anthropogenic inputs.

10.5.2 Sources and Processes Giving Rise to Elevated ARGs in Human-Impacted Environments

Beyond identification of sources of ARGs, the processes leading to elevated resistance in anthropogenically influenced rivers are of fundamental interest. At two extremes lie the phenomena of selection versus transport as the main hypothetical drivers of ARG proliferation (Fig. 10.3).

In the case of selection, the antibiotics known to be present as well as heavy metals and other undefined human-derived stressors impose selection pressure on native antibiotic-resistant river bacteria, which then proliferate along with their ARGs, relative to susceptible bacteria. In this case, the "source" of ARGs is the natural reservoir, which, as noted earlier in the chapter and elsewhere in this text, have been revealed by recent research to be more extensive than previously thought. If selection was indeed the dominant process in the Poudre River, then it would have been

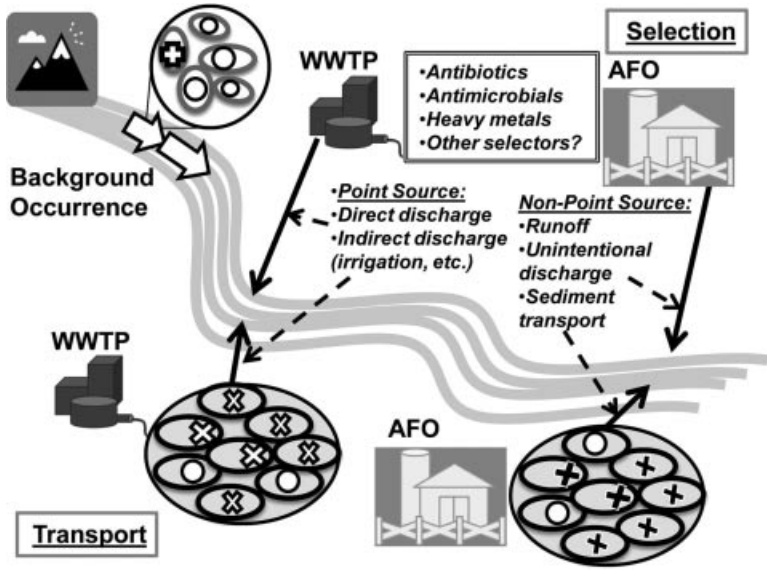

FIGURE 10.3 Illustration of the complementary phenomena of selection versus transport as the dominant processes driving amplified levels of ARGs in anthropogenically influenced riverine environments.

expected that the impacted river sites would have aligned closely with the pristine sites. However, neither the FOD nor the *tet*W phylogenetic component of the molecular signature aligned impacted river sites with the pristine sites. Thus, for this particular watershed, molecular evidence did not support selection as the dominant process driving the amplified ARG levels observed at the impacted sites.

In the case of transport, ARGs emanating from sources are carried to the river via physical processes. The similarity of the ARG molecular signatures of the impacted Poudre River and South Platte River sites with those of the putative source environments (AFOs and WWTPs) supports the hypothesis of transport as the dominant process giving rise to ARG elevation at the impacted river sites (Storteboom et al., 2010b). Further evidence that transport dominated over selection was that ARG FODs were actually found to be higher in the water than in the sediment (Storteboom et al., 2010b). If selection were the dominant process, then higher ARG FODs would have been anticipated in the sediment, given that bacterial and antibiotic concentrations (with the exception of sulfonamides) are higher in the sediment (Yang and Carlson, 2003; Kim and Carlson, 2007). Furthermore, Pei et al. (2006) failed to identify a positive correlation between antibiotic and ARG concentrations in Poudre River sediments. While the majority of the impacted river sites aligned most closely with WWTPs, the relative contributions of WWTPs and AFOs appear similar in the case of the most heavily impacted site, SP-3. Others have also speculated that physical transport (referred to as "transmission") of antibiotic-resistant bacteria and ARGs to impacted sites is likely a critical phenomenon driving elevated resistance in impacted environments (Singer et al., 2006).

10.5.3 General Insights into ARG Proliferation and Transport at the Watershed Scale

Application of the ARG molecular signature approach yielded new insight into the fundamental processes driving ARG proliferation in impacted riverine environments. Transport, rather than selection, appears to be the dominant process driving ARG proliferation in the South Platte River Basin. More specifically, WWTPs appear to be a major source of ARGs. The next question arises: Is transport universally the dominant process driving ARG proliferation in watersheds? The answer to this question requires further investigation. The South Platte River Basin is characterized by semiarid conditions, which may be less susceptible to runoff throughout many parts of the year. Would wet weather watershed runoff from AFOs have played a stronger role? Furthermore, selection may be more prevalent in extremely impacted rivers and streams, such as those under study in India (Fick et al., 2009) and China (Luo et al., 2010; Li et al., 2010). Thus, an important next step is to apply the overall ARG molecular signatures approach to other watersheds. While the precise ARG signatures may shift (e.g., which group of *tet* ARGs are associated with which sources), using the approach described, effective source signatures may be identified that are suitable to each watershed. The fact that *tet*W RFLP analysis yielded the same result as sequencing and phylogenetic analysis significantly reduces the cost and expertise required, making the method accessible to any lab capable of basic DNA extraction, PCR, restriction digest, and gel electrophoresis.

10.5.3.1 Point versus Nonpoint Transport Processes The results of the application of the ARG molecular signatures approach may provide further benefit by guiding future research efforts focused on key processes contributing to elevated antibiotic resistance levels in human-impacted environments. The fact that WWTPs have been implicated as important sources indicates that there is a need to measure ARG loading to the environment from WWTPs or, in other words, the mass flow rate of ARGs entering the "control volume" of interest. WWTPs typically directly or indirectly (via irrigation) discharge to rivers, thus this measurement is relatively straightforward. The concentrations of several ARGs measured by qPCR have been reported in recent studies. Auerbach et al. (2007) examined two different midwestern WWTPs and found that *tet*Q and *tet*G concentrations ranged from $\sim 1\times10^4$ to $\sim 1\times10^6$ gene copies per milliliter. Similarly, Böckelmann et al. (2009) investigated three different European WWTP effluents prior to artificial groundwater recharge and found that *tet*O was relatively widespread and ranged from $\sim 8.7\times10^2$ to 1.0×10^5 gene copies per milliliter. Further, up to 1.9×10^3 *erm*B, 5.7×10^2 *mec*A, 1.1×10^2 *bla*SHV-5, and 6.9×10^1 *amp*C gene copies per milliliter were detected in some WWTPs. *van*A was not detected in any of the WWTP effluents. Additional studies incorporating molecular tools and thus accessing the uncultured fraction of ARGs across a variety of WWTPs incorporating different treatment processes would be of value in order to comprehensively assess WWTP loading of ARGs to the environment.

In contrast to WWTPs, AFO-derived ARGs must make their way to the river via non-point-source processes, such as runoff or overland flow. Non-point-source pollution is much more challenging to characterize than point-source pollution,

but a significant body of literature exists on sediment transport that could help establish basic principles of ARG transport in the watershed (Davis and Fox, 2009). Of particular interest is the association of intracellular and extracellular ARGs with soil and sediment particles of various size fractions and characters and the subsequent mobility of such particles carrying ARGs. The fraction that is not bound to settleable material would then be assumed to be transported freely via overland flow, and loadings could be assessed in a more direct manner, as suggested for WWTPs. Interestingly, recent research has revealed that the water chemistry, in particular the presence of divalent cations, can enhance the binding of DNA to soil particles and potentially enhance their persistence (Lu et al., 2010). Furthermore, the conformation of the DNA and the fact that it was bound were not impediments to the transformation of recipient *Azotobacter vinelandii* cells. Thus, there is much to learn about the interactions both of antibiotic-resistant bacteria and ARGs with soil and sediment particles and the subsequent effects on fate, transport, horizontal gene transfer, and persistence.

10.5.3.2 Persistence Mechanisms Once transported to the river, various processes are of interest that may lead to long-term persistence of the ARGs. In this sense, it is important to note that selection and transport should not necessarily be considered as a dichotomy. The molecular signatures approach will provide an indication from where the dominant ARG forms originated, but it is not clear how long ARGs persist once relocated. Thus, the ARGs detected in the Poudre River study could well have established themselves in the river sediment many years prior to investigation. For this to be the case, there would likely be some form of selective advantage, or at least a lack of a disadvantage, once the ARGs are established in the river sediments. This is an interesting thought considering that the premise of the fecal indicator approach is that source bacteria possess poor survival rates relative to native bacteria, though numerous studies have demonstrated that they do, in fact, survive and replicate in the environment (Field and Samadpour, 2007). Processes of interest that could potentially support persistence of ARGs in river sediments include sorption and persistence of extracellular DNA, horizontal gene transfer to native river bacteria, selection of ARGs below the MIC thresholds, selection of ARGs by other nonantibiotic pollutants, and overall persistence in the absence of selection pressure.

Of the above-noted phenomena potentially driving ARG persistence in environmental reservoirs, the relationship between the presence of antibiotics and other selective agents with antibiotic resistance is at the forefront of consideration. To this end, Singer and colleagues (2006) have proposed an elegant landscape ecology-based approach for linking the presence of antibiotics and other selective agents to elevated antibiotic resistance levels in the environment. It would be of great interest to see the approach demonstrated at full scale. In lieu of more comprehensive examination, simple correlations between antibiotics and ARGs have been explored in several studies. For example, in a survey of eight lagoons at five cattle feedlots it was found that several tetracycline ARG (*tet*M, *tet*O, *tet*Q, *tet*W, *tet*B, *tet*L) were significantly higher in the lagoons corresponding to high antibiotic use relative to low and no antibiotic use (Peak et al., 2007). However, only a weak correlation was found between tetracycline and *tet* ARGs. Similarly, a comprehensive examination of ARG occurrence across lagoons of several livestock types (beef cattle, organic and conventional dairies, swine, and chicken) did reveal several significant correlations

between certain antibiotics and some tetracycline and sulfonamide ARGs, but the correlation coefficients were generally low and the correspondence between ARGs and antibiotics of the same class was poor (McKinney et al., 2010). Both studies suggested that the bulk of selection for antibiotic resistance likely takes place upstream in the animal gut and that subsequently antibiotics and ARGs are likely governed by different transport processes. Thus, correlations identified in receiving environments may often be incidental and not necessarily an indicator of selection taking place in the environment from which the sample was collected. The problem of co-transport of antibiotics and ARGs, as well as cross selection of ARGs by antibiotics or agents of different classes, was also noted by Singer and colleagues (2006). Interestingly, some of the strongest correlations observed in the McKinney study were between antibiotics and ARGs of different classes, for example, total sulfonamides and total *tet* ARGs ($R^2 = 0.77$) and lincomycin and total *sul* ARGs ($R^2 = 0.75$). Significant positive correlations were also identified between some metals and ARGs, but the R^2 values were generally lower, with the exception of Pb and *tet* ARG ($R^2 = 0.75$). Nevertheless, though antibiotics and other selective agents may not be exerting strong selective pressure, they could still be playing a role in stimulating bacteria at low levels (Davies et al., 2006) and thus contributing to long-term persistence.

Horizontal gene transfer is also of prime interest as a mechanism driving persistence. Though not examined previously in river sediments, manure amendment to soil provides a reasonable analogy. Similarly to exogenous bacteria transported to a river, manure bacteria do not survive well in soil. Nonetheless, Heuer and Smalla (2007) observed persistence of *sul*II ARGs in at least one soil following amendment with piggery manure, a phenomenon that was linked with a novel class of self-transferable plasmids carrying the *sul*II ARGs. Ghosh and LaPara (2007) similarly provided evidence that horizontal gene transfer played a role in persistence of tetracycline ARGs in a heavily manured soil. Correspondingly, a diverse array of broad host range plasmids, integrons, and gene cassettes encoding multiple antibiotic resistance have been found in piggery manure (Binh et al., 2008) and also in wastewater bacterial communities (Moura et al., 2009). This suggests that horizontal gene transfer may be a widespread driver of persistence of ARGs, serving to bridge the gap between environments and allowing ARGs to establish where their original host cells would not have fared so well.

10.6 MITIGATION IMPLICATIONS AND APPROACHES

Antibiotic resistance has been steadily increasing over several decades, representing one of the greatest human health challenges of our time. While diligent effort has been put forth in the clinical realm, there are still no clear solutions in sight. Prudent use of antibiotics is certainly critical to combating resistance, but it is becoming increasingly clear that a more comprehensive strategy is urgently needed. The traditional conceptual model of selection of resistance in the presence of the antibiotic and attenuation in the absence of the antibiotic is false in many instances, thus calling for new ways of thinking about the problem. For example, as we have seen in this chapter, sulfonamide resistance remains widespread to this day, in spite of the fact that sulfonamides have been largely phased out of use in human medicine

(Sköld, 2000). This does not bode well for reduction in antibiotic use as the sole strategy for combating antibiotic resistance. Thus, alternative strategies are urgently needed to slow the spread of anthropogenic and clinically relevant ARGs. In particular, a framework for quantifying the contribution of environmental reservoirs as sources and sinks for ARGs and associated genetic elements critical to human health would be of great value.

The mass balance concept central to the discipline of environmental engineering provides a central framework for accounting for the sources and processes contributing to ARG proliferation in human-impacted environments. Combined with the information obtained from the ARG molecular signatures approach, significant insight can be gained into the sources and processes at play in driving ARG fate and transport in the watershed. In this manner, "hot spots" for effective mitigation of ARGs may be identified. It should be noted that the intention is not to eliminate all ARGs everywhere, given the long evolutionary history of ARGs and the complex ecological role that they play in nature as this would be impossible. Rather, the focus is on slowing the spread of anthropogenic, clinically relevant pathogenic ARGs of concern to human health. As noted, tetracycline and sulfonamide ARGs have largely lost their value to human health, however, they serve as ideal models of study to guide future efforts to slow the spread of resistance to current and future antibiotics.

Assuming that transport holds true as the dominant ARG proliferation process across human-impacted watersheds, this has extremely poignant management implications. This suggests that efforts to contain antibiotic resistance will be most fruitful if aimed more toward processes that limit the mass loading of intracellular and extracellular DNA to receiving environments. To some degree, this shifts attention away from attempts to eliminate antibiotic contamination. However, it is believed that such efforts should not be abandoned as antibiotics are likely still an important factor in horizontal gene transfer and maintenance of ARGs upon establishing in environmental reservoirs.

In the characterization of the South Platte River Basin, application of the ARG molecular signatures approach indicated that WWTPs, and in one instance AFOs, were major sources of ARGs to the river water and sediment. The bright side of this finding is that WWTPs and AFOs may thus serve as critical nodes for minimizing the dissemination of ARGs. Relatively speaking, ARG release from WWTPs may be relatively straightforward to address, whereas AFOs will be more challenging because of the non-point-source nature of the source. The following sections provide a brief overview of potential biological and physical/chemical treatment options that may be applied on farms or in WWTPs for effective reduction in ARG loading to the environment.

10.6.1 Biological Treatment of ARGs: WWTP and on Farms

The abundance of antibiotic-resistant bacteria in WWTPs has been well documented. Of particular concern is that the conditions within the WWTP, particularly the high bacterial density combined with the stress of antibiotics and other anthropogenic agents, may act to select antibiotic-resistant bacteria and stimulate horizontal gene transfer (Bell et al., 1983; Dröge et al., 2000). Studies of resistance elements, including integrons and broad host range plasmids, indicate that they are abundant in wastewater bacterial communities (Moura et al., 2009). Thus, WWTPs themselves

may serve to amplify and disseminate ARGs. On the other hand, WWTPs may assist in reducing ARG concentrations. Possible mechanisms include biodegradation of the DNA encoding resistance, shift in environmental conditions relative to the human gut, and controlled microbial ecology within the WWTP.

Culture-based studies of the effects of wastewater treatment on antibiotic-resistant bacteria have yielded mixed results. Zhang et al. (2009) recently reported a selective increase in resistance of *Acinetobacter* spp. across eight antibiotics as well as an increase in multidrug resistance. A selective increase in ciprofloxacin-resistant enterococci has also been reported (da Silva et al., 2006). On the other hand, a decrease in multiantibiotic-resistant enterococci, by up to 4 log, has been observed (Garcia et al., 2007; Da Costa et al., 2006). Others have observed no measurable selective reduction in resistant *E. coli* (Lefkowitz and Duran, 2009), fecal coliforms, or enterococci (Vilanova et al., 2004). Importantly, none of these studies reported complete elimination of antibiotic-resistant bacteria. In one study, methicillin-resistant *S. aureus* were found in several influent wastewater and activated sludge samples but not in final effluent (Börjesson et al., 2010a). In another study, vancomycin- and erythromycin-resistant enterococci were observed to persist (Vilanova et al., 2004) and, even in cases where selective decreases were noted, resistant enterococci were present in the effluent on the order of 4.4×10^5 colony – forming units (CFU)/100 mL. Correspondingly, examination of the receiving waters has indicated increases in resistant *Acinetobacter* downstream from discharge (Zhang et al., 2009).

Relatively few culture-independent examinations of the effect of wastewater treatment on ARGs have been reported. In one study of two conventional activated sludge WWTPs in the midwestern United States, it was found that *tet*G and *tet*Q were reduced on a per volume basis but not when normalized to the DNA mass extracted. This indicates that the net reductions observed in ARGs were a result of bacterial reduction and not selective reduction of ARGs (Auerbach et al., 2007). Further, wastewater treatment did not appear to reduce the FODs of 10 *tet* ARGs investigated (except one sampling date). Börjesson et al. (2010b) similarly reported a reduction of *tet*A and *tet*B abundance during wastewater treatment likely as a result of overall bacterial reductions. One encouraging finding is that thermophillic anaerobic digestion might provide enhanced reduction of tetracycline ARGs (*tet*A, *tet*O, and *tet*X), relative to conventional mesophillic digestion (Ghosh et al., 2009).

Animal manure is well known to be enriched in ARGs and may be subjected to any combination of management practices, including lagoons, composting, or land application. McKinney et al. (2010) recently explored the potential of livestock lagoons for removal of ARGs by examining the treatment train of a cross section of livestock types, including conventional and organic dairies, swine, poultry, and beef cattle. An important finding was that, while a modest reduction of *tet*O and *tet*W ARGs was achieved in more extensive lagoon systems, a corresponding increase in *sul*I and *sul*II ARGs was observed. The same phenomenon was observed in lab-scale incubations of dairy lagoon water under varying aerobic/anaerobic and temperature conditions (Pei et al., 2007). This underscores a critical principle: There may not exist a "one size fits all" approach to biological treatment of all ARGs.

Thus, a general conclusion is that while biological treatment holds some potential, it may be too variable, and possibly even unrealistic, to control the microbial ecology in such a fashion to effectively eliminate ARGs.

10.6.2 Physical and Chemical Treatments of ARGs

Physical and/or chemical treatments of ARGs may present a more effective option, given the lack of reliability of biological treatment. However, relatively few such studies have been carried out. Perhaps the most promising is membrane treatment. For example, an examination of the ARGs present in the effluents produced by three European reclaimed water plants revealed that nearly all ARGs examined (*erm*B, *mec*A, *van*A, *bla*SHV-5, *amp*C, and *tet*O) were eliminated by reverse osmosis (Böckelmann et al., 2009). However, it was found that ultraviolet (UV) treatment and chlorination presented only a partial barrier to ARGs. A similar result was reported in another study examining the effect of seasonal UV treatment on *tet*A and *tet*G ARGs (Auerbach et al., 2007).

Similarly, structural conservation practices that are commonly implemented to abate fluxes of sediments, nutrients, and pesticides from diffuse sources could provide secondary benefits for ARG control. Farm practices for manure storage, tillage and residue management, wetlands, ponds, and lagoons are among these practices. For example, vegetative buffer strips have been shown to effectively reduce pathogens at the field scale between 60 and 90% (Coyne et al., 1998; Entry et al., 2000a, 2000b; Parajuli et al., 2008).

Though generally more costly than biological treatment, physical and chemical treatments are becoming more of a reality for wastewater treatment for other purposes, for example, to address pharmaceuticals and personal care products. Furthermore, recent studies have revealed the intrusion of ARGs into drinking water systems (Xi et al., 2009; Schwartz et al., 2003). Physical/chemical treatment processes are the principle form of drinking water treatment and thus could be optimized to remove or destroy ARGs. Treatment of ARGs in drinking water would represent the final barrier before they potentially go full cycle, back to be incorporated into the human biota. Thus, more detailed examination of the effect of physical and chemical treatment processes on ARGs would be of value.

10.7 SUMMARY AND FUTURE DIRECTIONS

In this chapter the concept of ARGs as contaminants and a basic framework for understanding their fate and transport in the environment has been put forth. The ARG molecular signatures approach represents a promising means not only to identify sources of ARGs but also to expose the key processes driving their proliferation. This can help to identify critical nodes for controlling the spread of anthropogenic sources of antibiotic resistance. Considering the vast diversity of resistance elements being revealed in various reservoirs, including the human gut (Sommer et al., 2009), future studies may benefit from advances in molecular techniques, such as metagenomic sequencing. This can assist in accessing the depth of diversity associated with resistance elements in the natural environment and may provide more precise delineation of anthropogenic sources versus the background resistance characteristics. Further studies into advanced physical/chemical destruction of ARGs are also recommended.

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REFERENCES

Aarestrup FM, Seyfarth AM, Emborg HD, Pedersen K, Hendriksen RS, Bager F (2001). Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark. *Antimicrob Agents Chemother* 45(7):2054–2059.

Aendekerk S, Diggle SP, Song Z, Hoiby N, Cornelis P, Williams P, Camara M (2005). The MexGHI-OpmD multidrug efflux pump controls growth antibiotic susceptibility and virulence in *Pseudomonas aeruginosa* via 4-quinolone-dependent cell-to-cell communication. *Microbiology* 151:1113–1125.

Agnelli A, Ascher J, Corti G, Ceccherini MT, Nannipieri P, Pietramellara G (2004). Distribution of microbial communities in a forest soil profile investigated by microbial biomass, soil respiration and DGGE of total and extracellular DNA. *Soil Biol Biochem* 36:859–868.

Allen HK, Moe LA, Rodbumrer J, Gaarder A, Handelsman J (2009). Functional metagenomics reveals diverse beta-lactamases in a remote Alaskan soil. *ISME J* 3:243–251.

Allen HK, Donato J, Huimi Wang H, Cloud-Hansen KA, Davies J, Handelsman J (2010). Call of the wild: Antibiotic resistance genes in natural environments. *Nat Rev* 8:251–259.

Alonso A, Sánchez P, Martínez JL (2001). Environmental selection of antibiotic resistance genes. *Environ Microbiol* 3(1):1–9.

Aminov RI (2009). The role of antibiotics and antibiotic resistance in nature. *Environ Microbiol* 11(12):2970–2988.

Aminov RI, Mackie RI (2007). Evolution and ecology of antibiotic resistance genes. *FEMS Microbiol Lett* 271:147–161.

Aminov RI, Garrigues-Jeanjean N, Mackie RI (2001). Molecular ecology of tetracycline resistance: Development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. *Appl Environ Microbiol* 67(1):22–32.

Aminov RI, Chee-Sanford JC, Garrigues N, Teferedegne B, Krapac IJ, White BA, Mackie RI (2002). Development validation and application of PCR primers for detection of tetracycline efflux genes of gram-negative bacteria. *Appl Environ Microbiol* 68(4):1786–1793.

Andersson DI, Levin BR (1999). The biological cost of antibiotic resistance. *Curr Opin Microbiol* 2(5):489–493.

Andrews JM (2001). Determination of minimum inhibitory concentrations. *J Antimicrob Chemother* 48:5–16.

Antunes P, Machado J, Sousa JC, Peixe L (2005). Dissemination of sulfonamide resistance genes (sul1, sul2, and sul3) in Portuguese *Salmonella enterica* strains and relation with integrons. *Antimicrob Agents Chemother* 49(2):836–839.

Auerbach EA, Seyfried EE, McMahon KD (2007). Tetracycline resistance genes in activated sludge wastewater treatment plants. *Water Res* 41:1143–1151.

Baker-Austin C, Wright MS, Stepanauskas R, McArthur JV (2006). Co-selection of antibiotic and metal resistance. *Trends Microbiol* 14(4):176–182.

Baquero F, Martinez J-L, Cantón R (2008). Antibiotics and antibiotic resistance in water environments. *Curr Opin Biotechnol* 19:260–265.

Bartoloni A, Bartalesi F, Mantella A, Dell'Amico E, Roselli M, Strohmeyer M, Barahona HG, Barron VP, Paradisi F, Rossolini GM (2004). High prevalence of acquired antimicrobial resistance unrelated to heavy antimicrobial consumption. *J Infect Dis* 189:1291–1294.

Beaber JW, Hocchhut B, Waldor MK (2004). SOS response promotes horizontal dissemination of antibiotic resistance genes. *Nature* 427:72–74.

Bell JB, Elliott GE, Smith DW (1983). Influence of sewage treatment and urbanization on selection of multiple resistance in fecal coliform populations. *Appl Environ Microbiol* 46:227–232.

Bergstrom CT, Lo M, Lipsitch M (2004). Ecological theory suggests that antimicrobial cycling will not reduce antimicrobial resistance in hospitals *Proc Nat Acad Sci USA* 101(36):13285–13290.

Binh CTT, Heuer H, Kaupenjohann M, Smalla K (2008). Piggery manure used for soil fertilization is a reservoir for transferable antibiotic resistance plasmids. *FEMS Microbiol Ecol*. 66(1):25–37.

Böckelmann U, Dörries H-H, Ayuso-Gabella MN, Salgot de Marcay M, Tandoi V, Levantesi C, Maciopinto C, Van Houtte E, Szewzyk U, Wintgens T, Grohmann E (2009). Quantitative PCR monitoring of antibiotic resistance genes and bacterial pathogens in three European artificial groundwater recharge systems. *Appl Environ Microbiol* 75(3):154–163.

Börjesson S, Matussek A, Melin S, Löfgren S, Lindgren PE (2010a). Methicillin-resistant *Staphylococcus aureus* (MRSA) in municipal wastewater: An uncharted threat? *J Applied Microbiol* 108: 1244–1251.

Börjesson S, Mattsson A, Lindgren P-E (2010b). Genes encoding tetracycline resistance in a full-scale municipal wastewater treatment plant investigated during one year. *J Water Health* 8(2):247–256.

Cardo D, Horan T, Andrus M, Dembinski M, Edwards J, Peavy G, Tolson J, Wagner D (2004). National nosocomial infections surveillance (NNIS) system report data summary from January 1992 through June 2004. *Am J Infect Control* 32:470–485.

Cattoir V, Poirel L, Mazel D, Soussy CJ, Nordmann P (2007). *Vibrio splendidus* as the source of plasmid-mediated QnrS-like quinolone resistance determinants. *Antimicrob Agents Chemother* 51:2650–2651.

Chee-Sanford JC, Aminov RI, Krapac IJ, Garrigues-Jeanjean N, Mackie RI (2001). Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Appl Environ Microbiol* 67(4): 1494–1502.

Coyne MS, Gilfillen RA, Villalba A, Zhang Z, Rhodes R, Dunn L, Blevins RL (1998). Fecal bacteria trapping by grass filter strips during simulated rain. *J Soil Water Conserv* 53(2): 140–145.

Crecchio C, Stotzky G (1998). Binding of DNA on humic acids: Effect on transformation of *Bacillus subtilis* and resistance to DNase. *Soil Biol Biochem* 30:1061–1067.

Crecchio C, Ruggiero P, Curci M, Colombo C, Palumbo G, Stotzky G (2005). Binding of DNA from *Bacillus subtilis* on montmorillonite-humic acids-aluminum or iron hydroxypolymers: Effects on transformation and protection against DNase. *Soil Sci Society Am J* 69:834–841.

da Costa PM, Vaz-Pires P, Bernardoc F (2006). Antimicrobial resistance in *Enterococcus* spp. isolated in inflow, effluent and sludge from municipal sewage water treatment plants. *Water Res* 40:1735–1740.

da Silva MF, Tiago I, Veríssimo A, Boaventura RAR, Nunes OC, Manaia CM (2006). Antibiotic resistance of enterococci and related bacteria in an urban wastewater treatment plant. *FEMS Microbiol Ecol* 55:322–329.

Dantas G, Sommer MOA, Oluwasegun RD, Church GM (2008). Bacteria subsisting on antibiotics. *Science* 320(5872):100–103.

Davies J (1994). Inactivation of antibiotics and the dissemination of resistance genes. *Science* 264:375–382.

Davies JE (1997). Origins acquisition and dissemination of antibiotic resistance determinants. *Ciba Found Symp* 207:15–27.

Davies J, Spiegelman GB, Yim G (2006). The world of subinhibitory antibiotic concentrations. *Curr Opin Microbiol* 9(5):445–453.

Davis CM, Fox JF (2009). Sediment fingerprinting: Review of the method and future improvements for allocating nonpoint source pollution. *J Environ Eng ASCE* 135(7):490–504.

Demaneche S, Jocteur-Monrozier L, Quiquampoix H, Simonet P (2001). Evaluation of biological and physical protection against nuclease degradation of clay-bound plasmid DNA. *Appl Environ Microbiol* 67:293–299.

Dröge M, Pühler A, Selbitschka W (2000). Phenotypic and molecular characterization of conjugative antibiotic resistance plasmids isolated from bacterial communities of activated sludge. *Mol Genomics Genet* 263:471–482.

Engemann CA, Keen PL, Knapp CW, Hall KJ, Graham DW (2008). Fate of tetracycline resistance genes in aquatic systems: Migration from the water column to peripheral biofilms. *Environ Sci Technol* 42:5131–5136.

Entry JA, Hubbard RK, Thies JE, Fuhrmann JJ (2000a). The influence of vegetation in riparian filter strips on coliform bacteria: I Movement and survival in soils. *J Environ Qual* 29(4):1215–1224.

Entry JA, Hubbard RK, Thies JE, Fuhrmann JJ (2000b). The influence of vegetation in riparian filter strips on coliform bacteria: II Survival in soils. *J Environ Qual* 29(4): 1215–1224.

Fick J, Soderstrom H, Lindberg RH, Phan C, Tysklind M, Larsson DGJ (2009). Contamination of surface ground and drinking water from pharmaceutical production. *Environ Toxicol Chem* 28(12):2522–2527.

Field KG, Samadpour M (2007). Fecal source tracking, the indicator paradigm, and managing water quality. *Water Res* 41(16):3517–3538.

Foucault ML, Depardieu F, Courvalin P, Grillot-Courvalin C (2010). Inducible expression eliminates the fitness cost of vancomycin resistance in enterococci. *Proc Nat Acad Sci USA* 107(39):16964–16969.

Fricke, WF, Wright MS, Lindell AH, Harkins DM, Baker-Austin C, Ravel J, Stepanauskas R (2008). Insights into the environmental resistance gene pool from the genome sequence of the multidrug-resistant environmental isolate *Escherichia coli* SMS-3-5. *J Bacteriol* 190(20): 6779–6794.

Garcia S, Wade B, Bauer C, Craig C, Nakaoka K, Lorowitz W (2007). The effect of wastewater treatment on antibiotic resistance in *Escherichia coli* and *Enterococcus* sp. *Water Environ Res* 79(12):2387–2395.

Ghosh S, LaPara TM (2007). The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. *ISME J* 1(3): 191–203.

Ghosh S, Ramsden SJ, LaPara TM (2009). The role of anaerobic digestion in controlling the release of tetracycline resistance genes and class 1 integrons from municipal wastewater treatment plants. *Appl Microbiol Biotechnol 2009*;84(4):791–796.

Gill SR, Fouts DE, Archer GL, Mongodin EF, DeBoy RT, Ravel J, Paulsen IT, Kolonay JF, Brinkac L, Beanan M, Dodson RJ, Daugherty SC, Madupu R, Angiuoli SV, Durkin AS, Haft DH, Vamathevan J, Khouri H, Utterback T, Lee C, Dimitrov G, Jiang LX, Qin HY, Weidman J, Tran K, Kang K, Hance IR, Nelson KE, Fraser CM (2005). Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin resistant *Staphylococcus epidermidis* strain. *J Bacteriol* 187(7):2426–2438.

Heuer H, Smalla K (2007). Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. *Environ Microbiol* 9(3):657–666.

Heuer H, Kopmann C, Binh CTT, Top EM, Smalla K (2009). Spreading antibiotic resistance through spread manure: Characteristics of a novel plasmid type with low %G plus C content. *Environ Microbiol* 11(4):937–949.

Kim SC, Carlson K (2006). Occurrence of ionophore antibiotics in water and sediments of a mixed-landscape watershed. *Water Res* 40(13):2549–2560.

Kim SC, Carlson K (2007). Temporal and spatial trends in the occurrence of human and veterinary antibiotics in aqueous and river sediment matrices. *Environ Sci Technol* 41:50–57.

Klare I, Badstubner D, Konstabel C, Bohme G, Claus H, Witte W (1999). Decreased incidence of van A-type vancomycin-resistant enterococci isolated from poultry meat and from fecal samples of humans in the community after discontinuation of avoparcin usage in animal husbandry. *Microb Drug Resist* 5:45–52.

Klevens RM, Edwards JR, Richards CL, Horan TC, Gaynes RP, Pollock DA, Cardo DM (2007). Estimating health care-associated infections and deaths in US hospitals 2002. *Public Health Rep* 122(2):160–166.

Knapp CW, Dolfing J, Ehlert PAI, Graham DW (2010). Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. *Environ Sci Technol* 44: 580–587.

Koike S, Krapac IG, Oliver HD, Yannarell AC, Chee-Sanford JC, Aminov RI, Mackie RI (2007). Monitoring and source tracking of tetracycline resistance genes in lagoons and groundwater adjacent to swine production facilities over a 3-year period. *Appl Environ Microbiol* 73(15):4813–4823.

Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT (2002). Pharmaceuticals hormones and other organic wastewater contaminants in US Streams 1999–2000: A national reconnaissance. *Environ Sci Technol* 36:1202–1211.

Krometis LAH, Characklis GW, Simmons III OD, Dilts MJ, Likirdopulos CA, Sobsey MD (2007). Intra-storm variability in microbial partitioning and microbial loading rates. *Water Res* 41:506–516.

Krometis LAH, Dillaha TA, Love NG, Mostaghimi S (2009). Evaluation of a filtration/dispersion method for enumeration of particle-associated *Escherichia coli*. *J Environ Qual* 38(3):980–986.

Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R, Chaudhary U, Doumith M, Giske CG, Irfan S, Krishnan P, Kumar AV, Maharjan S, Mushtaq S, Noorie T, Paterson DL, Pearson A, Perry C, Pike R, Rao B, Ray U, Sarma JB, Sharma M, Sheridan E, Thirunarayan MA, Turton J, Upadhyay S, Warner M, Welfare W, Livermore DM, Woodford N (2010). Emergence of a new antibiotic resistance mechanism in India Pakistan and the UK: A molecular biological and epidemiological study. *Lancet Infect Dis* 10(9):597–602.

Lang KS, Anderson JM, Schwarz S, Williamson L, Handelsman J, Singer RS (2010). Novel florfenicol and chloramphenicol resistance gene discovered in Alaskan soil by using functional metagenomics. *Appl Environ Microbiol* 76(15):5321–5326.

Leeb M (2004). Antibiotics: A shot in the arm. *Nature* 431:892–893.

Lefkowitz JR, Duran M (2009). Changes in antibiotic resistance patterns of *Escherichia coli* during domestic wastewater treatment. *Water Environ Res* 81:878–885.

Li D, Yu T, Zhang Y, Yang M, Li Z, Liu M, Qi R (2010). Antibiotic resistance characteristics of environmental bacteria from an oxytetracycline production wastewater treatment plant and the receiving river. *Appl Environ Microbiol* 76(11):3444–3451.

Lipsitch M, Bergstrom C, Levin B (2000). The epidemiology of antibiotic resistance in hospitals: Paradoxes and Prescriptions. *Proc Nat Acad Sci USA* 97:1938–1943.

Lu NX, Zilles JL, Nguyen TH (2010). Adsorption of extracellular chromosomal DNA and its effects on natural transformation of *Azotobacter vinelandii*. *Appl Environ Microbiol* 76(13): 4179–4184.

Luo Y, Mao DQ, Rysz M, Zhou DX, Zhang HJ, Xu L, Alvarez PJJ (2010). Trends in antibiotic resistance genes occurrence in the Haihe river China. *Environ Sci Technol* 44 (19):7220–7225.

Mackie RI, Koike S, Krapac I, Chee-Sanford J, Maxwell S, Aminov RI (2006). Tetracycline residues and tetracycline resistance genes in groundwater impacted by swine production facilities. *Anim Biotechnol* 17:157–176.

Martínez JL (2008). Antibiotics and antibiotic resistance genes in natural environments. *Science* 321:365–367.

McKinney CW, Loftin KA, Meyer MT, Davis JG, Pruden A (2010). tet and sul Antibiotic resistance genes in livestock lagoons of various operation type, configuration, and antibiotic occurrence. *Environ Sci Technol* 44: 6102–6109.

Moura A, Tacao M, Henriques I, Dias J, Ferreira P, Correia A (2009). Characterization of bacterial diversity in two aerated lagoons of a wastewater treatment plant using PCR-DGGE analysis. *Microbiol Res* 164:560–569.

Muller JF, Stevens AM, Craig J, Love NG (2007). Transcriptome analysis reveals that multidrug efflux genes are upregulated to protect *Pseudomonas aeruginosa* from pentachlorophenol stress. *Appl Environ Microbiol* 73(14):4550–4558.

Pallecchi L, Lucchetti C, Bartoloni A, Bartalesi F, Mantella A, Gamboa H, Carattoli A, Paradisi F, Rossolini GM (2007). Population structure and resistance genes in antibiotic-resistant bacteria from a remote community with minimal antibiotic exposure. *Antimicrob Agents Chemother* 51:1179–1184.

Pang Y, Brown BA, Steingrube VA, Wallace RJ, Roberts MC (1994). Tetracycline resistance determinants in *Mycobacterium* and *Streptomyces* species. *Antimicrob Agents Chemother* 38:1408–1412.

Parajuli PB, Mankin KR, Barnes PL (2008). Applicability of targeting vegetative filter strips to abate fecal bacteria and sediment yield using SWAT. *Agric Water Manag* 95:1189–1200.

Peak N, Knapp CW, Yang RK, Hanfelt MM, Smith MS, Aga DS, Graham DW (2007). Abundance of six tetracycline resistance genes in wastewater lagoons at cattle feedlots with different antibiotic use strategies. *Environ Microbiol* 9:143–151.

Pei RT, Kim SC, Carlson KH, Pruden A (2006). Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Res* 40:2427–2435.

Pei R, Cha J, Carlson KH, Pruden A (2007). Response of antibiotic resistance genes (ARG) to biological treatment in dairy lagoon water. *Environ Sci Technol* 41:5108–5113.

Poirel L, Rodriguez-Martinez JM, Mammeri H, Liard A, Nordmann P (2005). Origin of plasmid-mediated quinolone resistance determinant QnrA. *Antimicrobi Agents Chemother* 49:3523–3525.

Pote J, Ceccherini MT, Van VT, Rosselli W, Wildi W, Simonet P, Vogel TM (2003). Fate and transport of antibiotic resistance genes in saturated soil columns. *Eur J Soil Biol* 39:65–71.

Pruden A, Pei R, Storteboom H, Carlson KH (2006). Antibiotic resistance genes as emerging contaminants: Studies in northern Colorado. *Environ Sci Technol* 40:7445–7450.

Ram S, Vajpayee P, Shanker R (2007). Prevalence of multi-antimicrobial-agent resistant Shiga toxin and enterotoxin producing *Escherichia coli* in surface waters of river Ganga. *Environ Sci Technol* 41(21):7383–7388.

Rhodes G, Huys G, Swings J, McGann P, Hiney M, Smith P, Pickup RW (2000). Distribution of oxytetracycline resistance plasmids between aeromonads in hospital and aquaculture environments: Implication of Tn1721 in dissemination of the tetracycline resistance determinant Tet A. *Appl Environ Microbiol* 66:3883–3890.

Rowe-Magnus DA, Guerout AM, Ploncard P, Dychinco B, Davies J, Mazel D (2001). The evolutionary history of chromosomal super-integrons provides an ancestry for multi-resistant integrons. *Proc Natl Acad Sci USA* 98(2):652–657.

Schwartz T, Kohnen W, Jansen B, Obst U (2003). Detection of antibiotic-resistant bacteria and their resistance genes in wastewater surface water and drinking water biofilms. *FEMS Microbiol Ecol* 43(3):325–335.

Schwarz S, Kehrenberg C, Doublet B, Cloeckaert A (2004). Molecular basis of bacterial resistance to chloramphenicol and florfenicol. *FEMS Microbiol Rev* 28(5):519–542.

Shoemaker NB, Vlamakis H, Hayes K, Salyers AA (2001). Evidence for extensive resistance gene transfer among *Bacteroides* spp and among *Bacteroides* and other genera in the human colon. *Appl Environ Microbiol* 67:561–568.

Singer RS, Ward MP, Maldonado G (2006). Can landscape ecology untangle the complexity of antibiotic resistance? *Nat Rev* 4:943–952.

Sköld O (2000). Sulfonamide resistance: Mechanisms and trends. *Drug Resist Updat* 3:155–160.

Smalla K, Sobecky PA (2002). The prevalence and diversity of mobile genetic elements in bacterial communities of different environmental habitats: Insights gained from different methodological approaches. *FEMS Microbiol Ecol* 42:165–175.

Smalla K, Vanoverbeek LS, Pukall R, Vanelsas JD (1993). Prevalence of NPTII and TN5 in kanamycin-resistant bacteria from different environments. *FEMS Microbiol Ecol* 13:47–58.

Smalla K, Heuer H, Gotz A, Niemeyer D, Krogerrecklenfort E, Tietze E (2000). Exogenous isolation of antibiotic resistance plasmids from piggery manure slurries reveals a high prevalence and diversity of IncQ-like plasmids. *Appl Environ Microbiol* 66:4854–4862.

Smalla K, Heuer H, Gotz A, Niemeyer D, Krogerrecklenfort E, Tietze E (2001). Exogenous isolation of antibiotic resistance plasmids from piggery manure slurries reveals a high prevalence and diversity of IncQ-like plasmids (vol 66, pg 4854, 2000). *Appl Environ Microbiol 2000*;66:4854–4862.

Smalla K, Haines AS, Jones K, Krogerrecklenfort E, Heuer H, Schloter M, Thomas CM (2006). Increased abundance of IncP-1 beta plasmids and mercury resistance genes in mercury-polluted river sediments: First discovery of IncP-1 beta plasmids with a complex mer transposon as the sole accessory element. *Appl Environm Microbiol* 72:7253–7259.

Smith MS, Yang RK, Knapp CW, Niu Y, Peak N, Hanfelt MM, Galland JC, Graham DW (2004). Quantification of tetracycline resistance genes in feedlot lagoons using real-time PCR. *Appl Environ Microbiol* 70:7372–7377.

Sommer MOA, Dantas G, Church GM (2009). Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science* 325:1128–1131.

Speer BS, Salyers AA (1989). Novel aerobic tetracycline resistance gene that chemically modifies tetracycline. *J Bacteriol* 171:148–153.

Stepanauskas R, Glenn TC, Jagoe CH, Tuckfield RC, Lindell AH, McArthur JV (2005). Elevated microbial tolerance to metals and antibiotics in metal-contaminated industrial environments. *Environ Sci Technol* 39:3671–3678.

Storteboom H, Arabi M, Davis JG, Crimi B, Pruden A (2010a). Tracking antibiotic resistance genes in the South Platte River Basin using molecular signatures of urban, agricultural, and pristine sources. *Environ Sci Technol* 44:7397–7404.

Storteboom H, Arabi M, Davis JG, Crimi B, Pruden A (2010b). Identification of antibiotic-resistance-gene molecular signatures suitable as tracers of pristine river, urban, and agricultural sources. *Environ Sci Technol* 44:1947–1953.

Summers AO (2002). Generally overlooked fundamentals of bacterial genetics and ecology. *Clin Infecti Dis* 34:S85–S92.

Thaller MC, Migliore L, Marquez C, Tapia W, Cedeno V, Rossolini GM, Gentile G (2010). Tracking acquired antibiotic resistance in commensal bacteria of Galapagos land iguanas: No man no resistance. *PLoS ONE 2010*;5(2):e8989.

Van den Bogaard AE, Bruinsma N, Stobberingh EE (2000). The effect of banning avoparcin on VRE carriage in The Netherlands. *J Antimicrob Chemother* 46:146–148.

Vilanova X, Manero A, Cerdà-Cuéllar M, Blanch AR (2004). The composition and persistence of faecal coliforms and enterococcal populations in sewage treatment plants. *J Appl Microbiol* 96:279–288.

Volkmann H, Schwartz T, Bischoff P, Kirchen S, Obst U (2004). Detection of clinically relevant antibiotic-resistance genes in municipal wastewater using real-time PCR (TaqMan). *J Microbiol Methods* 56(2):277–286.

Xi C, Zhang Y, Marrs CF, Ye W, Simon C, Foxman B, Nriagu J (2009). Prevalence of antibiotic resistance in drinking water treatment and distribution systems. *Appl Environm Microbiol* 75(17):5714–5718.

Yang S, Carlson K (2003). Evolution of antibiotic occurrence in a river through pristine urban and agricultural landscapes. *Water Res* 37:4645–4656.

Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, Walsh TR (2009). Characterization of a new metallo-beta-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. *Antimicrob Agents Chemother* 53:5046–5054.

Zhang X, Wu B, Zhang Y, Zhang T, Yang L, Fang HHP, Ford T, Cheng S (2009a). Class 1 integronase gene and tetracycline resistance genes tetA and tetC in different water environments of Jiangsu Province China. *Ecotoxicology* 18:652–660.

Zhang Y, Marrs CF, Simon C, Xi C (2009b). Wastewater treatment contributes to selective increase of antibiotic resistance among *Acinetobacter* spp. *Sci Total Environ* 407:3702–3706.

11

ANTIBIOTIC RESISTANCE IN SWINE-MANURE-IMPACTED ENVIRONMENTS

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11.1 INTRODUCTION

An increasing body of evidence demonstrating entry of antibiotics and antibiotic resistance genes from anthropogenic sources into natural soil and water environments has raised even more questions about the lasting and future impacts consequent to drug resistance development in bacteria. These concerns stem, in part, from emerging knowledge about increased incidences, persistence, and diversity of antibiotic resistance (ABR) genes in soil and water environments with still very limited knowledge about the molecular microbial ecology of ABR occurring in situ in these natural systems. The practice of subtherapeutic doses of antibiotics for use in disease prophylaxis and growth promotion in animal livestock production has been the subject of particularly intense scrutiny, prompting bans of such uses completely in the European Union since 2006 and causing mounting concerns in the United State for more judicious use of antibiotics in food animals. Numerous articles over the last two decades have discussed in detail the various health and environmental aspects concerning the routine use of subtherapeutic antibiotic treatment in animal production (Gustafson and Bowen, 1997; Khachatourians, 1998; USGAO, 1999; Isaacson and Torrence, 2002; Séveno et al., 2002; Kümmerer, 2004; Shea, 2004; Chee-Sanford et al., 2009).

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It has been well documented that the occurrence of ABR bacteria increases in environments where antibiotics are in frequent use such as clinical settings (Gomez-Lus, 1998; Poole and Srikumar, 2001; Walsh 2003) and in animal production or veterinary settings (Witte, 2001; McEwen and Fedorka-Cray, 2002). More than one-half of all antibiotics produced in the United States are used in animal production and up to 80% are estimated for use in growth promotion (Levy, 1998; Hileman, 2001; Graham et al., 2007). It is generally acknowledged that the widespread use of antibiotics in this manner selects for antibiotic-resistant bacteria in enteric systems (Haack and Andrews, 2000), often occurring first in commensals and later in pathogens if present (Sørum and Sunde, 2001; Salyers et al., 2004). The high bacterial load in the excreted manure is a consequent source for ABR bacteria and their resident ABR genes. Estimates of 30–90% of orally administered antibiotics may also be excreted unchanged (Elmund et al., 1971; Halling-Sørensen et al., 1998; Boxall et al., 2004; Chander et al., 2005), posing further potential for drug resistance selection to occur among the resident bacteria in environmental compartments downstream of the animal gut.

Since before the mid-1970s, livestock farms were small, integrated production systems that efficiently managed animal waste onsite, often by applying manure to cropland owned by the farmers. The shift toward fewer but large (thousands of head) concentrated animal feeding operations (CAFOs) precluded such management practices and necessitated a means by which to locally dispose of high quantities of manure year-round. The current most common method to dispose of swine and feedlot cattle waste in the United States is collection in storage holds such as lagoons or pits, followed by land application of the manure effluent to adjacent crop fields that are often leased to provide sufficient land area to support the waste loads. The manure not only introduces high bacterial and chemical loads to the soil, the changes in chemical (Siddique and Robinson 2003; Plaza et al. 2006), biological (de Freitas et al., 2003; Sun et al., 2004; Wienhold, 2005; Hartmann et al., 2006), and physical (Wienhold 2005) soil properties may act to affect microbial populations and their consequent activities (Frostegard et al., 1997; Sun et al., 2004; Wienhold, 2005; Hartmann et al., 2006). In concert, the changes in the soil factors may have various effects on the microbial and molecular ecology (i.e., molecular phylogenetics) specifically associated with antibiotic resistances.

11.2 DETECTION OF ABR GENES AND BACTERIA IN NATURAL ENVIRONMENTS

It has been well documented over the years that many microorganisms of enteric origin survive the transition from effluent pit or lagoon into soil (Kibbey et al., 1978; Chandler et al., 1981; Stoddard et al., 1998; Bolton et al., 1999; Lee and Stotzky, 1999; Jiang et al., 2002; Guan and Holley, 2003; Boes et al., 2005). Their survival, together with the notion that many of these bacteria may also harbor ABR genes, had generated increased interest in environmental epidemiological tracking of ABR bacteria to assess the possibility of reentry to human and animal populations. Moreover, the extent of genetic transfer and acquisition of ABR genes in these natural environments remains unclear. The role of native soil and water microbial populations in the overall molecular ecology of ABR genes remains largely unknown.

The advent of molecular-based methods based on ABR genetic sequences has resulted in numerous reports of ABR genes in manure-impacted soil and water environments. Numerous independent studies have reported ABR gene detection associated specifically with swine production facilities, where 88% of U.S. swine producers routinely administer antibiotics in feed to grower/finisher pigs (USDA, 2001). The macrolide antibiotic tylosin is exclusively used in swine livestock along with tetracyclines, hence tetracycline resistance (tet^R) and tylosin resistance (erm^R) genes presented useful targets in the evaluation of swine-manure-impacted environments. Detection based on polymerase chain reaction (PCR) has been used to reveal mobility, persistence, and distribution of tet^R and erm^R genes in swine manure, swine-manure-applied soils, and groundwater underlying and down gradient from swine waste lagoons (Aminov et al., 2001, 2002; Chee-Sanford et al., 2001; Hund-Rinke et al., 2004; Schmitt et al., 2006; Smith et al., 2004; Koike et al., 2009). While these methods yield important data that aid in detection, tracking, and quantifying specific determinants within the genetic pool of a environmental sample's metapopulation without the need to culture, the methods rely on sequences from known genes and allow only limited interpretation about the exact mechanisms of how these ABR genes came to be and the identities of the populations harboring them. Further, the functional expression of the ABR genes that are detected with these methods is not readily determined in the natural environments where they were harbored.

Cultivation-based approaches have yielded isolates of ABR bacteria grown under antibiotic selection (Cotta et al., 2003; D'Costa et al., 2006; Onan and LaPara, 2003) and provide a limited means of assessing genetic expression of ABR genes in environmental samples. However, practical considerations usually do not allow exhaustive strategies to be undertaken to obtain growth or to use wide-range testing of drug concentrations in selection. The approach is further hampered by the lack of knowledge and measurement of the biologically active state of drug residues in manure-impacted soil and water environments. Consequently, it is unknown what may elicit a true drug resistance response from a bacterium in these environments where concentrations of antibiotics at clinical dosages may not be ecologically relevant, yet are often selected as the starting point for laboratory cultivation-based strategies. This is particularly challenging in natural soil and water environments, where most bacterial species have not been cultivated, knowledge of the biological activity exerted by exogenous drug residues is lacking, and the nature and extent of the interactions involving background biotic and abiotic factors is virtually unknown under current technology.

Several reports employing a combinatorial approach including both culture- and molecular-based detection, were used to examine a limited range of bacterial species (Schmidt et al., 2001; Jensen et al., 2002; Miranda et al., 2003; Kim et al., 2004; Agersø and Sandvang, 2005; Nikolakopoulou et al., 2005). This type of approach provides not only information about the species harboring the drug resistance trait but also confirmed the phenotypic expression of the activity. The genetic sequence information obtained from individual cultivated strains provided some ability to analyze phylogenetic relationships, with clues toward the possible origin and flow of the ABR determinant.

An interesting study that characterized the corresponding tet^R determinants in a collection of chlortetracycline-resistant soil bacteria isolated from several

manure-impacted soils and nonagricultural soils represented, to date, one of the only such studies that involved a relatively diverse set of nonenteric bacteria (Ghosh and LaPara, 2007). The collection encompassed several bacterial phyla with members representing a variety of commonly associated soil species, including multiple isolates in the genera *Bacillus, Streptomyces, Variovorax, Chryseobacterium*, and *Pseudomonas*. Of the 14 genes targeted, the most common harbored overall were the efflux determinants *tet*(L) and *tet*(A), with several instances of two tet^R determinants detected within an isolate. The commonality of the tet^R determinants detected among the phylogenetically distinct isolates suggested that this was a shared trait quite possibly acquired by lateral transfer, and more prominent in soils following manure exposure from swine production farms with histories of antibiotic usage. It is noteworthy in this study that regardless of the source of the soil, isolates of *Streptomyces*, a genus made up of several known species that produce tetracyclines, did not harbor any of the tet^R determinants that were tested. The results may not have precluded other sources of tet^R besides manure, however, finding relatively diverse bacteria harboring homologous genes did suggest the potential for genetic mobility over a wide range of bacteria within the soil compartment. The study also presented some interesting notions regarding the frequency of some tet^R determinants over others in diverse bacteria, for example, *tet*(L) and *tet*(A), genes specifically encoding efflux mechanisms. More insight into the predominant tet^R determinants along with the genetic structure in conjunction with genes such as plasmid carriers, transposon elements, or gene cassettes would be quite valuable. Increasing the number of similar studies that assess a wide range of bacteria, including commensal species of both enteric and soil origins, and their corresponding ABR determinants would nevertheless provide a larger basis by which to more accurately evaluate the dynamics of ABR genes in natural environments.

11.3 CASE STUDY: tet^R AND erm^R GENE DISTRIBUTION IN BACTERIA ISOLATED FROM SWINE MANURE PITS AND SOILS WITH AND WITHOUT HISTORY OF MANURE EXPOSURE

To further examine the tet^R and erm^R genes, the frequency of determinants harbored by bacteria, and the possible links of these genes to specific phylogenetic groups, the sites of two large swine production farms were investigated. The approach used was a cultivation- and PCR-based analysis of bacteria isolated from the manure holding pits and soil from adjacent crop fields with long histories (25 years or more) of land-applied manure originating from their respective sites. Upslope of the manure-applied fields, soils with no known exposure to swine manure were used to assess bacterial populations with ABR genes that could be attributed to resistances acquired under similar edaphic factors, excluding manure influences. Bacterial isolates were grown both aerobically and anaerobically on MR2A agar (Fries et al., 1994) under either tetracycline or tylosin selection (both 20 mg/L) from the pits and soils. Each isolate was analyzed for the presence of tet^R determinants conferring tetracycline resistance mechanisms of efflux [*tet* (B), (C), (D), (E), (G), (H), (J), (S), (T), (Y), and (Z)], ribosomal protection [*tet* (M), (O), (Q), and (W)], and the macrolide resistance genes *erm* (B), (F), (G), and (Q) using methods described previously (Aminov et al., 2001, 2002; Koike et al., 2009).

11.3.1 Phylogenetic Distribution of Tetracycline- and Tylosin-Resistant Bacterial Isolates

In total, 124 distinct isolates were identified on the basis of nearly full-length 16S ribosomal ribonucleic acid (rRNa) gene sequences and analyzed for their corresponding suite of *tet*^{R} and *erm*^{R} determinants These isolates phenotypically expressed either tetracycline or tylosin resistance and were presumed to represent only a fraction of the ABR bacterial population present. Figures 11.1–11.4 show the results of the identifications based on nearly complete ($\sim$1500 bp) 16S rRNA gene sequences according to the closest genera matched using the national center for biotechnology information (NCBI) GenBank BLAST series of tools (Madden et al., 1996). The following denotes the identities and characteristics of each isolate: genus and unique strain designation, tetracycline (TcR) or tylosin (TIR) resistance phenotype, *tet*^{R} and *erm*^{R} genotype (if detected), and GenBank accession number. The sources of the isolates are denoted by black (pit), white (nonmanured soil), and gray (manured soil) text. Posterior probabilities of clades (shown as node labels) were calculated using a Bayesian Markov chain Monte Carlo method with the MrBayes v.3.1.1 program (http://mrbayes.csit.fsu.edu/index.php). The scale bar shows expected changes per site.

Overall, the isolates obtained comprised four major bacterial phyla, including Actinobacteria, Firmicutes, Bacteroidetes, and the α, β, and γ subclasses of the Proteobacteria (Fig 11.1-11.4), generally consistent with the phylogenetic distributions of chlortetracycline-resistant strains found in the study by Ghosh and LaPara (2007). Recurrence of these phyla is, at least in part, a function of similar cultivation strategies and bias, although many of these species are prevalent in natural soil environments and thus, could comprise a significant reservoir of the ABR genes being circulated.

Under the growth selection conditions employed, the manure pit environments were dominated by species of Firmicutes (Fig. 11.2), followed by the majority of Actinobacteria isolated (Fig.11.1). Of the Proteobacteria, most of the species originating from the pits were represented in the γ subclass, with several enteric species recovered as represented by such common species as *Escherichia coli*, *Shigella* sp., *Enterobacter* sp., and *Serratia* sp. (Fig. 11.4). The survivability in the environment of species such as *E. coli* and the common enteric Firmicutes *Enterococcu*s has been well studied (Davies et al., 1995; Cools et al., 2001; Hartz et al., 2008), raising concerns that pathogenic strains of these species could circulate back to human and animal populations, along with exacerbating concerns that ABR traits have been found in these types of bacterial contaminants. The isolates from pits also comprised a large number of disparate species native to many natural soil and water environments, such as *Rhodococcus* and *Microbacterium*. Isolates from both untreated and manure-applied soils were largely comprised of a wide range of Proteobacteria (Fig. 11.1, 11.3, and 11.4), including many species that are frequently and easily cultivated from soil.

As possible vectors for the persistence and circulation of ABR genes, natural bacterial residents of soil may be highly significant due to their adaptive traits and probable growth in soil environments. Not unexpectedly, the relative higher diversity of ABR species was indeed obtained from environments where manure exposure occurred (pits and manured soils), but it is yet unknown if any existing concentrations of antibiotics may play a mechanistic role in drug resistance selection in these types of environments.

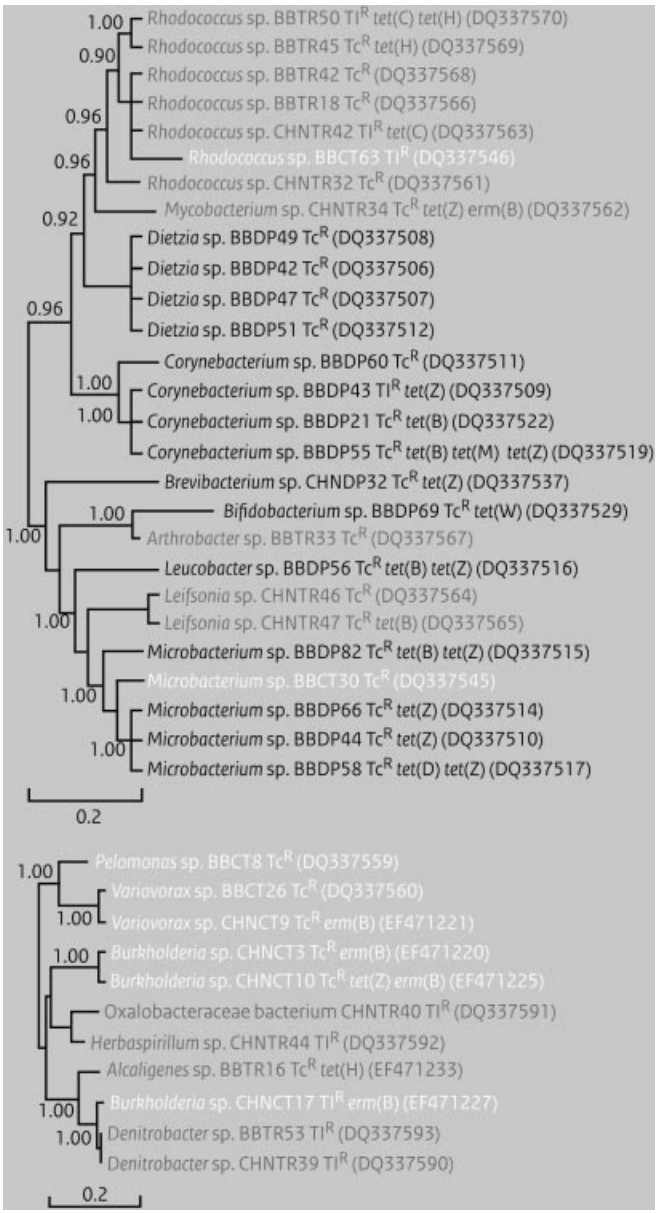

FIGURE 11.1 Phylogenetic relationships of tetracycline- or tylosin-resistant bacterial isolates belonging to the phylum Actinobacteria (upper image) and to the β subgroup of the phylum Proteobacteria (lower image) cultivated from manure holding pits and soils from two commercial swine production farms.

11.3.2 Distribution of *tet*^{R} and *erm*^{R} Genes among Isolates

The majority of the ABR genes targeted, namely *tet* (B), (C), (D), (E), (G), (H), (J), (M), (O), (Q), (W), (Y), (Z) and *erm* (B), (F), (G), were detected among the isolates, but numerous environmental species appeared to harbor unknown mechanisms of

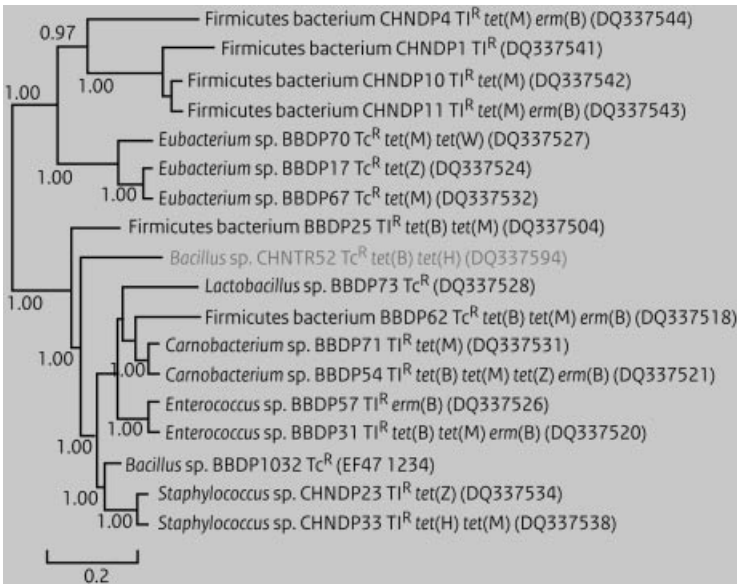

FIGURE 11.2 Phylogenetic relationships of tetracycline- or tylosin-resistant bacterial isolates belonging to the phylum Firmicutes cultivated from manure holding pits and soils from two commercial swine production farms.

tetracycline and tylosin resistances. Regardless of the history of manure exposure, 55–67% of the soil-derived isolates that exhibited either tetracycline- or tylosin-resistant phenotypes did not harbor any targeted *tet*^{R} or *erm*^{R} genes. These unknown determinants may comprise other genes found previously in other studies, such as *tet* (A), (K), (L), or [A(P)] but may also include novel resistance genes. Contrastingly, in pit environments, 72% of isolates selected on tetracycline and 93% of the isolates selected on tylosin did harbor known *tet*^{R} determinants that included *tet* (B), (D), (H), (M), (Q), (W), (Z). Pit-derived isolates, however, more frequently harbored multiple known *tet*^{R} genes, in some instances combinations of three or four different genes conferring both efflux and ribosomal protection mechanisms.

While incidences of multiple ABR strains within a single genus were isolated, also noteworthy were the different resistance determinants found harbored by closely related strains within a group. For example, *tet* (B), (G), and (H) were detected as individual determinants in closely related strains of the α-proteobacterium *Ochrobactrum* along with one additional strain harboring an unknown *tet*^{R} determinant (Fig. 11.3). The occurrence of different determinants suggested divergent processes leading to the circulation of these *tet*^{R} genes within this group.

Within the Actinobacteria, the three genera *Dietzia*, *Corynebacterium*, and *Microbacterium* appeared to comprise the main reservoir of ABR genes in the pits, harboring primarily *tet* (B) or *te*t (Z), with isolates expressing either tetracycline or tylosin resistances (Fig. 11.1). Aerobic coryneforms can be encountered in various ecosystems; in the human skin they harbor a considerable reservoir of tetracycline and erythromycin resistance determinants (Eady et al., 2000). Representatives of the other two genera, *Microbacterium* and *Dietzia*, are mostly isolated from environments such as soil and water, although the latter has been increasingly recognized as

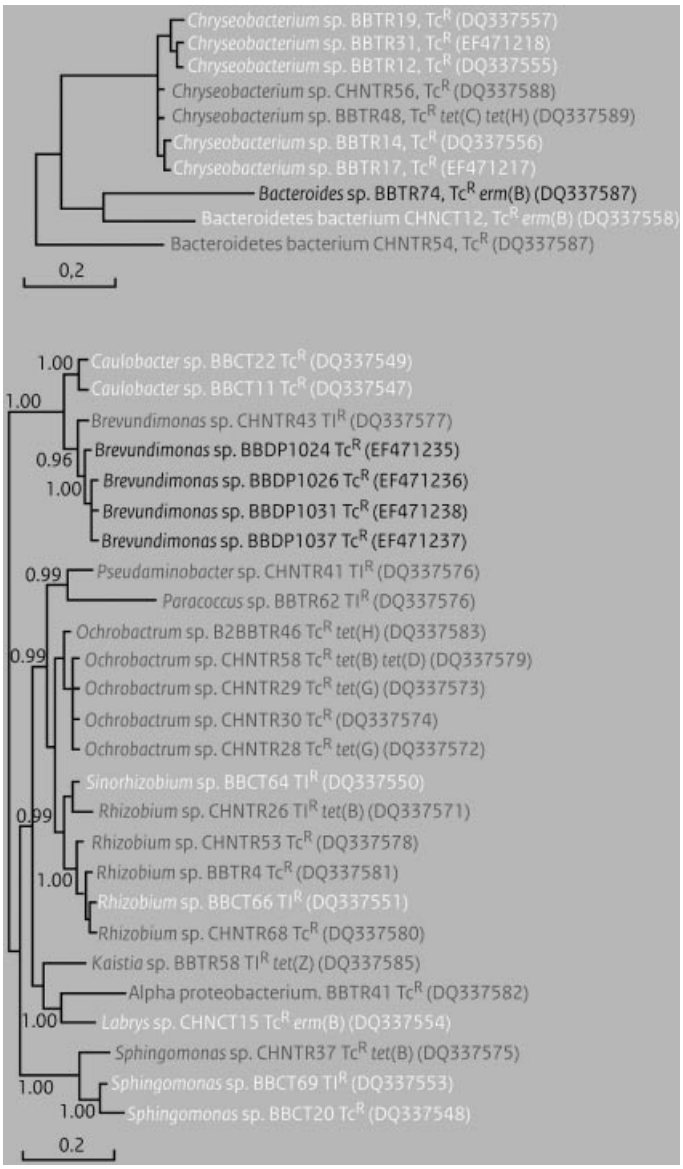

FIGURE 11.3 Phylogenetic relationships of tetracycline- or tylosin-resistant bacterial isolates belonging to the phylum Bacteroidetes (upper image) and the α subgroup of the phylum Proteobacteria (lower image) cultivated from manure holding pits and soils from two commercial swine production farms.

a potential human pathogen (Koerner et al., 2009). Very little is known about antibiotic resistance traits in these two genera, and these results are the first to describe tetracycline resistance determinants circulating in these bacteria.

In manured soil, the genus *Rhodococcus* dominated the isolates, with different strains exhibiting a variety of tet^R determinants (Fig. 11.1). It is interesting to note that *Rhodococcus*, common to soil and water environments, are known for their large

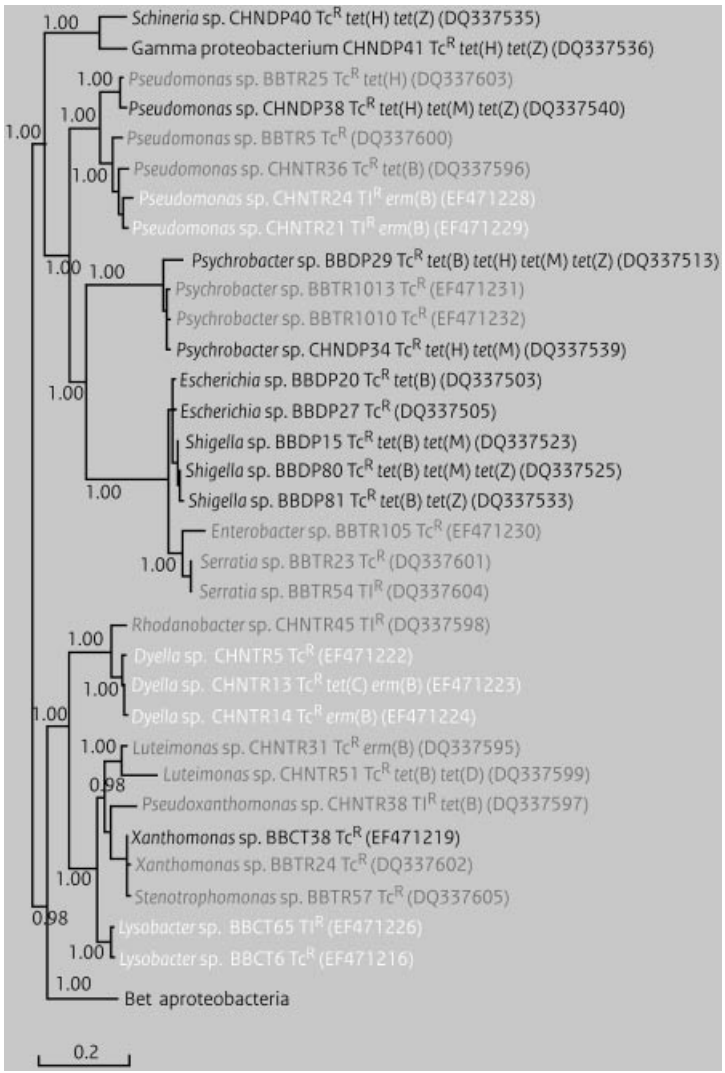

FIGURE 11.4 Phylogenetic relationships of tetracycline- or tylosin-resistant bacterial isolates belonging to γ subgroup of the phylum Proteobacteria cultivated from manure holding pits and soils from two commercial swine production farms.

genome size (e.g., *R. jostii* 9.7 Mb) and the presence of megaplasmids that carry large sets of genes (Martínková et al., 2009). Known strains of *Rhodococcus* can undergo a high frequency of recombination that confer new genes and metabolic adaptability, and, if the case is so with antibiotic resistance genes, this may be consistent with findings of multiple types of ABR determinants in these *Rhodococcus* isolates. The ABR genes, however, may not be readily expressed under laboratory conditions or confer resistance to high antibiotic concentrations tested (see, e.g., the *tet*^{R} genotype and phenotype of *Rhodococcus* sp. CHNTR42 in Fig. 11.1 and Table 11.1), but it cannot be excluded that they may do so in natural environments with modest antibiotic concentrations.

TABLE 11.1 Antibiotic Resistance Phenotypes[a] of Bacterial Isolates Cultivated from Swine Manure Pits, Manured-Soil, and Soil with No Known History of Manure Exposure

			Antibiotic[b]											
Isolate	Source[c]	Original Drug Selection	Tet	ChlTet	Cmp	Gent	Amp	Tyl	Ery	Bac	Pen	Van	Strp	Kan
Chryseobacterium sp. BBCT12	NMS	Tet	+	+	+	+	−	+	+	+	+	−	+	+
Chryseobacterium sp. BBTR48	MS	Tet	+	+	+	−	−	−	+	−	−	−	−	−
Burkholderia sp. CHNCT3	NMS	Tet	+	+	+	+	+	+	+	+	+	+	+	+
Burkholderia sp. CHNCT10	NMS	Tet	+	+	+/−	+	+	+	+	+	+	+	+	+
Serratia sp. BBTR23	MS	Tet	+	+	+	−	+	+	+	+	+	−	−	−
Ochrobactrum sp. CHNTR58	MS	Tet	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas sp. CHNTR36	MS	Tyl	+	+	+	−	+	+	+	+	+	+	+	+
Rhodococcus sp. CHNTR42	MS	Tyl	−	−	−	−	−	+	−	−	−	−	−	−
Leucobacter sp. BBDP56	PIT	Tet	+	+	−	−	−	+	−	−	−	−	−	+
Corynebacterium sp. BBDP55	PIT	Tet	+	+	−	−	−	+	+	−	−	−	+	−
Enterococcus sp. BBDP31	PIT	Tet	+	+	+	+	−	+	+	+	−	−	+	+
Staphylococcus sp. CHNDP33	PIT	Tet	+	+	−	−	−	−	+	+	−	−	+/−	−
Escherichia sp. BBDP20	PIT	Tyl	+	+	+/−	+/−	+	+	+	+	+	+	+/−	+
Psychrobacter sp. BBDP29	PIT	Tet	+	+	−	−	+	+	−	+	−	−	+	+
Shigella sp. BBDP80	PIT	Tet	+	+	−	−	−	+	−	−	−	+/−	−	−

[a] Bacterial isolates were tested on MR2A agar medium amended with 20 mg/L antibiotic compound. Growth in the presence of antibiotic was scored as (+) indicating resistance, (−) indicating non-resistance, and (+/−) indicating slight growth relative to growth on MR2A agar medium without antibiotic addition.

[b] Tetracycline (Tet), chlortetracycline (ChlTet), chloramphenicol (Cmp), gentamycin (Gent), ampicillin (Amp), tylosin (Tyl), erythromycin (Ery), bacitracin (Bac), penicillin (Pen), vancomycin (Van), streptomycin (Strp), and kanamycin (Kan).

[c] Isolate was originally obtained by growth under either tetracycline or tylosin selection from nonmanured soil (NMS), manured soil (MS), swine waste holding pit (PIT).

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Within the pit environments, the reservoirs of *tet*^{R} genes resided primarily in isolates belonging to the Firmicutes phylum, including isolates of enteric origins such as *Lactobacillus*, *Eubacterium, Carnobacterium*, and *Enterococcus* (Fig. 11.2). Their remarkable survivability outside of the gut environment in pits may contribute to further dissemination of ABR genes preselected and amplified in antibiotic-fed animals. The Tn*916* family of mobile genetic elements is found widely among many commensal and pathogenic bacteria, and primarily found in many Firmicutes (Roberts and Mullany, 2009). These elements contain *tet*(M) but may often include other accessory genes and are thought to be important vectors in movement of genetic traits among many bacteria. Not only do the elements facilitate horizontal gene transfer, they also cause genome rearrangements that may lead to natural selection and gene evolution events. Another important factor contributing to these genetic processes may be the residual tetracycline in pits, which can enhance transposon-mediated conjugal transfer (Showsh and Andrews, 1992). Thus, Firmicutes may be a key bacterial group with members that facilitate the acquisition of ABR traits among both commensals and pathogens in a variety of environments.

The *tet*^{R} gene pool residing within the Bacteroidetes may likely be comprised in part of unknown genes represented in the cultivated *Chryseobacterium* spp. (Fig. 11.3). These unknown *tet*^{R} determinants may include one or more naturally occurring genes already present in the background bacterial populations, where the influence of manure may not be the dominant factor in the selection of the tetracycline resistance trait. This is supported by a broader range of antibiotic resistances found in *Chryseobacterium* sp. BBCT12 from a non-manured soil compared to the strain BBTR48 from manured soil (Table 11.1). In contrast, the *Bacteroides* sp. BBDP74 isolated from a manure pit harbored multiple ribosomal protection genes such as *tet* (M), (Q), (W), which would be consistent with the consequences of higher selective pressure within its origin in the gastrointestinal system and in the concentrated pit environment. During the original cultivation selection, none of the isolates from this phylum cross selected on tylosin, albeit *erm*(B) was detectable in two isolates (Fig.11.3). Also, *Chryseobacterium* sp. BBCT12 exhibited tylosin resistance in subsequent phenotypic testing but did not harbor any of the *erm*^{R} genes used in our study.

A wide range of α-Proteobacteria representatives was mostly isolated from both manure-treated and untreated soils (Fig. 11.3). Similar α-Proteobacteria were reported by Ghosh and LaPara (2007). The only genus recovered from pit samples was *Brevundimonas*, with multiple strains harboring a diversity of *tet*^{R} and *erm*^{R} genes beyond the range covered by our set of 19 primers. The *Caulobacter* and *Sphingomonas* isolates from non-manured soils also did not produce any PCR amplified signals with the primer set in use, suggesting that these strains may contain naturally occurring reservoirs of novel tetracycline and tylosin resistance genes. The occurrence of several isolates of *Ochrobactrum* and *Rhizobium* from the manure-treated soil might represent indigenous soil strains with ABR acquisition potential, harboring a variety of ABR determinants. Species of *Ochrobactrum* are found associated with the rhizosphere but may also be important opportunistic human pathogens (Berg et al., 2005a). Multiple antibiotic resistances have been found in rhizosphere isolates of this genus, where the localized environment of the plant root often hosts high microbial abundances and contain the presence of diverse

antibiotics produced by root-associated microbes. A species of *Ochrobactrum* obtained from manured soil in this study demonstrated phenotypic resistances to 12 antibiotics representing 7 drug classes (Table 11.1).

Rhizobia, in particular, are known for housing large plasmids (100 kb to 2 Mb) that encode genes of nitrogen fixation along with other genes necessary for symbiotic relationships with plants. The conjugal transfer of these large plasmids is thought to play a major role in the evolution of rhizobia (Ding and Hynes, 2009). Isolates of the genus *Rhizobium*, with the exception of one strain, did not contain the known tet^R or erm^R genes tested, yet represented one of the more frequent strains recovered in our study (Fig. 11.3). Enhanced genetic transfer rates and competition for resources contribute to the development of high levels of natural resistances found in the rhizosphere environment, and one can also speculate that adding exogenous sources of ABR genes and drug residues via manure inputs would create an even more conducive set of conditions for ABR traits to proliferate.

Concerns for soil bacteria emerging as opportunistic human pathogens along with their selection and acquisition of ABR genes have highlighted the need to better understand the ecological links that contribute to the evolution and dynamics of circulating ABR reservoirs. Such opportunistic pathogens are distributed over a wide phylogenetic range. Within the β-Proteobacteria, the genus *Burkholderia* is also thought to comprise some species of opportunistic pathogens found in the rhizosphere. Two *Burkholderia* isolates were recovered from nonmanured soil under tetracycline selection, with both strains found to harbor *erm*(B), but only one harbored a known tet^R determinant, *tet* (Z) (Fig. 11.1), suggesting a largely unknown set of determinants may comprise the natural ABR reservoir circulating among this particular genus. Many Gram-negative bacteria are intrinsically resistant to hydrophobic macrolides such as tylosin (Nikaido, 1996), and thus a physiological basis may also be a major mechanism conferring drug resistance for many of the isolates obtained in the absence of known erm^R genes. Further, however, the two strains of *Burkholderia* (CHNCT3 and CHNCT10) from nonmanured soils with no known antibiotic exposure, were also phenotypically resistant to antibiotics representing five other drug classes in addition to tetracyclines and macrolides (Table 11.1), thus suggesting the existence of a profound natural diversity of ABR genes circulating among this bacterial group.

The largest diversity of tetracycline- and tylosin-resistant bacteria in terms of genera and species isolated was from the γ-Proteobacteria (Fig. 11.4). Accordingly, these representatives were almost uniformly recovered from all three ecological niches sampled, ranging from the enterobacteria in pits to *Pseudomonas, Dyella*, and *Lysobacter* in nonmanured soils. The genus *Pseudomonas* was unique in that the species were isolated from all three ecological compartments. *Pseudomonas aeruginosa* is a well-studied example of a versatile bacterium encountered in many environments, as well as a recognized opportunistic pathogen, exhibiting very unique high intrinsic resistance to many antibiotics. The intrinsic resistance of *P. aeruginosa* has been attributed to an unusually impermeable outer membrane, along with the concerted action of several basic physiological genes and mechanisms of multidrug efflux pumps (Normark and Normark, 2002; Fajardo et al., 2008). Homologs of the efflux pump mechanisms in *P. aeruginosa* have been found in species of *Burkholderia* and *Stenotrophomonas maltophilia*, the latter also belonging to the γ-Proteobacteria and thought to be an opportunistic pathogen found in a broad range

of environments. With the exception of gentamicin, *Pseudomonas* sp. CHNTR36 was resistant to all other antibiotics shown in the panel (Table 11.1).

In the background metagenome [i.e., genomic deoxyribonucleic acid (DNA) pool] of both farm manure pits, all 15 *tet*^{R} and 4 *erm*^{R} determinants used in the study were detected. Finding a wide array of resistance genes is consistent with many other reports of ABR gene diversity in waste-holding compartments such as swine lagoons and pits where the most concentrated inputs of bacteria and drug residues occur. These pits were monitored frequently over a 3–year period, and at any instance of sampling, the same 15 *tet*^{R} and 4 *erm*^{R} determinants were always detected, suggesting the stable maintenance of these genes over time. Not all 19 determinants were, however, represented in the collection of isolates obtained.

Of the known *tet*^{R} determinants in the pit metagenome, only *tet* (B), (D), (H), (M), (Q), (W), and (Z) and *erm* (B) were represented among the pit-derived isolates. In the metagenomic pool of manured soils, 8 of the 15 *tet*^{R} determinants were detected, including *tet* (B), (C), (H), (M), (O), (Q), (W), (Z) while all 4 tylosin resistance genes *erm* (B), (F), (G), (Q) were present. A nearly similar diverse range of known *tet*^{R} genes were present in nonmanured soils, including *tet* (C), (H), (M), (O), (W), and (Z), along with the presence of *erm* (B), (F), and (G). The presence of *tet*^{R} and *erm*^{R} genes typically thought to be of clinical relevance but found in soil environments that were presumed to have no impact from agricultural wastes raises the notion that these particular determinants may also be part of the natural background reservoir of ABR. These types of studies do bring to further light that accurate knowledge of a soil's prior history is critical for meaningful interpretation in such studies. The *tet*^{R} and *erm*^{R} genes that were unaccounted for in isolated strains could have resided in the uncultivated proportion of the bacterial populations. Further, there may be exogenous copies of these genes outside of living cells packed in transducing phage particles (Zeph et al., 1988) that were maintained in the genetic pool within these environments.

11.3.3 Molecular Ecology of *tet*^{R} and *erm*^{R} Genes in Manure Pits and Soils

The known genes encoding ribosomal protection proteins (RPP) are widely disseminated, occurring in both Gram-positive and Gram-negative bacteria (Chopra and Roberts, 2001; Roberts, 2005). The lateral transfer of these genes is evidenced by the broad phylogenetic distribution presently seen among bacteria harboring RPP genes as the content of RPP genes suggested the genes originated in Gram-positive bacteria. Consistent with a broad host range, *tet* (M) was detected among a number of isolates belonging to the Firmicutes, Actinobacteria, and γ-Proteobacteria, with one Bacteriodetes isolate, and all were from pit environments. Other genes encoding RPP genes such as *tet* (W) and *tet* (Q) had limited host ranges among the isolates obtained, with one notable *Bacteroides* sp., which harbored all three RPP genes detected, *tet* (M), (W), and (Q).

Among the genes encoding efflux, *tet* (B), (H), and (Z) were the most widely distributed among all the phyla represented, including Gram-negative and Gram-positive bacteria. The determinants *tet* (B), (C), (D), and (H) have previously been reported in association with Gram-negative organisms but were detected in both Gram-negative and Gram-positive organisms from both pit and soil environments. The tylosin resistance gene *erm* (B), a gene coding for methylase, was detected and

was distributed across all phylogenetic groups, however, most of the isolates exhibiting tylosin resistance did not harbor any of the four erm^R tested.

Many isolates were found to harbor multiple determinants for tetracycline resistance, of which 29% of pit-derived isolates were found to carry multiple tetracycline resistance determinants with as many as four different genes in one *Psychrobacter* sp. (Fig. 11.4). In manure-applied soils, 10% of the isolates carried multiple tet^R determinants, and none of the isolates from nonmanured soils exhibited multiple tet^R determinants. The major mechanisms of tetracycline resistance originated in the tetracycline-producing genus *Streptomyces* common to soil, and the number of new genera harboring known tet^R determinants has broadened presumably through genetic transfer events, with many found to contain more than one determinant (Roberts, 2005). Multiple tetracycline resistance genes have been found in *Streptomyces*, including clinically significant species, (Pang et al., 1994; Petković et al., 2006), yet despite its prevalence in soil, surprisingly, no tetracycline-resistant *Streptomyces* isolates were obtained from either pits or soils. This result may be attributed to the cultivation strategy used in our study, and perhaps a different isolation approach may have yielded *Streptomyces*.

The selection pressure within a concentrated environment containing drug residues, high cell numbers, and ABR genes such as an animal gut or waste storage compartment would likely be conducive to acquisition of multiple determinants. It is not known if the multiple genes harbored by a single cell are functional, nor is it known if one or more determinants are expressed at once or that these genes can be expressed in the host carrying the genes. For example, despite the detection of *tet* (B) in *Rhodococcus* sp. CHNTR42, this strain could not grow on 20 mg/L of tetracycline or chlortetracycline. Still, genes may be only weakly expressed in certain hosts and may provide adequate function in environments with low concentrations of antibiotics. Differential expression of genes may also be possible, conferring the ability of a species to express resistance depending on type of drug and drug concentrations present.

Concentrations of drug residues in soil following land application of manure are not well established, however, studies have shown tetracyclines can persist in biosolids following storage (Wu et al., 2009) along with the accumulation of tetracycline in soils from repeated land application of manure liquids (Hamscher et al., 2002). It is difficult to ascertain whether environmentally relevant concentrations of these compounds exert a selective pressure on bacteria, especially under in situ field conditions. Tetracycline concentrations ranging from 0.2 to 4 mg/L have been reported for manure-treated soils and lagoons (Campagnolo et al., 2002; Hamscher et al., 2002), and soil-bound residues have been shown to be biologically active (Halling-Sørensen et al., 2002; Aga et al., 2005; Chander et al., 2005). Tylosin, in contrast, breaks down rapidly in both manure and lagoon slurries (Kolz et al., 2005) and soil (Sassman et al., 2007), thus limiting its persistence. There may be selective advantage to possessing additional mechanisms of resistances to the same drug in environments containing higher drug concentrations. Perhaps selection of ABR traits due primarily to the effect of drug exposure is significant only in the concentrated manure environment of the pit, while following entry into soil, other mechanisms further leading to acquisition of resistances come to play.

Low levels of tetracycline have been shown to stimulate the transfer of tet^R genes (Torres et al., 1991; Showsh and Andrews, 1992; Clewell et al., 1995). Conjugal transfer of ABR determinants has been shown in both soil microcosms and native

soil environments (Lee and Stotzky, 1999; Andrews et al., 2004). Gotz and Smalla (1997) reported a 10-fold increase in plasmid transfer in soils receiving manure application relative to those that had not and suggested that elevated nutrient levels, often associated with repeated land application, may be a significant factor. Other soil conditions in the manure-applied soils, such as increased metal concentrations, greater cation exchange capacity, and organic matter sorption may also facilitate the acquisition of ABR determinants (Perron et al., 2004; Berg et al., 2005b; Kong et al., 2006).

Many of the isolates from this study that expressed phenotypic resistances did not appear to harbor any of the known determinants tested. These isolates, as well as others that harbored the targeted genes, may carry other tet^R or erm^R genes that were not tested such as *tet* (L) or the class of tetracycline resistance genes encoding enzymatic degradation such as *tet* (X) (Roberts, 2005). On the basis of several studies, determinants such as *tet* (M), *tet* (B), *tet* (Z), *tet* (L), and *erm* (B) may be genes that are more readily mobilized with potential for distribution across widespread bacterial phyla. It is not yet known what new, or as yet undiscovered, genes may explain the drug resistance abilities of many of the isolates.

The two swine farms used in this study were managed similarly with regard to antibiotic usage. The study conducted here evaluated only a single time point in decades-long histories of large animal production and manure land application. There were clearly differences in the ABR characteristics of bacteria obtained from pit environments in contrast to the soils at both sites. While the diversity of tet^R and erm^R determinants was lower in the nonmanured soils relative to the manured soils, the isolates exhibiting tetracycline resistance from the "unimpacted" soil environments occurred over a similar diverse range of phyla as the manured soils, and represented 17% of the total number of strains recovered. Finally, it is also noteworthy that several isolates representing disparate phyla and sources exhibited multiple drug resistances over a wide range of drug classes (Table 11.1). Interestingly, two closely related strains of *Chryseobacterium* exhibited very different drug resistance phenotypes. One isolate (*Chryseobacterium* sp. BBTR48) was from manured soil and was resistant to 4 of the 12 drugs, while the isolate from nonmanured soil (*Chryseobacterium* sp. BBCT12) was resistant to 10 of the 12 drugs. Such instances serve to demonstrate the possible myriad mechanisms of ABR occurrence, acquisition, and persistence that can occur in soil environments, with some that may not be directly linked to manure effects.

11.4 CONCLUDING REMARKS

Such cultivation- and molecular-based studies remain too few yet to speculate on the exact nature of ABR gene flow in natural environments. Ultimately, the original reservoirs of ABR genes are the natural ecosystems, where the genes have been involved in a number of functions not necessarily associated with resistance to antibiotics per se (Aminov, 2009). Due to continuous gene exchange between different ecological compartments, the subset of these genes was acquired and amplified in microbial ecosystems subjected to intensive antibiotic selection. This pool residing in commensal and pathogenic bacteria is reasonably well characterized. It is becoming more apparent, however, that the diversity of bacteria harboring ABR

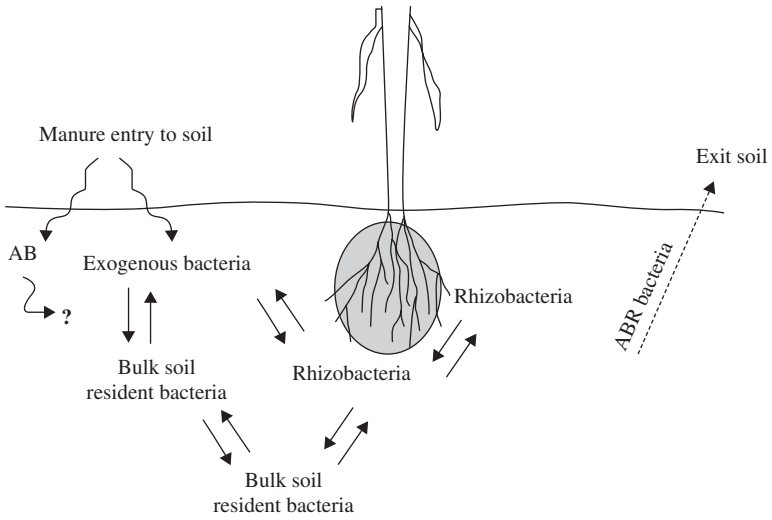

FIGURE 11.5 Schematic view showing potential for interactions among bacteria that may affect the dynamics of ABR development within a soil. An important point of entry into soil of nonnative (exogenous) ABR bacteria is shown as a consequent effect of manure land application. Antibiotic residues (AB) also enter the environment with unknown effects on the extant population of bacteria. The compartment of the rhizosphere (shaded) represents a unique environment of ABR development and exchange. Exit of ABR bacteria to other environments may occur via common mechanisms such as water runoff, leaching, and harvested plant associations.

genes may be much higher than imagined, even in environments with no known land application histories and many may exhibit multiple drug resistances. The unknown nature of determinants ABR in many isolates may also point to soil bacteria as important reservoirs of new antibiotic resistance genes (Riesenfeld et al., 2004; D'Costa et al., 2006). The types of bacterial species that became evident in cultivation studies also raise the importance of rhizobacteria in the overall molecular ecology of ABR genes in soil environments. As a soil subcompartment where gene transfer events may occur readily, rhizospheres have become apparent as significant reservoirs of resistance genes and gene movement (Fig. 11.5).

REFERENCES

Aga DS, O'Connor S, Ensley S, Payero JO, Snow D, Tarkalson D (2005). Determination of the persistence of tetracycline antibiotics and their degradates in manure-amended soil using enzyme-linked immunosorbent assay and liquid chromatography-mass spectrometry. *J Agric Food Chem* 53:7165–7171.

Agersø Y, Sandvang D (2005). Class 1 integrons and tetracycline resistance genes in *Alcaligenes*, *Arthrobacter*, and *Pseudomonas* spp. isolated from pigsties and manured soil. *Appl Environ Microbiol* 71:7941–7947.

Aminov RI (2009). The role of antibiotics and antibiotic resistance in nature. *Environ Microbiol* 11:2970–2988.

Aminov RI, Garrigues-Jeanjean N, Mackie RI (2001). Molecular ecology of tetracycline resistance: Development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. *Appl Environ Microbiol* 67:22–32.

Aminov RI, Chee-Sanford JC, Garrigues N, Teferedegne B, Krapac IJ, White BA, Mackie RI (2002). Development, validation, and application of PCR primers for detection of tetracycline efflux genes of gram-negative bacteria. *Appl Environ Microbiol* 68:1786–1793.

Andrews RE, Johnson WS, Guard AR, Marvin JD (2004). Survival of enterococci and Tn916-like conjugative transposons in soil. *Can J Microbiol* 50:957–966.

Berg G, Eberl L, Hartmann A (2005a). The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. *Environ Microbiol* 7:1673–1685.

Berg J, Tom-Petersen A, Nybroe O (2005b). Copper amendment of agricultural soil selects for bacterial antibiotic resistance in the field. *Lett Appl Microbiol* 40:146–151.

Boes J, Alban L, Bagger J, Møgelmose V, Baggesen DL, Olsen JE (2005). Survival of *Escherichia coli* and *Salmonella typhimurium* in slurry applied to clay soil on a Danish swine farm. *Preventative Vet Med* 69:213–228.

Bolton DJ, Byrne CM, Sheridan JJ, McDowell DA, Blair IS (1999). The survival characteristics of a non-toxigenic strain of *Escherichia coli* O157:H7. *J Appl Microbiol* 86:407–411.

Boxall ABA, Fogg LA, Blackwell PA, Kay P, Pemberton EJ, Croxford A (2004). Veterinary medicines in the environment. *Rev Environ Contam Toxicol* 180:1–91.

Campagnolo ER, Johnson KR, Karpati A, Rubin CS, Kolpin DW, Meyer MT, Esteban E, Currier RW, Smith K, Thu KM, McGeehin M (2002). Antimicrobial residues in animal water and water resources proximal to large-scale swine and poultry feeding operations. *Sci Total Environ* 299:89–95.

Chander Y, Kumar K, Goyal SM, Gupta SC (2005). Antibacterial activity of soil-bound antibiotics. *J Environ Qual* 34:1952–1957.

Chandler D, Farran I, Craven J (1981). Persistence and distribution of pollution indicator bacteria on land used for disposal of piggery effluent. *Appl Environ Microbiol* 42:453–460.

Chee-Sanford JC, Aminov RI, Krapac IJ, Garrigues-Jeanjean N, Mackie RI (2001). Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Appl Environ Microbiol* 67:1494–1502.

Chee-Sanford JC, Mackie RI, Koike S, Krapac IG, Lin Y-F, Yannarell AC, Maxwell S, Aminov RI (2009). Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. *J Environ Qual* 38:1086–1108.

Chopra I, Roberts M (2001). Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev* 65:232–260.

Clewell DB, Flannagan SE, Jaworski DD (1995). Unconstrained bacterial promiscuity: The Tn916-Tn1545 family of conjugative transposons. *Trends Microbiol* 3:229–236.

Cools D, Merckx R, Vlassak K, Verhaegen J (2001). Survival of *E. coli* and *Enterococcus* spp. derived from pig slurry in soils of different texture. *Appl Soil Ecol* 17:53–62.

Cotta MA, Whitehead TR, Zeltwanger RL (2003). Isolation, characterization and comparison of bacteria from swine faeces and manure storage pits. *Environ Microbiol* 5:737–745.

Davies CM, Long JA, Donald M, Ashbolt NJ (1995). Survival of fecal microorganisms in marine and freshwater sediments. *Appl Environ Microbiol* 61:1888–1896.

D'Costa VM, McGrann KM, Hughes DW, Wright GD (2006). Sampling the antibiotic resistome. *Science* 311:374–377.

de Freitas JR, Schoenau JJ, Boyetchko SM, Cyrenne SA (2003). Soil microbial populations, community composition, and activity as affected by repeated applications of hog and cattle manure in eastern Saskatchewan. *Can J Microbiol* 49:538–548.

Ding H, Hynes MF (2009). Plasmid transfer systems in the rhizobia. *Can J Microbiol* 55: 917–927.

Eady EA, Coates P, Ross JI, Ratyal AH, Cove JH (2000). Antibiotic resistance patterns of aerobic coryneforms and furazolidone-resistant Gram-positive cocci from the skin surface of the human axilla and fourth toe cleft. *J Antimicrob Chemother* 46:205–213.

Elmund GK, Morrison SM, Grant DW, Nevins MP (1971). Role of excreted chlortetracycline in modifying the decomposition process of feedlot waste. *Bull Environ Contam Toxicol* 6:129.

Fajardo A, Martínez-Martín N, Mercadillo M, Galán JC, Ghysels B, Matthijs S, Cornelis P, Wiehlmann L, Tümmler B, Baquero F, Martínez JL (2008). The neglected intrinsic resistome of bacterial pathogens. *PLoS ONE* 3:e1619.

Fries MR, Zhou J, Chee-Sanford J, Tiedje JM (1994). Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. *Appl Environ Microbiol* 60:2802–2810.

Frostegård A, Petersen S, Bååth E, Nielsen T (1997). Dynamics of a microbial community associated with manure hot spots as revealed by phospholipid fatty acid analyses. *Appl Environ Microbiol* 63:2224–2231.

Ghosh S, LaPara TM (2007). The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. *ISME J* 1:191–203.

Gomez-Lus R (1998). Evolution of bacterial resistance to antibiotics during the last three decades. *Int Microbiol* 1:279–284.

Gotz A, Smalla K (1997). Manure enhances plasmid mobilization and survival of *Pseudomonas putida* introduced into field soil. *Appl Environ Microbiol* 63:1980–1986.

Graham JP, Boland JJ, Silbergeld E (2007). Growth promoting antibiotic in food animal production: An economic analysis. *Public Health Rep* 122:79–87.

Guan TY, Holley RA (2003). Pathogen survival in swine manure environments and transmission of human enteric illness-A review. *J Environ Qual* 32:383–392.

Gustafson RH, Bowen RE (1997). Antibiotic use in animal agriculture. *J Appl Microbiol* 83:531–541.

Haack B, Andrews RE (2000). Isolation of Tn*916*-like conjugal elements from swine lot manure. *Can J Microbiol* 46:542–549.

Halling-Sørensen B, Nielsen SN, Lanzky PF, Ingerslev F, Holten Lützhøft HC, Jørgensen SE (1998). Occurrence, fate and effects of pharmaceutical substances in the environment—A review. *Chemosphere* 36:357--393.

Halling-Sørensen B, Sengeløv G, Tjørnelund J (2002). Toxicity of tetracyclines and tetracycline degradation products to environmentally relevant bacteria, including selected tetracycline-resistant bacteria. *Arch Environ Contam Toxicol* 42:263–271.

Hamscher G, Sczesny S, Höper H, Nau H (2002). Determination of persistent tetracycline residues in soil fertilized with liquid manure by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. *Anal Chem* 74:1509–1518.

Hartmann M, Fliessbach A, Oberholzer H-R, Widmer F (2006). Ranking the magnitude of crop and farming system effects on soil microbial biomass and genetic structure of bacterial communities. *FEMS Microbiol Ecol* 57:378–388.

Hartz A, Cuvelier M, Nowosielski K, Bonilla TD, Green M, Esiobu N, McCorquodale DS, Rogerson A (2008). Survival potential of *Escherichia coli* and Enterococci in subtropical beach sand: Implications for water quality managers. *J Environ Qual* 37:898–905.

Hileman B (2001). Furor over animal antibiotic use: Rising resistance to microbes in humans leads FDA to propose antibiotic livestock restrictions. *Chem Eng News* 79:47–52.

Hund-Rinke K, Simon M, Lukow T (2004). Effects of tetracycline on the soil microflora: Function, diversity, resistance. *J Soil Sediment* 4:11–16.

Isaacson RE, Torrence ME (2002). *The Role of Antibiotics in Agriculture*. American Academy of Microbiology, Washington, DC.

Jensen LB, Agersø Y, Sengeløv G (2002). Presence of *erm* genes among macrolide-resistant gram-positive bacteria isolated from Danish farm soil. *Environ Int* 28:487–491.

Jiang X, Morgan J, Doyle MP (2002). Fate of *Escherichia coli* O157:H7 in manure-amended soil. *Appl Environ Microbiol* 68:2605–2609.

Khachatourians GG (1998). Agricultural use of antibiotics and the evolution and transfer of antibiotic-resistant bacteria. *Can Med Assoc J* 159:1129–1136.

Kibbey HJ, Hagedorn C, McCoy EL (1978). Use of fecal streptococci as indicators of pollution in soil. *Appl Environ Microbiol* 35:711–717.

Kim SR, Nonaka L, Suzuki S (2004). Occurrence of tetracycline resistance genes *tet*(M) and *tet*(S) in bacteria from marine aquaculture sites. *FEMS Microbiol Lett* 237:147–156.

Koerner RJ, Goodfellow M, Jones AL (2009). The genus *Dietzia*: A new home for some known and emerging opportunist pathogens. *FEMS Immunol Med Microbiol* 55:296–305.

Koike S, Aminov RI, Yannarell AC, Gans H, Krapac IG, Chee-Sanford JC, Mackie RI (2009). Molecular ecology of macrolide-lincosamide-streptogramin B methylases in waste lagoons and subsurface waters associated with swine production. *Microb Ecol* 59(3):487–489.

Kolz AC, Moorman TB, Ong SK, Scoggin KD, Douglass EA (2005). Degradation and metabolite production of tylosin in anaerobic and aerobic swine-manure lagoons. *Water Environ Res* 77:49–56.

Kong WD, Zhu YG, Fu BJ, Marschner P, He JZ (2006). The veterinary antibiotic oxytetracycline and Cu influence functional diversity of the soil microbial community. *Environ Pollut* 143:129–137.

Kümmerer K (2004). Resistance in the environment. *J Antimicrob Chemother* 54:311–320.

Kümmerer K, Al-Ahmad A, Mersch-Sundermann V (2000). Biodegradability of some antibiotics, elimination of the genotoxicity and affection of waste water bacteria in a simple test. *Chemosphere* 40:701–710.

Lee G-H, Stotzky G (1999). Transformation and survival of donor, recipient, and transformants of *Bacillus subtilis in vitro* and in soil. *Soil Biol Biochem* 31:1499–1508.

Levy SB (1998). The challenge of antibiotic resistance. *Sci Am* 278:46–53.

Madden TL, Tatusov RL, Zhang J (1996). Applications of network BLAST server. *Methods Enzymol* 266:131–141.

Martínková L, Uhnáková B, Pátek M, Nešvera J, Křen V (2009). Biodegradation potential of the genus *Rhodococcus*. *Environ Int* 35:162–177.

McEwen SA, Fedorka-Cray PJ (2002). Antimicrobial use and resistance in animals. *Clin Infect Dis* 34:S93–S106.

Miranda CD, Kehrenberg C, Ulep C, Schwarz S, Roberts MC (2003). Diversity of tetracycline resistance genes in bacteria from Chilean salmon farms. *Antimicrob Agents Chemother* 47:883–888.

Nikaido H (1996). Outer membrane. In Neidhardt FC, Curtiss III, R, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, and Umbarger HE (Eds.), *Escherichia coli and Salmonella: Cellular and Molecular Biology*. ASM Press, Washington DC, pp. 29–47.

Nikolakopoulou T, Egan S, Van Overbeek L, Guillaume G, Heuer H, Wellington E, Van Elsas J, Collard J-M, Smalla K, Karagouni A (2005). PCR detection of oxytetracycline resistance genes *otr* (A) and *otr* (B) in tetracycline-resistant streptomycete isolates from diverse habitats. *Curr Microbiol* 51:211–216.

Normark BH, Normark S (2002). Evolution and spread of antibiotic resistance. *J Int Med* 252:91–106.

Onan LJ, LaPara TM (2003). Tylosin-resistant bacteria cultivated from agricultural soil. *FEMS Microbiol Lett* 220:15–20.

Pang Y, Brown BA, Steingrube VA, Wallace RJ, Roberts MC (1994). Tetracycline resistance determinants in *Mycobacterium* and *Streptomyces* species. *Antimicrob Agents Chemother* 38:1408–1412.

Perron K, Caille O, Rossier C, Van Delden C, Dumas J-L Kohler T (2004). CzcR-CzcS, a two-component system involved in heavy metal and carbapenem resistance in *Pseudomonas aeruginosa*. *J Biol Chem* 279:8761–8768.

Petković H, Cullum J, Daslav Hranueli D, Hunter IS, Perić-Concha N, Pigac J, Thamchaipenet A, Vujaklija D, Long PF (2006). Genetics of *Streptomyces rimosus*, the oxytetracycline producer. *Microbiol Mol Biol Rev* 70(3):704–728.

Plaza C, Hernandez D, Fernandez JM, Polo A (2006). Long-term effects of amendment with liquid swine manure on proton binding behavior of soil humic substances. *Chemosphere* 8:1321–1329.

Poole K, Srikumar R (2001). Multidrug efflux in *Pseudomonas aeruginosa*: Components, mechanisms and clinical significance. *Curr Topics Med Chem* 1:59–71.

Riesenfeld CS, Goodman RM, Handelsman, J (2004). Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. *Environ Microbiol* 6:981–989.

Roberts MC (2005). Update on acquired tetracycline resistance genes. *FEMS Microbiol Lett* 245:195–203.

Roberts AP, Mullany P (2009). A modular master on the move: The Tn*916* family of mobile genetic elements. *Trends Microbiol* 17:251–258.

Salyers AA, Gupta A, Wang Y (2004). Human intestinal bacteria as reservoirs for antibiotic resistance genes. *Trends Microbiol* 12:412–416.

Sassman SA, Sarmah AK, Lee LS (2007). Sorption of tylosin A, D, and A-aldol and degradation of tylosin in soils. *Environ Toxicol Chem* 26:1629–1635.

Schmidt AS, Bruun MS, Dalsgaard I, Larsen JL (2001). Incidence, distribution, and spread of tetracycline resistance determinants and integron-associated antibiotic resistance genes among motile aeromonads from a fish farming environment. *Appl Environ Microbiol* 67:5675–5682.

Schmitt H, Stoob K, Hamscher G, Smit E, Seinen W (2006). Tetracyclines and tetracycline resistance in agricultural soils: Microcosm and field studies. *Microb Ecol* 3:267–276.

Séveno NA, Kallifidas D, Smalla K, van Elsas, JD, Collard J-M, Karagouni AD, Wellington EMH (2002). Occurrence and reservoirs of antibiotic resistance genes in the environment. *Rev Med Microbiol* 13:15–28.

Shea KM (2004). Nontherapeutic use of antimicrobial agents in animal agriculture: Implications for pediatrics. *Pediatrics* 114:862–868.

Showsh SA, Andrews RE (1992). Tetracycline enhances Tn916-mediated conjugal transfer. *Plasmid* 28:213–224.

Siddique MT, Robinson JS (2003). Phosphorus sorption and availability in soils amended with animal manures and sewage sludge. *J Environ Qual* 32:1114–1121.

Smith MS, Yang RK, Knapp CW, Niu Y, Peak N, Hanfelt MM, Galland JC, Graham DW (2004). Quantification of tetracycline resistance genes in feedlot lagoons by real-time PCR. *Appl Environ Microbiol* 70:7372–7377.

Sørum H, Sunde M (2001). Resistance to antibiotics in the normal flora of animals. *Vet Res* 32:227–241.

Stoddard CS, Coyne MS, Grove JH (1998). Fecal bacterial survival and infiltration through a shallow agricultural soil: Timing and tillage effects. *J Environ Qual* 27:1516–1523.

Sun HY, Deng SP, Raun WR (2004). Bacterial community structure and diversity in a century-old manure-treated agroecosystem. *Appl Environ Microbiol* 70:5868–5874.

Torres OR, Korman RZ, Zahler SA, Dummy GM (1991). The conjugative transposon Tn925: Enhancement of conjugal transfer by tetracycline in *Enterococcus faecalis* and mobilization of chromosomal genes in *Bacillus subtilis* and *E. faecalis*. *Mol Gen Genetics* 225:395–400.

U.S. Department of Agriculture (USDA) (2001). Part I: Reference of swine health and management in the United States, 2000 N338.0801. National Animal Health Monitoring System, Washington, DC.

U.S. General Accounting Office (USGAO) (1999). *The Agricultural Use of Antibiotics and Its Implications for Human Health*. General Accounting Office, Washington, DC. Publication no. GAO-RCED 99–74.

Walsh C (2003). *Antibiotics: Actions, Origins, Resistance*. ASM Press, Washington, DC.

Wienhold BJ (2005). Changes in soil attributes following low phosphorus swine slurry application to no-tillage sorghum. *Soil Sci Soc Am J* 69:206–214.

Witte W (2001). Selective pressure by antibiotic use in livestock. *Int J Antimicrob Agents* 16:19–24.

Wu C, Spongberg AL, Witter JD (2009). Sorption and biodegradation of selected antibiotics in biosolids. *J Environ Sci Health Part A 2009* 44:454–461.

Zeph LR, Onaga MA, Stotzky G (1988). Transduction of *Escherichia coli* by bacteriophage P1 in soil. *Appl Environ Microbiol* 54:1731–1737.

12

ANTIMICROBIAL-RESISTANT INDICATOR BACTERIA IN MANURE AND THE TRACKING OF INDICATOR RESISTANCE GENES

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Fresh manure contains high amounts of the indicator bacteria *Escherichia coli* and *Enterococcus* spp. (Peu et al., 2006). Despite processes of self-hygienization such indicator bacteria are also highly concentrated in the manure, which is spread on the fields (Cotta et al., 2003; Peu et al., 2006). Livestock are therapeutically treated with antibiotics in the case of bacterial infection and subsequently excrete antibiotic-resistant bacteria (Han et al., 2009; Singer et al., 2008; Ghosh and Lapara, 2007, Levy et al., 1976a). We investigated a representative number of manure samples for contents of antibiotics (Chapter 16), heavy metals, and antimicrobial-resistant bacteria in order to detect factors that are associated with antimicrobial resistance of indicator bacteria (*E. coli* and enterococci) in manure. Antimicrobial resistance of these indicator bacteria was positively associated with the detection of tetracyclines, sulfonamides, zinc, and copper (Tables 1 and 2, Hölzel et al., 2010a, and unpublished results); moreover, antimicrobial resistance was negatively associated with the detection of lead and mercury. Besides the obvious—and expected—correlation of sulfonamide detection with sulfhonamide resistance and of tetracycline detection with doxycycline resistance, we want to emphasize the findings regarding chloramphenicol resistance. Apart from doxycycline resistance, no other investigated antibiotic resistance was associated with the tetracycline content as much as was chloramphenicol resistance (Table 12.1). In the European Union, chloramphenicol has been abolished for the treatment of animals since 1994 (EC No. 1430/94).

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TABLE 12.1 Wald Chi-Square (p Value) of Different Factors within a Linear Model Assessing the Association of These Factors with the Antimicrobial Resistance of *E. coli* ($n = 613$) Isolated from Liquid Pig Manure

	Antimicrobial Resistance against										
Factor	Ampicllin	Amoxicillin + Clavulanate	Cefaclor	Chloramphenicole	Cotrimoxazole	Doxycycline	Neomycin	Piperacillin	Spectinomycin	Streptomycin	Multiresistancea[a]
Copper (mg/kg)[b]	6.130	5.112	7,581	0.167	1.422	1.966	3.124	4.267	1.248	1.103	0.581
	(**0.013**)	(**0.024**)	(0.056)	(0.683)	(0.233)	(0.161)	(0.077)	(**0.039**)	(0.264)	(0.294)	(0.446)
Zinc (mg/kg)[b]	7.010	3.218	n.d.	2.577	1.021	19.927	2.861	11.407	0.406	2.383	13.805
	(0.072)	(0.359)	[c]	(0.461)	(0.796)	(**< 0.001**)	(0.414)	(**0.010**)	(0.939)	(0.497)	(**0.003**)
Lead (mg/kg)[b]	8.306	8.021	2,629	5.091	1.091	0.158	5.476	11.086	1.743	0.078	3.092
	(**0.016**)	(**0.018**)	(0.105)	(0.078)	(0.580)	(0.924)	(0.065)	(**0.004**)	(0.418)	(0.962)	(0.293)
Mercury (mg/kg)[b]	12.302	9.041	n.d. [c]	14.974	4.877	9.023	9.191	20.496	7.907	12.353	23.796
	(**0.002**)	(**0.011**)		(**0.001**)	(0.087)	(**0.011**)	(**0.010**)	(**< 0.001**)	(**0.019**)	(**0.002**)	(**< 0.001**)
Concentration of tetracyclines[d]	9.875	3,362	7,74	18.933	6.508	43.111	n.d.	4.776	6,786	15.485	49.948
	(**0.020**)	(0.339)	(**0.021**)	(**< 0.001**)	(0.089)	(**< 0.001**)	—[c]	(0.189)	(0.079)	(**0.001**)	(**< 0.001**)
Presence of sulfonamides[e]	1.085	0.504	n.d.[c]	2.145	14.172	2.324	n.d.[c]	0.331	2.414	0.608	5.502
	(0.297)	(0.478)		(0.143)	(**< 0.001**)	(0.127)		(0.565)	(0.120)	(0.435)	(**0.019**)
Time of sampling[f]	2.152	0.783	0.702	2.285	1.819	3.081	0.968	0.145	22.604	16.675	5.713
	0.142	(0.376)	(0.402)	(0.131)	(0.177)	(0.079)	(0.325)	(0.703)	(**<0.001**)	(**< 0.001**)	(**0.017**)

[a]Resistance to substances from different antimicrobial classes: none, one, two to three, or more than three. Values in **bold** indicate significant differences, $p < 0.05$.
[b]Manure, wet weight.
[c]Exluded from the model, as the omnibus test value indicated that the model including this factor was not superior to a model with fixed terms only (results of the omnibus test for cefaclor: likelihood chi-square value = 16.214, $p = 0.238$; for neomycin: chi-square = 21.541, $p = 0.063$).
[d] < 0.1 mg/kg, 0.1–1 mg/kg, 1–4 mg/kg, > 4 mg/kg.
[e]Negative/positive, detection limit 0.05 mg/kg.
[f]Autumn/spring.

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However, unlike avoparcin/vancomycin resistance, which diminished clearly after the ban of avoparcin (Aarestrup et al., 2001), chloramphenicol resistance is still very common in enterococci of pigs. In 2002/2003, 12.3% of *Enterococcus faecalis* from the manure of German pigs were chloramphenicol resistant (Hölzel et al., 2010b), whereas in the feces of diseased German pigs in 2005/2006 28.3% chloramphenicol-resistant *E. faecalis* isolates were found (S. Hörmansdorfer, G. Mölle, and P. Preikschat, personal communication). The latter finding was reproduced in 2007/2008 with 27.3% chloramphenicol-resistant *E. faecalis* from the same kind of material (C. Hölzel, M. Bischoff, G. Mölle, and P. Preikschat, unpublished results). All of these isolates are susceptible to florfenicol (a fenicole still in use in pigs).Thus in this case, it is improbable that florfenicole drives the maintenance of chloramphenicol resistance. Instead, our investigations indicate that tetracyclines select strains that are co-resistant to chloramphenicol. Resistance to tetracycline encoded by *tet*M and to chloramphenicol encoded by *cat* genes appears to be genetically linked on the pneumococcal transposon Tn5253 (Ayoubi et al., 1991), on the listerial plasmid pWDB100 (Hadorn et al., 1993), or on the enterococcal plasmid pICC8 (Straut et al., 1996), for example.

12.1 SELECTION OF TETRACYCLINE RESISTANCE GENES. PART 1: THE TRIAL

In fresh manure samples of pigs raised without any antibiotic contact, a basal *tet*M level of 10^9 copies per gram of manure was found by quantitative Polymerase chain reaction (PCR), corrected by the recovery rate of 22%. The corresponding basal levels of *tet*O and *tet*B were 10^8 and 10^7, respectively.Thus, among the tetracycline resistance genes *tet*M, *tet*O, and *tet*B, *tet*M is the most common tetracycline resistance gene in the bacterial community of liquid pig manure. The measured basal *tet* gene concentrations are one order of magnitude below the concentrations in pig manure collected during chlortetracycline therapy and the preslaughter interval (Table 12.2), indicating that a considerable part of the bacterial flora of pig manure either intrinsically possess chromosomal *tet* genes or maintains its mobile *tet* gene, containing elements without a need for any selective force. In an in vitro experiment, *Enterococcus* isolates from the pig manure samples maintained their *tet*L, *tet*M, or *tet*O genes at a steady level [measured as copies per 100 ng deoxyribonucleic acid (DNA)] in an antibiotic-free environment during the whole experimentation period of 500 generations (M. Bischoff and C. Hölzel, unpublished results). This is less surprising than it might seem at a first glance, since the isolates did not compete with susceptible strains. We know from the literature that the initial presence of competing susceptible isolates is crucial for any restoration of an antibiotic-susceptible population (Levy, 1997). De Gelder et al. (2004) observed a tetracycline-sensitive subpopulation growing from 0.1 to 7% in antibiotic-free medium within 500 generations. However, only 1 of 7086 initially resistant clones had lost its causative multiresistance plasmid, while the rest of the secondary-susceptible subpopulation lost resistance by a recombination event beneath the *tet* operon, but kept the plasmid (de Gelder et al., 2004). Under model conditions (subpopulations of 99.9% resistant and 0.1% susceptible strains), it would take two decades to lose the tetracycline resistance—but not the encoding gene—by mutation, and ages to lose the entire

TABLE 12.2 Prevalence of Antimicrobial Resistance in Indicator Bacteria Isolated from Manure with Different Tetracycline Concentrations

		Level of TET Contamination (mg/kg)[a]			
Species	Antibiotic Agent	Below Detection Limit Resistance Rates[a] (%)	≥ 0,1 ≤ 1	> 1 ≤ 4	> 4
E. coli ($n = 613$)		$n = 276$	$n = 169$	$n = 90$	$n = 78$
	Ampicillin	13.8	17.2	25.6	35.9
	Amoxicillin + clavulanate	8.0	13.0	12.2	16.7
	Piperacillin	9.1	10.1	17.8	20.5
	Streptomycin	51.8	54.4	64.4	69.2
	Spectinomycin	39.1	41.4	38.9	44.9
	Doxycycline	40.9	55.6	75.6	78.2
	Chloramphenicole	4.0	6.5	8.9	19.2
	Sulfamethoxazole + trimethoprim	11.2	16.0	23.3	26.9
	Cefaclor	4.0	4.7	5.6	11.5
E. faecalis ($n = 387$)		$n = 144$	$n = 107$	$n = 62$	$n = 68$
	Doxycycline	66.7	85.0	87.1	91.2
	Erythromycin	50.7	54.2	58.1	51.5
	Streptomycin (high level)	29.9	32.7	51.6	51.5
	Chloramphenicol	9.0	12.1	19.4	13.2
	Fosfomycin	11.1	10.3	6.5	1.5
	Rifampicin	56.9	55.1	46.8	42.6
	Tylosin	39.6	51.4	53.2	51.5
E. faecium ($n = 183$)		$n = 89$	$n = 52$	$n = 23$	$n = 18$
	Imipenem	6.7	19.2	30.4	22.2
	Doxycycline	11.2	40.4	52.2	38.9
	Ciprofloxacin	16.9	23.1	21.7	38.9
	Synercid	4.5	19.2	8.7	5.6
	Tylosin	6.7	21.2	17.4	22.2

[a]For breakpoints and concentration ranges see Hölzel et al. (2010a).

encoding genetic element, unless there would be selective disadvantage of carrying the gene or expressing the resistance phenotype (de Gelder et al., 2004). However, we have reason to assume that there is no such selective disadvantage in the case of most tetracycline resistance determinants since bacteria have evolved with these resistance determinants for ages. Bacteria that evolved with a *tet-cat* resistance plasmid for only 500 generations were superior to the nonevolved, plasmid-positive control, but also to bacteria that lost the *tet-cat* determinant spontaneously (Bouma and Lenski, 1988). Interestingly, subsequent experiments showed that this superiority was attributed only to the *tet*, not to the *cat* determinant (Lenski, 1998).

Though the basal *tet* gene content of liquid pig manure is already high, *tet*M, *tet*O, and *tet*B are further selectable by tetracycline treatment (Table 12.3). During

TABLE 12.3 Gene Concentrations (log copies/g) of Pig Manure Samples Collected in Three Different Experimental Designs

	Mean *tet*M		Mean *tet*O		Mean *tet*B	
	CTC[a]	Free	CTC[a]	Free	CTC[a]	Free
Trial	**10.2**	**9.7**	**9.2**	**8.5**	**8.1**	**7.1**
A	9.9	9.4	9.4	8.5	7.8	6.1
B	10.2	9.6	8.9	8.6	8.2	7.7
C	10.4	10.0	9.2	8.3	8.2	7.5

[a]Chlortetracycline (CTC): pigs fed 30 mg CTC/kg body weight for 21 days, housing see below. Free: control group sharing all housing and feeding conditions except CTC therapy. A, B: manure produced in two different years by 97 pigs (weight at the beginning: 8 kg) kept in a stable and fed CTC (concentrations see above). C: manure produced by 5 pigs (weight at the beginning: 46.4 kg) kept in metabolism crates and CTC for 30 days. Manure contained 46.7 (A), 9.1 (B), and 168.0 (C) mg CTC/kg ww.

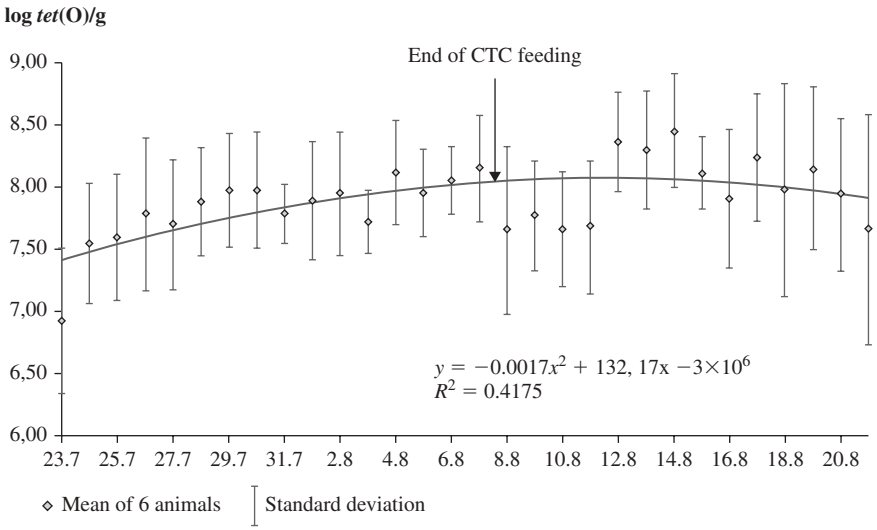

FIGURE 12.1 Shedding of *tet*O in the feces of pigs during and after feeding of 20 mg CTC / kg bodyweight.

antibiotic therapy, mean *tet*O contents in the feces increased from 7.57 log copies *tet*O per gram at the beginning to a maximum of 9.60 log copies per gram on day 9 of the preslaughter interval (Fig. 12.1). Changes in *tet*M and *tet*B contents were less pronounced: in general, mean log *tet*M copies per gram increased from 8.52 to a maximum of 9.45 on day 11 of the treatment period, and mean log copies *tet*B per gram increased from 6.49 to a maximum of 7.45 on day 6 of the treatment period. Interestingly, when we included a control group in a second study, *tet*M and *tet*O shedding also increased in this control group, with a certain delay and a mean delta to the treatment group of 1×10^1 for *tet*M (Fig. 12.2) and of 3×10^0 for *tet*O. The control group shared the same stable but had no direct contact with the treatment group since all pigs were separated in individual metabolism crates. The most probable explanation for this apparent parallelism seems a cross contamination

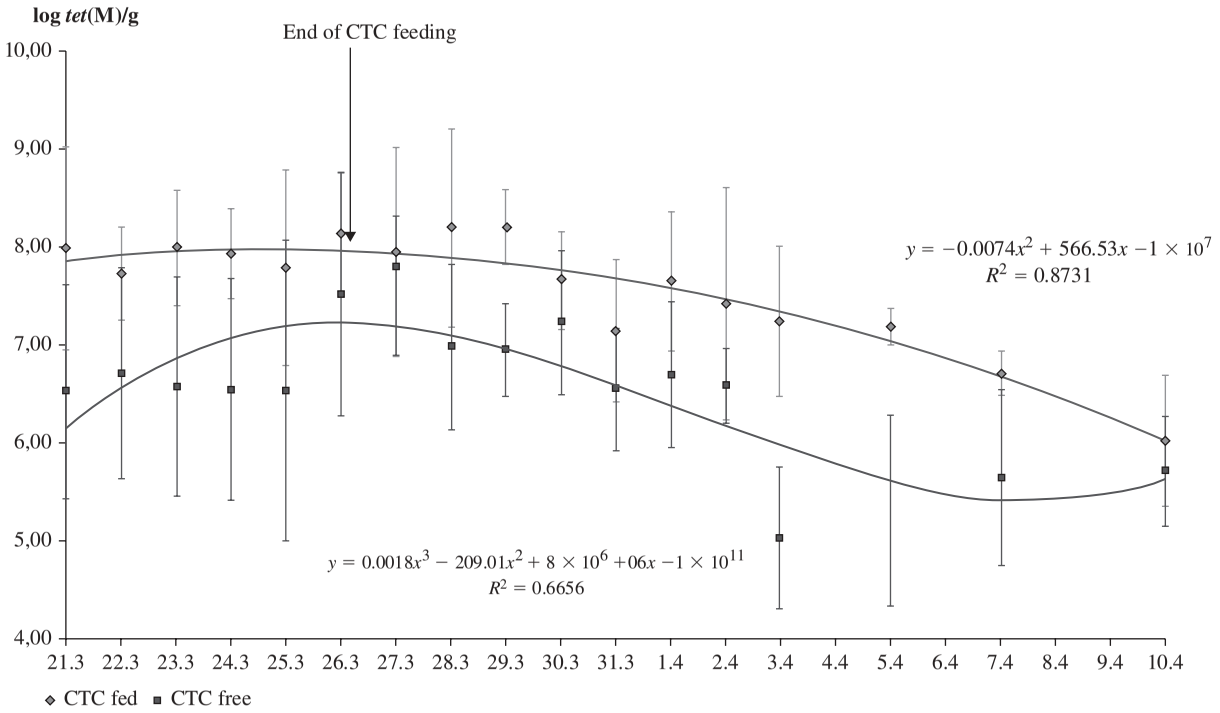

FIGURE 12.2 Shedding of *tet*M in the feces of pigs during and after feeding of 20 mg CTC/kg body weight. CTC-fed: treatment group. CTC-free: control group sharing all housing and feeding conditions (except CTC therapy) without direct contact to the treatment group.

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with tetracycline resistant bacteria—for example, by houseflies, which are common mechanical vectors of antimicrobial-resistant bacteria (Macovei et al., 2008), since the tetracycline treatment of the treatment group was the only variable factor within a comparatively standardized experimental situation (use of metabolism crates, standardized feeding, several weeks of adaptation to all experimental conditions except the tetracycline treatment). The transmission of resistant strains between animals of separated cages has been proven before (Levy et al., 1976b).

Hence, standardized pig manure contains high amounts of tetracycline resistance genes, and the concentration of these genes increases further if selected by antibiotic therapy. These observations raise one important question: What is the situation in the field and the fate of these shed resistance genes in the environment?

12.2 SELECTION OF *tet* GENES BY TETRACYCLINES. PART 2: THE FIELD

Tetracycline resistance genes *tet*M, *tet*O, and *tet*B were detected by quantitative PCR in all of 121 investigated manure samples within a monitoring project (Hölzel et al., 2010a; Chapter 16). Concentrations were 7.08–9.7 log copies of *tet*M, 5.41–8.66 log copies of *tet*O, and 4.31–8.18 log copies of *tet*B, each per gram of manure. The general storage practice of manure on farms includes continuous influx of fresh manure from the stable. Thus, the manure tanks always contained a certain portion of relatively fresh manure, despite the fact that the manure was collected for up to 12 months prior to its spreading to the fields. However, participating farmers were requested to automatically stir the contents of the manure tanks prior to the sampling, thereby minimizing the bias introduced by the addition of fresh fractions. Additionally, these manures—with their combination of older or fresher fractions—were subsequently spread to the fields and were, therefore, the adequate material for any attempt to assess the real risk posed by tetracycline resistance genes in manure.

As a cursory trial, we used the standardized CTC-containing manure samples B and C (see Table 12.3) to amend farmland and grassland with no known history of prior fertilization with pig manure. Chemical characteristics for the soils are given in Table 12.4. Both manures used in this trial (CTC-B, CTC-C) had similar contents of *tet* genes (Table 12.3). However, while manure B had a CTC content of 46.7 mg/kg, the CTC concentration in manure C was only 9.1 mg/kg. Interestingly, one week after fertilization (15 m^3/ha) followed by the seeding of corn, the 1- to 10-cm soil layer of farmland from trial B—which was analytically negative for *tet*M prior to the manuring—was still negative, while in trial C the *tet*M concentration of the same layer of similar farmland increased by the factor 10^5 within 8 days after

TABLE 12.4 Soil Characteristics of Three Soils Amended with CTC Containing Manure

Area	pH	Clay (%)	Silt (%)	Sand (%)	N_t (%)	Corg (%)	$CaCO_3$ (%)	Precipitation (mm)
Farmland I	7.2	39	52	9	0.44	3.33	56	739
Farmland II	7.3	28	64	8	0.52	5.59	31	
Grassland	6.8	31	40	29	0.53	4.96	n.d.	1265

the manuring. This second farmland area was already weakly positive for *tet*M prior to the manuring (29.1 m^3/ha). However, *tet*M could no longer be detected after day 22 postmanuring. Grassland that was amended with the same charge and amount of CTC-manure C (and was also weakly positive for *tet*M prior to the investigation) had a more moderate increase (factor 10^2) after manuring, but the *tet*M genes remained detectable for $\geq 51 < 89$ days. This period was shorter than the persistence of *tet*M in soil reported by Agersø et al. (2006), however, due to a low recovery rate (0.3–3 %) for *tet*M genes in soil, our limit of detection was quite high (10^3–10^4 copies *tet*M per gram of manure).

We did not determine whether the *tet*M genes occurred (i) as free DNA, (ii) bound to their original carriers, either from manure or from soil, or (iii) incorporated into new carriers. With regard to the latter, we should remember that the deposited CTC concentrations were higher in trial B while both the deposited *tet*M concentrations and the deposited bacterial load were higher in trial C. Thus, the third possibility seems less probable, as the selective conditions (= higher—but still subinhibitory—tetracycline concentrations per gram of soil) for the incorporation of *tet* genes would have been more favorable in trial B, in which we could not detect *tet* genes, compared to trial C, in which we did. The first two explanations for an increase of *tet*M after manuring—detection of free DNA or clonal spread of original carriers—are connected to a comparatively low risk, considering the fact that the *tet*M genes vanished within the investigation period, thus indicating that free DNA was not transformed into soil bacteria, and *tet*M carriers from pig manure were not able to survive in the soil. However, the fact that *tet*M was only detectable in trial C, where it was already detectable before manuring, might indicate that the application of CTC containing manure selected *tet*M carriers within the soil bacteria, which might stay within the population, albeit they decrease below the detection limit—just waiting for the next selection event.

Also, 3.9-fold higher contents of *tet*M and 3.4-fold higher contents of *tet*O were seen in manure samples with positive tetracycline findings (Hölzel et al., 2010a), analogous to the increase of *tet* genes during tetracycline therapy. Again, changes in the *tet* gene concentrations in a matrix can be explained in several ways. The proliferation of all bacteria would be the simplest reason but was excluded by relating the *tet* gene contents to the total bacterial load (colony-forming units). A second reason would be a shift within the bacterial diversity from one genus—or species—to another. Third, shifts might happen within each species increasing the *tet*M-positive subpopulation while lowering the *tet*M-negative subpopulation. Last (and presumably least), the number of *tet* gene copies per genome might increase. Apart from an increase of the total bacterial load, all events are selectable by tetracyclines and the observed increase might be best explained by a combination of intra- and interspecies shifts.

Interestingly, the most pronounced difference in *tet*M concentrations was seen between samples that were analytically negative for tetracyclines (< 0.1 mg/kg) and samples that contained 0.1–1 mg tetracycline per kg. Higher concentrations of tetracycline were not accompanied by further increased *tet*M concentrations. The *tet*M resistance gene is regularly found in tetracycline-susceptible bacteria (Schwaiger et al., 2009) and causes only moderate increases of MIC values (Schmitz et al., 2001; Ammor et al., 2008). Thus, it is counter-selected in the presence of high tetracycline contaminations, unless it is protected by the co-occurrence of other *tet* genes (Schwaiger et al., 2009). None of the 29 *E. faecalis* isolates from manure with

tetracycline concentrations >4 mg/kg carried *tet*M alone; 28 carried *tet*M and *tet*L together. By contrast, 19 of the 64 *E. faecalis* (30%) from manure with tetracyclines <0.1 mg/kg and 13 of 53 (25%) isolates of samples with 0.1–1 mg/kg carried *tet*M without further *tet* genes. However, the total prevalence of *tet*M (either alone or combined with other *tet* genes) increased in *E. faecalis* from 53% in negative samples, 76% in manures with 0.1–1 mg tetracyclines/kg, and 79% in manures with $>1 \leq 4$ mg/kg to 97% in manure >4 mg/kg, while no differences in the prevalence of enterococci in the different groups of manure were noted. However, *tet*M might be less often protected by other *tet* genes in other genera, which are less capable of exchanging genes than enterococci.

12.3 RESISTANCE GENES: ARE THE POOLS SEPARATED?

There is no doubt that large amounts of antimicrobial-resistant bacteria and antimicrobial resistance genes—possessed by these bacteria or existing as free DNA—are shed into the environment by farm animals. The question is whether these bacteria and genes find their way to humans. If they do, bacterial populations of humans and animals should be similar in terms of gene carriage and antimicrobial resistance. In this regard, it is of special interest to look at complex gene profiles since these allow us to draw epidemiologic conclusions on the exchange of mobile genetic elements, at least to a certain extent. Two other ways to investigate epidemiologic connections are very well justified, but do not render an analysis of gene profiles redundant. First, the analysis of the epidemiologic relationship of bacterial isolates (e.g., by pulsed-field gel electrophoresis or multilocus sequence typing) would detect the clonal spread of strains; however, it would not detect porcine-born bacterial genes that may have been introduced into the human bacterial flora by genetic exchange, without a need for their original bacterial carriers to remain present. Second, the analysis of single-gene prevalence in bacteria of humans and animals might already reveal differences; however, the discriminative power of complex gene profiles is higher. Additionally, a different prevalence of single genes does not necessarily indicate that the pools are separated since bacteria that can be swept from one pool into another (from animals to humans or vice versa) encounter different selective conditions in each. Different antibiotics are preferentially used in the therapy of humans and livestock, respectively. Certain genes or gene-carrying bacteria, though originating from livestock, might find more favorable conditions in humans. Thus these porcine-born bacterial genes would be more strongly selected in humans than in animals, reaching secondarily higher prevalence in humans, though they originated from animals. To illustrate this: The fact that *van* genes are (now) much more frequent in enterococci of humans compared to animals does not exclude that a certain part of these *van* genes originated from animals (Phillips, 2007). We assume that this would not be responsible for the majority of diverse resistance genes in the human bacterial flora; however, as in the case of vancomycin resistance genes, it might concern genes of particular risk.

We selected 11 candidate genes of *E. coli* to analyze the genetic profiles of human and porcine isolates and hypothesized that regional differences in the profiles of porcine or human isolates would be less pronounced than the source (human/pig) depending on differences. We found that the total prevalence of single resistance genes differed

significantly between porcine and human *E. coli* (Table 12.5), but not between northern and southern Bavaria. In detail, *tet*A was more prevalent in porcine isolates, while *tet*B, *sul*I, *sul*II, and *aad*A were more prevalent in human isolates (Schwaiger et al., 2010).

As a next step, we investigated the complex gene profiles formed by the candidate genes. Of the *E. coli* from humans and pigs 39% had unique profiles, which were only seen in one population. Of the isolates 29.3% would, in case of a population crossover, shift the frequency of profiles, whereas only 37.8% of the isolates would be imperceptibly exchangeable between populations. There is a certain temptation in the interpretation of genetic profiling data to overlook that frequent genes are more likely to coexist than rare genes, and that rare genes are more likely to coexist with frequent genes than alone. Thus, differences in gene profiles might only be a function of the different prevalence of single genes—a fact that does not impair the validity of gene-profile-based statements about population-related differences but would suggest that it is not worth the effort to present complex gene profiles instead of the prevalence of single genes. We need to discriminate between profiles that are based on an arbitrary combination of single genes and profiles that deviate from these probable combinations, in order to get any advantage from the gene profiling, compared to the analysis of the prevalence of single genes. Deviation between the expected and observed profiles might indicate the clonal spread of strains with distinct gene profiles, either within one population or across population borders, and it might further indicate the physical linkage of resistance genes.

We calculated the probability of the arbitrary combination of genes from the prevalence of single resistance genes in a certain population as expectation values and compared them with the observed distribution of gene profiles. Expectation values were calculated from the prevalence values of single genes in each population as $P(\mathrm{Gene_A}) \times P(\mathrm{Gene_B}) \times (\ldots) \times 1 - P(\mathrm{Gene_X}) \times 1 - P(\mathrm{Gene_Y}) \times (\ldots)$. The minimum expectation values were calculated from $P_{\text{lower limit}}(\mathrm{Gene_A}) \times (\ldots) \times 1 - P_{\text{upper limit}}(\mathrm{Gene_X}) \times (\ldots)$, whereas the maximum expectation values were calculated from $P_{\text{upper limit}} \times 1 - P_{\text{lower limit}}$.

The prevalence of eight different profiles differed significantly between human and porcine *E. coli* (Table 12.6). Five of these profiles were expected to differ since the expectation values (calculated from the prevalence of single genes) differed significantly between human and porcine isolates. Thus, these five profiles differed, but this information does not add new information to the known different prevalence of the single genes *tet*A, *tet*B, *sul*I, *sul*II, and *aad*A. However, three profiles—*tet*A-*sul*II-*str*A-*str*B, *tet*B-*str*A-*str*B, and *tet*A-*sul*I-*aad*A—had similar expectation values both in human and porcine enterococci but were found much more frequently in porcine enterococci.

Evidently, since our model treated all resistance genes as independent, it fitted rather poorly to the observed values in some cases, since (due to genetic linkage or incompatibility) many resistance genes are not independent, indeed. A considerable part of the resistance genes that we used in the model of gene profiling were correlated; in addition this correlation differed considerably between human and porcine isolates (Table 12.6). The linked genes *str*A and *str*B were positively correlated but stronger in porcine *E. coli* (correlation coefficient 0.96) than in human *E. coli* (0.86); *tet*A and *tet*B were negatively correlated but much stronger in *E. coli* from pigs (−0.87) than from humans (−0.47).The genes *sul*II and *str*B were correlated both in porcine (0.54) and human (0.62) *E. coli*, similar to *sul*II and *str*A, since *str*A and *str*B are usually

TABLE 12.5 Significant Correlations between the Prevalence of Resistance Genes in *E. coli* of Humans ($n = 152$) and Pigs ($n = 137$)[a]

	*tet*B	*str*A	*str*B	*aad*A	*sul*I	*sul*II	*sul*III	*tet*C	*tet*D	*tet*M
*tet*A	**(Pig)**, (Human)	(Pig)	(Pig), Human							
*tet*B		Pig								
*str*A			**Pig, Human**	(Pig)	Human	Pig, **Human**		Pig, (Human)	(Pig)	
*str*B				(Pig)	Human	**Pig, Human**		Pig	(Pig)	
*aad*A					Pig				Pig	
*sul*I						Human	(Human)	Pig	Pig	
*sul*II										
*sul*III										
*tet*C										
*tet*D										Pig

[a]**Bold:** correlation coefficient > 0.5. Only significant correlations are given in parentheses the table ( ) negative correlation.

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TABLE 12.6 Observed (Expected) Prevalence of Particular Gene Profiles that Differ Significantly between *E. coli* of Humans ($n = 152$) and Pigs ($n = 137$)

Gene Profile	Porcine % (%[a])	Human % (%[a])
*tet*A *sul*II *str*A *str*B	**11.7**[b] (0.43–4.93)	1.3 (0.16–2.23)
*tet*B *str*A *str*B	**11.0**[b](0.28–3.63)	1.3 (0.29–3.39)
*tet*A	**10.2**[b] (0.50–5.39)	0 (0.03–0.63)
*tet*A aadA	**9.5**[b] (0.38–4.49)	**2.6**[b] (0.04–0.86)
tetA *sul*I *aad*A	**3.7**[b] (0.04–0.93)	0 (0.03–0.60)
*tet*B *sul*I *sul*II *str*A *str*B *aad*A	**2.2**[b] (0.01–0.45)	**7.2**[b](0.47–4.9)
*tet*A *sul*I *sul*II *str*A *str*B aadA	**1.5**[b] (0.03–0.85)	**7.2**[b] (0.15–2.13)
tetB sulI sulII strA strB	0 (0.02–0.54)	3.3 (0.32–3.55)

[a]95% confidence interval, calculated from the single prevalences of resistance genes (% porcine enterococci/% human enterococci): *tet*A (57.7/32.2), *tet*B (38.7/55.9), *sul*I (12.4/39.5), *sul*II (40.1/61.8), *str*A (52.6/61.2), *str*B (54.7/63.8), are *aad*A (44.5/59.2).
[b] Beyond the 95% confidence interval of expectation values.

genetically linked. A couple of weak correlations were only seen in one of the investigation groups (human or porcine *E. coli*), whereas the correlations of *tet*A with *str*B and of *tet*C with *str*A were even contrary in the different investigation groups (Table 12.5). The resistance genes *tet*A and *tet*B are known to be negatively correlated, due to their presence on plasmids of different incompatibility groups (Boerlin et al., 2005; Kim et al., 2007; Rosengren et al., 2009). By contrast, *str*A and *str*B are usually linked (Chiou and Jones, 1993; Sundin and Bender, 1996). However—and interestingly—these associations known from the literature were stronger in *E. coli* isolates from pigs when compared to humans. This is reflected by the fact that the prevalence of most gene profiles in human *E. coli* was correctly predicted by our model, whereas the prevalence of several gene profiles in porcine *E. coli* was not. When we adapted the model by using linked probabilities for *str*A and *str*B (and linked improbabilities for *tet*A/*tet*B), the predictive value of the model increased (data not shown).

We know from the literature basically important facts on the genomic organization of particular strains, transposons, plasmids, or integrons, observed in very valuable studies on the single strain level. However, the nature of integrons is to catch (and maybe also to release) gene cassettes; the nature of transposons is to transpose. Thus, transposons join with other transposons, such as *Tn*3872, which is a fusion of *Tn*916 and *Tn*917 (Cochetti et al., 2007), or *Tn*5253, which is a fusion of *Tn*5251 and *Tn*5252 (Ayoubi et al., 1991). All these events lead to a frequent reorganization of mobile genetic elements. In other words, correlations between some resistance genes are well known, but how close these correlations are remains poorly understood: 13.9% of porcine and 40.8% of human *E. coli* carried type 1 integrons, as indicated by the simultaneous presence of *sul*I and *qac*EΔ1 (C. Hölzel and K. Schwaiger, unpublished results). However, these integrons were only weakly associated with other resistance genes, and the associated genes differed between human and porcine *E. coli* since *sul*I was associated with *str*A*str*B in human isolates and with *aad*A in porcine isolates [the latter is, for porcine isolates, also reported by Rosengren et al. (2009)].

As illustrated in the last sections, differences between resistance genes of human and porcine *E. coli* are pronounced since we see significant differences in (i) the prevalence of single resistance genes, (ii) the correlation between these genes, and (iii) the resulting

profiles formed by these genes. This suggests that the gene pools of human and porcine *E. coli* are relatively separated and that population crossover of genes or clones is rare. However, when we investigated enterococci, we found the gene profiles much less segregated. Of all isolates 7% had unique profiles, but 73% of the isolates would be imperceptibly exchangeable between populations, whereas the exchange of the remaining 20% would not alter the profile spectrum but would change the frequency of profiles (C. Hölzel and K. Schwaiger, unpublished results). This similarity of enterococcal gene profiles of humans and animals might be attributed to the fact that enterococci are better adapted to survive in the nonintestinal environment (Franz et al., 1999), resulting in a larger expansion of the contact-zone to both other microorganisms (for genetic transfer) and macroorganisms (for clonal spread).

12.4 CONCLUSIONS

Tetracycline resistance genes are highly concentrated in pig manure even if pigs are raised without antibiotic contact. The quantity of these resistance genes increases during antibiotic therapy, and the detection of antibiotics (tetracyclines, sulfonamides) in manure is associated with bacterial co-resistance to structurally unrelated substances (e.g., association of tetracyclines and resistance to chloramphenicol). Resistance gene profiles of *E. coli* from pig manure and humans differ intensely, indicating that the gene (and germ) pools of pigs and humans are largely separated. However, other bacteria (such as enterococci), which are better adapted to environmental survival than *E. coli*, might spread more easily between humans and animals.

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REFERENCES

Aarestrup FM, Seyfarth AM, Emborg HD, Pedersen K, Hendriksen RS, Bager F (2001). Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark. *Antimicrob Agents Chemother* 45:2054–2059.

Agersø Y, Wulff G, Vaclavik E, Halling-Sørensen B, Jensen LB (2006). Effect of tetracycline residues in pig manure slurry on tetracycline-resistant bacteria and resistance gene tet(M) in soil microcosms. *Environ Int* 32:876–882.

Ammor MS, Gueimonde M, Danielsen M, Zagorec M, Van Hoek AH, de Los Reyes-Gavilan CG, Mayo B, Margolles A (2008). Two different tetracycline resistance mechanisms, plasmid-carried tet(L) and chromosomally located transposon-associated tet(M), coexist in *Lactobacillus sakei* Rits 9. *Appl Environ Microbiol* 74:1394–1401.

Ayoubi P, Kilic AO, Vijayakumar MN (1991). Tn5253, the pneumococcal omega (cat tet) BM6001 element, is a composite structure of two conjugative transposons, Tn5251 and Tn5252. *J Bacteriol* 173:1617–1622.

Boerlin P, Travis R, Gyles CL, Reid-Smith R, Janecko N, Lim H, Nicholson V, McEwen SA, Friendship R, Archambault M (2005). Antimicrobial resistance and virulence genes of *Escherichia coli* isolates from swine in Ontario. *Appl Environ Microbiol* 71:6753–6761.

Bouma JE, Lenski RE (1988). Evolution of a bacteria/plasmid association. *Nature* 335:351–352.

Chiou CS, Jones AL (1993). Nucleotide sequence analysis of a transposon (Tn5393) carrying streptomycin resistance genes in *Erwinia amylovora* and other gram-negative bacteria. *J Bacteriol* 175:732–740.

Cochetti I, Tili E, Vecchi M, Manzin A, Mingoia M, Varaldo PE, Montanari MP (2007). New *Tn*916-related elements causing *erm*(B)-mediated erythromycin resistance in tetracycline-susceptible pneumococci. *J Antimicrob Chemother* 60:127–131.

Cotta MA, Whitehead TR, Zeltwanger RL (2003). Isolation, characterization and comparison of bacteria from swine faeces and manure storage pits. *Environ Microbiol* 5:737–745.

de Gelder L, Ponciano JM, Abdo Z, Joyce P, Forney LJ, Top EM (2004). Combining mathematical models and statistical methods to understand and predict the dynamics of antibiotic-sensitive mutants in a population of resistant bacteria during experimental evolution. *Genetics* 168:1131–1144.

Franz CM, Holzapfel WH, Stiles ME (1999). Enterococci at the crossroads of food safety? *Int J Food Microbiol* 47:1–24.

Ghosh S, Lapara TM (2007). The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. *ISME J* 1:191–203.

Hadorn K, Hachler H, Schaffner A, Kayser FH (1993). Genetic characterization of plasmid-encoded multiple antibiotic resistance in a strain of *Listeria monocytogenes* causing endocarditis. *Eur J Clini Microbiol Infect Dis* 12:928–937.

Han I, Congeevaram S, Park J (2009). Improved control of multiple-antibiotic-resistance-related microbial risk in swine manure wastes by autothermal thermophilic aerobic digestion. *Water Sci Technol* 59:267–271.

Hölzel CS, Harms KS, Küchenhoff H, Kunz A, Müller C, Meyer K, Schwaiger K, Bauer J (2010a). Phenotypic and genotypic bacterial antimicrobial resistance in liquid pig manure is variously associated with contents of tetracyclines and sulfonamides. *J Appli Microbiol* 108:1642–1656.

Hölzel CS, Schwaiger K, Harms KS, Küchenhoff H, Kunz A, Meyer K, Müller C, Bauer J (2010b). Sewage sludge and liquid pig manure as possible sources of antibiotic resistant bacteria. *Environ Res* 110:318–326.

Kim TE, Jeong YW, Cho SH, Kim SJ, Kwon HJ (2007). Chronological study of antibiotic resistances and their relevant genes in Korean avian pathogenic *Escherichia coli* isolates. *J Clin Microbiol* 45:3309–3315.

Lenski RE (1998). Bacterial evolution and the cost of antibiotic resistance. *Int Microbiol* 1:265–270.

Levy SB (1997). Antibiotic resistance: An ecological imbalance. *Ciba Found Symp* 207:1–9.

Levy SB, FitzGerald GB, Macone AB (1976a). Changes in intestinal flora of farm personnel after introduction of a tetracycline-supplemented feed on a farm. *N Engl J Med* 295: 583–588.

Levy SB, FitzGerald GB, Macone AB (1976b). Spread of antibiotic-resistant plasmids from chicken to chicken and from chicken to man. *Nature* 260:40–42.

Macovei L, Miles B, Zurek L (2008). Potential of houseflies to contaminate ready-to-eat food with antibiotic-resistant enterococci. *J Food Protect* 71:435–439.

Peu P, Brugere H, Pourcher AM, Kerouredan M, Godon JJ, Delgenes JP, Dabert P (2006). Dynamics of a pig slurry microbial community during anaerobic storage and management. *Appl Environ Microbiol* 72:3578–3585.

Phillips I (2007). Withdrawal of growth-promoting antibiotics in Europe and its effects in relation to human health. *Int J Antimicrob Agents* 30:101–107.

Rosengren LB, Waldner CL, Reid-Smith RJ (2009). Associations between antimicrobial resistance phenotypes, antimicrobial resistance genes, and virulence genes of fecal *Escherichia coli* isolates from healthy grow-finish pigs. *Appl Environ Microbiol* 75:1373–1380.

Schmitz FJ, Krey A, Sadurski R, Verhoef J, Milatovic D, Fluit AC (2001). Resistance to tetracycline and distribution of tetracycline resistance genes in European *Staphylococcus aureus* isolates. *J Antimicrob Chemother* 47:239–240.

Schwaiger K, Harms K, Hölzel C, Meyer K, Karl M, Bauer J (2009). Tetracycline in liquid manure selects for co-occurrence of the resistance genes *tet*(M) and *tet*(L) in *Enterococcus faecalis*. *Vet Microbiol* 139:386–392.

Schwaiger K, Hölzel C, Bauer J (2010). Resistance gene patterns of tetracycline resistant *Escherichia coli* of human and porcine origin. *Vet Microbiol* 142:329–336.

Singer RS, Patterson SK, Wallace RL (2008). Effects of therapeutic ceftiofur administration to dairy cattle on *Escherichia coli* dynamics in the intestinal tract. *Appl Environ Microbiol* 74:6956–6962.

Straut M, de Cespedes G, Horaud T (1996). Plasmid-borne high-level resistance to gentamicin in *Enterococcus hirae*, *Enterococcus avium*, and *Enterococcus raffinosus*. *Antimicrob Agents Chemother* 40:1263–1265.

Sundin GW, Bender CL (1996). Dissemination of the strA-strB streptomycin-resistance genes among commensal and pathogenic bacteria from humans, animals, and plants. *Mol Ecol* 5:133–143.

13

MUNICIPAL WASTEWATER AS A RESERVOIR OF ANTIBIOTIC RESISTANCE

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13.1 INTRODUCTION

Antimicrobials are an integral component of modern medicine, fighting a variety of infectious diseases. Their use has profoundly impacted medical practice, as these drugs have successfully eradicated many harmful bacterial infections. In spite of the role of antimicrobials in the protection of public health, the proliferation of antimicrobial-resistant bacteria represents one of the greatest public health challenges of the twenty-first century. Antimicrobial resistance has become particularly pertinent with the relatively recent development of pathogenic bacteria that are resistant to multiple antimicrobials. Health officials, and many scientists, now fear the antimicrobial era is nearing its end, and that currently treatable diseases will once again become fatal due to the ineffectiveness of first-line antimicrobials (Palumbi, 2001; Levin et al., 1999; Levy and Marshall, 2004; Levy, 2005).

While the problem of antimicrobial resistance among bacteria has long been known, the medical community and the public have been often cavalier concerning the use of antimicrobials. In the past, antimicrobials were indiscriminately prescribed for viral infections, such as influenza and the common cold, and substantial quantities of antimicrobials have been used in agriculture for growth promotion and prophylaxis (Harrison and Lederberg, 1998). In retrospect, the initial "solution" for the problem of antimicrobial resistance was relatively naïve; it was assumed that new antimicrobials would be discovered and/or developed at a rate faster than bacteria

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evolved resistance. However, since the 1960s, the discovery of new antimicrobials has substantially slowed and the evolution of bacterial resistance to antimicrobials has been unexpectedly rapid (Normack and Normack, 2002; Frost et al., 2005). The present solution for the problem of antimicrobial resistance is to limit the use of antimicrobials, although this will likely be largely inconsequential because the consequences of withholding antimicrobials to sick people can be dire and because antimicrobial use continues to be pervasive in agriculture in the United States.

Whereas the scientific community has made significant progress in elucidating the types and mechanisms of resistance, there is a relatively poor knowledge of the ecology of antimicrobial resistance, thus making it impossible to definitively identify the causes of and (more importantly) the solutions to the proliferation of antimicrobial resistance. That is, antimicrobial resistance is far better understood at the small scale (i.e., within an individual bacterium) than at the large scale (i.e., how is resistance spread over geographic distances?).

The general hypothesis driving our research is that the primary source of antimicrobial-resistant bacteria on the global scale is the gastrointestinal microbiota from human beings and animals ingesting antimicrobials for therapeutic and nontherapeutic purposes. Because these microorganisms are shed during defecation (and unaltered/partially altered antimicrobials are shed during urination), we postulate that municipal wastewater and animal waste are critical reservoirs of antimicrobial-resistant bacteria. Consistent with this view, agriculture has been repeatedly targeted as a source of antimicrobial resistance. Antimicrobial use in agriculture, in fact, has been extensively scrutinized; many have even demanded that nonveterinary use of antimicrobials in agriculture be curtailed or eliminated (FAAIR, 2002; Lipsitch et al., 2002).

In contrast, antimicrobial resistance in human waste has received far less attention. In our opinion, this is extraordinarily unfortunate because municipal wastewater treatment offers a tremendous but unrealized opportunity to slow the proliferation of antimicrobial resistance. Even though municipal wastewater has been clearly and repeatedly shown to contain substantial quantities of antimicrobial-resistant bacteria, the scientific community has generally failed to recognize municipal wastewater as a principal reservoir of antimicrobial resistance.

Why have municipal wastewater and municipal wastewater treatment been overlooked? Although this question cannot be answered with any certainty (in part because there are numerous reasons), it seems that scientists interested in antimicrobial resistance generally assume that municipal wastewater treatment protects the public from both microbial pathogens and antimicrobial-resistant bacteria. Conversely, the personnel who design and operate municipal wastewater treatment facilities likely lack knowledge of the ecology of antimicrobial resistance. The next part of this chapter will therefore describe the process of municipal wastewater treatment and its role in modern society. The subsequent section will review previous research on antimicrobial resistance in municipal wastewater and municipal wastewater treatment facilities. The fourth section of this chapter will suggest several opportunities to manipulate and/or alter the municipal wastewater treatment process such that it could be used to limit the proliferation of antimicrobial resistance. Finally, the concluding section will describe future research needs regarding the design of municipal wastewater treatment facilities intended to limit the spread of resistant bacteria and resistance determinants.

13.2 MUNICIPAL WASTEWATER AND MUNICIPAL WASTEWATER TREATMENT

Municipal wastewater is a complex mixture of literally anything that is flushed down a toilet or that is sent down a drain. Municipal wastewater also often includes industrial waste and stormwater, particularly if a municipality has combined storm and sanitary sewers. It is absolutely critical to emphasize that the primary component of municipal wastewater is water; the water content of municipal wastewater is typically greater than 99.9%. Because municipal wastewater has such a high water content, it is increasingly viewed as a resource rather than a waste, particularly in areas where water is a relatively scarce commodity.

Although municipal wastewater is often perceived by the general public as "toxic," it poses an environmental problem precisely because it is *not* toxic. (Note: Some industrial wastes are certainly toxic, but these wastes are not allowed to be sent to sanitary sewers.) Indeed, municipal wastewater is rapidly biodegradable in the environment, such that the discharge of untreated municipal wastewater to a surface water stimulates the growth of aerobic heterotrophic bacteria that metabolize the organic content of the waste. These biochemical reactions simultaneously deplete the dissolved oxygen levels in the surface water, which has numerous adverse effects (i.e., fish kills, noxious odors, etc.). Similarly, the nitrogen and phosphorus content of municipal wastewater can stimulate the growth of photosynthetic organisms, resulting in nuisance levels of algae, cyanobacteria (or blue-green algae), and/or aquatic plants.

The primary goal of municipal wastewater treatment, therefore, is to protect the environment from the adverse effects of the nutrients in municipal wastewater. This goal is achieved by a series of unit operations that systematically remove different components of the wastewater (Fig. 13.1). The first few unit operations (bar racks,

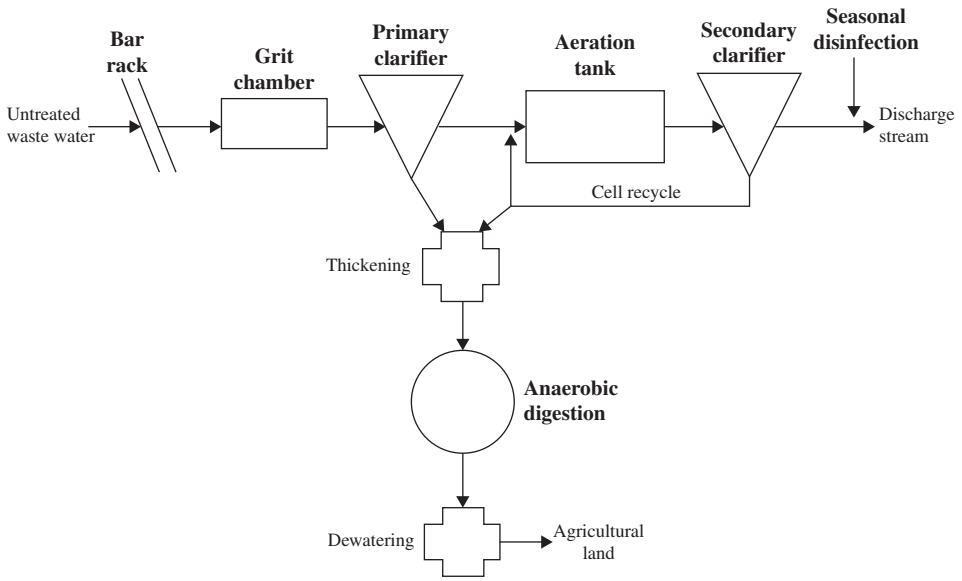

FIGURE 13.1 Schematic diagram of a conventional municipal wastewater treatment process.

screens, and grit chambers) are designed to remove relatively large debris (>2 cm) and coarse sand that would harm (or clog) the other unit operations. The primary clarifier, which is responsible for 30–50% of the total extent of treatment, is a quiescent chamber that allows organic particles to either settle or float, allowing them to be removed from the wastewater. Following the primary clarifier, there is almost always a biological treatment process that utilizes microbes to metabolize the soluble pollutants remaining in the wastewater. The most common technology, as shown in Figure 13.1, is the activated sludge process. The activated sludge process includes an aeration tank, which provides both mixing and oxygen to stimulate microbial growth, coupled to a secondary clarifier, which allows the microbes that grew in the aeration tank to be separated from the treated effluent. These microbes can then be either recycled back to the aeration tank (to help maintain a high concentration of microbes in that reactor) or disposed as a waste by-product. The water leaving the secondary clarifier is typically of very high quality (although it is not potable, it is often better quality than the surface water to which it is discharged), but it almost certainly contains human pathogens, so it is commonly disinfected prior to discharge.

The solids collected from the primary and secondary clarifiers also require treatment prior to disposal. Of primary importance is that these waste streams contain substantial quantities of both microorganisms and biodegradable organic carbon. These waste streams, therefore, are typically combined and thickened to remove some of the water, thereby increasing the solids content. The thickened waste stream is then typically sent to another treatment process, which essentially allows the microbes in the primary/secondary solids to metabolize the organic content. There are several of these processes, collectively known as "stabilization" processes (the term "stable" connotes that microbial activity has been reduced), of which the most commonly used technology is anaerobic digestion operated at 35–37 °C.

Following stabilization, the stabilized wastewater solids are then further dewatered to reduce the water content prior to ultimate disposal. Here again, there are a handful of options for disposal, but the application of these treated wastewater solids to agricultural land (as a soil conditioner and as a nutrient supplement) is perceived to be the most sustainable and environmentally friendly alternative. In the United States, wastewater solids can be applied to agricultural land used to grow crops not intended for human consumption if the solids have undergone a "process to significantly remove pathogens" (PSRP). Typically, the conventional anaerobic digestion process is sufficient to qualify as a PSRP. In contrast, treated wastewater solids that have undergone a "process to further remove pathogens" (PFRP) can be utilized almost without restriction. Conventional anaerobic digestion does not qualify as a PRFP, but thermophilic anaerobic digestion (operating temperature >55 °C) can qualify under certain circumstances. Alternatively, the wastewater solids must undergo a stringent stabilization/disinfection process, such as lime stabilization (incubation at pH > 11 for more than 2 hours) or pasteurization to inactivate microbial pathogens in the wastewater solids to qualify as a PFRP.

In conclusion, municipal wastewater treatment processes are primarily designed to protect surface water quality. It is merely a secondary goal of municipal wastewater treatment to limit risks to human health that might result from direct exposure to treated wastewater (as might occur with a surface water) or to treated wastewater solids applied to agricultural land. Because of the unit operations used to meet this secondary goal of protecting humans from exposure to pathogens, municipal

wastewater treatment certainly provides some degree of protection against the proliferation of antimicrobial resistance.

13.3 ANTIMICROBIAL-RESISTANT BACTERIA AND ANTIMICROBIAL RESISTANCE GENES IN MUNICIPAL WASTEWATER

Any discussion of antimicrobial-resistant bacteria in municipal wastewater must necessarily begin with a discussion of the methods used to characterize these organisms. In a clinical setting, a suspected pathogen is isolated and then phenotypically characterized with respect to its resistance to a handful of antimicrobials to identify an effective treatment. Whether this suspected pathogen is intrinsically resistant (i.e., it naturally lacks the intracellular target upon which the antimicrobial acts) or has evolved resistance (i.e., via point mutations or via the acquisition of a genetic determinant that confers resistance) is of little importance as long as an effective therapy can be identified.

In contrast, the characterization of antimicrobial resistance in municipal wastewater is substantially more complicated. It is possible to isolate a broad diversity of strains, but it is well known that microbiological media are capable of growing only a very small fraction of the total bacterial community (Amann et al., 1995). Extending beyond the so-called "great plate count anomaly" (Staley and Konopka, 1985), the characterization of a bacterium isolated from wastewater cannot merely depend on its phenotype with respect to resistance to various antimicrobials. It is absolutely critical to determine an organism is intrinsically resistant (no risk of participating in future lateral gene transfer events), has evolved resistance via point mutations (little risk of participating in future lateral gene transfer events), or has evolved resistance by acquiring a gene (or genes) encoding resistance (high risk of participating in future lateral gene transfer events).

The need to genotypically characterize bacterial isolates can be particularly problematic when analyzing organisms that have not been extensively studied. It is often difficult to a priori determine whether the organism has a suitable target; it is even more difficult to determine whether the organism has evolved resistance via a point mutation. It is somewhat easier to determine whether an organism has acquired a gene (or genes) encoding resistance because there are typically numerous genes known to encode resistance to a particular antimicrobial. Identifying these genes can nonetheless be cumbersome (e.g., there are more than 40 known genes that encode resistance to tetracycline; Chopra and Roberts, 2001) or extremely difficult for the case of organisms that harbor novel genes that have yet to be discovered.

One valid approach to studying antimicrobial resistance in wastewater, therefore, is to focus on a specific, well-known bacterial species (e.g., *Escherichia coli*). Walter and Vennes (1985) determined that between 0.35 and 5% of coliforms were resistant to multiple antimicrobials, of which as many as 75% were capable of lateral gene transfer via conjugation. Gallert et al. (2005) isolated more than 750 fecal coliforms, most of which were resistant to multiple antimicrobials. Da Costa et al. (2006) isolated more than 900 strains of enterococci from municipal wastewater, about half of which were resistant to multiple antimicrobials. Łuczkiewicz et al. (2010) isolated more than 350 strains of fecal coliforms and enterococci, of which 9 and 29%, respectively, were resistant to multiple antimicrobials.

An alternative cultivation-based approach to studying antimicrobial resistance in wastewater is to focus on resistance to broad-spectrum antimicrobials for which there should be few (if any) intrinsically resistant bacteria. We used this approach to study more than 150 strains isolated on tetracycline-amended and ciprofloxacin-amended growth media inoculated with biomass from the aeration tanks at a municipal wastewater treatment facility (Ramsden et al., 2010). Phenotypically, these bacteria were all resistant to multiple antimicrobials and about half of them harbored a gene encoding resistance to tetracycline, including those bacteria isolated on ciprofloxacin-amended growth media. It was particularly noteworthy that most of these bacteria also harbored an integron, which is a genetic element that allows a bacterium to incorporate an exogenous gene into its genome and control its expression (Mazel, 2006). The frequent occurrence of integrons and multiple antimicrobial resistance and genes encoding resistance to tetracycline suggested that the bacteria isolated from municipal wastewater had previously participated in numerous lateral gene transfer events.

Cultivation-independent approaches are also becoming more common in the analysis of antimicrobial resistance in municipal wastewater. This approach usually involves targeting a gene (or genes) by polymerase chain reaction (PCR) or quantitative PCR (qPCR). This approach is advantageous because it directly targets the genotype, rather than the phenotype. Cultivation-independent approaches have also been used to study the plasmid metagenome of wastewater, revealing that plasmids obtained from wastewater samples encoded resistance to all major classes of antimicrobials (Szczepanowski et al., 2008, 2009).

The disadvantages of cultivation-independent approaches include the anonymity of the organisms that harbor the genes (i.e., there is no opportunity to analyze the host for multiple antimicrobial resistance, its ability to transfer the genes via conjugation, etc.) and the inability to detect novel genes for resistance. It is also important to consider that the PCR/qPCR approach tracks genes that might not be functional (i.e., if merely a gene fragment is present) or might not be functional in the specific host. This latter scenario would seem to be uncommon, although research in our lab has identified Gram-positive bacteria that harbor *tet*(A) (a gene encoding an efflux protein that anchors in the Gram-negative cell wall) and Gram-negative bacteria that harbor *tet*(L) (a gene encoding an efflux protein that anchors in the Gram-positive cell wall) (Ghosh and LaPara, 2007).

Relatively little data is available regarding the fate of antimicrobial resistance genes in municipal wastewater treatment facilities. Auerbach et al. (2007) demonstrated that the diversity of tetracycline resistance determinants was greater in municipal wastewater than in natural lake water samples and that ultraviolet (UV) disinfection had no impact on the quantity of resistance genes in the treated wastewater effluent. It has also been demonstrated that conventional wastewater treatment (i.e., activated sludge) tends to make bacterial populations in treated effluent more frequently resistant, resistant to a larger variety of antimicrobials, and more likely to be resistant to multiple antimicrobials compared to those in raw influent (da Silva et al., 2006, 2007). A more recent study determined that a high temperature ($T > 50\,^{\circ}\mathrm{C}$) anaerobic digestion process ($>50\,^{\circ}\mathrm{C}$) was more effective at reducing the quantities of four genes (three tetracycline resistance determinants as well as the integrase of class 1 integrons) than a conventionally operated anaerobic digestion process ($T = 35$–$37\,^{\circ}\mathrm{C}$) (Ghosh et al., 2009). This latter result was then

confirmed in a laboratory study in which anaerobic digestion at 55 °C achieved statistically significant reductions in several tetracycline resistance genes and the integrase gene of class 1 integrons, whereas anaerobic digestion at 37 °C did not (Diehl and LaPara, 2010).

In conclusion, municipal wastewater is clearly a source of numerous antimicrobial-resistant bacteria and antimicrobial resistance genes. Different researchers have reported widely different values for multiple antimicrobial resistance, but it is clear that multiple antimicrobial resistance is common among bacteria in municipal wastewater. Furthermore, many resistance genes discovered in wastewater have been recently identified in clinical isolates, indicating that resistance genes have been exchanged between clinical and wastewater environments (Szczepanowski et al., 2009). In addition to this genetic exchange between clinical and wastewater domains, resistance genes are highly mobile among phylogenetically distant species (Heuer et al., 2002). This means that a wastewater treatment plant, which contains a rich diversity of pathogens, commensals, and environmental microorganisms, may provide a unique opportunity for resistance determinants to exchange horizontally with bacteria typically not considered clinically relevant.

13.4 MUNICIPAL WASTEWATER TREATMENT: SLOWING THE PROLIFERATION OF RESISTANCE?

In the two sections above, we reviewed the research describing the presence of antimicrobial resistant bacteria and antimicrobial resistance genes in municipal wastewater as well as the process by which municipal wastewater is treated. A pertinent question, therefore, is: how can municipal wastewater treatment be improved to reduce the proliferation of antimicrobial resistance? There should be a conceptual difference in the design of a municipal wastewater treatment facility intended to protect surface water and to protect against accidental exposure to pathogenic microorganisms (i.e., the current goals and design concept) compared to the design of an analogous facility intended to thwart the proliferation of antimicrobial resistance. The routes of pathogen exposure are implicit components of the laws regulating the discharge of treated municipal wastewater or the application of wastewater solids to agricultural land. The presence of pathogens in treated wastewater and in wastewater solids is of little risk because human beings rarely consume surface water without additional treatment other than via accidental ingestion and because wastewater solids processed by a PSRP are not used to grow crops intended for human consumption. In contrast, antimicrobial resistant bacteria in treated municipal wastewater or in treated wastewater solids could somehow become a source of resistant bacteria as well as genes encoding resistance that could then arise (via lateral gene transfer) in pathogenic organisms.

In considering the conceptual re-design of municipal wastewater treatment, it is first important to ponder the routes by which antimicrobial resistant bacteria are likely to leave a municipal wastewater treatment facility. Antimicrobial resistant bacteria could, in theory, be aerosolized in the aeration tanks, but this seems substantially less important than the bacteria that could leave in the treated effluent or in the treated wastewater solids. Of these latter two avenues, the treated wastewater effluent is far less substantial regarding the quantity of bacteria (i.e., the majority of

antimicrobial-resistant bacteria in municipal wastewater will be in the wastewater solids), but far more impactful with respect to mobility after escaping a treatment facility (i.e., antimicrobial-resistant bacteria in surface waters are far more mobile than those in wastewater solids applied to agricultural soils).

There are two modifications or additions that could be reasonably made to the municipal wastewater treatment process to reduce the release of antimicrobial-resistant bacteria with the treated wastewater. The first approach would be to impose far more stringent disinfection technologies to the wastewater effluent. The implementation of more stringent disinfection technologies, however, might be logistically complicated because additional application of chlorine will result in the additional production of carcinogenic disinfection by-products, and alternative disinfection technologies are substantially more costly. Rapid sand filtration could also be used to reduce the release of antimicrobial-resistant bacteria from municipal wastewater; this process would be advantageous in that it would not require additional chemical use and it would not generate harmful disinfection by-products.

There are likely numerous opportunities to substantially reduce the quantities of antimicrobial-resistant bacteria during the treatment of wastewater solids. Most of the technologies for the treatment of wastewater solids focus on stabilization (i.e., the reduction of organic content and microbial activity) rather than disinfection. There are numerous technologies that could be used to disinfect wastewater solids, but the most practical appears to be thermophilic anaerobic digestion. This technology can simultaneously stabilize wastewater solids while achieving noteworthy reductions in antimicrobial resistance determinants at a cost comparable to conventional anaerobic digestion. (Note: Anaerobic digestors are typically heated using methane produced during the digestion process; higher anaerobic digestor temperatures, therefore, can be achieved with minimal extra cost.)

13.5 SUMMARY AND FUTURE RESEARCH NEEDS

In conclusion, municipal wastewater contains substantial quantities of antimicrobial-resistant bacteria. Unlike other reservoirs of antimicrobial resistance, however, the resistant microbes in municipal wastewater could be eliminated by subtly manipulating or modifying the wastewater treatment process to account for this "emerging environmental pollutant" (Pruden et al., 2006). The impetus for better wastewater treatment in which antimicrobial-resistant bacteria are specifically targeted will require substantial policy shifts, requiring substantial additional governmental regulation. Current regulations on treated wastewater effluent and treated wastewater solids are not particularly onerous with respect to microbial pathogens (an analog of antimicrobial-resistant bacteria) because direct ingestion of treated wastewater and/or wastewater solids is rare and almost always unintentional.

Additional research is needed to further characterize the antimicrobial-resistant bacteria in municipal wastewater as well as the ability of the various municipal wastewater treatment processes to eliminate these organisms. It is critically important to asses the potential ability of the different technologies used to treat wastewater solids because wastewater solids undoubtedly contain a very large fraction of the resistant bacteria found in the untreated wastewater.

REFERENCES

Amann RI, Ludwig W, Schleifer K-H (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev* 59:143–169.

Auerbach EA, Seyfried EE, McMahon KD (2007). Tetracycline resistance genes in activated sludge wastewater treatment plants. *Water Res* 41:1143–1151.

Chopra I, Roberts M. (2001). Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev* 65:232–260.

da Costa PM, Vaz-Pires P, Bernardo F (2006). Antimicrobial resistance in *Enterococcus* spp. isolated in inflow, effluent and sludge from municipal sewage water treatment plants. *Water Res* 40:1735–1740.

da Silva MF, Tiago I, Veríssimo A, Boaventura RAR, Nunes OC, Manaia CM (2006). Antibiotic resistance of *Enteroccoci* and related bacteria in an urban wastewater treatment plant. *FEMS Microbiol Ecol* 55:322–329.

da Silva MF, Vaz-Moreira I, Gonzalez-Pajuelo M, Nunes OC, Manaia CM (2007). Antimicrobial resistance patterns in *Enterobacteriaceae* isolated from an urban wastewater treatment plant. *FEMS Microbiol Ecol* 60:166–176.

Diehl DL, LaPara TM (2010). Effect of temperature on the fate of genes encoding tetracycline resistance and the integrase of class 1 integrons within anaerobic and aerobic digesters treating municipal wastewater solids. *Environ Sci Technol* 44:9128–9133.

FAAIR Scientific Advisory Panel (2002). Policy recommendations. *Clin Infect Dis* 34:S76–S77.

Frost LS, Leplae R, Summers AO, Toussaint A (2005). Mobile genetic elements: The agents of open source evolution. *Nat Rev Microbiol* 3:722–732.

Gallert C, Fund K, Winter J (2005). Antibiotic resistance of bacteria in raw and biological treated sewage an in groundwater below leaking sewers. *Appl Microbiol Biotechnol* 69: 106–112.

Ghosh S, LaPara TM (2007). The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. *ISME J* 1:191–203.

Ghosh S, Ramsden SJ, LaPara TM (2009). The role of anaerobic digestion in controlling the release of tetracycline resistance genes and class 1 integrons from municipal wastewater treatment plants. *Appl Microbiol Biotechnol* 84:791–796.

Harrison PF, Lederberg J (Eds.) (1998). *Antimicrobial Resistance: Issues and Opinions*. Workshop report. National Academy Press, Washington DC.

Heuer H, Krögerrecklenfort E, Wellington EMH, Egan S, Van Elsas JD, Van Overbeek L, Collard JM, Guillaume G, Karagouni AD, Nikolakopoulou TL, Smalla K (2002). Gentamicin resistance genes in environmental bacteria: Prevalence and transfer. *FEMS Microbiol Ecol* 42:289–302.

Levin BR, Lipsitch M, Bonhoeffer S (1999). Population biology, evolution, and infectious disease: convergence and synthesis. *Science* 283:806–809.

Levy SB (2005). Antibiotic resistance—The problem intensifies. *Adv Drug Del Rev* 57:1446–1450.

Levy SB, Marshall B (2004). Antibacterial resistance worldwide: Causes, challenges and responses. *Nat Med* 10:S122–S129.

Lipsitch M, Singer RS, Levin BR (2002). Antibiotics in agriculture: When is it time to close the barn door? *Proc Nat Acad Sci USA* 99:5752–5754.

Łuczkiewicz A, Jankowska K, Fudala-Książek S, Olańczuk-Neyman K (2010). Antimicrobial resistance of fecal indicators in municipal wastewater treatment plant. *Water Res* 44 (17):5089–5097.

Mazel D (2006). Integrons: Agents of bacterial evolution. *Nat Rev Microbiol* 4:608–620.

Normack BH, Normack S (2002). Evolution and spread of antibiotic resistance. *J Intern Med* 25:91–106.

Palumbi SR (2001). Evolution-humans and the world's greatest evolutionary force. *Science* 293:1786–1790.

Pruden A, Pei R, Storteboom H, Carlson KH (2006). Antibiotic resistance genes as emerging contaminants: Studies in Northern Colorado. *Environ Sci Technol* 40:7445–7450.

Ramsden SJ, Ghosh S, Bohl LJ, LaPara TM (2010). Phenotypic and genotypic analysis of bacteria isolated from three municipal wastewater treatment plants on tetracycline-amended and ciprofloxacin-amended growth media. *J Appl Microbiol* 109(5):1609–1618.

Staley JT, Konopka A (1985). Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial environments. *Ann Rev Microbiol* 39:321–346.

Szczepanowski R, Bekel T, Goesmann A, Krause L, Krömeke H, Kairser O, Eichler W, Pühler A, Schlüter A (2008). Insight into the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to antimicrobial drugs analysed by the 454-pyrosequencing technology. *J Biotechnol* 136:54–64.

Szczepanowski R, Linke B, Krahn I, Gartemann KH, Gutzkow T, Eichler W, Pühler A, Schlüter A (2009). Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. *Microbiol—SGM* 155:2306–2319.

Walter MV, Vennes JW (1985). Occurrence of multiple-antibiotic-resistant enteric bacteria in domestic sewage and oxidation lagoons. *Appl Environ Microbiol* 50:930–933.

14

STRATEGIES TO ASSESS AND MINIMIZE THE BIOLOGICAL RISK OF ANTIBIOTIC RESISTANCE IN THE ENVIRONMENT

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14.1 ROLE OF SEWAGE TREATMENT PLANTS IN THE TRANSFER OF RESISTANCE GENES

Pharmaceuticals can enter the aquatic environment via improper disposal of waste from humans and animals. Human pharmaceuticals, such as antibiotics, first enter municipal sewage treatment plants with the wastewater from private households and hospitals. During wastewater treatment, however, they are not eliminated completely and, hence, reach the surface waters. The volume of antibiotics released in effluents from hospitals and households indicates a selection pressure on bacteria (Kümmerer and Henninger, 2003). In this context, it is very difficult at the moment to estimate whether the antibiotics occurrence in clarified wastewaters or waters from receiving bodies contributes also to the spreading of resistances in potentially human pathogenic microorganisms.

The effect of an antibiotic is based on the inhibition of a specific process in the bacterial cell, for instance, by binding to an enzyme or substrate of bacterial metabolism. Genetically mediated resistance to antibiotics or biocides is based on various strategies. Some directly target the antibiotics (cleavage, inactivation, or discharge), others are based on modification or reconstruction of the point of attack. Bacterial resistance is understood to be the resistance of microorganisms against antibiotics and biocides. It is distinguished between primary (natural) resistance as

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the inherent property of a germ and secondary (acquired) resistance. Microorganisms have "learned" by evolution to defy the effect of antibiotics. Resistance genes may be transferred very rapidly and widely to other bacteria via mobile genetic elements such as plasmids or transposons. Gene transfer exceeds the limits of species and genera (horizontal gene transfer).

Resistance may also be caused by the modification of functional bacterial genes (mutation). Such mutations in the bacterial chromosome can then be transferred to the subsequent generation (vertical gene transfer). Since the introduction of antibiotics for the treatment of infections, a constant increase in microbial antibiotic resistance in bacteria found in hospitals and the exterior environment has been observed.

The largest problems result from microorganisms that have become resistant against antibiotics from various classes of active agents. Pathogenic microorganisms with multiple resistances to various antibiotics are not modified in terms of their pathogenicity to humans and animals, but the possibilities of successful medical therapy against infection are severely limited. Infections with resistant pathogens are characterized by a more complicated course of the disease and may even cause death under certain circumstances. In the case of such infections, these patients may not be treatable or the therapy is possible with an accompanying increased cost. Increased resistance against antibiotics such as vancomycin is observed in *Enterococcus faecium* and *Enterococcus faecalis* in hospitals. Transfer of vancomycin resistance genes to other bacteria, including staphylococci causing a high fraction of nosocomial infections, cannot be excluded (González-Zorn and Courvalin, 2003).

Tracking the main input sources of resistant bacteria can lead from hospitals ultimately to the sewage treatment plants. Hospitals with intensive care units and the connected municipal sewage treatment plants are discussed as major sources of input of resistant bacteria and hot spots of the transfer of resistance genes among aquatic bacteria. Some of the microbes of most concern can be eliminated in the activated sludge basin of the biological treatment stage, but in many cases those microorganisms that have resistance genes are incompletely removed. Every activated sludge floc represents a microuniverse with millions of various species of bacteria. Here, the resistance genes are free to exchange their carriers. They are frequently located on plasmids, which are passed on among bacteria, not only within a family but also to foreign bacterial species. In this way, the resistance genes can enter microorganisms that have had no prior exposure to an antibiotic.

High cell densities and cell diversities and the increased calcium and magnesium concentrations occurring in wastewaters and sewage treatment plants favor such a gene transfer. Nutrient fluctuations caused by fluctuating organic pollutions of wastewaters are considered another environmental stimulus of gene exchange. Consequently, the clear water may contain a number of bacteria that now carry resistance genes. They enter rivers and spread further. What exactly happens in nature remains to be discovered. From the rivers, it is possible for bacteria with antibiotic resistance genes present to come in contact with humans in drinking water.

According to studies of resistance to antibiotics used for a longer time, the number of multiresistant bacteria increases. Moreover, *Escherichia coli* and coliform bacteria resistant to six to eight antibiotics at the same time have been found in wastewater and surface water (Feuerpfeil et al., 1999). Multiresistant *Pseudomonas aeruginosa* isolates being resistant to five and up to seven therapeutically relevant antibiotics

have also been found in sewage and receiving waters (Schwartz et al., submitted for publication). Currently, there is little data available regarding the effects of realistic mixtures of antibiotics on the bacteria present in the aeration tanks of sewage treatment plants.

Sewage overflows, which can occur after heavy rainfall, are likely to spread antibiotic resistance in the environment. This was confirmed in a case study in which antibiotic resistance was frequently detected in mud samples from flooded areas after heavy flooding in 2002 of the Elbe and Mulde rivers in Germany. In this study on the effects of facultative pathogenic microorganisms, high cell counts were found in bacterial isolates for multiple antibiotic resistance (MAR) in the river mud in flooded cellars, playgrounds, and streets one year after the flooding. Such MAR cell counts for the river itself were much lower. The likely cause of the high cell counts was the overflow of nearby wastewater treatment plants (WTPs) (Abraham and Wenderoth, 2005).

Specific studies of the Karlsruhe Institute of Technology (KIT) and partners focused on *E. faecium*/*faecalis* resistance to vancomycin and *P. aeruginosa* resistance to ciprofloxacin and imipenem (Schwartz et al., 2003, 2006; Volkmann et al., 2004). Both *E. faecium* and *P. aeruginosa* are so-called facultative pathogenic bacteria, which means that they may cause infections primarily in immune-deficient patients.

14.1.1 Detection of Vancomycin-Resistant Enterococci

Different genotypes of glycopeptide resistance exist in enterococci. Of these the *van*A genotype is the most frequent in central Europe (Iversen et al., 2002; Witte et al., 1999; Courvalin, 1990). The expression of glycopeptide resistance in enterococci can be induced by glycopeptides but not by other antibiotics (Leclercq et al., 1999).

A resistance to glycopeptide vancomycin of *E. faecium* and *E. faecalis* is mediated by the gene *vanA* that can be detected with molecular biology methods [polymerase chain reaction (PCR), sequencing, etc.]. We have studied wastewater samples from various wastewater partial flows and sewage treatment plants (Table 14.1).

Following the extraction of the deoxygribonucleic acid (DNA) from the wastewater samples, *E. faecium*/*faecalis* as a potential carrier of the resistance gene *vanA* was detected directly in 61% of the samples without previous enrichment with synthetic nutrients. In addition, wastewater samples were subjected to an artificial enrichment of these bacteria prior to DNA extraction. In this case, enterococci were detected in 100% of these samples. The resistance gene *vanA* was found in 22% of the original wastewater samples and 80% of the enriched wastewater samples. In some samples,

TABLE 14.1 PCR Detection of *E. faecium*, *E. faecalis*, and the Vancomycin-Specific Antibiotic Resistance Gene *van*A in Municipal Wastewaters

	Genomic DNA from Wastewater	Enterococci-Specific Enrichment
Number of samples	18	15
Enterococci positive	11	15
% of positive samples	61	100
*van*A gene positive	4	12
% *van*A positive samples	22	80

no enterococci were detected, but the resistance gene *vanA* was. This may indicate that the resistance gene *vanA* that initially existed in enterococci only has already been transferred horizontally to other bacterial species.

14.1.2 Detection of Rsistant and Multiple Resistant *P. aeruginosa*

Resistance against the antibiotic ciprofloxacin frequently used in hospitals is not caused by acquired genes but by mutations. Resistance is mediated by specific exchanges of certain bases of the DNA. Such modifications can be detected by molecular biology methods. The targets of ciprofloxacin are considered to be the type II topoisomerases (DNA gyrase and topoisomerase IV), which are essential enzymes for controlling the topological state of DNA during replication and transcription. Alterations in DNA gyrase and topoisomerase IV caused by mutations appear to play a major role in fluoroquinolone resistance in clinical isolates of *P. aeruginosa*. As previously shown, an amino acid replacement in the gyrase loci GyrA (Thr-83 to Ile or Ala, or Asp-87 to Asn, Gly or Thy), Gyr B (Asp-426 or Lys-447), and ParC (Ser-87 to Leu), leading to a fluoroquinolone resistance (Akasaka et al., 2001). We analyzed such mutations in environmental isolates using minisequencing in combination with capillary electrophoreses (Schwartz et al., 2006). Ciprofloxacin-resistant *P. aeruginosa* strains were isolated from clinical wastewaters. Most of these bacteria carried mutations in position *gyr*A83, with an amino acid change from Thr to Ile and in *par*C97 with an amino acid change from Ser to Leu. As presented in Table 14.2, all environmental ciprofloxacin-resistant isolates carried mutations at *gyr*A codon positions 87 and 91 and in lower quantities at *par*C codon position 87 and 91. No mutation was found at *gyr*B codon position 466. In contrast to clinical isolates in other studies, no mutations at *gyr*A codon position 87 were detected in wastewater *P. aeruginosa* (Table 14.2). Ciprofloxacin-resistant *P. aeruginosa* strains carrying these point mutations were enriched from wastewaters in all cities under investigation. Furthermore, these bacteria passed the wastewater treatment plant and were also detected in downstream river water. The results indicate that humans under antibiotic therapy might be a possible origin of ciprofloxacin-resistant *P. aeruginosa* dissemination into the environment. As a consequence, these resistant bacteria occur in aquatic environments even with presumably low antibiotic selection pressure. Some antibiotics, such as fluoroquinolones and β-lactams in subinhibitory concentrations, have been shown to increase the frequency of mutants by inducing the SOS response is meaning the induction of cascades of stress responses in bacteria and/or

TABLE 14.2 Percentage of Fluoroquinolone-Resistance-Mediating Mutations in *n* Resistant *P. aeruginosa* Isolates from Clinical Patients and Wastewater Samples

Resistant Clinical Isolates from Patients (n=116)[a]	Resistant Isolates from Wastewater (n=120)[b]
94% mutation in *gyr*A	100% mutation in *gyr*A
13% mutation in *gyr*B	0% mutation in *gyr*B
84% mutation in *par*C	86% mutation in *par*C

[a]From Akasaka et al. (2001).
[b]From Schwartz et al. (2006).

has been recently described as a stimulator of recombination between divergent DNA sequences including horizontal transfer of DNA sequences (Tattevin et al., 2009; López and Bláques, 2009). When concerned with horizontal gene transfer, antibiotic stress induces transformability via competence (Prudhomme et al., 2006), and antibiotic pressure may also select for bacterial cells with an increased frequency of mutation (hypermutators) (Mao et al., 1997).

In this regard, clinical as well as municipal wastewaters are important reservoirs in which antibiotic-resistant organisms and/or determinants persist and are released to the environment. By linking different environmental compartments, including municipal sewage and surface water, wastewater treatment plants may facilitate the spread of antibiotics, antibiotic resistance genes, and resistant bacteria. Furthermore, the high microbial density and diversity of biofilms and activated sludge may facilitate genetic exchange in wastewater treatment plants (Zhang et al., 2009, Schlüter et al., 2007).

However, we have demonstrated the persistence of multiresistant *P. aeruginosa* carrying up to seven acquired therapeutically relevant antibiotic resistance mechanisms in clinical and downstream municipal wastewater systems of two German cities (Schwartz et al., 20xx) (Table 14.3). Agar diffusion testing as well as molecular biology analyses confirmed their multiresistant phenotypes. Pulsed field gel electrophoresis (PFGE) genotyping resulted in different genogroups of *P. aeruginosa* isolates depending on sampling points and numbers of resistances. Furthermore, multiresistant *P. aeruginosa* were also released from municipal wastewater treatment plants to the receiving body (Schwartz et al., 20xx). This result demonstrated that wastewater treatment is not sufficient to retard antibiotic-resistant pathogens released from hospital environment. Pirnay et al. (2005) found no real difference between *P. aeruginosa* strains released from clinical to environmental habitats. The pattern of biodiversity within clinical samples, and aquatic samples from a Belgium river, resembled that of all global samples (clinical and environmental).

Furthermore, multiresistant *P. aeruginosa* isolates were found mainly in hospital wastewaters or wastewaters influenced by hospitals. Along the wastewater pathway, these microorganisms were detected up to the sewage treatment plant and even beyond in the receiving water (Table 14.4). Hence, hospitals are one of the hot spots of

TABLE 14.3 Detection of Multiresistant *P. aeruginosa* along the Wastewater Pathway from Hospital to Receiving Body[a]

	Numbers of Resistances in One Isolate		
Source of Samples	5	6	7
WW hospital A	+	+	+
WW hospital B	−	−	+
WTP influent	+	+	+
WTP effluent	−	−	+
River at WTP	−	+	+
River before WTP	−	−	−
River after WTP	−	−	−

[a]Multi-resistant means more than five clinically relevant antibiotic resistances in one isolate. WW: wastewater; WTP: wastewater treatment plant.

TABLE 14.4 Detection of Multiresistant *P. aeruginosa* in Wastewater and Surface Water Compartments of Three German Cities

	City A	City B	City C
Housing area	−	−	−
Hospital	+	+	+
Inflow WTP	+	+	+
Effluent WTP	+	−	−
Receiving water	+	−	−

the emission of these multiresistant bacteria. Antibiotic-resistant bacteria, including *P. aeruginosa*, were also found in domestic wastewaters without hospital influence, but there were no representatives with multiresistance to more than five antibiotics.

14.2 FATE OF ANTIBIOTIC-RESISTANT BACTERIA AND THEIR RESISTANCE DETERMINANTS

The study of fecal indicators has dominated many studies of antibiotic-resistant bacteria in the aquatic environment, but little work has been done to assess the prevalence of drug-resistant bacteria in treated drinking water and their relationships to antibiotic-resistant microorganisms in untreated source waters (Xi et al., 2009; Armstrong et al., 1981). Such studies have found increased rates of resistant bacteria in drinking water within the distribution network by standard plate count experiments and have concluded that the treatment of raw water and its subsequent distribution selects antibiotic-resistant bacteria. In agreement with these data, increased phenotypic resistance rates were also detected at the drinking water sampling points in our studies. Additionally, genotypical investigations concerning the underlying resistance mechanisms were performed to distinguish between intrinsic and acquired antibiotic resistance. The occurrence of the *van*A and *amp*C resistance genes in heterotrophic bacteria confirmed the influence of the water sources on the genotype of the drinking water bacteria. Furthermore, most of the available literature data concerning resistant bacteria and their genotypes in the environment are from bulk water analysis, food samples, and patient isolates. In the present study, biofilms were analyzed for resistant bacteria and their genotypes.

Genetic techniques were established to detect resistance genes directly in biofilm populations without cultivation, based on the assumption of gene carriage equaling resistance. Strong amplicon DNA bands specific to *van*A and *amp*C, respectively, were found in hospital biofilms and in the sludge of the municipal sewage treatment plant (Schwartz et al., 2003, 2006; Volkmann et al., 2004). Quantitative real-time-PCR analyses targeting of ampicillin (*amp*C), vancomycin (*van*A), and imipenem (bla_{vim}) resistance genes were performed on water samples from wastewater pathways of three different German cities. Without any enrichment of selected bacteria, the resistance genes could be amplified from previously extracted genomic DNA from entire wastewater populations (Table 14.5). Variable abundances of resistance genes were quantified for the different cities and sampling points. High quantities for all resistance genes were found in clinical wastewaters; the ampicilin resistance genes were detected in all sampling points including effluent water of the treatment plant.

TABLE 14.5 Abundances of Ampicillin (*amp*C), Vancomycin (*van*A), and Imipenem (*bla*VIM) Antibiotic Resistance Genes in Total Genomic DNA from Wastewater Samples of Three German Cities[a]

	City A			City B			City C		
Wastewater Pathways	*amp*C	*van*A	bla_{vim}	*amp*C	*van*A	bla_{vim}	*amp*C	*van*A	bla_{vim}
Housing area	8.9×10^4	—	3.0×10^0	1.8×10^4	—	—	8.8×10^3	—	1.2×10^1
Clinical wastewater	1.5×10^4	3.3×10^2	4.9×10^4	9.9×10^2	*9.0×10^2*	9.0×10^0	1.8×10^3	—	2.3×10^1
Inflow WTP	4.6×10^4	—	1.8×10^3	2.2×10^2	*8.2×10^1*	6.0×10^0	2.2×10^3	—	1.0×10^1
Effluent WTP	1.6×10^3	—	1.6×10^4	5.4×10^1	1.0×10^0	1.8×10^1	3.9×10^2	—	—

[a]Numbers are cell equivalents/100 ng total DNA.

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TABLE 14.6 Detection of Clinical Relevant Antibiotic Resistance Genes in Total DNA from Natural Biofilms as Well as Enriched Bacteria

	*amp*C gene		*van*A gene		*mec*A gene	
Biofilms of Water Cycle	In Entero bacteriaceae	Total DNA	In Enterococci	Total DNA	In Staphylococci	Total DNA
In clinical wastewater	+	+	+	+	+	+
In wastewater treatment plant	+	+	+	+	−	−
In receiving water	+	+	+	+	−	−
In drinking water biofilm	−	+	−	+	−	−

In some cases, vancomycin as well as imipenem resistance were detected in bacterial community genomes from effluent wastewaters, indicating the dissemination of resistance genes into the downstream aquatic environments.

Moreover, clinically relevant resistance determinants were detected in biofilms of a drinking water distribution system. It is possible that they had been transferred to drinking water bacteria by horizontal gene transfer and, thus, passed the processing barriers (Table 14.6) (Schwartz et al., 2003). The vancomycin resistance gene *van*A and the β-lactam resistance gene *amp*C were amplified from the genomic DNA of various drinking water biofilms of a public distribution system, whereas no enterococci or Enterobacteriaceae were cultivated. Also, ribosomal ribonucleic acid (rRNA)-related sequences, characteristic for enterococci, could not be found, which also indicates the absence of these bacteria. The molecular biological analysis based on the detection of ribosomal DNA (rDNA)-specific sequences confirmed the result that, despite the presence of *van*A, no enterococcal ribosomal amplicons were found in parallel PCR assays. It is clear that both the *van*A resistance gene and ribosomal DNA must be amplified from drinking water biofilms if enterococci are present in these biofilms independent of their physiological state. In the following the deductions are summarized:

- The homolog resistance genes *van*A and *amp*C found in drinking water biofilms are not located in enterococci or Enterobacteriaceae (since these were absent) but also not on the culturable heterotrophic bacteria. Therefore, other (nonculturable heterotrophic) bacteria must account for the signal in the PCR.
- The resistance genes *van*A and *amp*C were not found in the culturable heterotrophes; the observed resistance in these culturable heterotrophic drinking water bacteria to selected antibiotics is apparenty not mediated via *van*A and *amp*C. Thus, this resistance is due to other mechanisms, for example, other genes.
- The presence of the *van*A and *amp*C resistance genes in the drinking water system biofilms may be the result of horizontal gene transfer from enteric bacteria to autochtonous aquatic bacteria, given the high degree of homology. This transfer may have happened on several occasions between the presumed source (wastewater) and sink (surface water), although an autochthonous origin of this gene cannot be excluded.

Great diversity of microorganisms coupled with a high density of immobilized biomass support the exchange of resistance genes by horizontal gene transfer. Both aspects are found, especially in biofilms from aquatic systems. About 60–90% of Gram-negative bacteria are able to exchange plasmids carrying resistance genes (R plasmids) via horizontal gene transfer (Davison, 1999).

In addition to the transfer of resistant bacteria to the aquatic system, antibiotics in the aquatic environment could have an influence on the resistance situation. Recent studies have demonstrated the occurrence of various antimicrobial compounds in wastewaters and in wastewater treatment plants (Hirsch et al., 1999; Giger et al., 2003). Although the antimicrobial concentrations found in wastewater are generally lower than the concentrations necessary to inhibit the growth of bacteria, they may affect susceptible bacteria and determine selection in favor of resistant bacteria. Thus, investigations relating to the fate and occurrence of antibiotics resistance complement the results found for antibiotic agents in the environment.

14.3 BIOEFFECTIVITIES OF LOW CONCENTRATED ANTIBIOTICS

Antibiotics that enter the wastewater and surface water via numerous anthropogenic sources due to their increasing use possibly affect the resistance of bacteria and are therefore of particular importance. However, chemical and analytical studies lack the biological analysis of their effects on microorganisms in terms of resistance formation and subsequent reproduction of clinically relevant resistant bacteria in microbial communities in the aquatic environment. Although antibiotic concentrations found in wastewaters were for the most part, considerably lower than the minimum inhibitor concentrations valid in the medical therapy, they are postulated to affect sensitive bacteria and, thus, select resistant microorganisms in the aquatic environment (Davison, 1999). Davies et al. (2006) described effects of subinhibitory concentrations of antibiotics in a wide range of genes not related to target functions, indicating functional interactions with many other cellular processes. Such phenomena are known as "hormesis," where low doses of antibiotics stimulate different biological responses than high-dose applications. For example, low concentrations of antibiotics influence the gene expression in biofilm populations (Ohlsen et al., 1998). Rachid et al. (2000) describe effects on polysaccharide synthesis in biofilms of *Staphylococcus aureus*. The comprehensive study of Goh et al. (2002) confirmed that antibiotics in subinhibitory concentration ranges have a modulatory effect on the transcription of 5% of 6500 gene promoters studied.

Bactericidal antibiotics can also stimulate bacteria to produce reactive oxygen species (ROS) (Kohanski et al., 2007; Dwyer et al., 2009), which are highly deleterious molecules that can interfere with the normal functions of oxygen-respiring organisms. Certain ROS, such as hydroxyl radicals, can directly damage DNA and lead to an accumulation of mutations. Kohanski et al. (2010) demonstrated that sublethal antibiotic treatment can lead to multidrug resistance in *S. aureus* and *E. coli*, which has important implications for the widespread use and misuse of antibiotics. The effect of low-concentrated antibiotics on bacteria has hardly been studied with respect to the specific induction of resistance mechanisms in the environment so far.

Due to their effectiveness against enterococci, glycopeptide antibiotics (vancomycin and teicoplanin) are very important for the treatment of severe infections in

TABLE 14.7 Gene Expression Analyses of the Vancomycin Resistance Gene *van*A in *E. faecium* B7634 Being Resistant Against Vancomycin[a]

Clinical Wastewater Spiked with Vancomycin	Expression of the *van* A Resistance Gene
0 mg/L	No expression
0.032 mg/L	Weak expression
0.32 mg/L	Weak expression
3.20 mg/L	Strong expression
32.0 mg/L	Strong expression

[a]*Van*A mRNA was detected via Northern blot hybridization.

humans. Expression of a glycopeptide resistance in enterococci can only be induced by the glycopeptide itself (Patel et al., 1997) and not by other antibiotics (Leclercq et al., 1999). In biofilms of hospital wastewater, enterococci with the vancomycin resistance-mediating gene *vanA* reached a fraction of 25% of the total enterococci population (Schwartz et al., 2003). At the Karlsruhe Institute of Technology, a dose-dependent expression of the resistance gene *vanA* was measured on the messenger RNA (mRNA) level. In this biological test, it was shown that even low concentrations of 0.032 mg/L vancomycin, far below the clinically defined minimum inhibitory concentration (i.e., 32 mg/L according to Clinical and Laboratory Standards Institute (CLSI) for enterococci, resulted in an induction of *van*A gene expression. The concentration that induced gene expression was lower than the clinically used antibiotics concentration by a factor of 1000 (Table 14.7) (Obst et al., 2006). These data confirmed that low concentrated vancomycin had a molecular biological effect in reference bacteria. The relevance for a selective pressure under environmental conditions is still an open question.

Thus, selective pressure refers also to environmental conditions especially in case of low-level resistant bacteria and other environmental factors such as other drugs or environmental pollutants (Bacquero et al., 1998). Transcriptome and proteome analyses have revealed that the changes in expression profiles induced by different antibiotics are very diverse and that they can be detected in a wide range of genes not related to the target functions (Davies et al., 2006). The effect of subinhibitory concentrations of antibiotics on a range of general responses in the cell have been identified and exhibited changed properties when compared to high concentrations (Yim et al., 2007).

14.4 REDUCTION OF THE DISSEMINATION OF ANTIBIOTIC-RESISTANT BACTERIA

Many studies demonstrated that clinical wastewaters are often heavily charged with organic compounds as well as pathogenic bacteria. These wastewaters are known to be one important source of the dissemination of infectious agents, including antibiotic multiresistant pathogens, into the downstream aquatic systems. Although reductions of pathogens were observed during sewer passage and more significantly during the treatment at the municipal plants, hazardous microorganisms nevertheless reached the aquatic environments. So far, the disinfection techniques applied have demonstrated disadvantages such as toxic by-products during chemical disinfection

or reduced efficiency in case of ultraviolet (UV) disinfection owing to the high particle load of the untreated wastewaters. Within the framework of our studies, an alternative local wastewater disinfection technique was applied for clinical wastewater treatment (Rieder et al., 2008; Gusbeth et al., 2009). The pulsed electric field (PEF) technology is directed against membranes of biological cells. The bielectrical breakthrough of the phosphorus lipid double layer in biological membranes induced by pulsed electric fields is known to be one of the major principles of this technique for cell disruption. Owing to the nonchemical treatment of clinical wastewater during PEF application, no technique-induced by-products are expected. Hospital wastewater was treated with the PEF technology. The inactivation efficiencies of bacteria were successfully monitored with real-time PCR. As the differentiation between living and dead bacterial cells is important for the determination of the disinfection efficiency, propidium monoazide (PMA) was applied (Nocker et al., 2007). PMA selectively penetrates cells with compromised membranes and intercalates into the DNA inhibiting a subsequent PCR amplification. The rates of reduction were examined for specific pathogens, including antibiotic-resistant bacteria and wastewater populations using PCR-denaturing gradient gel electrophoresis. The results demonstrated that the main part of the bacterial population could be inactivated efficiently with the PEF treatment (Rieder et al., 2008). Moreover, it was demonstrated that naturally occurring nuclease activities were not affected by the PEF treatment in contrast to a thermal treatment. The results indicated that the PEF treatment is an appropriate alternative disinfection concept for the treatment of clinical wastewaters and surpass the disadvantages of other disinfection methods and to minimize the dissemination of antibiotic-resistant bacteria to the environment.

14.5 STRATEGY TO EVALUATE THE RESISTANCE SITUATION AND ITS BIOLOGICAL RISK

The increase in antibiotic-resistant pathogens was recognized by the World Health Organization (WHO) to be an increasing problem that will make the health care systems of the world's population face big challenges in the future. Even resistance against new antibiotics can be found in bacteria. Pathways of spreading resistant bacteria are only partly understood.

The concept for the determination of resistance situations in aquatic systems developed by the Karlsruhe Institute of Technology allows an identification of hot spots of the introduction of resistant and above all multiresistant bacteria. This concept is based on the quantitative determination of resistance factors, indicators of multiresistance, and resistance gene induction by water ingredients (Fig.14.1). This is the basis of a biological assessment of an aquatic biotope in terms of antibiotic resistance. Horizontal gene transfer events that are particularly relevant to wastewater and biofilms play an important role in the spreading of clinically relevant resistance. Thus, molecular biological approaches should be used to study the total biocenoses. In the individual case, recommendations for further wastewater processing can be derived to minimize the release of resistance genes or multiresistant bacteria into the receiving environment. In any case, the changes of diversities and abundances of microorganisms in water and sediment populations under wastewater influence induced by antibiotics-resistant bacteria should be considered a primary effect.

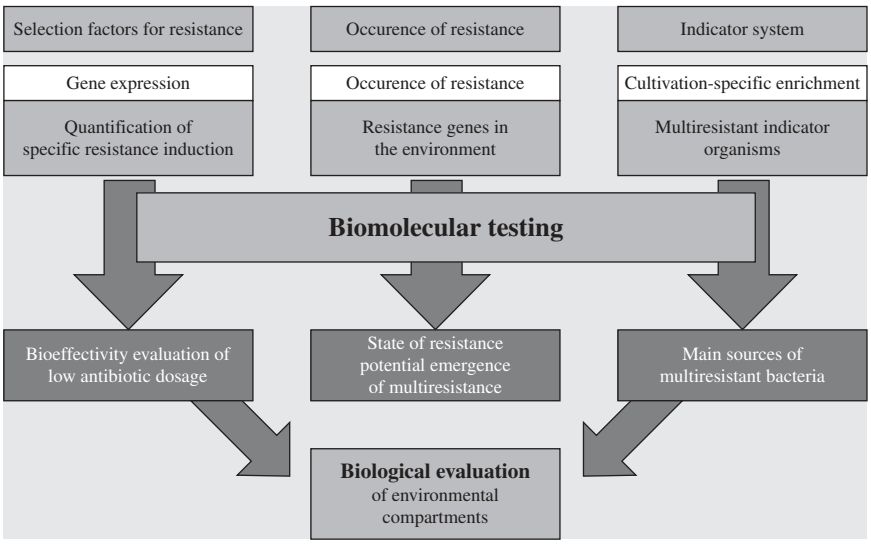

FIGURE 14.1 Strategy for biological determination of resistance situations with microbiology and molecular biology methods.

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REFERENCES

Abraham WR, Wenderoth DF (2005). Fate of facultative pathogenic micro organisms during and after the flood of the Elbe and Mulde rivers in August 2002. *Acta Hydroch Hydrob* 33:449–454.

Akasaka T, Tanaka M, Yamaguchi A, Sato K (2001). Type II topoisomerase mutations in fluoroquinolone-resistant clinical strains of *Pseudomonas aeruginosa* isolated in 1998 and 1999: Role of target enzyme in mechanism of fluorochinolone resistance. *Antimicrob Agents Chemother* 45:2263–2268.

Armstrong J, Shigeno D, Calomiris J, Seidler R (1981). Antibiotic-resistant bacteria in drinking water. *Appl Environ Microbiol* 42:277–283.

Bacquero F, Negri MC, Morosini MI, Blázquez J (1998). Antibiotic-selective environments. *Clin Infect Dis* 27:5–11.

Courvalin P (1990). Resistance of enterococci to glycopeptides. *Antimicrob Agents Chemother* 36:2201–2203.

Davies J, Spiegelman G, Yim G (2006). The world of subinhibitory antibiotic concentration. *Curr Opin Microbiol* 9:445–453.

Davison J (1999). Genetic exchange between bacteria in the environment. *Plasmid* 42:73–91.

Dwyer D, Kohansky M, Collins J (2009). Role of reactive oxygen species in antibiotic action and resistance. *Curr Opin Microbiol* 12:482–489.

Feuerpfeil I, López-Pila J, Schmidt R, Schneider E, Szewzyk R (1999). Antibiotic resistant bacteria and antibiotics in the environment. *Bundesgesundheitsblatt-Gesundheitsforschung-Gesundheitsschutz* 42:37–50.

Giger W, Alder A, Golet E, Kohler HP, McArdell C, Molnar E Siegrist HR, Suter M (2003). Occurrence and fate of antibiotics as trace contaminants in wastewater, sewage sludges, and surface waters. *Chimica* 77:485–491.

Goh E, Yim G, Tsui W, McClure J, Surette M, Davies J (2002). Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics. *Proc Natl Acad Sci USA* 99:17025–17030.

González-Zorn B, Courvalin P (2003). *Van*A-*mediated high level glycopeptide resistance in* MRSA. *Lancet* 3:67–68.

Gusbeth C, Frey W, Volkmann H, Schwartz T, Bluhm H (2009). Pulsed electric field treatment for bacteria reduction and its impact on hospital wastewater. *Chemosphere* 75:228–233.

Hirsch R, Ternes T, Haberer K, Kratz KL (1999). Occurrence of antibiotics in the aquatic environment. *Sci Total Environ* 225:109–118.

Iversen A, Kühn I, Franklin A, Möllby R (2002). High prevalence of vancomycin-resistant enterococci in Swedish sewage. *Appl Environ Microbiol* 68:2838–2842.

Kohanski M, Dwyer D, Hayete B, Lawrence C, Collins J (2007). A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* 130:797–810.

Kohanski M, DePristo M, Collins J (2010). Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. *Mol Cell* 37:311–320.

Kümmerer K, Henninger A (2003). Promoting resistance by the emission of antibiotics from hospitals and households into effluent. *Clin Microbiol Infect* 9:1203–1214.

Leclercq R, Dutka-Malen S, Brisson-Noel A, Molinas C, Derlot E, Arthur M, Duval J, Witte W, Klare I, Werner G (1999). Selective pressure by antibiotics as feed additives. *Infection* 27:35–38.

López E, Blázques J (2009). Effect of subinhibitory concentrations of antibiotics on intrachromosomal homologues recombination in *Escherichia coli*. *Antimicrob Agents Chemother* 53:3411–3415.

Mao E, Lane L, Lee Miller J (1997). Proliferation of mutators in a cell population. *J Bacteriol* 179:417–422.

Nocker A, Sossa-Fernandez P, Burr M, Camper A (2007). Use of propidium monoazide for live/dead distinction in microbial ecology. *Appl Environ Microbiol* 73:5111–5117.

Obst U, Schwartz T, Volkmann H (2006). Antibiotic resistant pathogenic bacteria and their resistance genes in bacterial biofilms. *J Artificial Organs* 29:387–394.

Ohlsen K, Ziebuhr W, Koller KP, Hell W, Wichelhaus T, Hacker J (1998). Effects of subinhibitory concentrations of antibiotics on alpha-toxin (HLA) gene expression of methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* isolates. *Antimicrob Agents Chemother* 42:2817–2823.

Patel R, Uhl J, Kohner P, Hopkins M, Cockerill F (1997). Multiplex PCR detection of *van*A, *van*B, *van*C-1, and *van*C-2/3 genes in enterococci. *J Clin Microbiol* 35:703–707.

Pirnay JP, Matthijs S, Colak H, Chablain P, Bilocq F, Van Eldere J, De Vos D, Zizi M, Triest L, Cornelis P (2005). Global *Pseudomonas aeruginosa* biodiversity as reflected in a Belgian river. *Environ Microbiol* 7:969–980.

Prudhomme M, Ataich L, Sanchez G, Martin B, Claverys J (2006). Antibiotic stress induces genetic transformability in the human pathogen *Streptococcus pneumoniae*. *Science* 313:89–92.

Rachid S, Ohlsen K, Witte W, Hacker J, Ziebuhr W (2000). Effect of subinhibitory antibiotic concentrations on polysaccharide intercellular adhesin expression in biofilm-forming *Staphylococcus epidermidis*. *Antimicrob Agents Chemother* 44:3357–3363.

Rieder A, Schwartz T, Schön-Hölz K, Marten SM, Süß J, Gusbeth C, Kohnen W, Swoboda W, Obst U, Frey W (2008). Molecular monitoring of inactivation efficiencies of bacteria during pulsed electric field treatment of clinical wastewater. *J Appl Microbiol* 105:2035–2045.

Schlüter A, Szczepanowski R, Pühler A, Top E (2007). Genomic of IncP-1 antibiotic resistance plasmids isolated from wastewater treatment plant provides evidence for a widely accessible drug resistance gene pool. *FEMS Microbiol Rev* 31:449–477.

Schwartz T, Kohnen W, Jansen B, Obst U (2003). Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS Microbiol Ecol* 43:325–336.

Schwartz T, Volkmann H, Kirchen S, Kohnen W, Schön-Hölz K, Jansen B, Obst U (2006). Real-time PCR detection of *Pseudomonas aeruginosa* in clinical and municipal wastewater and genotyping of the ciprofloxacin-resistant isolates. *FEMS Microbiol Ecol* 57:158–167.

Schwartz T, Schön-Hölz K, Ott E, Kohnen W (20xx). Multi-resistant *Pseudomonas aeruginosa* in clinical and municipal wastewater systems. Submitted for publication.

Tattevin P, Basuino L, Chambers H (2009). Subinhibitory fluoroquinolone exposure selects for reduced beta-lactam susceptibility in methicillin-resistant *Staphylococcus aureus* and alterations in the SOS-mediated response. *Res Microbiol* 160:187–192.

Volkmann H, Schwartz T, Bischoff P, Kirchen S, Obst U (2004). Detection of clinically relevant antibiotic-resistance genes in municipal wastewater using real-time PCR (TaqMan). *J Microbiol Methods* 56:277–286.

Witte W, Wirth R, Klare I (1999). Enterococci. *Chemother* 45:135–145.

Xi C, Zhang Y, Marrs C, Ye W, Simon C, Foxman B, Nriagu J (2009). Prevalence of antibiotic resistance in drinking water treatment and distribution system. *Appl Environ Microbiol* 75:5714–5718.

Yim G, Wang HH, Davies JE (2007). Antibiotics as signalling molecules. *Philos Trans R Soc Lond B Biol Sci* 362:1195–1200.

Zhang Y, Marrs C, Simon C, Xi C (2009). Wastewater treatment contributes to selective increase of antibiotic resistance among *Acinetobacter* spp. *Sci Total Environ* 407:3702–3706.

15

ANTIBIOTIC RESISTANCE IN ANIMALS—THE AUSTRALIAN PERSPECTIVE

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In accord with changes in other parts of the world due to antibiotic resistance, antibiotic use in food-producing animals has changed significantly over the last 10 years in Australia. In addition, some antibiotics widely used overseas are not registered for use in food-producing animals. On the other hand, the range of antibiotics available for use in companion animals has increased. No antibiotics are registered for use in aquaculture at this time, however, off-label use is common. These factors impinge on the antibiotic sensitivity of isolates from animals and environmental sources. Treatment failure in animals due to antibiotic resistance appears to be a rare event as resistance to animal pathogens is uncommon, although there are significant exceptions including widespread resistance in enterotoxigenic *Escherichia coli* from pigs, enteric isolates from calves, *Staphylococcus aureus* from mastitic milk from cattle, and skin and ear isolates from dogs. The tight controls on antibiotics used in livestock appear to have had a significant impact on resistance in some human pathogens, for example, fluoroquinolone resistance in *Campylobacter*, which is still at very low levels.

15.1 REGULATORY CONTROL OF ANTIBIOTIC USE

15.1.1 Background

The increasing prevalence of antibiotic resistance in bacteria that cause diseases in humans has sparked much public and governmental concern in most developed

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countries. The implications for the effectiveness of antibiotics in treating human diseases and treatment failure impose a huge health cost on the economy. The rate at which antibiotic resistance emerges and new antimicrobials are developed is one that is not equivalent.

For the purpose of this chapter, particular emphasis is placed on bacterial disease agents. One of the means of controlling diseases, especially those caused by bacteria, is with the use of antibiotics. Antibiotics are used in animals to treat, or as a prophylactic to prevent, infections. Antibiotics have also been used for growth promotion or production-enhancing purposes in food-producing animals. The use of antibiotics, however, comes with other issues such as bacteria resistance to antibiotics.

There has been a significant amount of debate, arguments, and controversies about the use of antimicrobials in animals, especially food-producing animals. Many believe that resistance to antimicrobials important to human medicine has been generated in animals and is spread to humans with the potential to cause major harm (Threlfall et al., 2000; Luo et al., 2003) while others believe that the evidence is minimal or nonexistent (Phillips et al., 2004). However, the consensus view is that antibiotic use in food-producing animals has influenced the emergence of resistance in human pathogens, particularly in the case of zoonotic enteric pathogens such as salmonella and *Campylobacter* and enterococci and coliforms ((Hammerum and Heuer, 2009; Davies and Davies, 2010). More recently, it has become clear that methicillin-resistant *Staphylococcus aureus* (MRSA) can pass between humans and animals and *vice versa*, thus direct transfer of resistant organisms as well as food-borne transfer must be taken into account (Morgan, 2008). The challenges posed by antimicrobial resistance have led to the formation of expert groups that have issued various reports in some developed countries. The focus of these reports has been upon the use of antibiotics in animals and, in particular, their use as feed additives for the purpose of growth promotion.

Many countries have been implementing programs for surveillance of antibiotic resistance and antibiotic use to monitor these factors in food-producing animals, for example, Denmark DANMAP (Emborg et al., 2004), Sweden SVARM (Bengtsson et al., 2004), Norway NORM/NORM-VET (Blix et al., 2004), the Netherlands (NETHMAP, 2009), Canada CIPARS (Bair et al., 2003), United States-NARMS (Fedorka-Cray et al., 1998), and Japan JVARM (http://www.nval.go.jp/taisei/taisei.html, 1999). In Australia, the Minister for Health and Aged Care and the Minister for Agriculture, Fisheries and Forestry established a Joint Expert Technical Advisory Committee on Antibiotic Resistance (JETACAR) in 1998 that included experts from human health, veterinary medicine, and primary industry. Their job was to assess the scientific evidence for a link between the use of antibiotics in food-producing animals and the emergence of antibiotic-resistant bacteria in humans. They also develop evidence-based recommendations for the appropriate future management of antibiotic use in food-producing animals. Many of the recommendations were implemented, but 10 years later and progress has halted, and there appears to be no government interest in developing antimicrobial resistance surveillance programs similar to those in other countries.

Use of agricultural and veterinary chemicals (including antibiotics) is regulated through the Australian Pesticides and Veterinary Medicines Authority (APVMA). In response to JETACAR, APVMA revised its requirements regarding applications for registration of antibiotics to include special data relating to antibiotic resistance,

including a risk assessment relating to the use of antibiotics in both food-producing and non-food-producing animals (APVMA, 2009). Another outcome from JETACAR was the scheduling by the National Drugs and Poisons Scheduling Committee of all antibiotics used in animals (except for some products used in home aquaria and in aviary birds and some of the growth-promoting products such as ionophores, avilamycin, flavophospholipol, and olaquindox) as prescription-only medicines (http://www.tga.gov.au/ndpsc/index.htm) (NDPSC, 2003).

15.1.2 Use of Antibiotics in Food-Producing Animals in Australia

This conservative approach to registration of antibiotics for use in animals has resulted in the prohibition or severe limitation of the use of certain antibiotics. For example, use of fluoroquinolones and gentamicin has never been permitted in food-producing animals and use of chloramphenicol, streptomycin, and nitrofurans has been prohibited for a number of years. This makes Australia something of a test case in assessing the impact of animal use of antibiotics on antibiotic resistance in human pathogens. One striking result is the low rate of fluoroquinolone resistance in human isolates of *Campylobacter* (Unicomb et al., 2006).

15.2 ANTIBIOTIC RESISTANCE IN BACTERIA IN THE ENVIRONMENT

In recent years there has been increasing interest in antibiotic resistance in the environment. While most attention has focused on resistance that is linked to the use of antibiotics in animals and humans, it is important to recognize that soil organisms, particularly those that produce antimicrobial substances, can be intrinsically resistant to some antibiotics as soil organisms appear to be the source of many antibiotic resistance genes (Davies, 1997; Martinez, 2009; Allen et al., 2010). Indeed, some researchers have reported finding class 1 integrons in soil and sediments not exposed to antibiotics (Stokes et al., 2006) and class 1 and other integrons in biofilms in unpolluted freshwater (Gillings et al., 2009). However, the main concern is the frequent reporting of antibiotic resistance genes in enteric bacteria (particularly *E. coli*) that have contaminated soil and water from wastewater and from farming activities [see reviews by Bacqero et al. (2008) and Kümmerer (2009a, 2009b)]. There are limited reports from Australia. Boon and Cattanach (1999) found that resistance to some antibiotics such as ampicillin, chloramphenicol, nalidixic acid, and some aminoglycosides was more prevalent in native heterotrophic bacteria than in *E. coli* in various sites along a river in southeastern Australia. A study of *E. coli* isolates from surface waters receiving effluent from wastewater treatment plants in southeast Queensland found that resistance to a number of antibiotics was common, but that by comparing resistance patterns with those of isolates from humans, pets, livestock, and native animals, it was possible to determine that most isolates in rural areas were of nonhuman origin, whereas human isolates predominated in urbanized area (Carroll et al., 2005). Watkinson et al. (2007) also reported on significant levels of resistance in *E. coli* isolates in river waters exposed to wastewater discharges. A much higher detection rate for methicillin, sulfonamide, gentamicin, and vancomycin resistance genes was found in reclaimed effluent water in comparison with river water samples (Barker-Reid et al., 2010).

15.3 ANTIBIOTIC RESISTANCE IN LIVESTOCK

15.3.1 Antibiotic Resistance in Cattle

In Australia, infectious conditions in cattle are therapeutically treated with antibiotics. However, this is mostly on an individual animal basis except during occasional disease outbreak in a herd (Table 15.1). Cattle that are extensively raised are never exposed to antibiotics (JETACAR, 1999).

There is relatively little published information on antibiotic resistance in Australian cattle. Frost (1981) had reported that penicillin and streptomycin resistance was present in isolates of *S. aureus* from bovine mastitis but no methicillin resistance and little resistance to other antibiotics available for use in cattle. Barton et al. (2003) noted that these findings were still applicable at that time. Unfortunately, there has been no systematic investigation of antibiotic resistance in bovine mastitis isolates since the mid-1990s.

There have been several reports on antibiotic sensitivity in *E. coli*, mostly commensal isolates from healthy animals. Isolates from diagnostic submissions were found to be resistant mainly to ampicillin, streptomycin, and sulfathiazole (Bettelheim et al., 2003). In a population-based study of commensal isolates, Jordan et al. (2005) found 91% of the 10,279 isolates assessed expressed no resistance, 7.3% were resistant to sulfamethoxazole, 3.6% to tetracycline, 2.2% to ampicillin, and 0.09% to gentamicin. Class 1 and class 2 integrase genes were found to be more common in lot-fed cattle compared with grass-fed cattle with the lowest prevalences found in organic cattle (Barlow et al., 2009).

The results of a small pilot surveillance program (DAFF, 2007) revealed 18% of feedlot cattle isolates were resistant to tetracyclines (1.5% of grass-fed cattle) and 1.5% of both lot-fed and grass-fed cattle were resistant to florfenicol. With reference to enterococci, 14% of *Enterococcus faecium* isolates from lot-fed cattle were resistant to virginiamycin (17% from grass-fed cattle) and the same percentages were resistant to erythromycin.

TABLE 15.1 Types of Conditions and Antibiotics Used in Cattle

Conditions	Antibiotics
Respiratory infection in cattle	Tetracyclines, tylosin, tilmicosin, ceftiofur, erythromycin, neomycin, trimethoprim–sulfonamide combinations
Mastitis in dairy herds	β-Lactams, tetracyclines, lincomycin, trimethoprim–sulfonamide combinations
Dry cow therapy for mastitis control in dairy herd	β-Lactams, tetracyclines, neomycin
Control of lactic acidosis and bloat in feedlot cattle	Virginiamycin and polyethers
Coccidiosis in young animals	Polyethers
Enteric infections	Tetracyclines, neomycin
Hoof infections such as footrot	Penicillin, tetracyclines, ceftiofur, trimethoprim–sulfonamide combination

Source: (JETACAR (1999).

Resistance in *Salmonella* isolates has been monitored by the Australian Salmonella Reference Centre since 2000 (Australian Salmonella Reference Centre, 2001). Resistance rates vary between the years, but resistance to fluoroquinolones and gentamicin and cefotaxime resistance has been very uncommon. In 2009, the highest rate of resistance was to streptomycin (5%), although in earlier years resistance rates for streptomycin were as high as 43%, and significant rates of resistance to ampicillin, tetracycline, sulfonamides, and trimethoprim were reported (Australian Salmonella Reference Centre, 2009). Multiple resistance in isolates has also declined sharply to 1.5% in 2009, compared with 18% in 2004 (Australian Salmonella Reference Centre, 2004). No isolates of *Salmonella* Typhimurium DT 104 or multiple-resistant strains of *Salmonella* Newport have been reported in Australian cattle. The lack of exposure of Australian cattle to fluoroquinolones and restricted use of ceftiofur may have contributed to this. In contrast, multidrug-resistant *Salmonella* in cattle are not uncommon in other parts of the world (Hoelzer et al., 2010). Most Australian beef cattle are grass-fed and hence rarely treated with antibiotics, although treatment in feedlot beef cattle and dairy cattle would be more common, thus the relatively low rates of resistance reported in Australian cattle can be explained. Checkley et al. (2010) in a Canadian study noted that resistance rates to tetracyclines, sulfamethoxazole, and ampicillin increased when these antibiotics were used in feedlot cattle. However, other studies have reported that antibiotic resistance is more of an issue in calves and that resistance rates in adult cattle, regardless of management system, can be quite low (Call et al., 2008).

15.4 ANTIBIOTIC RESISTANCE IN COMPANION ANIMALS

15.4.1 Staphylococcus

Since its first isolation in 1961, MRSA has become a major nosocomial pathogen worldwide (Voss and Doebbeling, 1995). MRSA was initially associated with hospital-acquired infections, however, it is now increasingly seen in the community (Vandenesch et al., 2003). In recent years, MRSA has been increasingly reported as an emerging problem in veterinary medicine, particularly in small-animal and equine practices (Leonard and Markey, 2008; Abbott et al., 2010). The strains reported to date from dogs and cats have been hospital-acquired human strains.

Many countries have reported the occurrence of MRSA in dogs and cats (Morris et al., 2006; Sasaki et al., 2007; Abbott et al., 2010; Faires et al., 2010; Floras et al., 2010). In Australia, there have been a few studies that reported on the occurrence of MRSA in companion animals. Malik et al. (2006) reported the identification of MRSA ST239 from dogs.

15.4.2 Other Bacteria

A comparative retrospective study compared the minimum inhibitory concentrations (MICs) of ciprofloxacin, enrofloxacin, and amoxicillin–clavulanate for archived canine and feline *E. coli* and canine *Staphylococcus intermedius* isolates collected 10–20 years earlier (during the 1980s and early 1990s.) with those for recently collected isolates. All feline *E. coli* isolates and recently collected canine *S. intermedius* isolates were susceptible to all fluoroquinolones. There was a statistically significant increase in the

MIC range of ciprofloxacin and enrofloxacin for recently collected *E. coli*, and in the MIC range of amoxycillin–clavulanate for recently collected *S. intermedius* isolates compared to archived isolates. Twelve of 59 recently collected canine *E. coli* isolates were resistant to both ciprofloxacin and enrofloxacin. Resistant canine *E. coli* isolates were associated with complicating host or infection site factors (Gottlieb et al., 2008).

There has been a series of studies reporting on multidrug resistance in *E coli* isolated from extra-intestinal infections in dogs and cats. Most of the isolates were resistant to fluoroquinolones and produced AmpC β-lactamases (Sidjabat et al., 2006; Gibson et al., 2010a). The same group has also investigated multidrug-resistant and AmpC β-lactamases in *Enterobacter* spp. from extra-intestinal infections in dogs (Sidjabat et al., 2007; Gibson et al., 2010b).

15.5 ANTIBIOTIC RESISTANCE IN HORSES

Horses can be regarded as either livestock or companion animals. The range of antibiotics that can be used in horses is quite extensive. Systematic studies of antibiotic resistance are rare, but the recognition of MRSA associated with horses has increased interest in antibiotic resistance in this species.

Equine MRSA infections have been documented in a number of countries (Weese et al., 2004; Baptiste et al., 2005; O'Mahony et al., 2005; Cuny et al., 2006), with wound infections and postoperative infections tending to be the most common manifestations similar to the situation in dogs. Initially, the route of transmission is thought to be the hands of veterinary staff within the veterinary hospital setting and the hospital environment (Weese et al., 2004). The number of reports on MRSA colonization and infections in horses is on the increase and is now also being associated with private practices and the community (Weese and van Duijkeren, 2010).

In the Netherlands, the percentage of MRSA found in equine clinical samples increased from 0% in 2002 to 37% in 2008, with MRSA of *spa* types t011 and t2123, both belonging to the livestock-associated MLST ST398 being predominant (van Duijkeren et al., 2010). In a German study to characterize the equine MRSA stains, 19 MRSA strains derived from horses belonged to five sequence types, clustering into three different genetic groups, which are Clonal complex 8 (CC8), including sequence type 8 (ST8) and ST254, and CC22, containing ST22 and ST1117 and ST398. CC8 was found to be predominant (Walther et al., 2009).

As yet there are no published studies of MRSA in horses in Australia. However, MRSA has been identified as a cause of significant illness and death in neonatal foals (Jane Axon, personal communication), and MRSA carriage has been found in Australian equine veterinary practitioners (Jordan et al., 2011). There are relatively few studies reporting on antibiotic resistance in enteric organisms in horses. Dunowska et al., (2006) noted that antimicrobial resistance in *E. coli* in hospitalized horses was significantly higher than in nonhospitalized horses. Resistance to sulfonamides or trimethoprim–sulfamethoxazole, gentamicin, and tetracyclines was the most prevalent resistance. A recent study has found the multidrug resistance is common in commensal *E. coli*, with more than 90% of isolates resistant to chloramphenicol, ampicillin, tetracycline, ciprofloxacin, and trimethoprim. Interestingly, there was negligible resistance to apramycin, nalidixic acid, or florfenicol

(Ahmed et al., 2010). Class 1 integrons have been found in *E. coli* and other Enterobacteriaceae (van Duijkeren et al., 2005; Kadlec and Schwarz, 2008). Multidrug resistance in *Salmonella* isolates has been frequently reported (Hartmann and West, 1995; Ward et al., 2005; Niwa et al., 2009).

In relation to isolates from clinical cases, a study by Esink et al., (1993) found antimicrobial resistance to all the major classes of antimicrobials in various isolates including *Salmonella* Typhimurium, *Staphylococcus* spp., *E. coli*, *Klebsiella* spp. and *Proteus* spp. Albihn et al., (2003) investigated uterine isolates from mares with fertility problems and commented that the majority of streptococcal isolates were resistant to aminoglycosides and some to tetracyclines but that *E. coli* isolates were resistant to a wider range of antimicrobials. However, a more recent work from Canada commented that resistance in equine isolates was not common, although their results were similar to those of Albihn and co-workers (Clark et al., 2008). Australian data is sparse. Multidrug resistance has been reported in *Salmonella* and *E. coli* (Bucknell et al., 1997; Amavisit et al., 2001; Gibson et al., 2010a).

15.6 ANTIBIOTIC RESISTANCE IN AQUACULTURE

Aquaculture is a rapidly growing and diverse industry with operations spread throughout all states in Australia (Love and Langenkamp, 2003). The importance of aquaculture as a percentage of total fisheries production has been increasing over the past 10 years. In 1995–1996, aquaculture represented 22% of the total value of fishery production, increasing to 29% in 2004–2005 (Tedesco and Szakiel, 2006). The Australian aquaculture industry has estimated that it could triple its yearly sales to $2.5 billion by 2010 if it can successfully exploit its competitive advantages to meet increasing domestic and global demand for fishery and aquaculture products (Love and Langenkamp, 2003).

Disease outbreaks, as well as other environmental issues, have been recognized as the major problems facing the aquaculture industry (Verschuere et al., 2000; Austat, 2003; Nematollahi et al., 2003; Treasurer et al., 2007). As a result, antibiotics are being used to reduce the impact of bacterial diseases (Le et al., 2005; Alday et al., 2006; Cabello, 2006; Moriarty, 1997), and there are reports that the use of antibiotics has resulted in increased antibiotic resistance in bacteria from aquaculture environments (DePaola et al., 1988, 1995; Samuelsen et al., 1992; Kerry et al., 1995; Rhodes et al., 2000; Miranda and Zemelman, 2002; Alcaide et al., 2005; Jacobs and Chenia, 2007; Nawaz et al., 2006).

The Australian animal health authorities carried out a pilot study of antibiotic resistance in food-producing animals in 2005, and, although JETACAR (1999) recommended inclusion of aquaculture in such a surveillance program, this did not occur. There are no antibiotics registered for aquaculture use in Australia, but there are indications that antibiotics registered for use in food-producing animals (such as oxytetracycline) might be in use off-label, and there may well be illegal use of other antibiotics such as streptomycin and sulfamethoxazole. On occasion, the Australian Veterinary Pesticides and Medicines Authority has issued permits for use of oxolinic acid for treatment of furunculosis in goldfish (M.D. Barton, personal communication) and just recently for oxytetracycline and florfenicol for the treatment of diseases in salmon (Darby, 2007).There is also a new report indicating that the use of

antibiotics in salmon and trout farms has risen from about 12 kg a year a decade ago to almost 8 tonnes in the first 3 months of 2007 (Darby, 2007). This is an indication that there could well be more issues with antibiotic resistance in aquaculture in Australia.

A limited amount of work has been carried out to investigate antibiotic resistance associated with Australian aquaculture. A study by Akinbowale et al., (2006) concentrated on assessment of antibiotic resistance in some bacteria that are of importance in aquaculture such as *Aeromonas* spp., *Vibrio* spp., and *Pseudomonas* spp. Some isolates of other bacteria from aquaculture environments were also included. Although a reasonable number of isolates were obtained to provide a baseline, there were some constraints that limited the scope and extent of the study. The results obtained in the study suggested that there are some antibiotic resistance issues as indicated by the resistance phenotypes of the isolates, the widespread distribution of resistance genes, and the demonstrated capacity for transfer of resistance genes between different bacterial genera.

In the first study, 100 Gram-negative and 4 Gram-positive wild-type bacterial strains that were considered to be of diagnostic significance were collected from diagnostic and research laboratories from different states in Australia; these strains had been isolated from aquaculture settings and included freshwater and marine fishes, crustaceans, and environmental isolates. The isolates were tested against antibiotics that have been reported as used in aquaculture (in countries other than Australia) or in livestock production in Australia (and therefore available for off-label use in aquaculture) or of interest in human medicine.

The results obtained from this study showed resistance to some penicillin and some of the cephalosporins, oxytetracycline, tetracycline, and sulfamethoxazole. A few isolates were also resistant to kanamycin, chloramphenicol, florfenicol, erythromycin, trimethoprim–sulfamethoxazole, nalidixic acid, oxolinic acid, and ciprofloxacin, although fluoroquinolones are not registered for livestock production in Australia. Single isolates were also resistant to gentamicin and trimethoprim. Some of the low-level resistance found occurred with antibiotics that are not registered for use in livestock production in Australia. A moderate level of resistance was observed for tetracyclines even though oxytetracycline, in particular, is widely used for livestock production in Australia. Plasmid-mediated antibiotic resistance in aquaculture has been well documented (Inglis et al., 1993; Son et al., 1997; Adams et al., 1998; Schmidt et al., 2001b; Agersø et al., 2007); and about three quarters of isolates in this study were found to possess plasmids, suggesting the potential for involvement of plasmids in the resistance observed (Akinbowale et al., 2006).

There are reports of tetracycline resistance genes in aquaculture and aquatic environments from different geographical locations (Aoki et al., 1987; Andersen and Sandaa, 1994; Adams et al., 1998; Schmidt et al., 2001; Miranda et al., 2003; Kim et al., 2004; Nawaz et al., 2006; Agerso et al., 2007; Jacobs and Chenia, 2007). In the Australian study, tetracycline resistance determinants, *tet*A, *tet*D, *tet*E, and *tet*M, were detected in isolates while *tet*B, *tet*C, *tet*G, *tet*K, *tet*L, *tet*O, *tet*S, and *tet*34 were not detected in any of the strains. The study also reported for the first time the occurrence of *tet*M in *Edwardsiella tarda* (Akinbowale et al., 2007). The *tet* genes detected in isolates from this study are similar to those that have been reported elsewhere except that *tet*M was the most commonly identified gene in this study,

rather than *tet*A, *tet*B, *tet*D, and *tet*E, which have been most common elsewhere (Aoki and Takahashi, 1987; Andersen and Sandaa, 1994; Furushita et al., 2003; Nawaz et al., 2006; Jacobs and Chenia, 2007). Using conjugation as a means of transfer of genetic information, the transfer of some *tet* genes from *Aeromonas* and *Vibrio* spp. to *E. coli* strains from chicken, pig, and humans was demonstrated (Akinbowale et al., 2007).

Based on the results from the initial studies, further investigations were carried out on bacteria isolated from fish and sediment samples from different rainbow trout farms in Victoria. Forty two *Aeromonas* and 85 *Pseudomonas* spp. were from sediment, and 48 *Aeromonas* and 44 *Pseudomonas* were isolated from fish. The isolates were tested for their sensitivity to a number of antibiotics. In contrast to what was found in the initial studies, there was no widespread resistance to penicillins and cephalosporins in both *Aeromonas* and *Pseudomonas* spp. Although in most cases they had high MICs, the wild-type distribution and cut-off classified them as wild-type susceptibility. Most of the *Pseudomonas* species were only classified as resistant to ticarcillin, whereas the *Aeromonas* species were resistant to more antibiotics with tetracycline being the highest. Oxolinic acid, ciprofloxacin, and gentamicin resistance was not found in the *Aeromonas* sp. Fluoroquinolones are not registered for use in livestock in Australia, and this probably explains the lack of resistance to ciprofloxacin, with only 5 isolates being resistant from the initial studies. Oxytetracycline resistance was observed in many *Aeromonas* spp. Most of the *Pseudomonas* isolates were resistant to multiple antibiotics, however, only 2 *Pseudomonas* sp. were resistant to tetracycline.

What must be borne in mind throughout is the fact that most *Pseudomonas* spp. are intrinsically resistant to many chemical agents (Russsel and Day, 1996) and have different mechanisms for getting rid of many substrates, one such mechanism being the use of efflux pumps (Stover et al., 2000).

There is the concern that metal contamination functions as a selective agent in the proliferation of antibiotic resistance (Baker-Austin et al., 2006), and heavy metals can be taken up into foods, particularly fish and shellfish (Turner et al., 2005). In this study resistance to seven heavy metals (Cd^{2+}, Cr^{3+}, Co^{2+}, Mn^{2+}, Pb^{2+}, Zn^{2+}, Cu^{2+}) was investigated in selected isolates. The study indicated that the isolates tested were tolerant to different concentrations of heavy metals as evidenced by their minimum inhibitory concentration ranging from 6.25 μg/mL to >3200 μg/mL. However, it appears that this is the natural behavior of the wild-type population.

The cadmium-resistant gene *cad*A was detected in more than half of the isolates with cadmium MIC of >200 μg/mL. One of the efflux systems *mex*AB–*opr*M encoding a tripartite pump was investigated in this study because it exports a wide range of antibiotics. The major integral inner membrane efflux component of the *mex*AB–*opr*M pump, *mex*B, was detected in about three quarters of the isolates. Multidrug efflux systems have been recognized as efficient mechanisms of resistance in *Pseudomonas* spp. (Ramos et al., 1998; Aires et al., 1999; Rojas et al., 2001; Pournaras et al., 2005).

The involvement of integrons as mechanisms of resistance was also investigated in the *Pseudomonas* and the *Aeromonas* isolates. Integrase gene *Int*1 was detected in less than one quarter of the *Pseudomonas* and slightly more than one quarter of the *Aeromonas* isolates. Streptomycin resistance *aad*A gene was detected in half of

the integrase-positive *Pseudomonas* and in three quarters of the integrase-positive *Aeromonas* isolates; this gene is usually associated with integrons. The *aad*A gene was found to be present in some isolates that were classified as being phenotypically susceptible to streptomycin. This points to one of the problems of setting breakpoints for interpreting susceptibility results. It could as well be that more phenotypically susceptible isolates are carrying resistance genes.

Other integron-associated genes, *sul*1 and *qac*Δ1, were also detected in *Aeromonas* isolates but not in the *Pseudomonas*. The involvement of integrons in antibiotic resistance in aquaculture and aquatic organisms has been previously reported from other geographical locations (Petersen et al., 2000; L'Abee-Lund and Sorum, 2001; Schmidt et al., 2001; Sørum et al., 2003; Henriques et al., 2006; Khan et al., 2006; Mukherjee and Chakraborty, 2006; Jacobs and Chenia, 2007).

Tetracycline resistance determinants, *tet*C and *tet*A, were detected in tetracycline-resistant *Aeromonas* isolates. All tetracycline-resistant isolates were found to harbor *tet*C while half carried the *tet*A gene. These results are different from the initial study where *tet*C was not detected at all but some of the isolates also carried *tet*A. Many of the isolates for the initial study were from northern Australia and from a variety of aquaculture settings, whereas the trout isolates were from southern Australia freshwater trout farms.

15.6.1 Salmonella

Antibiotic-resistant *Salmonella* infections are rare in Australia. Musto and co-workers (2006) investigated an increase in multidrug-resistant *Salmonella paratyphi* B biovar Java (S. Java) infections in Australia during 2003–2004. Eighty-two percent (18/22) of S. Java cases enrolled into the study reported that they had been in contact with aquariums housing fish during their incubation period. Seventy-two percent (13/18) of cases were infected with strains that were resistant to ampicillin, streptomycin, tetracycline, chloramphenicol, sulfonamides, and spectinomycin. Case reports revealed common high-risk behaviors, such as cleaning aquaria with bare hands and discarding the water in household sinks. Sixty-one percent (11/18) of cases reported that fish in their aquarium had been sick or died in the week prior to their illness, and *S. paratyphi* Java was isolated from the water or gravel of 5 cases. These antibiotic strains are being spread internationally and may become endemic in countries importing tropical fish or result in transfer of resistance to other more common *Salmonella* serotypes and other hosts (Musto et al., 2006).

15.7 ANTIBIOTIC RESISTANCE IN PIGS

The pig industry in Australia is relatively small with approximately 2500 producers farming 5 million animals (DAFF, 2007). Intensive pig production often requires therapeutic and prophylactic use of antibiotics to counter enteric and respiratory diseases (JETACAR, 1999). Antibiotics incorporated in feeds at subtherapeutic levels are used for control of chronic diseases such as swine dysentery and porcine proliferative enteropathy (PPE), rather than specifically for growth promotion. Antibiotic use in pigs is strictly controlled and must be authorized by a prescription from a veterinarian. Frequently used antibiotics are listed in Table 15.2.

TABLE 15.2 Frequently Used Antibiotics in Pigs

Condition	Treatment
Enterotoxigenic *E. coli* in piglets (unweaned) and weaner pigs (first 2 weeks postweaning)	Amoxicillin, trimethoprim, spectinomycin, apramycin, and neomycin
Mycoplasma pneumonia	Tetracycline, lincosamide, tylosin, tiamulin
Pleuropneumonia (*Actinobacillus pleuropneumonia*)	Procaine penicillin, amoxicillin, tetracyclines, trimethoprim, tilmicosin
Proliferative enteritis (*Lawsonia intracellularis*)	Olaquindox, tetracyclines, lincomycin, tylosin
Colitis (*Serpulina* spp.)	Tiamulin, lincomycin

15.7.1 Escherichia coli

Published reports indicate that worldwide resistance to antimicrobials is very common in pig isolates of *E. coli*. Multidrug resistance is the norm, and resistance to all available classes of antimicrobials has been reported. Recent reviews (Barton, 2000; Aarestrup et al., 2008; Harada and Asai, 2010) report a strong relationship between widespread use of antimicrobials and resistance in pigs in many countries.

In Australia, a survey was conducted in the 1970s by the then Animal Health Committee (AHC) (Barton et al., 2003). The isolates were obtained from different sources in different years, which make the interpretation of results difficult. What is obvious, however, is that resistance to streptomycin and tetracycline in pigs was prevalent in Australia about 30 years ago. A number of the isolates were resistant to more than one antibiotic, with the co-resistant combinations of tetracycline and streptomycin (6%) or tetracycline, streptomycin, and ampicillin (2%) being the most common (Barton et al., 2003). A more recent pilot surveillance study (DAFF, 2007) found 13% isolates sensitive to all antibiotics and 22% resistant to one antibiotic (mostly tetracycline)—the remainder of isolates were resistant to up to six classes of antibiotics, with resistance to tetracyclines, fenicols, trimethoprim–sulfamethoxazole, and ampicillin particularly common. There was no resistance to ceftiofur or ciprofloxacin and resistance to gentamicin was rare. This is another example of how restricting antimicrobial use in food-producing animals can influence the antimicrobial resistance patterns in enteric organisms.

15.7.2 Campylobacter

Resistance to fluoroquinolones and/or erythromycin in *Campylobacter* isolates is of human public health concern. Clearly, these resistance patterns are found in pig isolates in many parts of the world, along with resistance to tetracyclines (Padungton and Kaneene, 2003; Tadesse et al., 2010). In Australia, an unpublished 1999 study of antibiotic resistance in 216 nonspeciated *Campylobacter* from pigs (R. Pratt and M.D. Barton, personal communication) found that 50% of the isolates were resistant to erythromycin, 47% resistant to clindamycin, 43% to tetracycline, 32% to ampicillin, and 3% to gentamicin. No resistance to chloramphenicol or ciprofloxacin was detected. A subsequent study of carcass swabs from pigs (Hart et al., 2004) reported that 2–3% of isolates were resistant to ampicillin and neomycin, 18–24%

resistant to clindamycin, 76–87% to erythromycin, 84–87% to lincomycin, 80–87% to tylosin, and around 70% to tetracycline. There was no resistance to gentamicin or ciprofloxacin. Widespread use of tylosin, lincomycin, and tetracyclines in pigs no doubt drives the increased resistance levels seen in these isolates.

A study involving the sampling of pig carcasses and pig meat to assess the level of resistance in zoonotic enteric bacteria of concern to human health was carried out in South Australian pigs. Thermophilic *Campylobacter* species showed widespread resistance (60–100%) to tylosin, erythromycin, lincomycin, ampicillin, and tetracycline. No resistance was seen to ciprofloxacin. The enterococci demonstrated little resistance (0–30%) to vancomycin or virginiamycin, but the overall results from the antibiotic sensitivity testing of the enterococci have demonstrated how widespread their resistance has become. *Escherichia coli* strains showed widespread resistance to tetracycline and moderately common resistance (30–60%) to ampicillin and sulfadiazine. Resistance to more than one antibiotic was common. Pigs from New South Wales were also sampled, and differences in resistance patterns were noted, perhaps reflecting different antibiotic use regimens in that state (Hart et al., 2004).

15.7.3 Enterococci

Resistance to glycopeptide antibiotics encoded by the *van*A gene is a major human health concern. Feeding of avoparcin to pigs (and other animal species) has led to the spread of vancomycin-resistant *E. faecium* and *Enterococcus faecalis* to humans in many parts of the world. However, glycopeptides have never been registered for use in the United States and, as a consequence, *van*A vancomycin resistant enterococci (VRE) had not been isolated from livestock or retail meat (Hershberger et al., 2005) until the report of Donabedian et al. (2010) describing the detection of *van*A *E. faecium* in pigs. The use of avoparcin was banned in many countries in 1997, and the prevalence of *van*A enterococci in animals declined sharply in most countries (Wegener, 2003), although resistance has persisted (Biavsco et al., 2007; de Jong et al., 2009), presumably due to co-location of resistance determinants with antibiotics still used in livestock. Similarly, there has been concern about use of streptogramins in animals and the potential for resistance to compromise use of streptogramins in human medicine. Streptogramin resistance has been reported in livestock isolates (de Jong et al., 2009). It is less prevalent in pig isolates compared with poultry isolates (Jackson et al., 2007), reflecting greater use in chickens. High-level gentamicin resistance has also been reported from pig enterococcal isolates from several countries (Jackson et al., 2005; De Graef et al., 2007; Han et al., 2010; Larsen et al., 2010). Australian studies have demonstrated little resistance to vancomycin or virginiamycin, and no high-level gentamicin resistance has been found (Hart et al., 2004).

15.7.4 Salmonella

There was a surveillance program between 1990 and 1997 by the National Enteric Pathogen Surveillance Scheme. The testing was on the bovine, chicken, and porcine strains of *Salmonella*. The results of the study suggested that there has been an increase in resistance, and also the prevalence of resistance in the various hosts was different from the results obtained in 1970 (Table 15.3).

TABLE 15.3 Frequency of Resistance in *Salmonella* (1990–1997)

Chemotherapeutic Concentration	Bovine (396)	Chicken (108)	Porcine (51)
Ampicillin 32 μg/mL	31	17	35
Chloramphenicol 10 μg/mL	18	5	10
Streptomycin 25 μg/mL	86	5	10
Tetracycline 20 μg/mL	47	44	92
Sulphathiazole 550 μg/mL	70	19	41
Trimethoprim 50 μg/mL	29	17	35
Kanamycin 10 μg/mL	28	15	31
Nalidixic acid 50 μg/mL	0.5	0	0
Spectinomycin 50 μg/mL	0.6	4	5
Gentamicin 25 μg/mL	0.6	4	5
Ciprofloxacin 0.06 μg/mL	0	5	7

Source: From (Barton et al., 2003).

15.8 ANTIBIOTIC RESISTANCE IN POULTRY

The Australian poultry industry is divided into several sectors, which includes the chicken meat and egg industries and, on a much smaller scale, turkey and game bird production. The method of production is largely intensive with the industries located mainly in rural–urban fringes (ABARE, 2006).

Generally, poultry production is faced with the problems of disease caused by bacteria, viruses, protozoan, parasite, and fungi. These causative agents are involved in different types of poultry disease, which could be on the skin and feather, digestive, respiratory, and nervous system. Diseases lead to huge economic losses and, as such, various means are used to control the level and the impact of diseases.

In Australia, the use of antibiotics in poultry is strictly regulated and can only be given upon veterinary prescription (JETACAR, 1999). Only a few antibiotics can be given to egg layers to avoid residues in eggs that could make them unsuitable for human consumption. Other means of controlling bacterial diseases in the poultry industry include vaccines, biosecurity procedures, and hygiene methods.

The principal microbiological pathogens of concern on poultry meat in Australia are *Campylobacter* spp. and *Salmonella* spp (FSANZ, 2006). There is worldwide concern about antibiotic resistance in *Campylobacter* and *Salmonella* and *E. coli* and enterococci.

15.8.1 Campylobacter

Overseas studies have reported resistance to erythromycin, tetracycline, ampicillin, nalidixic acid, and ciprofloxacin (de Jong et al., 2009; Deckert et al., 2010). These studies all note differences in resistance patterns between *Campylobacter jejuni* (the predominant species in chickens) and *Campylobacter. coli.*

Campylobacter is the most common bacterial cause of food-borne disease in Australia (OzFoodNet, 2005). There are a few Australian studies on the levels of antibiotic resistance in animal isolates of *Campylobacter*. A study of 79 chicken isolates found widespread resistance to erythromycin and significant resistance to doxycycline but no resistance to enrofloxacin (Korolik et al., 1996). A later study

(Barton and Wilkins, 2001), 216 *Campylobacter* (142 *C. jejuni*, 74 *C. coli*) were isolated from chicken samples from three sources. There were significant differences in resistant rates between the three sources, presumably reflecting differences in antibiotic use practices in the different companies. Resistance to ampicillin in *C. jejuni* varied between 50 and 61%, to lincomycin and tylosin from 4 to 28%, to tetracycline from 15 to 37%, and erythromycin from 0 to 11%. Resistance to neomycin and gentamicin was negligible, and one isolate from one source was resistant to ciprofloxacin. In the case of *C. coli*, ampicillin resistance was around 35%, resistance to tetracycline varied from 16 to 36%, and lincomycin from 4 to 30%, to erythromycin from 0 to 17%, and to tylosin from 1 to 17%. Resistance to neomycin and gentamicin was negligible and two isolates from one source were resistant to ciprofloxacin (Barton and Wilkins, 2001).

Subsequently, Pratt and Korolik (2005) reported high-level tetracycline resistance encoded by *tet*O in both *C. jejuni* and *C. coli* isolates. The most noteworthy difference in Australia is the absence of fluoroquinolone resistance in chicken isolates of *Campylobacter*, resulting in little fluoroquinolone resistance in human isolates in this country (Unicomb et al., 2006).

One hundred and twenty-five *C. jejuni* and 27 *C. coli* isolates collected from 39 broiler farms in southeast Queensland were included in the antimicrobial sensitivity studies conducted by Miflin and co-workers (2007). Ampicillin, chloramphenicol, ciprofloxacin, erythromycin, nalidixic acid, and tetracycline were the antibiotics tested. *Campylobacter jejuni* isolates had the highest level of resistance to tetracycline (19.2% by MIC and 18.4% by disk) and ampicillin (19.2% by MIC and 17.6% by disk). All the isolates were sensitive to ciprofloxacin and chloramphenicol, as well as nalidixic acid, for *C. coli*. All *C. jejuni* isolates were sensitive to erythromycin.

In the pilot surveillance program conducted by the Department of Agriculture, Fisheries, and Forestry (DAFF, 2007), 131 *Campylobacter* isolates from chickens were included. Tetracycline resistance was found in 19.7% of isolates and erythromycin resistance in 9.8% of isolates. Two of the isolates (1.5%) were resistant to both antimicrobials, and all isolates were sensitive to gentamicin, ciprofloxacin, and nalidixic acid (DAFF, 2007).

15.8.2 Salmonella

Poultry are a major source of human *Salmonella* infections. Resistance to antibiotics varies widely between *Salmonella* serovars and between geographic regions. Resistance to a wide range of antibiotics is seen in poultry, and multidrug-resistant strains are commonly reported; however, resistance prevalence is lower than that seen in pigs (de Jong et al., 2009; Gyles, 2008).

There has been an increase in resistance of chicken *Salmonella* isolates between 1990 and 1997 (Table 15.4). Results from the Australian Salmonella Reference Centre indicate a difference in resistance patterns between egg-producing and meat-producing chickens (Australian Salmonella Reference Centre, 2009), with resistance to streptomycin, tetracycline, sulfonamides, and ampicillin of 31, 10, 7 and 6%, respectively, in 1475 meat chicken isolated, whereas 2, 4, 2, and 5% of 265 egg-producing hen isolates were resistant to those antibiotics, respectively. There was no resistance to fluoroquinolones or ceftiofur in any of the isolates. Three percent of the meat chicken isolates and 0.4% of the egg production isolates were resistant to 4 or more antibiotics.

TABLE 15.4 Summary of Poultry *Campylobacter* Resistance Profile (1996–2007)

Resistance	Negligible to No Resistance
Erythromycin	Enrofloxacin
Doxycycline	Ciprofloxacin
Tetracycline	Nalidixic acid
Ampicilin	Neomycin
Tylosin	Gentamicin
	Chloramphenicol

15.8.3 Enterococcus spp.

Concerns about resistance in chicken isolates are the same as those related to pig isolates. A recent study has reported a low prevalence of vancomycin in European chickens (de Jong et al., 2009) in contrast to studies that occurred prior to the withdrawal of avoparcin (Bate et al., 1994; Aarestrup et al., 2000). Interestingly, vancomycin-resistant enterococci have recently been reported from Swedish poultry even though avoparcin has not been used since the mid-1980s (Nilsson et al., 2009). Streptogramin resistance has been found to be more prevalent than vancomycin resistance in recent studies (Hayes et al., 2004; de Jong et al., 2009).

In terms of Australian studies, Barton and Wilkins (2001) found widespread resistance to virginiamycin in *E. faecium* isolates. These isolates had been collected prior to the withdrawal of avoparcin from the Australian market in 2000, and so *van*A-encoded vancomycin was found in 34% of 270 enterococcal isolates. Seventy-four of the resistant isolates were *E. faecium*, 9 were *Enterococcus hirae*, and one was *E. faecalis*, and 8 were from miscellaneous *Enterococcus* spp. All but 2 resistant *E. hirae* isolates came from one poultry company, suggesting that avoparcin use was largely restricted to that company. *van*C-encoded intrinsic resistance was also detected in a number of isolates.

Two hundred and thirty-eight *Enterococcus* spp. from chickens were included in the DAFF pilot surveillance program for antibiotic sensitivity testing. Sixty-one of the isolates were *E. faecium*, which were found to be resistant to ampicillin (4.9%), erythromycin (45.9%), and virginiamycin (26.2%). Seven of these isolates were resistant to 2 antibiotics while 2 were resistant to 3 antibiotics. In the 123 *E. faecalis* isolates tested, resistance was found to erythromycin (77.3%), vancomycin (0.8%) (low-level *van*C), and virginiamycin (92.8%). Eighty-seven of these isolates (70.7%) were resistant to 2 antibiotics while 1 of the isolates was resistant to 3 antibiotics.

Resistance to erythromycin and virginiamycin were observed in 75.7 and 33.4% of *E. hirae* and *Enterococcus. casseliflavus* isolates. All the enterococci tested were sensitive to gentamicin and teicoplanin (DAFF, 2007).

15.8.4 Eschevichia coli

Antibiotic resistance in chicken isolates of *E. coli* is of increasing interest with the recognition of the link between extra-intestinal human infections and avian pathogenic strains of *E. coli* (APEC) (Ewers et al., 2009). However, most antimicrobial resistance studies have focused on commensal isolates from healthy chickens, although there are probably APEC strains among these. Resistance in commensal

E. coli isolates is widespread (Gyles 2008; de Jong et al., 2009). Some Australian studies have also found resistance in avian strains of *E. coli*.

An earlier study with frozen chickens obtained in different food stores in Australia (Caudry and Stanisich, 1979) found that resistance to mercury(II) or to at least one antibiotic comprised 13.7% of the *E. coli* isolated from one carcass but between 65 and 90% of the *E. coli* isolated from the other four samples. The most common resistance found was to streptomycin, sulfathiazole, and tetracycline, which occurred in approximately 10–40% of the *E. coli* tested from each sample. In two of the samples tested, chloramphenicol resistance was 23%, and resistance to ampicillin or mercury(II) varied from just a few to 20% in each sample. There was very low resistance to kanamycin, gentamicin, and trimethoprim.

An account of the resistance pattern of avian *E. coli* from three chicken meat production companies (T. Grimes, personal communication) was included in the JETACAR report (JETACAR, 1999). Widespread resistance to tetracycline and significant resistance to ampicillin and sulfonamides–trimethoprim was indicated (JETACAR, 1999). Barton and Wilkins (2001) found significant resistance to ampicillin, tetracyclines, streptomycin, and trimethoprim in isolates, with difference in resistance rates between poultry companies. No resistance to fluoroquinolones was found, and there was negligible resistance to gentamicin. More than 50% of isolates were resistant to three or more classes of antibiotics.

In the pilot surveillance program conducted by the Department of Agriculture, Fisheries, and Forestry (DAFF, 2007), 269 *E. coli* isolates were subjected to

TABLE 15.5 Summary of Poultry *Campylobacter*, *Enterobacter*, and *E. coli* Resistance Profile (1996–2007)

Resistance	Negligible to No Resistance
CAMPYLOBACTER	
Erythromycin	Enrofloxacin
Doxycycline	Ciprofloxacin
Tetracycline	Nalidixic acid
Ampicillin	Neomycin
Tylosin	Gentamicin
	Chloramphenicol
ENTEROBACTER	
E. faecium	Gentamicin
Ampicillin	Teicoplanin
Erythromycin	
Virginiamycin	
E. faecalis, E.hirae*, and *E. casseliflavus	
Erythromycin	Vancomycin
Virginiamycin	Gentamicin
	Teicoplanin
E. COLI	
Streptomycin	Cefotaxime
Sulfathiazole	Ceftiofur
Tetracycline	Gentamicin
Ampicillin	Florfenicol
Trimethoprim/sulfamethoxazole	chloramphenicol

antibiotic sensitivity testing, and the study found resistance to ampicillin (33.1%), chloramphenicol (1.8%), ciprofloxacin (0.4%), florfenicol (3.4%), nalidixic acid (1.9%), tetracycline (44.3%), and trimethoprim–sulfamethoxazole (27.2%). Multiple antibiotic resistance was found in 93 isolates (34.6%), which were resistant to 2 or more antimicrobials with 7 isolates resistant to 4 antimicrobials. These chicken isolates were sensitive to cefotaxime, ceftiofur, and gentamicin. A summary of resistance profiles for three indicator bacteria in poultry is presented in Table 15.5.

15.8.5 Staphylococcus aureus

One hundred and twenty-six *S. aureus* isolated from 2 poultry farms in western Australia were found to have varying levels of resistance to tetracycline, penicillin, streptomycin, erythromycin, lincomycin, and trimethoprim. However, none was methicillin resistant (Bertolatti et al., 2003).

15.9 WILD AUSTRALIAN MAMMALS

A retrospective analysis was conducted of 946 strains of Enterobacteriaceae isolated from wild Australian mammals between 1993 and 1997 (Sherley et al., 2000). The prevalence of resistance to fixed concentrations of 32 antimicrobial agents was determined, and the respective roles that the taxonomic family of the host, state of origin, and bacterial species play in defining prevalence and range of resistance were investigated. The results demonstrated a low but widespread prevalence of antimicrobial resistance in wild isolates. Only amikacin, ciprofloxacin, meropenem, and gentamicin inhibited growth in all 946 samples. There was extensive variation in the combination of antibiotics to which isolates were resistant, and multiple antibiotic resistance was common. Geographical location and host group significantly influenced the antibiotic resistance profile of an isolate, whereas bacterial species influenced both the resistance profile of an isolate and the number of antibiotics to which it was resistant. However, there was no correlation between resistance prevalence and intensity of agriculture or density of human habitation. The prevalence of antimicrobial resistance in Enterobacteriaceae isolated from wild Australian mammals was generally lower than the values reported for Australian human clinical isolates.

The role of these factors in determining observed antibiotic resistance profiles suggests that any study measuring resistance in wild isolates should include the broadest possible range of bacterial species, host species, and sampling locations.

15.10 SUMMARY

This chapter has provided an overview of the current scientific knowledge related to antimicrobial resistance in animals in Australia. While the results of several of the scientific studies in Australia are aligned with observations in other regions of the world, the issue of limiting the opportunities for the spread of antimicrobial resistance in humans, companion animals, livestock, fish, and the natural environment remains an important issue of concern.

REFERENCES

Aarestrup FM, Kruse H, Tast E, Hammerum AM, Jensen LB (2000). Associations between the use of antimicrobial agents for growth promotion and the occurrence of resistance among *Enterococcus faecium* from broilers and pigs in Denmark, Finland and Norway. *Microbial Drug Resistance* 6:63–70.

Aarestrup FM, Duran CO, Burch DG (2008). Antimicrobial resistance in swine production. *Anim Health Res Rev* 9:135–148.

Abbott Y, Leggett B, Rossney AS, Leonard FC, Markey BK (2010). Isolation rates of methicillin-resistant *Staphylococcus aureus* in dogs, cats and horses in Ireland. *Vet Rec* 166 (15):451–455.

Adams CA, Austin B, Meaden PG, McIntoch D (1998). Molecular characterisation of plasmid-mediated oxytetracycline resistance in *Aeromonas salmonicida*. *Appl Environ Microbiol* 64:4194–4201.

Agerso Y, Bruun MS, Dalsgaard I, Larsen, JL (2007). The tetracycline resistance gene tet(E) is frequently occurring and present on large horizontally transferrable plasmids in *Aeromonas* spp. from fish farms. *Aquaculture* 266(1–4):47–52.

Ahmed MO, Clegg PD, Williams NJ, Baptiste K, Bennett M (2010). Antimicrobial resistance in equine faecal *Escherichia coli* isolates from North West England. *Ann Clin Microbiol and Antimicrob* 9:12.

Aires JR, Kohler T, H Nikaido H, Plesiat P (1999). Involvement of an active efflux system in the natural resistance of *Pseudomonas aeruginosa* to aminoglycosides. *Antimicrob Agents Chemother* 43:2624–2628.

Akinbowale OL, Peng H, Barton MD (2006). Antimicrobial resistance in bacteria isolated from aquaculture sources in Australia. *J Appl Microbiol* 100(5):1103–1113.

Akinbowale OL, Peng H, Barton M (2007). Diversity of tetracycline resistance genes in bacteria from aquaculture sources in Australia. *J Appl Microbiol* 103:2016–2025.

Albihn A, Båverud V, Magnusson, U (2003). Uterine microbiology and antimicrobial susceptibility in isolated bacteria from mares with fertility problems. *Acta Vet Scand* 44:121–129.

Alcaide E, Blasco MD, Esteve C (2005). Occurrence of drug resistant bacteria in two European eel farms. *Appl Environ Microbiol* 71:3348–3350.

Alday V, Guichard B, Smith P, Uhland, C (2006). Towards a risk analysis of antimicrobial use in aquaculture. Joint FAO/WHO/OIE expert consultation on Antimicrobial use in aquaculture and antimicrobial resistance, Seoul, South Korea.

Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J (2010). Call of the wild: Antibiotic resistance genes in natural environments. *Nat Rev Microbiol* 8:251–259.

Amavisit P, Markham PF, Lightfoot D, Whithear KG, Browning GF (2001). Molecular epidemiology of *Salmonella Heidelberg* in an equine hospital. *Vet Microbiol* 80:85–98.

Andersen SR, Sandaa RA (1994). Distribution of tetracycline resistance determinants among gram-negative bacteria isolated from polluted and unpolluted marine sediments. *Appl Environ Microbiol* 60:908–912.

Aoki T, Takahashi A (1987). Class D tetracycline resistance determinants of R-plasmids from fish pathogens *Aeromonas hydrophila, Edwardsiella tarda* and *Pasteurella piscisida*. *Antimicrob Agents Chemother* 31:1278–1280.

Aoki T, Satoh T, Kitao T (1987). New teracyline resistace determinat on R-plasmids from *Vibrio anguillarum*. *Antimicrob Agents Chemother* 31:1446–1449.

APVMA (2009). *Veterinary Manual of Requirements and Guidelines*, Vol. 3 (data requirements). Australian Pesticides and Veterinary Medicines Authority. Available: http://www.apvma.gov.au/morag_vet/.

Austat (2003). Feature article—Aquaculture and the environment. *Forestry and Fishing—Year Book*, Australia.

Australian Salmonella Reference Centre (2001). *Annual report*. Institute of Medical and Veterinary Science, Adelaide, South Australia, p. 3.

Australian Salmonella Reference Centre (2004). *Annual report*. Institute of Medical and Veterinary Science, Adelaide, South Australia, pp. 4–5.

Australian Salmonella Reference Centre (2009). *Annual report*. Institute of Medical and Veterinary Science, Adelaide, South Australia, pp. 4–6.

Bacqero F, Martínez JL, Cantón R (2008). Antibiotic and antibiotic resistance in water environments. *Curr Opin Biotechnol* 19:260–265.

Bair C, Ahmed R, Avery B, Boerlin P, Daignault D, Doré K, Dutil L, Irwin R, Léger D, Long K, Martin L (2003). CIPARS—Canadian Integrated Program for Antimicrobial Resistance Surveillance: Working towards the preservation of effective antimicrobials for humans and animals. http://dsp-psd.pwgsc.gc.ca/Collection/H39-1-3-2003E.pdf

Baker-Austin C, Wright MS, Stepanauskas R, McArthur JV (2006). Co-selection of antibiotic and metal resistance. *Trends Microbiol* 14(4):176–182.

Baptiste KE, Williams K, Willams NJ, Wattret A, Clegg PD, Dawson S, Corkill JE, O'Neill T, Hart CA (2005). Methicillin-resistant staphylococci in companion animals. *Emerg Infect Dis* 11:1942–1944.

Barker-Reid F, Fox EM, Faggian R (2010). Occurrence of antibiotic resistance genes in reclaimed water and river water in the Werribee Basin, Australia. *J Water Health* 8:521–531.

Barlow RS, Fegan N, Gobius KS (2009). Integron-containing bacteria in faeces of cattle from different production systems at slaughter. *J Appl Microbiol* 107:540–545.

Barton MD (2000). Antibiotic use in animal feed and its impact on human health. *Nutr Res Rev* 13:279–299.

Barton MD, Wilkins J (2001). *Antibiotic Resistance in Bacteria Isolated from Poultry*. Rural Industries Research and Development Corporation, Canberra.

Barton MD, Pratt R Hart WS (2003). Antibiotic resistance in animals. *Commun Dis Intell* 27 (Suppl):S121–S126.

Bate J, Jordens JZ, Griffiths DT (1994). Farm animals as a putative reservoir for vancomycin-resistant enterococcal infection in man. *J Antimicrob Chemother* 34:507–514.

Bengtsson B, Anders F, Greko C, Pringle M, Odensvik K (2004). SVARM—Swedish Veterinary *Antimicrobial Resistance Monitoring*. C. G. a. M. P. Björn Bengtsson, National Veterinary Institute.

Bertolatti D, O'Brien FG, Grubb WB (2003). Characterization of drug-resistant *Staphylococcus aureus* isolated from poultry processing plants in Western Australia. *Int J Environ Health Res* 13:43–54.

Bettelheim KA, Hornitzky MA, Djordjevic SP, Kuzevski A (2003). Antibiotic resistance among verocytotoxigenic *Escherichia coli* (VTEC) and non-VTEC isolated from domestic animals and humans. *J Med Microbiol* 52:155–162.

Biavsco F, Foglia G, Paoletti C, Zandri G, Magi G, Guaglianone E, Sundsfjord A, C. Pruzzo G, Donelli G, Facinelli B (2007). vanA-type enterococci from humans, animals and food: Species distribution, population structure, Tn1546 typing and location, and virulence determinants. *Appl Environ Microbiol* 73:3307–3319.

Blix HS, Grave K, Hofshagen M, Iversen B, Kløvstad H, Kruse H, Lassen J, Mannsåker T, Norström M, Simonsen GS, Steinbakk M, Sunde M. (2004). NORM/NORM-VET: Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway.

Boon PJ, Cattanach, M (1999). Antibiotic resistance of native and faecal bacteria isolated from rivers, reservoirs and sewage treatment facilities in Victoria, south-eastern Australia. *Lett Appl Microbiol* 28:164–168.

Bucknell DG, Gasser RB, Irving A, Whihear K (1997). Antimicrobial resistance in salmonella and *Escherichia coli* isolated from horses. *Austral Vet J* 75:355–356.

Cabello FC (2006). Heavy use of prophylactic antibiotics in aquaculture: A growing problem for human and animal health and for the environment. *Environ Microbiol* 8 (7):1137–1144.

Call DR, Davis MA, Sawant AA (2008). Antimicrobial resistance in beef and dairy cattle production. *Anim Health Res Rev* 9:159–167.

Carroll S, Hargreaves M, Goonetilleke A (2005). Sourcing faecal pollution from onsite wastewater treatment systems in surface waters using antibiotic resistance analysis. *J Appl Microbiol* 99:471–482.

Caudry SD, Stanisich VA (1979). Incidence of antibiotic-resistant *Escherichia coli* associated with frozen chicken carcasses and characterization of conjugative R plasmids derived from such strains. *Antimicrob Agents Chemother* 16(6):701–709.

Checkley SL, Campbell JR, Chirino-Trejo M, Janzen ED, Waldner CL (2010). Associations between antimicrobial use and the prevalence of antimicrobial resistance in fecal *Escherichia coli* from feedlot cattle in western Canada. *Can Vet J* 51:853–861.

Clark CA, Greenwood S, Boison S, Chirino-Trejo M, Dowling PM (2008). Bacterial isolates from equine infections in western Canada (1998–2003). *Can Vet J* 49:153–160.

Cuny C, Kuemmerle J, Stanek C, Willey B, Strommenger B, Witte W (2006). Emergence of MRSA infections in horses in a veterinary hospital: Strain characterisation and comparison with MRSA from humans. *Eurosurveillance* 11(1):44–47.

DAFF (2007). Pilot surveillance program for Antimicrobial resistance in bacteria of animal origin. Australian Government Department of Agriculture, Fisheries and Forestry, Canberra.

Darby A (2007). Risk to humans as salmon antibiotics soar. *Sydney Morning Herald* 52, 965: 1–24.

Davies, J. E. (2007) Origins, Acquisition and dissemination of antibiotic resistance determinants, in ciba foundation symposium 207-Antibiotic Resistance: Origins, Evolution, Selection and Spread (eds D. J. Chadwick and J. Goode), John Wiley & Sons, Ltd., Chichester, UK. doi: 10.1002/9780470515358.

Davies J, Davies D (2010). Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev* 74:417–433.

Deckert A, Valdiviesco-Garcia A, Reid-Smith R, Tamblyn S, Seliske P, Irwin R, Dewey C, Boerlin P, McEwen SA (2010). Prevalence and antimicrobial resistance in *Campylobacter* spp isolated from retail chicken in two health units in Ontario. *J Food Protect* 73: 1317–1324.

De Graef EM, Decostere A, De Leener E, Goossens H, Baele M, Haesebrouck F (2007). Prevalence and mechanism of resistance against macrolides, lincosamides and streptogramins among *Enterococcus faecium* isolates from food-producing animals and hospital patients in Belgium. *Microb Drug Resis* 13:135–141.

De Jong A, Bywater R, Butty P, Deroover E, Godinho K, Klein U, Marion H, Simjee S, Smets K, Thomas V, Vallé M, Wheadon A (2009). A pan-European survey of antimicrobial susceptibility towards human-use antimicrobial drugs among zoonotic and commensal enteric bacteria isolated from healthy food producing animals. *J Antimicrob Chemother* 63:733–744.

DePaola A, Flynn PA, McPhaerson RM, Levy SB (1988). Phenotypic and genotypic characterisation of tetracycline resistant *Aeromonas hydrophila* from cultured channel catfish (*Ictalurus punctatus)* and their environments. *Appl Environ Microbiol* 54:1861–1863.

DePaola A, Peeler JT, Rodrick GE (1995). Effect of oxytetracycline-medicated feed on antibiotic resistance of gram-negative bacteria in catfish ponds. *Appl Environ Microbiol* 61:2335–2340.

Donabedian SM, Perri MB, Abdujamilova N, Gordoncillo MJ, Naqvi A, Reyes KC, Zervos MJ, Bartlett P (2010). Characterisation of vancomycin-resistant *Enterococcus faecium* isolated from swine in three Michigan counties. *J Clin Microbiol* 48:4156–4160.

Dunowska M, Morley PS, Traub-Dargatz JL (2006). Impact of hospitalisation and antimicrobial drug administration on antimicrobial susceptibility patterns of commensal *Escherichia coli* isolated from feces of horses. *J Am Vet Med Assoc* 228:1909–1917.

Emborg HD, Larsen PB, Heuer OE, Jensen VF, Hammerum AM, Bagger-Skjøt L, Brandt C, Frimodt-Møller N, Monnet DL (2004). DANMAP—Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, foods and humans in Denmark. O. E. H. Hanne-Dorthe Emborg, Per Bundgaard Larsen, Danish Zoonosis Centre, Danish Institute for Food and Veterinary Research, and D.-S. Mørkhøj Bygade 19, Statens Serum Institut, Danish Veterinary and Food Administration, Danish Medicines Agency, Danish Institute for Food and Veterinary Research. http://www.danmap.org/pdfFiles/Danmap_2004.pdf

Esink JM, Van Klingeren B, Houwers DJ, Klein WR, Vulto AG (1993). In-vitro susceptibility to antimicrobial drugs of bacterial isolates from horses in The Netherlands. *Equine Vet J* 25:309–314.

Ewers C, Antão EM, Diehl I, Phillipp HC, Wieler LH (2009). Intestine and environment of the chicken as reservoirs for extraintestinal pathogenic *Escherichia coli* strains with zoonotic potential. *Appl Environ Microbiol* 75:184–192.

Faires MC, Traverse M, Tater KC, Pearl DL, Weese JS (2010). Methicillin-resistant and -susceptible *Staphylococcus aureus* infections in dogs. *Emerg Infect dis* 16(1):69–75.

Fedorka-Cray PJ, Miller M, Dargatz DA (1998). NARMS-National Antimicrobial Susceptibility Monitoring Program—Veterinary Isolates, (U.S. Food and Drug Administration, U. S. Department of, Agriculture, Centers for Disease Control, Washington, DC.

Floras A, Lawn K, Slavic D, Golding GR, Mulvey MR, Weese JS (2010). Sequence type 398 methicillin-resistant *Staphylococcus aureus* infection and colonisation in dogs. *Vet Rec* 166 (26):826–827.

Frost, A. J. (1981). The resistance to antimicrobial agents of *Staphylococcus aureus* isolated from the bovine udder. *Australian Veterinary Journal* 57(6):262–267.

FSANZ (2006). *Public Health and Safety of Poultry Meat in Australia*. Food Standards Australia New Zealand, Canberra.

Furushita M, Shiba T, Maeda T, Yahata M, Kaneoka A, Takahashi Y, Torii K, Hasegawa T, Ohta M (2003). Similarity of tetracycline resistance genes isolated from fish farm bacteria to those from clinical isolates. *Appl Environ Microbiol* 69(9):5336–5342.

Gibson JS, Cobbold RN, Trott DJ (2010a). Characterisation of multi-drug resistant *Escherichia coli* isolated from extraintestinal clinical infections in animals. *J Med Microbiol* 59:592–598.

Gibson JS, Cobbold RN, Heisig P, Sidjabat HE, Kyaw-Tanner M, Trott DJ (2010b). Identification of Qnr and AAC(61)-1-br-cr plasmid-mediated fluoroquinolone resistance determinants in multi-drug resistant *Enterobacter* spp. isolated from extraintestinal infections in companion animals. *Vet Microbiol* 143:329–336.

Gillings MR, Holley MP, Stokes HW (2009). Evidence for dynamic exchange of qac gene cassettes between class 1 integrons and other integrons in freshwater biofilms. *FEMS Microbiol Lett* 296:282–288.

Gottlieb S, Wigney DI, Martin PA, Norris JM, Malik R, Govendir M (2008). Susceptibility of canine and feline *Escherichia coli* and canine *Staphylococcus intermedius* isolates to fluoroquinolones. *Austral Vet J* 86(4):147–152.

Hammerum AM, Heuer OE (2009). Human health hazards from antimicrobial-resistant *Escherichia coli* of animal origin. *Clin Infect Dis* 48:916–921.

Han D, Unno T, Jang J, Lim K, Lee SN, Ko G, Sadowsky MJ, Hur HG (2010). The occurrence of virulence traits among high-level aminoglycosides resistant *Enterococcus* isolates obtained from faeces of humans, animals and birds in South Korea. *Int J Food Microbiol*.

Harada K, Asai T (2010). Role of antimicrobial selective pressure and secondary factors on antimicrobial resistance prevalence in *Escherichia coli* from food-producing animals in Japan. *J Biomedi Biotechnol*.

Hart WS, Heuzenroeder MW, Barton MD (2004). Antimicrobial resistance in *Campylobacter* spp., *Escherichia coli* and *Enterococci* associated with pigs in Australia. *J Vet Med B Infect Dis Vet Public Health* 51(5):216–221.

Hartmann FA, West SHE (1995). Antimicrobial susceptibility profiles of multi-drug resistant *Salmonella* anatum isolated from horses. *J Vet Diagn Invest* 7:159–161.

Hayes JR, English LL, Carr LE, Wagner DD, Joseph SW (2004). Multiple antibiotic resistance of *Enterococcus* spp isolated from commercial poultry production environments. *Appl Environ Microbiol* 70:6005–6011.

Henriques IS, Fonseca F, Alves A, Saavedra MJ, Correia A (2006). Occurrence and diversity of integrons and β-lactamase genes among ampicillin-resistant isolates from estuarine waters. *Res Microbiol* 157:938–947.

Hershberger E, Oprea SF, Donabedian SM, Perri M, Bozigar P, Bartlett P, Zervos MJ (2005). Epidemiology of antimicrobial resistance in enterococci of animal origin. *J Antimicrob Chemother* 55:127–130.

Hoelzer K, Soyer Y, Rodriguez-Rivera LD, Cumming KJ, McDonough PL, Schoonmaker-Bopp DJ, Root TP, Dumas NB, Warnick LD, Gröhn YT, Wiedman M, Baker KNK, Besser TE, Hancock DD, Davis MA (2010). The prevalence of multi-drug resistance is higher among bovine than human *Salmonella enterica* serotype Newport, Typhimurium and 4,5,13:i:- isolated in the United States but differs by serotype and geographic region. *Appl Environ Microbiol* 76:5947–5959.

Inglis V, Yimer E, Bacon EJ, Ferguson S (1993). Plasmid-mediated antibiotic resistance in *Aeromonas salmonicida* isolated from atlantic salmon, *Salmo salar* L. in Scotland. *J Fish Dis* 16:593–600.

Jackson CR, Fedorka-Cray PJ, Barrett JB, Ladely SR (2005). High-level gentamicin resistant enterococci isolated from swine. *Epidemiol Infect* 133:367–371.

Jackson CR, Fedorka-Cray PJ, Barrett JB, Hiott LM, Woodley TA (2007). Prevalence of streptogramin resistance in enterococci from animals: Identification of vatD from animal sources in the USA. *Int J Antimicrob Agents* 30:60–66.

Jacobs L, Chenia HY (2007). Characterization of integrons and tetracycline resistance determinants in *Aeromonas* spp. isolated from South African aquaculture systems. *Int J Food Microbiol* 114(3):295–306.

JETACAR (1999). The use of antibiotics in food-producing animals: Antibiotic-resistant bacteria in animals and humans. Commonwealth of Australia, Canberra.

Jordan D, Morris SG, Gill P, Andersen LM, Chowdhury A, Stevenson AE & Spence SA (2005). Mass screening for antimicrobial resistant Escherichia coli in dairy cows in northern New South Wales. *Australian Veterinary Journal* 83:688–694.

Jordan DJ, Simon J, Fury S, Moss S, Giffard P, Maiwold M, Southwell P, Barton MD, Axon JE, Morris SG, Trott DJ (2011). Carriage of methicillin-resistant *Staphylococcus aureus* by veterinarians in Australia. *Austral Vet*, in press.

JVARM (1999). The Japanese Veterinary Antimicrobial Resistance Monitoring System. National Veterinary Assay Laboratory, Ministry of Agriculture, Forestry and Fisheries. Available: http://www.nval.go.jp/taisei/taisei.html.

Kadlec K, Schwarz S (2008). Analysis and distribution of class 1 and class 2 integrons and associated gene cassettes among *Escherichia coli* isolates from swine, horses, cats and dogs collected in the BfT-Germ Vet monitoring study. *J Antimicrob Chemother* 62:469–473.

Kerry J, Hiney M, Coyne R, NicGabhainn S, Gilroy D, Cazabon D, Smith P (1995). Fish feed as a source of oxytetracycline-resistant bacteria in the sediments under fish farms. *Aquaculture* 131:101–113.

Khan AA, Cheng CM, Van KT, West CS, Nawaz MS, Khan SA (2006). Characterization of class 1 integron resistance gene cassettes in *Salmonella enterica* serovars Oslo and Bareily from imported seafood. *J Antimicrob Chemother* 58(6):1308–1310.

Kim SR, Nonaka L, Suzuki S (2004). Occurrence of tetracycline resistance genes tet(M) and tet (S) in bacteria from marine aquaculture sites *FEMS Microbiol Lett* 237(1):147–156.

Korolik V, Chang J, Coloe PJ (1996). Variation in antimicrobial resistance in *Campylobacter* spp isolated in Australia from humans and animals in the last five years. In D.G Newell, JM Ketley, and RA Feldman (Eds.) *Campylobacters, Helicobacters, and Related Organisms*. Plenum, New York, pp. 393–398.

Kümmerer K (2009a). Antibiotics in the aquatic environment—A review—Part I. *Chemosphere* 75:417–434.

Kümmerer K (2009b). Antibiotics in the aquatic environment—A review—Part II. *Chemosphere* 75:435–441.

L'Abee-Lund TM, Sorum H (2001). Class I integrons mediated antibiotic resistance in the fish pathogen *Aeromonas salmonicida* world-wide. *Microb Drug Resist* 7:263–272.

Larsen J, Schoønheyder HC, Lester CH, Plsen SS, Porsbo LJ, Garcia-Migura L, Jensen LB, Bisgaard M, Hammerum AM (2010). Porcine-origin gentamicin-resistant *Enterococcus faecalis* in humans, Denmark. *Emerg Infect Dis* 16:682–684.

Le TX, Munekage Y, Kato S (2005). Antibiotic resistance in bacteria from shrimp farming in mangrove areas. *Sci Total Environ* 349:95–105.

Leonard FC, Markey BK (2008). Methicillin-resistant *Staphylococcus aureus* in animals: A review. *Vet J* 175(1):27–36.

Love G, Langenkamp D (2003). Australian aquaculture: Industry profiles for selected species. ABARE *e*Report. Australian Bureau of Agricultural and Resource Economics.

Luo N, Sahin O, Lin J, Michel LO, Zhang Q (2003). In vivo selection of *Campylobacter* isolates with high levels of fluoroquinolone resistance associated with gyrA mutations and the function of the CmeABC efflux pump. *Antimicrob Agents Chemother* 47(1): 390–394.

Malik SG, Coombs GW, O'Brien FG, Peng H, Barton MD (2006). Molecular typing of methicillin-resistant staphylococci isolated from cats and dogs. *J Antimicrob Chemother* 58:428–431.

Martinez J. (2009). The role of natural environments in the evolution of resistance traits in pathogenic bacteria. *Proc R Soc B* 276:2521–2530.

Miflin JK, Templeton JM, Blackall PJ (2007). Antibiotic resistance in *Campylobacter jejuni* and *Campylobacter coli* isolated from poultry in the South-East Queensland region. *J Antimicrob Chemother* 59(4):775–778.

Miranda CD, Zemelman R (2002). Bacterial resistance to oxytetracycline in Chilean salmon farms. *Aquaculture* 212:31–47.

Miranda CD, Kehrenberg C, Ulep C, Schwarz S, Roberts MC (2003). Diversity of tetracycline resistance genes in bacteria from Chilean salmon farms. *Antimicrob Agents Chemother* 47:883–888.

Morgan M (2008). Methicillin-resistant *Staphylococcus aureus* and animals: Zoonosis or humanosis? *J Antimicrob Chemother* 62:1181–1187.

Moriarty D (1997). The role of microorganisms in aquaculture ponds. *Aquaculture* 151: 333–349.

Morris DO, Mauldin EA, O'Shea K, Shofer FS, Rankin SC (2006). Clinical, microbiological, and molecular characterization of methicillin-resistant *Staphylococcus aureus* infections of cats. *Am J Vet Res* 67(8):1421–1425.

Mukherjee S, Chakraborty R (2006). Incidence of class 1 integrons in multiple antibiotic-resistant Gram-negative copiotrophic bacteria from the River Torsa in India. *Res Microbiol* 157(3):220–226.

Musto J, Kirk M, Lightfoot D, Combs BG, Mwanri L (2006). Multi-drug resistant *Salmonella Java* infections acquired from tropical fish aquariums, Australia, 2003–04. *Commun dis Intell* 30(2):222–227.

Nawaz M, Sung K, Khan SA, Khan AA, Steele R (2006). Biochemical and molecular characterization of tetracycline-resistant *Aeromonas veronii* isolates from catfish. *Appl Environ Microbiol* 72(10):6461–6466.

NDPSC (2003). Record of Reasons, 39th Meeting, October 14–16, 2003, pp. 53–62. National Drugs and Poisons Schedule Committee. Available: http://www.tga.gov.au/ndpsc/record/rr200310a.pdf.

Nematollahi A, Decostere A, Pasmans F, Haesebrouck F (2003). *Flavobacterium psychrophilum* infection in salmonid fish. *J Fish Dis* 26:563–574.

NETHMAP (2009). Consumption of antimicrobial agents and antimicrobial resistance among medically important bacteria in the Netherlands. SWAB-(Stichting Werkgroep Antibiotica Beleid)-Foundation of the Dutch Working Party on Antibiotic Policy. http://www.swab.nl/swab/cms3.nsf/uploads/1D61A8F6E60555F3C125763900414B7B/$FILE/nethmap2009_21-9-2009.pdf.

Nilsson O, Greko C, Top J, Franklin A, Bengtsson B (2009). Spread without known selective pressure of a vancomycin-resistant clone of *Enterococcus faecium* among broilers. *J Antimicrob Chemother* 63(5):868–872.

Niwa H, Anzai T, Izumiya H, Morita-Ishihara T, Watanabe H, Uchida I, Tozaki T, Hobo S (2009). Antimicrobial resistance and genetic characteristics of *Salmonella typhimurium* isolated from horses in Hokkaido Japan. *J Vet Med Sci* 71:1115–1119.

O'Mahony R, Abbott Y, Leonard FC, Markey BK, Quinn PJ, Pollock PJ, Fanning S, Rossney AS (2005). Methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from animals and veterinary personnel in Ireland. *Vet Microbiol* 109(3–4):285–296.

OzFoodNet (2005). Reported foodborne illness gastroenteritis in Australia: Annual report of the OzFoodNet network 2004. *Commun Dis Intell* 29:164–191.

Padungton P, Kaneene JB (2003). *Campylobacter* spp in humans, chickens, pigs and their antimicrobial resistance. *J Med Vet Sci* 65:161–170.

Petersen A, Guardabassi L, Dalsgaard A, Olsen JE (2000). Class I integrons containing a dhfr1 trimethoprim resistance gene cassette in aquatic *Acinetobacter* spp. *FEMS Microbiol Lett* 182:73–76.

Phillips I, Casewell M, Cox T, De Groot B, Friis C, Jones R, Nightingale C, Preston R, Waddell J (2004). Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *J Antimicrob Chemother* 53(1):28–52.

Pournaras S, Maniati M, Spanakis N, Ikonomidis A, Tassios PT, Tsakris A, Legakis NJ, Maniatis AN (2005). Spread of efflux pump-overexpressing, non-metallo-β-lactamase-producing, meropenem-resistant but ceftazidime-susceptible *Pseudomonas aeruginosa* in a region with bla_{VIM} endemicity. *J Antimicrob Chemother* 56:761–764.

Ramos J L, Duque E, Godoy P, Segura A (1998). Efflux pumps involved in toluene tolerance in *Pseudomonas putida* DOT-T1E. *J Bacteriol* 180(13):3323–3329.

Rhodes G, Huys G, Swings J, McGann P, Hiney M, Smith P, Pickup RW (2000). Distribution of oxytetracycline resistance plasmids between aeromonads in hospitals and aquaculture environments: Implication of Tn1721 in dissemination of the tetracycline resistance determinant Tet A. *Appl Environ Microbiol* 66:3883–3890.

Rojas A, Duque E, Mosqueda G, Golden G, Hurtado A, Ramos JL and Segura A (2001). Three efflux pumps are required to provide efficient tolerance to toluene in *Pseudomonas putida* DOT-T1E. *J Bacteriol* 183(13):3967–3973.

Russsel AD, Day MJ (1996). Antibiotic and biocide resistance in bacteria. *Microbios* 85:45–65.

Samuelsen OB, Torsvik Vand Ervik A (1992). Long-range changes in oxytetracycline concentration and bacterial resistance towards oxytetracycline in a fish farm sediment after medication. *Sci Total Environ* 114:25–36.

Sasaki T, Kikuchi K, Tanaka Y, Takahashi N, Kamata S, Hiramatsu K (2007). Methicillin-resistant *Staphylococcus pseudintermedius* in a veterinary teaching hospital. *J Clini Microbiol* 45(4):1118–1125.

Schmidt AS, Bruun MS, Dalsgaard I, Larsen JL (2001a). Incidence, distribution and spread of tetracycline resistance determinants and integron-associated antibiotic resistance genes among motile aeromonads from fish farming environment. *Appl Environ Microbiol* 67:5675–5682.

Schmidt AS, Bruun MS, Larsen JL, Dalsgaard I (2001b). Characterisation of class 1 integrons associated with R-plasmids in clinical *Aeromonas salmonocida* isolates from variuos geographical areas. *J Antimicrob Chemother* 47:735–743.

Sherley M, Gordon DM, Collignon PJ (2000). Variations in antibiotic resistance profile in Enterobacteriaceae isolated from wild Australian mammals. *Environ Microbiol* 2(6):620–631.

Sidjabat HE, Townsend KM, Lorentzen M, Gobius KS, Fegan N, Chin JJC, Bettelheim KA, Hanson ND, Bensink JC, Trott DJ (2006). Emergence and spread of two distinct clonal groups of multi-drug resistant *Escherichia coli* in a veterinary teaching hospital in Australia. *J Med Microbiol* 55:1125–1134.

Sidjabat HE, Hanson ND, Smith-Moland E, Bell JM, Gibson JS, Filippich LJ, Trott DJ (2007) Identification of plasmid-mediated extended-spectrum and AmpC Î²-lactamases in Enterobacter spp. isolated from dogs. Journal of Medical Microbiology 56: 426–434.

Son R, Rusul G, Sahilah AM, Zainuri A, Raha AR, Salmah I (1997). Antibiotic resistance and plasmid profile of *Aeromonas hydrophila* isolates from cultured fish (*Telapia mossambica*). *Lett Appl Microbiol* 24:479–482.

Sørum H, L'Abée-Lund TM, Solberg A, Anette W (2003). Integron-containing IncU R plasmids pRAS1 and pAr-32 from the fish pathogen *Aeromonas salmonicida*. *Antimicrob Agents Chemother* 47(4):1285–1290.

Stokes HW, Nesbø CL, Holley MP, Bahl MI, Gillings MR, Boucher Y (2006). Class 1 integrons potentially predating the association with Tn402-like transposition genes are present in a sediment microbial community. *J Bacteriol* 188:5722–5730.

Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FSL, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrock-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Smith K, Spencer D, Wong GKS, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock REW, Lory S, Olson MV (2000). Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature* 406:959–964.

Tadesse DA, Bahnson PB, Funk JA, Thakur S, Morrow WE, Wittum T, Degraves F, Rajala-Schultz P, Gebrayes WA (2010). Prevalence and antimicrobial resistance profile of *Campylobacter* spp. isolated from conventional and antimicrobial-free swine production systems from different US regions. *Foodborne Pathogen Dise*.

Tedesco L, Szakiel S (2006 August). Indigenous people in aquaculture. ABARE research report 06.9. Prepared for the Australian Government Department of Agriculture, Fisheries and Forestry, Canberra, pp 1–84.

Threlfall EJ, Ward LR, Frost JA, Willshaw GA (2000). Spread of resistance from food animals to man—The UK experience. *Acta Vet Scand* 93(Suppl):63–68.

Treasurer W, Birkbeck TH, Laidler LA, Cox DI (2007). Atypical *Aeromonas salmonicida* infection in naturally- and laboratory-challenged farmed haddock, *Melanogrammus aeglefinus* (L.) *J Fish Dis* 30(5):313–318.

Turner N, Cressey P, Lake R, Whyte R (2005). Review of non-commercial wild food in New Zealand. New Zealand Food and Safety Authority, pp. 1–182.

Unicomb L, Ferguson J, Stafford RJ, Ashbolt R, Kirk MD,Becker NG, Patel MS, Gilbert GL, Valcanis M, Mickan L, ACSS Group (2006). Low level fluoroquinolone resistance among *Campylobacter jejuni* in Australia. *Clin Infect Dis* 42:1368–1374.

Van Duijkeren E, Box ATA, Schellen P, Houwers DJ, Fluit AC (2005). Class 1 integrons in Enterobacteriaceae isolated from clinical infections of horses and dogs in The Netherlands. *Microb Drug Resist* 11:383–386.

Van Duijkeren E, Moleman M, Sloet Van Oldruitenborgh-Oosterbaan MM, Multem J, Troelstra A, Fluit AC, Van Wamel WJB, Houwers DJ, de Neeling AJ, Wagenaar JA (2010). Methicillin-resistant *Staphylococcus aureus* in horses and horse personnel: An investigation of several outbreaks. *Vet Microbiol* 141(1–2):96–102.

Vandenesch F, Naimi T, Enright MC, Lina G, Nimmo GR, Heffernan H, Liassine, Bes M, Greenland T, Reverdy ME, Etienne J (2003). Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: Worldwide emergence. *Emerg Infect Dis* 9(8):978–984.

Verschuere L, Rombaut G, Sorgeloos P, Verstraete W (2000). Probiotic bacteria as biological control agents in aquaculture. *Microbiol Mol Biol Rev* 64:655–671.

Voss A, Doebbeling BN (1995). The worldwide prevalence of methicillin-resistant Staphylococcus aureus. *Int J Antimicrob Agents* 5(2):101–106.

Walther B, Monecke S, Ruscher C, Friedrich AW, Ehricht R, Slickers P, Soba A, Wleklinski CG, Wieler LH, Lubke-Becker A (2009). Comparative molecular analysis substantiates zoonotic potential of equine methicillin-resistant *Staphylococcus aureus*. *J Clin Microbiol* 47 (3):704–710.

Ward MP, Brady TH, Couëtil LL, Liljeblke K, Maurer JJ, Wu C (2005). Investigation and control of salmonellosis caused by multi-drug resistant *Salmonella typhimurium* in a population of hospitalised horses. *Vet Microbiol* 107:233–240.

Watkinson AJ, Micalizzi GB, Graham GM, Bates JB, Constanzo SD (2007). Antibiotic resistant *Escherichia coli* in wastewaters, surface waters and oysters from an urban riverine system. *Appl Environ Microbiol* 73:5667–5670.

Weese JS, van Duijkeren E (2010). Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus pseudintermedius* in veterinary medicine. *Vet Microbiol* 140(3–4):418–429.

Weese JS, DaCosta T, Button L, Goth K, Ethier M, Boehnke K (2004). Isolation of methicillin-resistant *Staphylococcus aureus* from the environment in a veterinary teaching hospital. *J Vet Internal Med* 18(4):468–470.

Wegener HC (2003). Antibiotics in animal feed and their role in resistance development. *Curr Opin Microbiol* 6:439–445.

PART III

ANTIMICROBIAL SUBSTANCES AND RESISTANCE

16

DETECTION AND OCCURRENCE OF ANTIBIOTICS AND THEIR METABOLITES IN PIG MANURE IN BAVARIA (GERMANY)

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16.1 INTRODUCTION

16.1.1 Amounts of Antibiotics Used in Veterinary Medicine

Antibiotics represent one of the most important therapeutic groups used in veterinary medicine. The treatment of infectious diseases and the prevention of transmission of zoonotic agents to humans justify the use of these pharmaceuticals. In 1998, 3902 tons of antibiotics were used in veterinary medicine in Europe (FEDESA, 1999). A survey of BfT (2005) estimated a total amount of 668.8 tons administered by veterinarians in Germany.

The amount of prescribed antibiotics differs from animal species to animal species. Investigations done by Rassow and Schaper (1996) in the Weser-Ems region in Germany showed that 75% of the production orders were used for pigs, 24% for poultry, and only 1% for cattle and other animals. According to a study made by Grafe (2000) for this area, 90% of all manufacturing orders were intended for pigs; similar results were presented by Ungemach (2000). The frequent production orders for pigs may be due to the common occurrence of infectious diseases in livestock on

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the one hand and the easy administration of antibiotics with feed or drinking water on the other hand. In addition, the use of antibiotics for metaphylactic purposes in the context of herd management contributes to the usage of high amounts in livestock production. For example, newly formed stock groups were frequently treated on a routine base with antibiotics due to the different origin of the piglets (Blaha, 1996; Schwarz et al., 2000; Ungemach, 2000).

There are no detailed and actual data about the amount of antibiotics used in livestock in Germany. Surveys by FEDESA (1999) and studies from Lower Saxony (Germany) by Winckler and Grafe (2000) confirm results from Rassow and Schaper (1996), showing that tetracyclines are the most common class of compounds. Studies on antibiotic use in France, Denmark, and Great Britain showed a large variation regarding the consumption levels and the classes of compounds. However, these studies also underline the importance of tetracyclines and sulfonamides (Thiele-Bruhn, 2003).

16.1.2 Occurrence of Antibiotics in Manure

The occurrence of antibiotic residues in manure requires that they are only slightly or not metabolized in the animal and that the stability of the compound in manure is high. β-lactams or cephalosporins, for instance, are important in terms of the amount used, but because of their relative instability they are, however, of minor importance. In contrast, the therapeutic group of tetracyclines (e.g., chlortetracycline, tetracycline), sulfonamides (e.g. sulfadiazine, sulfamethazine), and pleuromutilins (e.g., tiamulin) are relatively stable compounds and thus of greater relevance (Christian et al., 2003; Göbel et al., 2004; Hirsch et al.,1998, 1999; Schlüsener et al., 2006; Xiu-Sheng et al., 2004).

Manure removal handling, storage capacity for manure (also duration of storage), and liquid manure treatment process (addititives for manure, aeration) also influence the stability and thus the possible residue loads (Langhammer, 1989). Persistent residues of antibiotics in manure may result in a contamination of soil and water and may influence the terrestrial and aquatic microbiota (Halling-Sørensen et al., 1998; Daughton and Ternes, 1999; Jørgensen and Halling-Sørensen, 2000; Kümmerer, 2003; Kay et al., 2004; Watanabe et al., 2010). Manure is an important factor in agriculture; in 2009, 152 million tons accrued in Germany (cattle and pig production). In Bavaria 30.1 million tons annually (2009) were used for fertilization or further purposes (e.g., biogas production), with 7.9 million tons originating from pigs (Schwab and Döhler, 2010). Up to now only a couple of studies analyzed the presence of residues of antibiotics in manure in Germany.

A screening of slurry of farms in North Rhine–Westphalia ($N = 181$) by Grafe (2000) was focused on tetracyclines; 43 positive results in concentrations from 0.6 mg/kg (limit of determination) up to 66 mg/kg were found. Residues of tiamulin and salinomycin (0.04/0.01 mg/kg) were detected in one of four manure samples by Schlüsener et al. (2003). As higher levels were expected, the authors assumed that these substances are less persistent in manure. A study in the Weser-Ems region in Lower Saxony ($N = 168$, 1999/2000, $N = 176$, 2000/2001) measured residues of antibiotics in manure samples. Tetracyclines were determined in up to 49.4% of the samples (limit of determination of 0.8 mg/kg) with maximum concentration

of 46 mg/kg. Sulfonamides (sulfamethazine and sulfadiazine) occurred in a similar frequency; however, maximum concentrations of 235 mg/kg were analyzed (Engels, 2004).

Haller et al. (2001, 2002) concentrated on the analysis of sulfonamides under environmental conditions. In six fresh manure samples (dry matter 3.3%) taken from farms in Switzerland, in which medicated feed was applied, sulfamethazine (8.7 mg/kg) and its N4-acetyl metabolite (2.6 mg/kg), as well as sulfathiazole (12.4 mg/kg) were detected. The synergist trimethoprim, however, which is also widely used, could only be analyzed in trace concentrations (limit of determination 0.1 mg/kg); the authors discussed this result with irreversible sorption effects in the gut or a rapid degradation in manure.

Little is known about the fate of antibiotics in manure during storage. Boxall et al. (2003, 2004) stated half-lives of about 30 days for sulfonamides in manure. However, for some sulfonamides a reactivation in manure could be observed. For example, N4-acetyl sulfamethazine (metabolite from phase II) can be converted back in manure by bacterial turnover into the original sulfamethazine (Berger et al., 1986; Langhammer, 1989). Studies of Arikan et al. (2007) showed that composting of manure (with sawdust) of beef cattle, therapeutically treated with oxytetracycline, can reduce the levels of extractable antibiotic residues.

The existing studies considered only a small spectrum of antibiotic residues in manure. Thus, a method for analyzing 23 different antibiotic substances was developed and used to conduct a comprehensive field study in Bavaria (Fig. 16.1), where about one third of German livestock is located.

(a)

FIGURE 16.1 *(See color insert.)*

(b)

(c)

FIGURE 16.1 (Continued)

16.2 MATERIALS AND METHODS

16.2.1 Sampling (Cross-Field Study)

One manure sample per farm ($N = 380$) was taken in the autumn of 2002 or in the spring of 2003 from pig farms at the time of its distribution on the fields. All administrative districts of Bavaria (Germany) were represented commensurate with their livestock quantities. Representation was achieved by defining the desired number of farms per administrative district in advance and taking the randomized sample individually for each administrative district. Farms were divided into three farming types (fattening pig herds, combined farms, and breeding farms) and two classes of size were built from each type. According to the Bavarian structure of pig farming, size limits for "large" farms were chosen quite low, at >30/45 sows

(combined/breeding farms) or >220 fattening pigs. Using these criteria, farms were chosen randomly from an agricultural database using a random generator and the anonymous monitoring was based on volunteer participation. In accordance with the objectives of sampling, the collected samples were equally distributed between all three farming types: breeding farms ($n = 133$), fattening farms ($n = 132$), and combined farms ($n = 115$). This equal distribution was not possible regarding farm size (small/large); higher sample numbers were achieved from "large" farms ($n = 248$) compared to "small" ones ($n = 132$), mainly due to the higher percentage of litter use among small farms, leading to separated dung and slurry samples instead of desired homogenized manure.

16.2.2 Method for the Determination of Antibiotics in Manure

For validation, manure was artificially contaminated with antibiotics [chlortetracycline (CTC), tetracycline (TC), oxytetracycline, doxycycline, sulfadiazine, sulfadimethoxine, sulfadoxin, sulfaguanidine, sulfamerazine, sulfameter, sulfamethoxypyrazine, sulfamethoxazole, sulfaphenazole, sulfapyridine, sulfathiazole, sulfamethazine, N4-acetylated-sulfamethazine, sulfisomedine, sulfachloropyridazine, trimethoprim, enrofloxacin, tiamulin, florfenicol] in graded concentrations (0.05, 0.1, 0.5, and 5.0 mg/kg). Metabolites of N4-acetylated sulfadiazine, sulfamerazine, and sulfathiazole were synthesized according to Pfeifer et al. (2002). All analytical steps were carried out in polypropylene (PP) tubes to avoid complexation reactions (tetracyclines). Before extraction, a buffer was added (citric buffer, pH 4.7, for tetracyclines and Sorensen buffer, pH 6.0, for sulfonamides and other antibiotics). The samples were extracted twice with ethylacetate. Lipophilic components were separated by liquid–liquid cleanup steps with hexane. Finally, solid-phase extraction (StrataX, Phenomenex) was used as the second cleanup step. These methods consisted of robust procedures to extract manures with highly variable pH and dry matter content.

16.2.3 High-Performance Liquid Chromatography – Mass Spectrometry (HPLC–MS) Analysis

For identification and quantification an HPLC system (WATERS, 2690 Separations Module, Milford, MA) in combination with a quadrupole mass-spectrometer (VG Platform 2) with an electrospray (ESP) ionization source and the software of MassLynxTM Datasystem (Micromass, Altrichamn UK) has been used. Separation was performed with an RP C8/C18 column (Luna RP C8, 2.0×150 mm, 3.0 μm, PHENOMENEX; Atlantis RP C18, 2.1×150 mm, 3.0 μm, WATERS) and using an acetonitrile/water gradient. Identification was based on retention time and relative intensity of the selected ions. For quantification, the area of the quasi-molecular ion peak was compared with that of an external standard.

Chromatographically the 6-iso- and 4-epimers of chlortetracycline were not separated from the parent substance. As the chromatograms of artificial contaminated samples were similar to those from "naturally contaminated" samples, it could be suggested that these compounds were formed during sample preparation. The confirmation of iso- and epimerization could be also observed in vivo or during storage (Grote et al., 2004). In this study the summarized chlortetracycline content

was specified. Estimations of relevant chromatograms showed a fraction of about 40% unchanged chlortetracycline.

16.2.4 Method Validation

Accuracy and precision were determined in recovery experiments with fortified samples. Four different spike values were measured ($n = 5$): 5.0, 0.5, 0.1, and 0.05 mg/kg. Mean recovery rates ranged from 61.5 to 105.4%. The developed methods were capable of detecting concentrations above 0.1/0.05 mg/kg (limit of determination defined as the smallest analyte content for which the method was validated with the specified accuracy and precision) with satisfying correctness and robustness.

16.2.5 Trials to Determine the Fate of Antibiotics During Storage (in Laboratory Scale)

The stability of 12 sulfonamides as well as trimethoprim, tiamulin, enrofloxacin, and florfenicol during manure storage was investigated. Therefore, antibiotic-free manure (6.4% dry matter, pH 6.1; organic matter: 79.7% of dry matter, N: 6.52% dry matter, NH_4–N: 4.17% dry matter, P: 27.52 g/kg dry matter, K: 36.06 g/kg dry matter, total aerobe count of bacteria: log 9.36 colony-forming units/g) was artificially contaminated and stored in polyethylene bottles for up to 16 weeks at −20 °C, 7 °C, and room temperature (20–22 °C). Thus, aliquots (each $n = 5$) were analyzed at particular times (0, 1, 3 days and 1, 2, 3, 4, 12, 16 weeks). Manure samples, which were "naturally contaminated" with chlortetracycline, were stored at 7 °C and frozen (−20 °C) for 5 months.

16.2.6 Statistics

Statistical analysis for data evaluation was carried out using chi-squared test ($\alpha = 0.05$).

16.3 RESULTS AND DISCUSSION

16.3.1 Laboratory Storage Experiment

Chlortetracycline content in manure did not change during 5 months at storage temperatures of 20 or 7 °C. The results regarding the stability of sulfonamides in manure at different storage conditions are summarized in Table 16.1. Surprisingly, not all sulphonamides behaved in the same way. No reduction of sulfadiazine, sulfaguanidine, sulfaisomedin, sulfamerazine, sulfamethazine, sulfamethoxypyrazine, and sulfapyridine could be observed at any temperature. Other compounds, however, were reduced during these 16 weeks, at least partially. At room temperature this happened usually faster than at lower storage temperatures (7 °C); in the frozen state (−20°C) mostly no reduction was observed–expect for enrofloxacin; Figure 16.2 shows the example of sulfamethoxazole.

These results demonstrate that the degradation of antibiotics in manure depends on various factors that are likely specific to storage temperature and to compound. Knowledge of the physical-chemical behavior of the group of antibiotics alone does not suffice to estimate the fate of a specific antibiotic in manure.

TABLE 16.1 Degradation (%) of Sulfonamides and Further Antibiotics in Manure within 16 Weeks at Different Storage Temperatures

Antibiotic Substance	Degradation [median, $n = 5$, (%)]		
	−20 °C	7 °C	Room Temperature (20–22 °C)
Sulfadiazine	0	0	0
Sulfadimethoxine	0	20	30
Sulfachloropyridazine	5	10	10
Sulfaguanidine	0	0	0
Sulfisomedine	0	0	0
Sulfamerazine	0	0	0
Sulfamethazine	0	0	0
N4-acetyl-Sulfamethazine	0	60	65
Sulfamethoxazole	0	80	80
Sulfamethoxypyrazine	0	0	0
Sulfaphenazole	0	25	30
Sulphapyridine	0	0	0
Sulphathiazole	0	15	30
Enrofloxacine	20	80	80
Florfenicole	0	75	80
Tiamulin	0	10	25
Trimethoprim	0	70	70

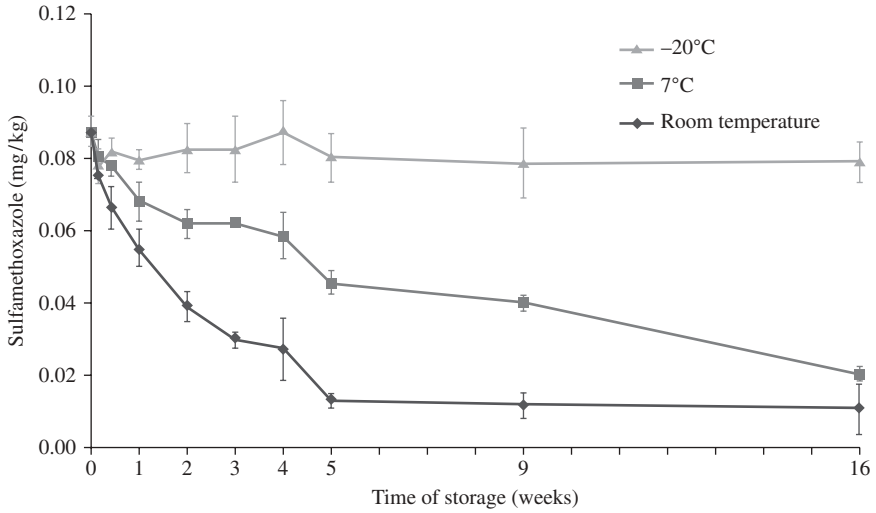

FIGURE 16.2 Degradation of sulfamethoxazole in manure during storage (laboratory scale) at different temperatures (± SD, $n = 5$).

16.3.2 Analysis of Field Samples of the Cross-Field Study

In the analyses of 380 pig manure samples, 112 samples (29.5%) did not contain concentrations of the specific target compounds above the limit of determination; 135 samples (35.5%) contained 1; and 76 samples (20.0%) contained 2 antibiotics. In 46

samples (12.1%) 3 of the screened compounds were defected, and in 9 samples (2.4%) 4 of the screened antibiotics could be detected. Two samples (0.5%) contained even 5 antibiotic compounds.

Table 16.2 gives an overview of the antibiotic residues in pig manures. As expected, tetracyclines and sulfonamides were the most frequently analyzed drug groups. Although maximum concentrations of about 50 mg/kg chlortetracycline or 38 mg/kg sulfamethazine were detected, the medians of the positive findings were below 0.8 mg/kg. A more precise analysis of the concentration–distribution of chlortetracycline, tetracycline, sulfadiazine, and sulfamethazine shows that most samples (87–98%) were below 1.0 mg/kg (Fig 16.3). Co-occurrence of tetracyclines

TABLE 16.2 Detected Antibiotic Residues in Pig Manure ($N = 380$)

		Concentration [mg/kg]	
Antibiotic Substance	Positive Findings [a](%)	Median[b]	Range (min–max)
Chlortetracycline	37	0.34	0.1–50.8
Tetracycline	29	0.68	0.1–46.0
Oxytetracycline	4	0.14	0.1–0.9
Doxycycline	1	0.38	0.1–0.7
Sulfamethazine	48	0.12	0.05–38
N4-acetyl-sulfamethazine	31	0.10	0.05–27
Sulfadiazine	5	0.78	0.05–5
Sulfamerazine	2	0.70	0.05–0.9
Sulfamethoxazole	1	0.05	0.05–0.05
Sulfathiazole	1	0.07	0.05–0.1
Sulfadimethoxine	1	0.19	0.05–0.6

[a]Tetracyclines > 0.1 mg/kg; sulfonamides > 0.05 mg/kg.
[b]Positive findings.

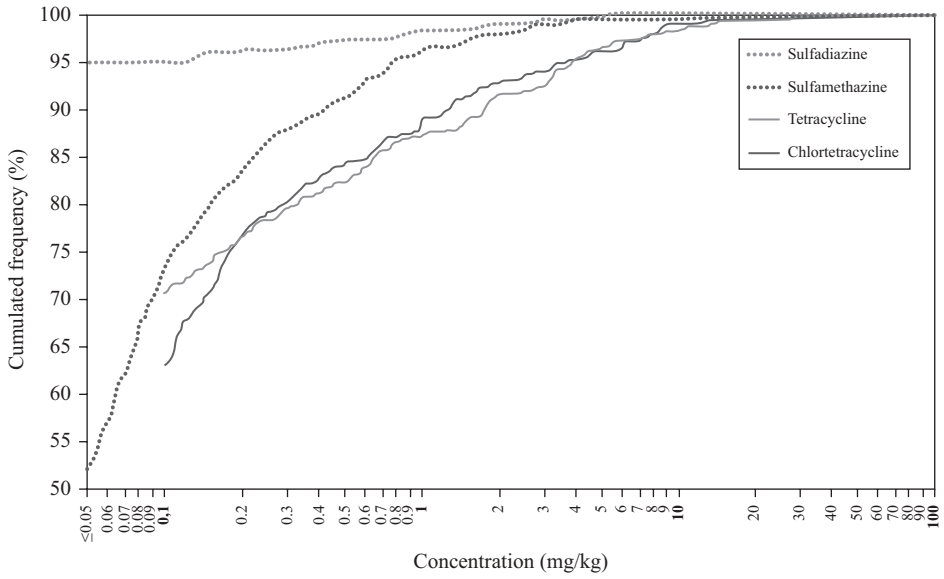

FIGURE 16.3 Cumulated frequency (%) of detected antibiotics in pig manure ($N = 380$).

and sulfonamides was observed in 29.2% of the samples. Interestingly, 23 of 25 samples, which contained both drug groups in concentration> 0.5 mg/kg, originated from large scale farms.

16.3.2.1 Differential Analysis of the Tetracycline Results For a better evaluation of these results four concentration classes (<0.1 mg/kg, 0.1 ≤1.0 mg/kg, >1.0 ≤ 4.0 mg/kg, and> 4.0 mg/kg) were formed. The criteria for this classification were the limit of determination of tetracyclines in pig manure and the breakpoints (minimal inhibitory concentrations, MIC) used for characterisation of bacteria as "sensitive," "intermediate," or "resistant" in the sensitivity test for bacterial pathogens to antibiotics DIN (2004).

In this study, 9.7% of the samples showed tetracycline contents above 4 mg/kg. This concentration should allow the growth of only bacteria classified as resistant (Kayser et al., 2001; Feuerpfeil et al., 1999)—provided that tetracyclines are as effective in manure as in standardized medium. It must be noted that a shift of the ratio between sensitive to resistant bacteria occurs mainly in the gastrointestinal tract of the animals, where growth conditions are more favorable for many bacteria than in manure. If tetracycline concentrations are diluted with antibiotic-free fractions of manure during storage, a further selection effect would not be expected. The action of tetracyclines is bacteriostatic, which means that susceptible bacteria can recover once the concentrations of antibiotics fall below the MIC of these susceptible bacteria. Of manure samples 13.2% showed tetracycline concentrations >1.0 ≤ 4.0 mg/kg (MIC range for bacteria classified as intermediate). Also these concentrations should be taken into account because they are able to activate the exchange of plasmids and transposons (containing resistances genes) and enhance adaption processes (Corpet et al., 1989; Doucet-Populaire et al., 1991).

Tetracycline-sensitive bacteria can only grow in a medium with tetracycline concentration ≤1.0 mg/L. Of the samples 28.2% contained measurable tetracycline concentration in this dimension. It is well known that low amounts of antibiotic residues are able to enhance low-resistant populations compared to the susceptible flora; adaptation processes to rising concentrations can lead to high resistance levels of some individual bacteria (George and Levy, 1983; Mittler and Lenski, 1992; Baquero et al., 1997). For these reasons, concentrations that range between 0.1 and ≤ 1.0 mg/kg cannot be safely discounted as being problematic. This is confirmed by the fact that manure samples with tetracycline concentrations in the discussed range contained significantly more tetracycline genes (*tet*M, *tet*O) than samples with tetracycline concentrations below the limit of determination (Hölzel et al., 2009). For further information see also Chapter 12.

The box plot in Figure 16.4 shows the range of the chlortetracycline and tetracycline concentrations from the different farm types and sizes. In most cases, the median (positive findings) of the large-scale farm is higher than the median of smaller farms.

Regarding the farm size (large or small), it could be determined that the percentage of findings with high concentrations (> 4 mg/kg) were higher in large-scale farms than in smaller ones. This result indicates an even greater use of tetracyclines per animal in large farms, which may be the result of a higher risk of infection. Clear differences between farm types had not been recognized (Table 16.3).

Interestingly it was notable that samples acquired in the spring showed a higher percentage of samples (10.2%) with high residues (> 4.0 mg/kg) than those collected

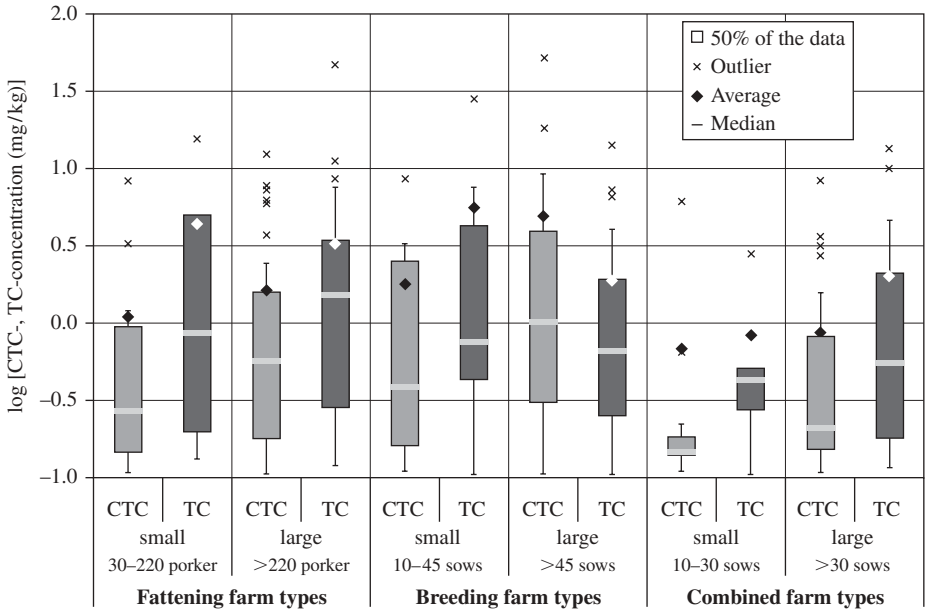

FIGURE 16.4 Contents of chlortetracycline (CTC) and tetracycline (TC) in regard to farm type and farm size (box plot).

TABLE 16.3 Tetracycline Findings Classified to Breakpoint Ranges Regarding Farm Type and Farm Size[a]

			Percentage Classified in Concentration Classes[b] (%)			
Farm Type	Farm Size	Antibiotic	<0.1 mg/kg	0.1 ≤ 1.0 mg/kg	> 1.0 ≤ 4 mg/kg	>4 mg/kg
Fattening farms	Small ($n = 43$)	CTC	67.4[a]	25.6	4.7	2.3
		TC	90.7[d]	4.7	2.3	2.3
	Large ($n = 89$)	CTC	48.3[e]	34.8	10.1	6.7
		TC	55.1[d]	21.3	18.0	5.6
Breeding farms	Small ($n = 40$)	CTC	77.5	15.0	5.0	2.5
		TC	82.5[d]	10.0	2.5	5.0
	Large ($n = 75$)	CTC	65.3	17.3	10.7	6.7
		TC	58.7[d]	25.3	12.0	4.0
Combined farms	Small ($n = 49$)	CTC	75.5[c]	22.4	2.0	0
		TC	89.8[d]	8.2	2.0	0
	Large ($n = 84$)	CTC	60.7[c]	29.9	8.3	1.2
		TC	71.4[d]	16.7	9.5	2.4

[a]CTC chlortetracycline, TC tetracycline.
[b]According to MIC (minimal inhibitory concentration)—classes of DIN 58940-4.
[c]Significant difference of farms size within the farm type (chi^2-squared, $\alpha = 0.1$).
[d]Significant difference of farms size within the farm type (chi^2-squared, $\alpha = 0.05$).

in the autumn (2.6%). However, a significant difference could only be shown between the proportion of tetracycline free (< 0.1 mg/kg) between autumn and spring. This effect could be explained with the need for more antibiotic treatment in winter.

16.3.2.2 Differential Analysis of the Sulfonamide results The collected manure samples showed positive findings for sulfamethazine (and N4-acteylated sulfamethazine), sulfadiazine (N4-acetylated sulfadiazine), sulfamerazine (N4-acetylated sulfamerazine), sulfathiazole, sulfamethoxazole, and sulfadimethoxine (Table 16.2). The concentrations of the most frequently detected sulfonamides (sulfamethazine, N4-acetylated sulfamethazine, and sulfadiazine) ranged mainly from 0.05 to 2.0 mg/kg. The concentration of the N4-acetylated metabolite of sulfamethazine was below the parent substance (Fig. 16.5).

In general, the content of sulfamethazine was below the concentrations of tetracyclines. The breakpoint for sulfonamides (64 mg/kg, DIN 58940-4) was reached in only one sample, thus the risk of selection effects for resistant bacteria does not seem to be serious. However, more investigations are needed to evaluate the consequences of low concentrations of sulfonamides and other antibiotics in the environment. Regarding the farm type, it could be recognized that only 2.2% of large-scale fattening farms showed sulfamethazine concentrations above 4 mg/kg. Significant differences in antibiotic-free proportion between the herd sizes of the particular farm type could only be observed for combined farms.

Comparing these results with those of the Weser-Ems area (Engels, 2004), the limit of determination must be taken into account. A limit of determination of 0.8 mg/kg—like in the analysis of Engels—would reduce the percentage of positive finding in this study from 48 to only 4.7%. Thus it can be stated that in the Bavarian pig manure samples less sulfonamide residues were found than in Weser-Ems (49.3%), also the maximum residues were lower (present study: 38 mg/kg;

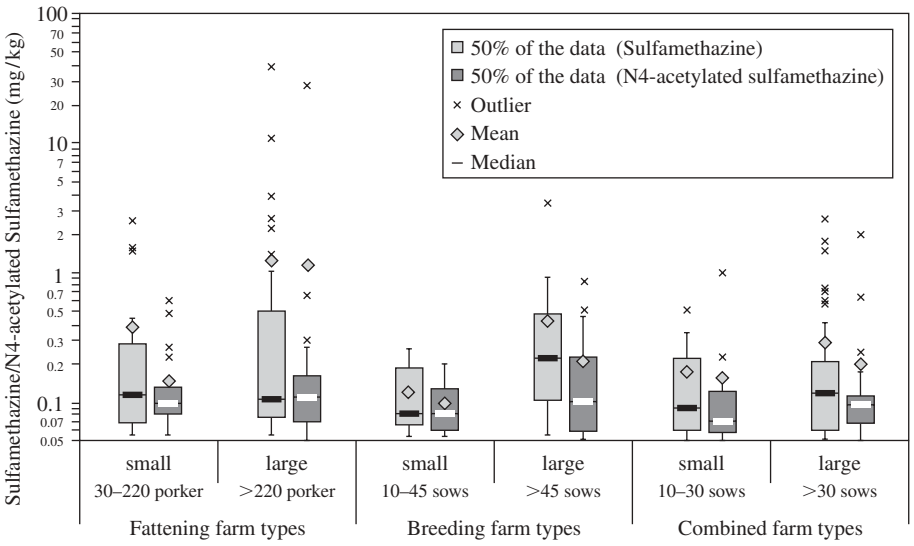

FIGURE 16.5 Concentration of sulfamethazine and N4-acetyl-sulfamethazine regarding farm types and farm sizes (box plot).

Weser-Ems: 235.1/167.0 mg/kg). These relatively low contents of sulfonamides in Bavaria may be the result of its restricted application in recent years in combination with residues of previous treatment (K. Heinritzi, personal communication, 2005).

The absence of the widely used synergist of sulfonamides, trimethoprim, can possibly be explained by its reduced stability in manure (see Table 16.1).

16.4 CONCLUSIONS

Tetracyclines could be detected in 51% of the acquired field samples. Reasons for this are their frequent use (especially in the large-scale livestock facilities) and their persistence in manure. Assuming an application of 30 m^3 manure/ha, tetracycline concentrations at the limit of determination (0.1 mg/kg) would lead to an application of 3 g/ha arable land; the detected maximum concentration would lead to 1.8 kg/ha (calculated with a concentration of 59.8 mg/kg: maximum detected content of 46 mg/kg adjusted to analytical recovery rate of 70.3%). For sulfonamides such as sulfamethazine, a maximum load of 1.1 kg/ha could be calculated. Sulfonamides appear to have a high potential to resist degradation in manure—as shown in own (16.3.1) initial studies—and may be transferred into aquatic environment because of their hydrophilic nature (Haller et al., 2002).

As antibiotic residues in manure may also influence the composition and activity of soil bacteria, the development of technologies to reduce antibiotic residues should be promoted. Already Langhammer (1989) elaborated on possible solutions to improve the degradation of relatively stable antibiotic residues. Examples for this were the improving of the microbial potential by aeration (stirring), fresh manure supply, and higher storage temperatures (Kühne et al., 2000).

Recent studies from Alvarez et al. (2010) indicate a degradation of oxytetracycline and chlortetracycline during the anaerobic digestion of pig manure. However, further investigations would be of great interest, such as the use of biogas power plants or other manure treatments such as composting.

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REFERENCES

Arikan O, Sikora L, Mulbry W, Khan S, Foster G (2007). Composting rapidly reduces levels of extractable oxytetracycline in manure from therapeutically treated beef calves. *Bioresour Technol* 98:169–176.

Alvarez J, Otero L, Lema J, Omil F (2010). The effect and fate of antibiotics during the anaerobic digestion of pig manure. *Bioresour Technol* 101 (22): 8581–8586.

Baquero F, Negri M, Morosini M, Blázques J (1997). The antibiotic selective process: Concentration specific amplification of low-level resistant population. In DJ Chadwick and J Goode (Eds.), *Antibiotic Resistance: Origin, Evolution and Spread*, Ciba Foundation Symposium. Wiley, Chichester.

Berger K, Petersen B, Büning-Pfaue H (1986). Persistenz von Gülle-Arzneistoffen in der Nahrungsmittelkette. *Archiv für Lebensmittelhygiene* 37:99–102.

BfT (Bundesverband für Tiergesundheit, e.V.) (2005, February). Arzneimittel in der Umwelt. Tiergesundheit—Berichte aus der Tiergesundheitsindustrie in Europa. *Tiergesundheit im Blickpunkt*, Ausgabe 47.

Blaha T (1996). Gesundheits-und Umweltrisiken von Antiinfektiva und Antiparasitika in der Nutztierhaltung—Vermeidungsstrategien und Auswege. *Deutsche Tierärztliche Wochenschrift* 103:278–280.

Boxall A, Kolpin D, Halling-Sørensen B, Tolls J (2003). Are veterinary medicines causing environmental risks? *Environ Sci Technol* 37(15):286A–294A.

Boxall A, Fogg L, Blackwell P, Kay P, Pemberton E, Croxford A (2004). Veterinary medicines in the environment. *Rev Environ Contamin Toxicol* 180:1–91.

Christian T, Schneider R, Fäber H, Skutlarek D, Meyer M, Goldbach H (2003). Determination of antibiotics residues in manure, soil and surface waters. *Acta Hydrochim Hydrobiol* 31(1):36–44.

Corpet D, Lumeau S, Corpet F (1989). Minimum antibiotic levels for selecting a resistance plasmid in a gnotobiotic animal model. *Antimicrob Agents Chemother* 33(4):535–540.

Daughton C, Ternes T (1999). Pharmaceuticals and personal care products in the environment: Agents of subtle change? *Environ Health Perspe* 107:907–938.

DIN (Deutsches Institut für Normung e.V.) (2004). Bewertungsstufen der minimalen Hemmkonzentration von antimikrobiellen Wirkstoffen (Teil 4). *Methoden zur Empfindlichkeitsprüfung von mikrobiellen Krankheitserregern gegen Chemotherapeutika*, Beuth Verlag, Berlin, 58940-4–supplement 1:2004-02 (2004).

Doucet-Populaire F, Trieu-Cuot P, Dosbaa I, Andremont A, Courvalin P (1991). Inductible transfer of conjugative transposons Tn1545 from *Enterococcus faecalis* to *Listeria monocytogenes* in the digestive tract of gnotobiotic mice. *Antimicrob Agents Chemother* 35(1):185–187.

Engels H (2004). Verhalten von ausgewählten Tetrazyklinen und Sulfonamiden in Wirtschaftsdüngern und in Böden [dissertation]. Georg-August-Universität, Göttingen. Available: http://deposit.ddb.de/cgi-bin/dokserv?idn=982290071&dok_var=d1&dok_ext=pdf&filename=982290071.pdf.

FEDESA (1999) Antibiotics for animals—A FEDESA perspective on antibiotics, animal health and the resistance debate. Available: http://www.fedesa.be or http://www.ifahsec.org, http://www.ifahsec.org/europe/topics/antibio/pdf/Dossier9.pdf.

Feuerpfeil I, López-Pila J, Schmidt R, Schneider E, Szewzky R (1999). Antibiotikaresistente Bakterien und Antibiotika in der Umwelt. *Bundesgesundheitsblatt—Gesundheitsforschung und—schutz* 42:37–50.

George A, Levy S (1983). Amplifiable resistance to tetracycline, chloramphenicol and other antibiotics in *Escherichia coli*: Involvement of a non-plasmid-determined efflux of tetracycline. *J Bacteriol* 155(2):531–540.

Göbel A, McArdell C, Suter M, Giger W (2004). Trace determination of macrolide and sulfonamide antimicrobials, a human sulfonamide metabolite, and trimethoprim in wastewater using liquid chromatography coupled to electrospray tandem mass spectrometry. *Anal Chem* 76:4756–4764.

Grafe A (2000). Untersuchungen zum Einsatz pharmakologisch wirksamer Stoffe in der Veredelungswirtschaft unter besonderer Berücksichtigung der Tetrazykline (dissertation). Georg-August-Universität, Göttingen.

Grote M, Vockel A, Schwarze D, Mehlich A, Freitag M (2004). Fate of antibiotics in food chain and environment originating from pig fattening. *Fresenius Environ Bull* 13 (11b):1216–1224.

Haller M, Müller S, Stoob K, McArdell C (2001). Antibiotika in Hofdünger—Analytik und erste Resultate. Jahresbericht der EAWAG, Schweiz, 33–34. available: http://e-collection.ethbib.ethz.ch/eserv/eth:22384/eth-22384-05.pdf.

Haller M, Müller S, McArdell C, Alder C, Suter M (2002). Quantification of veterinary antibiotics (sulfonamides and trimethoprim) in animal manure by liquid chromatography-mass spectrometry. *J Chromatogr A* 952: 111–120.

Halling-Sørensen B, Nielsen S, Lanzky P, Ingerslev F, Holten Lützhøft HC, Jørgensen S (1998). Occurrence, fate and effects of pharmaceutical substances in the environment—A review. *Chemosphere* 36(2):357–393.

Hirsch R, Ternes T, Haberer K, Mehlich A, Ballwanz F, Kratz K (1998). Determination of antibiotics in different water compartments via liquid chromatography electro-spray tandem mass spectrometry. *J Chromatogr A* 815:213–223.

Hirsch R, Ternes T, Haberer K, Kratz K (1999). Occurrence of antibiotics in the aquatic environment. *Sci Total Environ* 225:109–118.

Hölzel C, Harms K, Küchenhoff H, Kunz A, Müller C, Meyer K, Schwaiger K, Bauer J (2009). Phenotypic and genotypic bacterial antimicrobial resistance in liquid pig manure is variously associated with contents of tetracyclines and sulfonamides. *J Appl Microbiol* 108 (5):1642–1656.

Jørgensen S, Halling-Sørensen B (2000). Drugs in the environment. *Chemosphere* 40:691–699.

Kay P, Blackwell P, Boxall A (2004). Fate of veterinary antibiotics in a macroporous tile drained clay soil. *Environ Toxicol Chem* 23(5):1136–1144.

Kayser FH, Bienz K, Eckert J, Zinkernagel R (2001). *Medizinische Mikrobiologie*, Vol. 10. Auflage , Georg Thieme Verlag, Stuttgart.

Kümmerer K (2003). Significance of antibiotics in the environment. *J Antimicrob Chemother* 52:5–7.

Kühne M, Ihnen D, Möller M, Agthe O (2000). Stability of tetracycline in water and liquid manure. *J Vet med A* 47:379–384.

Langhammer, J-P (1989). Untersuchungen zum Verbleib antimikrobiell wirksamer Arzneistoffe als Rückstände in Gülle und im landwirtschaftlichen Umfeld [dissertation]. Friedrich-Wilhelms-Universität, Bonn.

Mittler J, Lenski, L (1992). Experimental evidence for an alternative to directed mutation in the bgl operon. *Nature* 356(6368):446–448.

Pfeifer T, Tuerk J, Bester K, Spiteller M (2002). Determination of selected sulfonamide antibiotics and trimethoprim in manure by electrospray and atmospheric pressure chemical ionization tandem mass spectrometry. *Rapid Commun Mass Spectrom* 16:663–669.

Rassow D, Schaper H (1996). Zum Einsatz von Fütterungsarzneimitteln in Schweine- und Geflügelbeständen in der Region Weser-Ems. *Deutsche tierärztliche Wochenschrift* 103 (7):237–284.

Schlüsener M, Bester K, Spiteller M (2003). Determination of antibiotics such as macrolides, ionophores and tiamulin in liquid manure by HPLC-MS/MS. *Anal Bioanal Chem* 375: 942–947.

Schlüsener M, von Arb M, Bester K (2006). Elimination of macrolides, tiamulin, and salinomycin during manure storage. *Arch Environ Contam Toxicol* 51:21–28.

Schwab M, Döhler H (2010). Livestock manure—annual production in the Federal Republic of Germany. *Landtechnik* 65(5):354–356.

Schwarz S, Kehrenberg C, Walsh R (2000). Review: Use of antimicrobial agents in veterinary medicine and food animal production. *Int J Antimicrob Agents* 17:431–437.

Thiele-Bruhn S (2003). Pharmaceutical antibiotic compounds in soils—A review. *J Plant Nutr Soil Sci* 166:145–167.

Ungemach F (2000). Figures on quantities of antibacterials used for different purposes in the EU countries and interpretation. *Acta Agric Scand* supply 93:89–97.

Watanabe N, Bergmaschi B, Loftin B., Meyer M Harter T (2010). Use and environmental occurrence of antibiotics in freestall dairy farms with manured forage fields. *Environ Sci Technol* 44:6591–6600.

Winckler C, Grafe A (2000). Stoffeintrag durch Tierarzneimittel und pharmakologische Futterzusatzstoffe unter besonderer Berücksichtigung der Tetrazykline. UBA Texte 44/00, Umweltbundesamt, Umweltforschungsplan des BMU, Forschungsbericht 29733911:1–145.

Xiu-Sheng M, Bishay F, Chen M, Metcalfe C (2004). Occurrence of antimicrobials in the final effluents of wastewater treatment plants in Canada. *Environ Sci Technol* 38:3533–3541.

17

FATE AND TRANSPORT OF ANTIBIOTICS IN SOIL SYSTEMS

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17.1 INTRODUCTION

Following administration to humans or animals, antibiotics are absorbed and in some instances may be metabolized. The parent compound(s) and any metabolites may then be released into the soil environment directly, for example, due to excretion by pasture animals or indirectly due to the application of animal manure or sewage sludge to fields as a fertilizer (Halling-Sørensen et al., 1998; Boxall et al., 2003, 2004a; Boxall, 2004; Sarmah et al., 2006). This chapter reviews the present state of understanding of the inputs of antibiotics to the soil environment and synthesizes the available information on the fate and transport of antibiotics in soil systems. Gaps in current knowledge are highlighted and recommendations made for future research.

17.2 ROUTES OF INPUT TO THE SOIL ENVIRONMENT

The main routes of input of antibiotics to the soil environments and subsequent transport routes are illustrated in Figure 17.1.

For veterinary antibiotics, the major route of entry of the compounds into the environment is probably via excretion following use and the subsequent application of contaminated manure onto land as a fertilizer (Metcalfe et al., 2008). Intensively reared farm animals are usually housed indoors for long periods at a time. Consequently, large quantities of farmyard manure, slurry, or litter are produced that are then disposed of at high application rates onto land (Boxall et al., 2004a).

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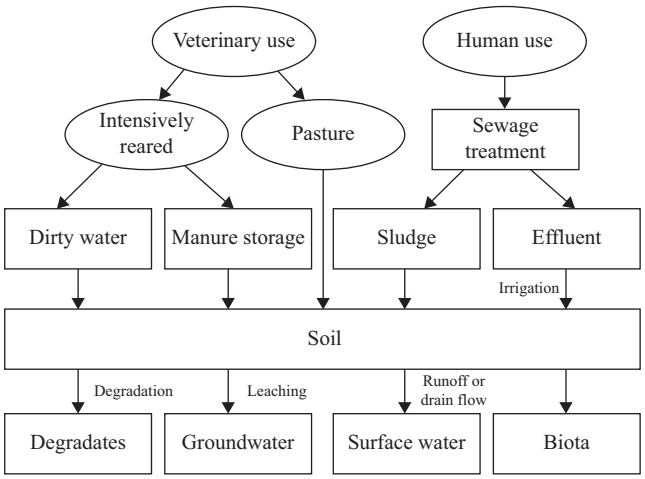

FIGURE 17.1 Routes of input and distribution of antibiotics in soil systems.

Manure or slurry will typically be stored before it is applied to land. During this storage time, it is possible that residues of veterinary antibiotics will be degraded. A wide range of antibiotics has been detected in manures for land application including tetracyclines, sulfonamides, macrolides, 2,4-diaminopyrimidines, fluoroquinolones, and ionophores (Furtula et al., 2009; Martinez-Carballo et al., 2007; Hu et al., 2010b; Zhao et al., 2010). Drugs administered to grazing animals or animals reared intensively outdoors may be deposited directly to land in dung or urine, exposing soil organisms to high local concentrations (Halling-Sørensen et al., 1998).

For human-use antibiotics, following therapy, the compounds and their metabolites will be released to the sewage system. The sewage will typically be treated before release to surface water bodies. During the treatment process, antibiotics may sorb to particulate organic matter in the activated sludge treatment process (McClellan and Halden, 2010). The particulate matter is then separated from the liquid phase and, in a number of countries, following treatment, it is used as a soil fertilizer. This fertilizer material is referred to as biosolids or sewage sludge. A range of antibiotic classes have been reported in biosolids, including fluoroquinolones, macrolides, sulfonamides, tetracyclines, and 2,4-diaminopyrimidines (McClellan and Halden, 2010). Other antimicrobial compounds such as triclosan and miconazole, which are used in the home as biocides, have also been detected in biosolids. Antibiotics that do not sorb to sludge may also enter the soil environment if treated wastewater is used for irrigation purposes (Ternes et al., 2007).

17.3 OCCURRENCE IN SOIL SYSTEMS

While a large number of studies have now investigated the occurrence of antibiotics in surface waters around the world (e.g., see the review of Monteiro and Boxall, 2010), much less information is available on the levels of antibiotics in soil systems. Antibiotics from the tetracycline, sulfonamide, fluoroquinolone, 2,4-diaminopyrimidine, and macrolide classes have been detected in soils in Europe,

North America, and Asia (Table 17.1). Typically, maximum concentrations observed in these studies for the different classes of antibiotics are in the tens to hundreds of micrograms per kilogram range. Most studies in soil systems have looked at antibiotics arising from veterinary use, although some data are available on concentrations in soil following sludge application (Golet et al., 2002) and resulting from wastewater irrigation (Kinney et al., 2006). While the data on human-use antibiotics is limited, the available data indicate that veterinary inputs are probable more significant in terms of soil exposure.

TABLE 17.1 Antibiotics Detected in Soils in Monitoring Studies Performed across the World

Antibiotic	Countries Monitored	References
2,4-Diaminopyrimidines		
Trimethoprim	United Kingdom	Boxall et al. 2006b
Fluoroquinolones		
Ciprofloxacin	China, Switzerland, Turkey	Hu et al. 2010b, Golet et al. 2002, Uslu et al. 2008
Enrofloxacin	China, Turkey	Hu et al. 2010b, Uslu et al. 2008
Norfloxacin	Switzerland	Golet et al. 2002
Ofloxacin	China	Hu et al. 2010b
Pefloxacin	China	Hu et al. 2010b
Macrolides		
Lincomycin	China, United Kingdom	Boxall et al. 2006b, Hu et al. 2010b
Sulfonamides		
Sulfachloropyridazine	China	Hu et al. 2010b
Sulfadiazine	United Kingdom	Boxall et al. 2006b
Sulfadimethoxine	United States	Watanabe et al. 2010
Sulfadoxine	China	Hu et al. 2010b
Sulfamethazine	Canada, China	Hu et al. 2010b, Watanabe et al. 2010
Sulfamethoxazole	United Kingdom	Watanabe et al. 2010, Kinney et al. 2006
Tetracyclines		
Chlortetracycline	Canada, China, Germany, Spain, Turkey,	Hu et al. 2010b, Aust et al. 2008, Andreu et al. 2009, Cengiz et al. 2010, Hamscher et al. 2002, Jacobsen et al. 2004
Doxycycline	Spain	Andreu et al. 2009
Oxytetracycline	China, Spain, Turkey, United Kingdom	Boxall et al. 2006b, Hu et al. 2010b, Andreu et al. 2009, Cengiz et al. 2010
Tetracycline	China, Germany, Spain, United States	Hu et al. 2010b, Watanabe et al. 2010, Andreu et al. 2009, Hamscher et al. 2002

17.4 FATE AND TRANSPORT IN THE SOIL ENVIRONMENT

Once an antibiotic is released to the soil environment, it may persist in the soil or degrade, be transported to surface waters or groundwater, or be taken up into biota. The behavior of the antibiotic will be determined by its underlying physical properties (including water solubility, lipophilicity, volatility, and sorption potential). In the following sections information on the fate and transport of antibiotics in the environment is reviewed.

17.4.1 Sorption in Soil

The degree to which antibiotics may adsorb to soils varies widely with reported sorption coefficients (K_d) between soils and water varying in some instances by more than four orders of magnitude, depending on the soil type (Table 17.2). The available data indicate that sulfonamide antibiotics will be mobile in the soil environment and move to surface waters and groundwater, whereas tetracycline, macrolide, and fluoroquinolone antibiotics will exhibit low mobility. The large variability in sorption behavior of antibiotics in different soil systems is explained by the fact that most antibiotics are ionizable substances with pK_a values in the pH range of natural soils. Antibiotics can therefore occur in the soil environment as negative, neutral, zwitterionic, and positively charged species (Ter Laak et al., 2006a, 2006b). The ratio of these species will primarily be driven by the pH of the soil system. Each of the species will interact with soil particles in different ways, for example, neutral species will exhibit hydrophobic interactions with the organic carbon in the soil, cationic species will be attracted to the negatively charged surfaces of soil particles, and the anionic species will be repelled by the negative charges (Ter Laak, 2006b). Anionic species can also associate with soil particles through cation bridging (Gao and Pedersen, 2005).

A number of recent studies have attempted to understand the behavior of the individual species of antibiotics in soils. For example, Lertpaitoonpan et al. (2009) studied the sorption of sulfamethazine at different pH values. They concluded that the antibiotic associated with soil via hydrophobic interactions at pH values <7.4 due to compound being in the unionized form and that at higher pHs, the compound was in the anionic form, and therefore electrostatic repulsion occurred with negatively charged soil surfaces resulting in lower soprtion. Kahle and Stamm (2007) studied the sorption of sulfathiazole in different solid matrices at different pH values. They concluded that sorption of cationic species of the molecule was greatest followed by neutral and then anionic species. The sorption behavior of antibiotics is therefore strongly influenced by the pH, cationic exchange capacity, and organic carbon content and the nature of the organic carbon of the soil (Jones et al., 2005; Sassman and Lee, 2005; Strock et al., 2005; Ter Laak et al., 2006b; Kahle and Stamm, 2007). In addition other soil properties, including metal oxide content, and ionic strength appear to be important. A range of empirical sorption models have been proposed to provide an estimation of the sorption of antibiotics in a particular soil type based on a range of soil parameters (including the organic carbon, cation exchange capacity, and oxide content of the soil) (Figueroa-Diva et al., 2010; Ter Laak et al., 2006b), these models may be invaluable in the future in estimating the sorption behavior for antibiotics across a range of soil types. Other approaches have

also been proposed for predicting sorption of antibiotics and related compounds in soils, including the use of mammalian pharmacology data (Williams et al., 2009) and the use of neural networks based on antibiotic and solid matrix properties (Baron et al., 2009).

Most studies into the sorption behavior of antibiotics have studied distribution between soil and a water phase only. It is also important to recognize that antibiotics will reach soils through the application of sewage sludge and manure to fields and that the sorption of the compounds may be affected by the presence of the sludge or manure matrix. Boxall et al. (2002) showed that the addition of pig slurry to soil increased the sorption of a sulfonamide antibiotic. In contrast, Thiele-Bruhn and Aust (2004) observed a decrease in the sorption of a group of sulfonamides following addition of slurry. The addition of sewage sludge to soil generally decreases the sorption of sulfonamide antibiotics and other human-use pharmaceuticals. The observed changes in sorption behavior were explained by changes in either the pH of the system or the dissolved organic carbon concentration of the supernatant (Thiele-Bruhn and Aust, 2004; Boxall et al., 2002).

17.4.2 Persistence in Soil

The main route for degradation of antibiotics in soils is via aerobic soil biodegradation, although, in some instances, other mechanisms such as photodegradation may be important (Wolters and Steffens, 2005). Degradation of antibiotics is affected by environmental conditions such as temperature and pH and the presence of specific degrading bacteria that have developed to degrade groups of medicines (Ingerslev and Halling-Sørensen, 2001). As well as varying significantly between chemical classes (e.g., see Table 17.2), dissipation rates for antibiotics also vary within a chemical class. The presence of manure of sewage sludge also has an influence on the persistence and can either decrease or increase the persistence of an antibiotic in the soil environment (Wang et al., 2006).

The degradation processes may well result in the formation of degradation products. For example, Halling-Sørensen et al. (2003) investigated the degradation of oxytetracycline in soil interstitial pore water and identified a range of transformation products, including 4-epi-oxytetracycline, α-apo-oxytetracycline, and β-apo-oxytetracycline. Studies with sulfadiazine (Kasteel et al., 2010) have identified two main transformation products, namely *N*1-2-(4-hydroxypyrimidinyl) benzenesulfanilamide (4-OH-SDZ) and 4-(2-iminopyrimidin-1(2H)-yl)aniline. In some instances, these degradation products may be of greater environmental concern than the parent compound as some may have similar or greater toxicity, some are more persistent, and some are more mobile (Boxall et al., 2004b). It is, therefore, important that the fate of the degradation products in soils is considered when assessing the impact of an antibiotic on the environment.

There is an increasing body of evidence showing that the observed dissipation of many antibiotics is not due to degradation of the antibiotic molecule but due to irreversible binding of the molecule to soil particles (Heise et al., 2006). Once sequestered in the soil, the compounds are thought to become entrapped and may persist in soil for several years (Forster et al., 2009). Currently, there is a significant amount of debate over whether these nonextractable residues are bioaccessible to soil organisms or may become bioaccessible in the future, and hence they pose a risk

TABLE 17.2 Measured Sorption Coefficients (K_d) and Dissipation Half-Lifes for Antibiotic Substances in Soils

	K_d	Half-life in Soil	References
2,4-Diaminopyrimidines			
ormetoprim	1.3–58.3	—	Sanders et al. (2008)
Fluoroquinolones			
Ciprofloxacin	74–54,600	> 65	Figueroa-Diva et al. (2010), Bayer (1997)
Danofloxacin	—	87–143	Chen et al. 1997
Enrofloxacin	54–33,600	359–696	Figueroa-Diva et al. (2010), Bayer (1997)
Norfloxacin	41–36,400	—	Figueroa-Diva et al. (2010)
Ofloxacin	309–3,554	—	Drillia et al. (2005), Nowara et al. (1997)
Ionophores			
Monensin	—	<4	Sassman and Lee (2007)
Lasalocid	—	<4	Sassman and Lee (2007)
Macrolides			
Efrotomycin	8.3–290	—	Yeager and Halley (1990)
Erythromycin	—	11	Schlusener and Bester (2006)
Tylosin	5.4–66,900	95–97	Hu and Coats (2009), Rabolle and Spliid (2000), Sassman and Lee (2007b), Blackwell et al. (2005)
Tylosin A aldol	516–7,740	—	Sassman et al. (2007)
Pleuromutilins			
Tiamulin	—	26	Schlusener and Bester (2006)
Quinolones			
Olaquindox	0.69–1.67	5.8–8.8	Rabolle and Spliid (2000), Ingerslev and Halling-Sϕrensen (2001)
Sulfonamides			
Sulfamethoxazole	0.23–37.6	—	Drillia et al. (2005)
Sulfadiazine	1.4–2.8	12–18	Thiele Bruhn et al. (2004), Yang et al. (2009a)
Sulfanilamide	1.5–1.7	—	Thiele Bruhn et al. (2004)
Sulfamethazine	0.23–98.25	18.6	Lertpaitoonpan et al. (2009), Accinelli et al. (2007)
Sulfapyridine	1.6–7.4	21.3	Thiele (2000), Accinelli et al. (2007)
Sulfachloropyridazine	0.9–1.8	2.8–3.5	Boxall et al. (2002), Blackwell et al. (2005)
Sulfadimethoxine	0.4–25.8	—	Sanders et al. (2008), Thiele-Bruhn et al. (2004)
Tetracyclines			
Chlortetracycline	1,280–2,386	30	Gavalchin and Katz (1994)
Oxytetracycline	417–1026	16–56	Rabolle and Spliid (2000), Blackwell et al. (2005), Yang et al. (2009), Wang and Yates (2008)
Tetracycline	1,147–2,370	—	

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to ecosystems. Heise et al. (2006) explored the bioacessibility of nonextractable residues of sulfamethoxazole to plants and earthworms. They demonstrated that only a small fraction of the residues was taken up by both test organisms. Much more work is required to assess whether this observation also holds true for other antibiotics.

17.5 TRANSPORT IN SOIL SYSTEMS

Antibiotics applied to soil can be transported to aquatic systems in surface runoff, subsurface flow, and drain flow. The extent of transport via any of these processes is determined by a range of factors, including the solubility, sorption behavior, and persistence of the antibiotic; the physical structure, pH, organic carbon content, and cation exchange capacity of the soil matrix; and climatic conditions such as temperature and rainfall volume and intensity. A number of studies have explored the transport of veterinary and human-use antibiotics in soil systems. Lysimeter, field-plot, and full-scale field studies have investigated the transport of antibiotics from the soil surface to field drains, ditches, streams, rivers, and groundwater (Kay et al. 2004, 2005a, 2005b, 2005c; Burkhard et al., 2005; Hamscher et al., 2005; Kreuzig and Holtge, 2005; Blackwell et al., 2007, 2009). A range of experimental designs and sampling methodologies has been used. These investigations are described in more detail below.

17.5.1 Leaching to Groundwater

A number of laboratory-based column leaching studies have been performed to assess the leaching of antibiotics through soil (Hu and Coats, 2009; Unwold et al., 2010; Wu et al., 2010). These studies have looked at the effects of a range of variables (including soil type, antibiotic loading, the presence of manure/biosolids, and water flow) on leaching behavior of antibiotics. Wu et al. (2010) explored the leaching behavior of clindamycin and four other human-use pharmaceuticals in soils with different properties. The results indicated that the selected pharmaceuticals have low mobility in soils, although small portions of the applied pharmaceuticals were recovered in the leachates, possibly due to dissolved organic-matter-facilitated transport. Unwold et al. (2010) demonstrated that the leaching of sulfonamides is affected by the input concentration of the antibiotic and the flow of water through the column. Hu and Coats (2009) showed that the leachability of tylosin is dependent on soil properties and manure amendment.

Blackwell et al. (2009) used more realistic semifield lysimeter experiments to explore the influence of slurry amendment and incorporation and the effects of climate on leaching behavior of sulfachloropyridazine, oxytetracycline, and tylosin. While sulfachloropyridazine was found to leach, oxytetracycline and tylosin were not detected in any leachate samples. These differences in behavior were explained by the sorption and persistence characteristics of the compounds. Changes in rainfall timing and the presence/absence of manure had little effect on the sulfonamide leaching. Comparison of the experimental measurements with simulations from a leaching model, which is routinely used by regulators and industry to assess the risk of veterinary medicines to groundwater indicated that the model greatly underestimates

the transport of antibiotics to groundwater. This raises questions over the application of these models in the regulatory risk assessment process.

A series of controlled studies and monitoring studies have also been done at the field scale. Aust et al. (2010) explored the leaching of sulfonamide antibiotics using field lysimeter studies over a number of years. Antibiotics were detected in the majority of leachate samples taken and were detected 23 months after application, demonstrating that these compounds can persist in the natural environment for some time. Soil management, such as tillage and cropping, were found to affect the leaching behavior of the antibiotics. The movement of sulfonamides and tetracyclines in soil profiles has also been investigated at the field scale using suction probes (Hamscher et al., 2005; Blackwell et al., 2007). In these studies, sulfonamides were found at depth but the tetracyclines were not, which is most likely due to the high potential for tetracyclines to sorb to soil. In a 3-year monitoring study, Dolliver and Gupta (2008) quantified the leaching of antibiotics arising from treatment of cattle and poultry.

Chlortetracycline was not detected in leachate samples but monensin and tylosin were detected. There are only a few reports of veterinary medicines in groundwater itself (Hirsch et al., 1999; Watanabe et al., 2010). In an extensive monitoring study conducted in Germany, a large number of groundwater samples were collected from agricultural areas in order to determine the extent of contamination by antibiotics (Hirsch et al., 1999). The data show that in most areas with intensive livestock breeding, no antibiotics were present above the limit of detection (0.02–0.05 μg/L), and sulfonamide residues were, however, detected in four samples. While the source of contamination of two of these is considered to be attributable to irrigation with sewage, the authors conclude that sulfamethazine, detected at concentrations of 0.08 and 0.16 μg/L, could possibly have derived from veterinary applications, since it is not used in human medicine. Watanabe et al. (2010) found sulfadimethoxine, sulfamethazine, and lincomycin in shallow groundwater directly down gradient from manure storage lagoons on farms in the United States. In a study in Canada, Kuchta et al. (2009) assessed the potential of lincomycin to contaminate groundwater, but concentrations were below limits of detection.

17.5.2 Runoff

Transport of antibiotics in runoff (i.e., overland flow) has been observed for tetracycline antibiotics (i.e., oxytetracycline), sulfonamide (sulfadiazine, sulfamethazine, sulfathiazole, sulfamethoxazole, and sulfachloropyridazine), ionophores (monensin), and macrolide antibiotics (lincomycin, erythromycin, and tylosin) (Kay et al., 2005a; Kreuzig et al., 2005; Davis et al., 2006; Topp et al., 2008; Stoob et al., 2007; Kuchta et al., 2009). Just like leaching, the transport of these substances is influenced by the sorption behavior of the compounds, the presence of manure in the soil matrix, and the nature of the land to which the manure is applied. Runoff of highly sorptive substances, such as tetracyclines, is typically lower than the more mobile compounds such as sulfonamides (Kay et al., 2005a; Davis et al., 2006). For selected compounds (e.g., macrolides and tetracyclines), particle-associated transport appears to be important in determining the degree of transport in runoff (Davis et al., 2006)—erosion control practices could, therefore, be used to reduce losses of these compounds from agricultural fields to surface waters. The presence of manure and slurry has been shown to increase the transport of sulfonamides via

runoff by 10–40 times in comparison to runoff following direct application of these medicines to soils (Burkhard et al., 2005). Possible explanations for this observation include physical "sealing" of the soil surface by the slurry and/or a change in pH as a result of manure addition, which alters the speciation and fate of the medicines (Burkhard et al., 2005). Other factors such as the nature of the agricultural field, agricultural practices, and the presence/absence of snow melt also appear to affect the degree of transport of antibiotics in runoff. For example, overland transport of antibiotics from ploughed soils has been shown to be significantly lower than runoff from grasslands (Kreuzig et al., 2005) and higher runoff of antibiotics has been observed during periods of high snowmelt compared to periods of minimal snowmelt (Dolliver and Gupta, 2008). Antibiotic losses are generally higher from the no-tillage systems compared with losses from ploughed or injected systems (Dolliver and Gupta, 2008; Topp et al., 2008). Larsbo et al. (2008) evaluated the MACRO model, which is routinely used by regulators and industry to assess the risks of pesticides, for predicting movement of antibiotics from soils to surface waters in runoff. Results showed that the model could accurately simulate concentrations of sulfadimidine in runoff, indicating that the models that have been developed and used for pesticide and nutrient risk assessment might also be appropriate for assessment of environmental exposure to antibiotics.

17.5.3 Drain Flow

The transport of a range of antibacterial substances (i.e., tetracyclines, macrolides, sulfonamides, and trimethoprim) has been investigated using field-based studies in tile-drained clay soils (Kay et al., 2004; Boxall et al. 2006a; Edwards et al., 2009). Kay et al. (2004) studied the movement of sulfachloropyridazine and oxytetracycline via drain flow to surface waters. Concentrations of the sulfonamide in the drain flow water were an order of magnitude higher than the tetracycline, even though the spiking concentrations for the test compounds were similar. Like the runoff results, these differences are again likely to be due to differences in sorption behavior of the two compounds. In a subsequent investigation at the same site (Kay et al., 2004), in which the soil was tilled, much lower concentrations were observed in the drain flow, suggesting that tillage may be a useful mitigation strategy in the event that an antibiotic is found to pose a risk to aquatic systems. Edwards et al. (2010) demonstrated the transport of sulfamethoxazole and a range of other human pharmaceuticals from a biosolid-treated field via tile drains to surface waters. In a UK study, Boxall et al. (2006b) continuously monitored surface waters in streams receiving tile drainage inputs from farmland treated with manure from animals that had received a range of antibiotics (including oxytetracycline, lincomycin, sulfadiazine, and trimethoprim). Maximum concentrations of antibiotics in stream water ranged from 0.02 μg/L (trimethoprim) to 21.1 (lincomycin) μg/L, and concentrations of antibacterials in stream sediments ranged from 0.5 to 813 μg/kg.

17.6 UPTAKE INTO BIOTA

Antibiotics may also be taken up from soil into biota, and in the last few years the potential uptake of antibiotics and other pharmaceuticals into terrestrial organisms,

in particular plants, has received increasing attention (Migliore et al., 2003; Kumar et al., 2005; Boxall et al., 2006b). Studies with a range of compounds and plant species show that a number of antibiotics (including tetracyclines, sulfonamides, macrolides, and fluoroquinolones) are taken up by plants following exposure to antibiotic contaminated soil at environmentally realistic concentrations (Kumar et al., 2005; Boxall et al., 2006b; Dolliver et al., 2007; Kong et al., 2006; Hu et al., 2010b; Table 17.3). The levels in plants are, however, very low and are unlikely to pose a risk to humans (Boxall et al., 2006b). The mechanisms driving uptake into plants have not yet been characterized, and this is an area that warrants further research (Boxall et al., 2006b).

Very little data are available on uptake of antibiotics from soils into other organisms. Some information is available on the uptake of nonextractable residues of sulfonamides into earthworms (Heise et al., 2006). The uptake of a range of human pharmaceuticals, including the antibacterial trimethoprim, from biosolid-amended soils into earthworms has also been assessed (Kinney et al., 2008), but in this study no medical use antibiotics were detected in the earthworm tissue.

TABLE 17.3 Antibiotics Shown to Be Taken up by Different Plant Species

Plant Type	Antibiotics Taken up	References
Alfalfa	Oxytetracycline	Kong et al. (2007)
Cabbage	Chlortetracycline	Kumar et al. (2005)
Carrot	Enrofloxacin, florfenicol, trimethoprim	Boxall et al. (2006b)
Celery	Chlortetracycline, tetracycline, sulfadimethoxine, sulfachloropyridazine, ofloxacin, pefloxacin, lincomycin	Hu et al. (2010b)
Coriander	Oxytetracycline, chlortetracycline, tetracycline, sulfadimethoxine, sulfachloropyridazine, ofloxacin, lincomycin	Hu et al. (2010b)
Corn	Chlortetracycline, sulfamethazine	Dolliver et al. (2007), Kumar et al. (2005)
Lettuce	Sulfamethazine, trimethoprim, florfenicol	Dolliver et al. (2007), Boxall et al. (2006b)
Onion	Chlortetracycline	Kumar et al. (2005)
Potato	Sulfamethazine	Dolliver et al. (2007)
Radish	Oxytetracycline, chlortetracycline, tetracycline, sulfamethazine, sulfadimethoxine, sulfachloropyridazine, ofloxacin, pefloxacin, lincomycin, chloramphenicol	Hu et al. (2010b)
Rape	Oxytetracycline, tetracycline, chlortetracycline, sulfamethazine, sulfadimethoxine, ofloxacin, lincomycin, chloramphenicol	Hu et al. (2010b)
Soybean	Oxytetracycline, norfloxacin	Boonsaner and Hawker (2010)

There is also the possibility that antibiotics may move from soils into food chains and affect wildlife. In a recent study, residues of fluoroquinolone antibiotics were detected in the eggs of vultures and red kites in Spain, and the presence of these residues have been associated with fatal embryo chondral damage (Lemus et al., 2008). The fluoroquinolones were most likely taken up due to consumption of contaminated animal carcasses, but this does highlight the need to develop a better understanding of the exposure of organisms to antibiotics in the terrestrial environment.

17.7 RECOMMENDATIONS FOR FUTURE RESEARCH

Over the past decade, there has been increasing research on the fate and behavior of antibiotics in the soil environment, and there is now a much better understanding about how these compounds interact with soils, how they persist in soils, and their potential to be transported to surface waters. However, there are still a number of uncertainties that require addressing before there can be a full understanding of the environmental risks of antibiotics. Areas requiring further research include:

- More work is needed on human antibiotics and other classes of veterinary antibiotics in soils—Most work on antibiotics in soil systems has focused on veterinary medicines from the tetracycline, fluoroquinolone, and sulfonamide classes. It would be valuable to gain an understanding of the inputs, occurrence, fate, and behavior of some of the classes that have been less well studied. Some of the human antibiotics may be much more important in terms of potential impacts of environmental exposure on human health.
- More monitoring of antibiotics in soils is required—most monitoring studies have focused on pharmaceuticals in surface waters and emissions from wastewater treatment plants. It would be valuable to generate information on the occurrence and persistence of a range of antibiotics in agricultural systems for a range of geographical areas.
- Better integration of knowledge on fate and transport in order to develop models for assessing environmental exposure to antibiotics at the field and scales.
- Assessment of the environmental risks of nonextractable residues of antibiotics—There is an increasing body of data showing that the dissipation of many antibiotics in soils is as a result of the formation of nonextractable residues. We need to better understand the potential for these residues to be taken up by organisms (including microbes) as well as the potential for the compounds to be remobilized in the future (e.g., due to changes in the properties of the environment).
- Uptake into terrestrial organisms and food chains—A number of studies have demonstrated the uptake of antibiotics into plants; however, we do not yet understand the processes determining uptake. A systematic program of work is required to develop models for predicting uptake of antibiotics into plants as well as uptake into other terrestrial organisms. The development of an understanding of the potential for antibiotics to move through terrestrial food chains would also be valuable.
- Assessment of the nature and fate of antibiotic transformation products in soil systems.

REFERENCES

Accinelli C, Koskinen WC, Becker JM, Sadowsky MJ (2007). Environmental fate of two sulfonamide antimicrobial agents in soil. *J. Agric. Food Chem.* 55(7):2677–2682.

Andreu V, Vasques-Roig P, Blasco C, Pico Y (2009). Determination of tetracycline residues in soil by pressurized liquid extraction and liquid chromatography tandem mass spectrometry. *Anal Bioanal Chem* 394(5):1329–1339.

Aust MO, Godlinski F, Travis GR, Hao XY, McAllister TA, Leinweber P, Thiele-Bruhn S (2008). Distribution of sulfamethazine, chlortetracycline and tylosin in manure and soil of Canadian feedlots after subtherapeutic use in cattle. *Environ Pollut* 156(3):1243–1251.

Aust MO, Thiele-Bruhn S, Seeger J, Godlinski F, Meissner R, Leinweber P (2010). Sulfonamides leach from sandy loam soils under common agricultural practice. *Water Air Soil Pollut* 211(1–4):143–156.

Bayer (1997). Baytril 10% injection: Safety Datasheet 345354/01. Bayer, Newbury, UK.

Blackwell PA, Boxall ABA, Kay P, Noble, H (2005). Evaluation of a lower tier exposure assessment model for veterinary medicines. *J Agric Food Chem* 53:2192–2201.

Blackwell PA, Kay P, Boxall ABA (2007). The dissipation and transport of veterinary antibiotics in a sandy loam soil. *Chemosphere* 67(2):292–299.

Blackwell PA, Kay P, Ashauer R, Boxall ABA (2009). Effects of agricultural conditions on the leaching behaviour of veterinary antibiotics in soils. *Chemosphere* 75(1):13–19.

Boonsaner M, Hawker DW (2010). Accumulation of oxytetracycline and norfloxacin from saline soil by soybeans. *Sci Total Environ* 408(7):1731–1737.

Boxall ABA (2004a). The environmental side effects of medication. *EMBO Rep* 5(12):1110–1116.

Boxall ABA, Blackwell PA, Cavallo R, Kay P, Tolls J (2002). The sorption and transport of a sulphonamide antibiotic in soil systems. *Toxicol Lett* 131:19–28.

Boxall ABA, Kolpin DW, Halling-Sorensen B, Tolls J (2003). Are veterinary medicines causing environmental risks? *Environ Sci Technol* 37(15):2003, 286A.

Boxall ABA, Fogg LA, Kay P, Blackwell PA, Pemberton EJ, Croxford A (2004a). Veterinary medicines in the environment. *Rev Environ Contam Toxicol* 180:1–91.

Boxall ABA, Sinclair CJ, Fenner K, Kolpin DW, Maund S (2004b). When synthetic chemicals degrade in the environment. *Environ Sci Technol* 38(19):369A–375A.

Boxall A, Fogg L, Baird D, Telfer T, Lewis C, Gravell A, Boucard T (2006a). Targeted monitoring study for veterinary medicines. R&D technical report. Environment Agency, Bristol, United Kingdom.

Boxall ABA, Johnson P, Smith EJ, Sinclair CJ, Stutt E, Levy LS (2006b). Uptake of veterinary medicines from soils into plants. *J Agric Food Chem* 2006b; 54(6):2288–2297.

Burkhard M, Stamm S, Waul C, Singer H, Muller S (2005). Surface runoff and transport of sulfonamide antibiotics on manured grassland. *J Environ Qual* 34:1363–1371.

Cengiz M, Balcioglu IA, Otuc HH, Cengiz TG (2010). Evaluation of the interaction between soil and antibiotics. *J Environ Sci Health Part B* 45(3):183–189.

Davis JG, Truman CC, Kim SC (2006). Antibiotic transport via runoff and soil loss. *J Environ Qual* 35(6):2250–2260.

Dolliver H, Gupta S (2008). Antibiotic losses in leaching and surface runoff from manure-amended agricultural land. *J Environ Qual* 37(3):1227–1237.

Dolliver H, Kumar K, Gupta S (2007). Sulfamethazine uptake by plants from manure-amended soil. *J Environ Qual* 36(4):1224–1230.

Drillia P, Stamatelatou K, Lyberatos G. (2005) Fate and mobility of pharmaceuticals in solid matrices. *Chemosphere* 60(8):1034–1044.

Edwards M, Topp E, Metcalfe CD, Li H, Gottschall N, Bolton P, Curnoe W, Payne M, Beck A, Kleywegt S, Lapen DR (2009). Pharmaceutical and personal care products in tile drainage following surface spreading and injection of dewatered municipal biosolids to an agricultural field. *Sci Total Environ* 407(14):4220–4230.

Figueroa-Diva RA, Vasudevan D, MacKay AA (2010). Trends in soil sorption coefficients within common antimicrobial families. *Chemosphere* 79(8):786–793.

Forster M, Laabs V, Lamshoft M, Groenweg J, Zuhlke S, Spiteller M, Krauss M, Kaupenjohann M, Amelung W (2009). Sequestration of manure-applied sulfadiazine residues in soils. *Environ Sci Technol* 43(6):1824–1830.

Furtula V, Huang L, Chambers PA (2009). Determination of veterinary pharmaceuticals in poultry litter and soil by methanol extraction and liquid chromatography-tandem mass spectrometry. *J Environ Sci Health Part B* 44(7):717–723.

Gao JA, Pedersen JA (2005). Adsorption of sulfonamide antimicrobial agents to clay minerals. *Environ. Sci. Technol.* 39(24):9509–9516.

Gavalchin J, Katz SE (1994). The persistence of fecal-borne antibiotics in soil. *J AOAC Int* 77:481–485.

Halling-Sørensen B, Nors Nielsen S, Lanzky PF, Ingerslev F, Holten Lützhøft HC, Jorgensen SE (1998). Occurrence, fate and effects of pharmaceutical substances in the environment: A review. *Chemosphere* 36:357–393.

Halling-Sørensen B, Lykkeberg A, Ingerslev F, Blackwell P, Tjornelund J (2003). Characterisation of the abiotic degradation pathways of oxytetracyclines in soil interstitial water using LC-MS-MS. *Chemosphere* 50(10):1331–1342.

Hamscher G, Pawelzick HT, Hoper H, Nau H (2005). Different behaviour of tetracyclines and sulfonamides in sandy soils after repeated fertilization with liquid manure. *Environ Toxicol Chem* 24(4):861–868.

Heise J, Holtge S, Schrader S, Kreuzig R (2006). Chemical and biological characterization of non-extractable sulfonamide residues in soil. *Chemosphere* 65(11):2352–2357.

Hirsch R, Ternes T, Haberer K, Kratz KL (1999). Occurrence of antibiotics in the aquatic environment. *Sci. Tot. Environ.* 225(1–2):109–118

Hu DF, Coats JR (2009). Laboratory evaluation of mobility and sorption for the veterinary antibiotic, tylosin, in agricultural soils. *J Environ Monitor* 11(9):1634–1638.

Hu XG, Zhou QX, Luo Y (2010b). Occurrence and source analysis of typical veterinary antibiotics in manure, soil, vegetables and groundwater from organic vegetable bases, northern China. *Environ Pollut* 158(9):2992–2998.

Ingerslev F, Halling-Sørensen B (2001). Biodegradability of metronidazole, olaquindox and tylosin and formation of tylosin degradation products in aerobic soil/manure slurries. *Chemosphere* 48:311–320.

Jones AD, Bruland GL, Agrawal SG, Vasudevan D (2005). Factors influencing the sorption of oxytetracycline to soils. *Environ Toxicol Chem* 24(4):761–770.

Kahle M, Stamm C (2007). Sorption of the veterinary antimicrobial sulfathiazole to organic materials of different origin. *Environ Sci Technol* 41(1):132–138.

Kasteel R, Mboh CM, Unold M, Groeneweg J, Vanderborght J, Vereecken H (2010). Transformation and sorption of the veterinary antibiotic sulfadiazine in two soils: A short-term batch study. *Environ Sci Technol* 44(12):4651–4657.

Kay P, Blackwell PA, Boxall ABA (2004). Fate and transport of veterinary antibiotics in drained clay soils. *Environ Toxicol Chem* 23:1136–1144.

Kay P, Blackwell PA, Boxall ABA (2005a). A lysimeter experiment to investigate the leaching of veterinary antibiotics through a clay soil and comparison with field data. *Environ Pollut* 134:333–341.

Kay P, Blackwell PA, Boxall ABA (2005b). Column studies to investigate the fate of veterinary antibiotics in clay soils following slurry application to agricultural land. *Chemosphere* 60(4):497–507.

Kay P, Blackwell PA, Boxall ABA (2005a). Transport of veterinary antibiotics in overland flow following the application of slurry to arable land. *Chemosphere* 59(7):951.

Kinney CA, Furlong ET, Werner SL, Cahill JD (2006). Presence and distribution of wastewater-derived pharmaceuticals in soil irrigated with reclaimed water. *Environ Toxicol Chem* 25(2):317–326.

Kinney CA, Furlong ET, Kolpin DW, Burkardt MR, Zaugg SD, Werner SL, Bossio JP, Benotti MJ (2008). Bioaccumulation of pharmaceuticals and other anthropogenic waste indicators in earthworms from agricultural soil amended with biosolid or swine manure. *Environ Sci Technol* 42(6):1863–1870.

Kong WD, Zhu YG, Liang YC, Zhang J, Smith FA, Yang A (2007). Uptake of oxytetracycline and its phytotoxicity to alfalfa (Medicago sativa L.). *Environ Pollut* 147(1):187–193.

Kreuzig R, Holtge S (2005). Investigations on the fate of sulfadiazine in manured soil: Laboratory experiments and test plot studies. *Environ Toxicol Chem* 24(4):771–776.

Kreuzig R, Holtge S, Brunotte J, Berenzen N, Wogram J, Sculz R (2005). Test-plot studies on runoff of sulfonamides from manured soils after sprinkler irrigation. *Environ Toxicol Chem* 24(4):777–781.

Kuchta SL, Cessna AJ, Elliot JA, Peru KM, Headley JV (2009). Transport of lincomycin to surface and groundwater from manure-amended cropland. *J Environ Qual* 38 (4):1719–1727.

Kumar K, Gupta SC, Baidoo SK, Chander Y, Rosen CJ (2005). Antibiotic uptake by plants from soil fertilized with animal manure. *J Environ Qual* 34(6):2082–2085.

Larsbo M, Fenner K, Stoob K, Burhardt M, Abbaspour K, Stamm C (2008). Simulating sulfadimidine transport in surface runoff and soil at the microplot and field scale. *J Environ Qual* 37(3):788–797.

Lemus JÁ, Blanco G, Grande J, Arroyo B, García-Montijano M, Martinez F (2008). Antibiotics threaten wildlife: Circulating quinolone residues and disease in avian scavengers. *PLoS ONE* 2008; 3(1):e1444.

Lertpaitoonpan W, Ong SK, Moorman TB (2009). Effect of organic carbon and pH on soil sorption of sulfamethazine. *Chemosphere* 76(4):558–564.

Martínez-Carballo E, González-Barreiro C, Scharfa S, Gans O (2007). Environmental monitoring study of selected veterinary antibiotics in animal manure and soils in Austria. *Environ Pollut* 148(2):570–579.

McClellan K, Halden RU (2010). Pharmaceuticals and personal care products in archived US biosolids from the 2001 EPA national sewage sludge survey. *Water Res* 44(2):658–668.

Metcalfe C, Boxall A, Fenner K, Koplin D, Servos M, Silberhorn E, Staveley J (2008). Exposure assessment of veterinary medicines in aquatic systems. In Crane M, Boxall A, and Barrett K (Eds) Veterinary medicines in the environment. CRC Press, USA.

Migliore L, Cozzolino S, Fiori M (2003). Phytotoxicity and uptake of enrofloxacin in crop plants. *Chemosphere* 52:1233–1244.

Monteiro SC, Boxall ABA (2010). Occurrence and fate of human pharmaceuticals in the environment. *Rev Environ Contam Toxicol* 202:53–154.

Nowara A, Burhenne J, Spiteller M (1997). Binding of fluoroquinolone carboxylic acid derivatives to clay minerals. *J Agric Food Chem* 45:1459–1463.

Rabølle M, Spliid NH (2000). Sorption and mobility of metronidazole, olaquindox, oxytetracycline and tylosin in soil. *Chemosphere* 40:715–722.

Sanders SM, Srivastava P, Feng Y, Dane JH, Basile J, Barnett MO (2008). Sorption of the veterinary antimicrobials sulfadimethoxine and ormetoprim in soil. *J Environ Qual* 37(4):1510–1518.

Sarmah AK, Meyer MT, Boxall ABA (2006). A global perspective on the use, sales, exposure pathways, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere* 65(5):725–759.

Sassman SA, Lee LS (2005). Sorption of three tetracyclines by several soils: Assessing the role of pH and cation exchange. *Environ Sci Technol* 39:7452–7459.

Sassman SA, Lee LS (2007). Sorption and degradation in soils of veterinary ionophore antibiotics: Monensin and lasalocid. *Environ Toxicol Chem* 26(8):1614–1621.

Sassman SA, Sarmah AK, Lee LS (2007b). Sorption of tylosin A, D, and A-aldol and degradation of tylosin A in soils. *Environ. Toxicol. Chem.* 26(8):1629–1635.

Sarmah AK, Meyer MT, Boxall ABA (2006). A global perspective on the use, sales, exposure pathways, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere* 65(5):725–759.

Schlusener MP, Bester K (2006). Persistence of antibiotics such as macrolides, tiamulin and salinomycin in soil. *Environ Pollut* 143(3):565–571.

Stoob K, Singer HP, Mueller SR, Schwarzenbach RP, Stamm CH (2007). Dissipation and transport of veterinary sulphonamide antibiotics after manure application to grassland in a small catchment. *Environ Sci Technol* 41(21):7349–7355.

Strock TJ, Sassman SA, Lee LS (2005). Sorption and related properties of the swine antibiotic carbadox and associated *n*-oxide reduced metabolites. *Environ Sci Technol* 39:3134–3142.

Ter Laak TL, Gebbink WA, Tolls J (2006a). Estimation of sorption coefficients of veterinary medicines from soil properties. *Environ Toxicol Chem* 25(4):933–941.

Ter Laak TL, Gebbink WA, Tolls J (2006b). The effect of pH and ionic strength on the sorption of sulfachloropyridazine, tylosin and oxytetracycline to soil. *Environ Toxicol Chem* 25(4):904–911.

Ternes TA, Bonerz M, Herrmann N, Teiser B, Andersen HR (2007). Irrigation of treated wastewater in Braunschweig, Germany: An option to remove pharmaceuticals and musk fragrances. *Chemosphere* 66(5):894–904.

Thiele S (2000). Adsorption of the antibiotic pharmaceutical compound sulfapyridine by a long-term differently fertilized loess Chernozem. *J. Plant Nut. Soil Sci.* 163(6); 589–594.

Thiele-Bruhn S, Aust M (2004). Effects of pig slurry on the sorption of antibiotics in soil. *Arch Environ Contam Toxicol* 47:31–39.

Thiele-Bruhn S, Seibicke T, Schulten HR, Leinweber P. (2004). Sorption of sulfonamide pharmaceutical antibiotics on whole soils and particle-size fractions. *J. Environ. Qual.* 33(4):1331–1342.

Topp E, Monteiro SC, Beck A, Ball Coehlo B, Boxall ABA, Duenk PW, Kleywegt S, Lapen DR, Payne M, Sabourin L, Metcalfe CD (2008). Runoff of pharmaceuticals and personal care products following application of biosolids to an agricultural field. *Sci. Tot. Environ.* 396(1):52–59.

Unwold M, Kasteel R, Groenweg J, Vereecken H (2010). Transport of sulfadiazine in undisturbed soil columns: Effects of flow rate, input concentration and pulse duration. *J Environ Qual* 39(6):2147–2159.

Uslu MO, Yediler A, Balcioglu IA, Schulte-Hostede S (2008). Analysis and sorption behavior of fluoroquinolones in solid matrices. *Water Air Soil Pollut* 190(1–4):55–63.

Wang QQ, Yates SR (2008). Laboratory study of oxytetracycline degradation kinetics in animal manure and soil. *J Agric Food Chem* 56(5):1683–1688.

Wang QQ, Guo MX, Yates SR (2006). Degradation kinetics of manure-derived sulfadimethoxine in amended soil. *J Agric Food Chem* 54(1):157–163.

Watanabe N, Bergamaschi BA, Loftin KA, Meyer MT, Harter T (2010). Use and environmental occurrence of antibiotics in freestall dairy farms with manured forage fields. *Environ Sci Technol* 44(17):6591–6600.

Williams M, Ong PL, Williams DB, Kookana RS (2009). Estimating the sorption of pharmaceuticals based on their pharmacological distribution. *Environ. Toxicol. Chem.* 28(12):2572–2579.

Wolters A, Steffens M (2005). Photodegradation of antibiotics on soil surfaces: Laboratory studies on sulfadiazine in an ozone-controlled environment. *Environ Sci Technol* 39:6071–6078.

Wu CX, Sponberg AL, Witter JD, Fang M, Czajkowski KP, Ames A (2010). Dissipation and leaching potential of selected pharmaceutically active compounds in soils amended with biosolids. *Arch Environ Contam Toxicol* 59(3):343–351.

Yang JF, Yng CG, Zhou LJ, Liu S, Zhao JL (2009). Dissipation of oxytetracycline in soils under different redox conditions. *Environ Pollut* 157(10):2704–2709.

Yang JF, Ying GG, Yang LH, Zhao JL, Liu F, Tao R, Yu ZQ, Peng P. (2009a). Degradation behavior of sulfadiazine in soils under different conditions. *J. Environ. Sci. Hlth. B.* 44(3):241–248.

Yeager RL, Halley BA (1990). Sorption/desorption of [14C] efrotomycin with soils. *J. Agric. Food. Chem.* 38:886–890.

Zhao L, Dong YH, Wang H (2010). Residues of veterinary antibiotics in manures from feedlot livestock in eight provinces of China. *Sci Total Environ* 408(5):1069–1075.

18

ANTIBIOTICS IN THE AQUATIC ENVIRONMENT

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18.1 INTRODUCTION

This contribution gives a very short overview of the presence of antibiotics in the aquatic environment. It addresses the input, occurrence, fate, and effects of antibiotics in the environment and some open questions. As for details the reader is referred to other contributions in chapters of this book as well as in other publications (Sarmah et al., 2006; Kümmerer 2008, 2009). Although antibiotics have been used in large quantities for some decades, it is only in recent years that a more complex investigation of antibiotic substances has been undertaken in order to permit an assessment of the environmental risks they may pose. Within the last decade, an increasing number of studies covering antibiotic input, occurrence, fate and, effects have been published, but there is still a lack of understanding and knowledge about the fate and effects of antibiotics in the aquatic environment despite the numerous studies performed. In contrast to the properties and effects from their therapeutic application, these same properties are often disadvantageous for target *and* nontarget organisms in the environment. A review of pharmaceuticals including antibiotics for veterinary use and application within fattening of animals on a global scale was recently published by Sarmah et al. (2006). Therefore, this aspect is not fully covered in the following review. A detailed review of analytical methods for the determination of antibiotics in the aquatic environment has been published recently. Measurements conducted during the last decade showed that the concentrations of antibiotics in municipal sewage, hospital effluents, influent and effluent of sewage treatment plants (STPs), surface water, and groundwater, although decreasing in that

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order, are mostly in the same range. Therefore, as for the concentration of antibiotics in the aquatic environment, only some of the recent literature published on the topic is cited in the following sections.

Antibiotics can be grouped by chemical structure or by their mechanism of action. They are a diverse group of chemicals that can be divided into subgroups such as β-lactams, quinolones, tetracyclines, macrolides, sulfonamides, and others. They are often complex molecules that may possess different functionalities within the same molecule. Therefore, under different pH conditions these molecules can be neutral, cationic, anionic, or zwitterionic. Because of the different functionalities within one molecule, their physicochemical and biological properties may change with pH levels (Cunningham, 2008; Trivedi and Vasudevan, 2007). For ceftazidime, which forms an inner salt, different chemical species are expected to form depending on pH (Fig. 18.1). Ciprofloxacin, for example, possesses both basic and acidic functionalities. The acid constants are 6.16 and 8.63. At a pH of 7.04, the isoelectric point of ciprofloxacin, the molecule carries both a negative and a positive charge, that is, it is neutral as an entity despite these charges within the molecule. Solubility, hydrophobictiy, and hydrophilicity, and therefore log K_{ow} or log K_D, is dependent upon pH. Ciprofloxacin zwitterions appeared to interact via both carboxylate oxygen atoms to form bidentate chelate and bridging bidentate complexes within colloidal iron oxide–ciprofloxacin precipitates, and bidentate chelates on the goethite surface (Trivedi and Vasudevan, 2007). However, the structure of the

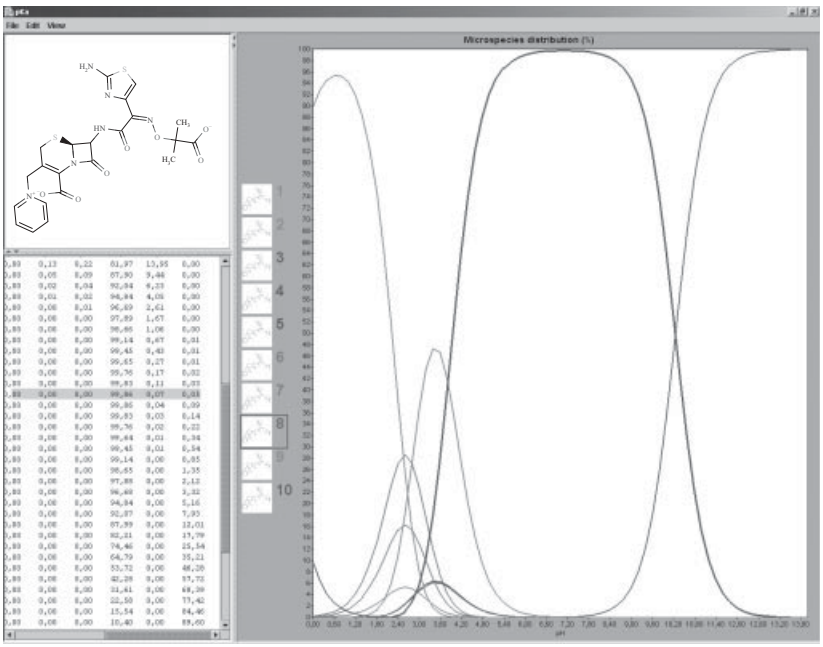

FIGURE 18.1 Internal salt and zwitterionic character of ceftazidime (top). Depending on pH additional different chemical species can be formed by internal protonation, that is, shift of protons between the basic amino functions and acidic carboxyl groups (bottom). (Calculator Plugins were used for structure property prediction and calculation, Marvin 4.1.1, 2006, ChemAxon (http://www.chemaxon.com) (Kümmerer, 2008). *(See color insert.)*

aqueous ferric–ciprofloxacin complexes remained unclear. Sorption showed a strong pH-dependent behavior when following the fraction of zwitterionic species over the entire pH range studied. Analysis indicated that different types of ciprofloxacin surface complexes are formed.

18.2 SOURCES OF ANTIBIOTICS IN THE ENVIRONMENT

18.2.1 Natural Background

The question of natural background concentrations of antibiotics is important for the risk assessment of antibiotics. Several antibiotics such as some β-lactams, streptomycins, aminoglycosides, and others are produced by soil bacteria. The antibiotic activity from local soil samples is variable and requires the examination of several samples to find a few that produce zones of inhibition. To the authors' best knowledge, there have been no findings of tetracyclines in soils that have not been fertilized with manure containing tetracyclines. This situation may be different in tropical soils as the bacteria producing tetracycline occur naturally in higher density in such soils. Up to now, there has been no report of the production of antibiotics in sediments or the aquatic environment.

18.2.2 Production and Manufacturing

Emissions from production plants have been thought of as being of minor importance. However, only recently it has been found that in some Asian countries concentrations of up to several milligrams/liter could be found in effluents for single compounds (Larsson et al., 2007; Li et al., 2008a,b) including antibiotics. In developed countries a manufacturing plant can also make a significant contribution to total pharmaceutical concentration in the influent of an STP, as has been shown only recently for the VEAS STP in Oslo (Thomas, 2008) and in the United States (Phillips et al., 2010).

18.2.3 Consumption—Some General Aspects

Antibiotics are used extensively in human and veterinary medicine, as well as in aquaculture, for the purpose of preventing (prophylaxis) or treating microbial infections. Several hundred different antibiotic and antimycotic substances are used in human and veterinary medicine, for example, more than 250 in Germany (Kümmerer and Henninger, 2003). Internationally, comparable data on antibiotic consumption is scarce, and whatever information is available is highly variable. Usage patterns may be different in different countries (Kümmerer, 2008, 2009). In the United States, for instance, the use of streptomycin in fruit growing is widespread, whereas its use for this purpose is banned in other countries such as Germany. Wise (2002) estimated antibiotic consumption worldwide to range between 100,000 and 200,000 t (metric ton) per annum. In 1996, about 10,200 t of antibiotics were used in the European Union (EU), of which approximately 50% was applied in veterinary medicine and as growth promoters. According to data supplied by the European Federation of Animal Health (FEDESA, 2001), in 1999 there were a total of 13,216 t of

antibiotics used in the European Union and Switzerland, 65% of which was applied in human medicine. A recent report estimated that U.S. livestock producers use approximately 11,200 t of antimicrobials for nontherapeutic purposes, primarily to promote the growth of cattle, hogs, and poultry. Clinical uses are estimated at about 10% of total antimicrobial use (Union of Concerned Scientists, 2001).

18.2.4 Use in Human Medicine

Consumption for humans in total, per capita and the individual share of each compound, varies from country to country. Antibiotic prescription rates and intake without prescription vary markedly between countries. Varying levels of use of single compounds is quite common. Vancomycin, for example, is heavily used in the United States, whereas in Germany it is only used in cases in which all other possible compounds that have proven to be ineffective due to resistance. Data on the country-specific use for groups of antibiotics in different countries are available from different sources but mostly as DDD [defined daily dose according to the World Health Organization (WHO)]. Country-specific consumption for groups of antibiotics in human medicine in DDDs can be found for Europe on the European Surveillance homepage of the European Surveillance of Antimicrobial Consumption (ESAC) (http://www.esac.ua.ac.be/main.aspx?c=*ESAC2&n=1066l). Antibiotic use (expressed as DDD per day and capita) ranges from 8.6 to 36 in Europe. β-lactam antibiotics, including the subgroups of penicillins, cephalosporins, and, as a marginal fraction, carbapenems and others, make up the largest share of human-use antibiotics in most countries. They account for approximately 50–70% of total antibiotic use. In most countries, sulfonamides, macrolides, and fluoroquinolones follow in decreasing order of use (http://www.esac.ua.ac.be/main.aspx?c=*ESAC2&n=1063). Sales of antibiotics vary strongly between countries. Regional and local consumption also may be different within a single country: For the European Union, in total 22 g per capita and year would be due to medical use. For the United States it is estimated to be approximately 17 g per capita and year, when calculated from the available data for use in human medicine (Kümmerer, 2004). However, these data include some uncertainty. In Germany nationwide average use of antibiotics in 1998 was 4.95 g per capita and year, whereas it was 2.9 g per capita and year in a small town (9000 inhabitants, no hospital present but two elderly peoples' homes). If the share of the average use in hospitals in Germany is added, the result would be 3.85 g per capita and year. These data demonstrate that there should be a potential for a reduction in antibiotic use without negative health consequences. If antibiotics are sold over the counter (OTC), that is, without any prescription, consumption could be still higher. Excretion rates for the unchanged active compound cover a broad range (10–90%, ceftazidime, for example, less than 10%). On average, if the volume for all antibiotics used is totaled the metabolic rate is estimated to be 30% (Kümmerer and Henninger, 2003), that is, 70% of the used active pharmaceutical ingredients (APIs) is excreted unchanged into wastewater. The data presented above show that, at least at the level of specific compounds, general data on the consumption of antibiotics may be misleading. Evaluation on a case-by-case basis may be necessary to assess substance flows of antibiotics.

In contrast to general expectation, hospitals are not the main source of pharmaceuticals in municipal sewage (Kümmerer, 2008; Schuster et al., 2008). De Wirth

et al., (2004) found that on the basis of DDDs, the use of antibiotics in hospitals accounts for 5–20% of the total antibiotic use in European hospitals. In total, 412 t of antibiotics were used in Germany in 1998. Taking the compound-specific metabolization rates into account, 305 t are emitted into wastewater, of which 92 t were due to hospitals (Kümmerer and Henninger, 2003). The data demonstrate that there are no point sources, but foremost there is diffuse input by the general public through STPs (Zuccato et al., 2010).

18.3 PRESENCE IN THE AQUATIC ENVIRONMENT

Antibiotics can be more or less extensively metabolized by humans and animals. After administration, antibiotics for human use or their metabolites are excreted into the effluent and reach the STP. The nonmetabolized fraction is excreted as a still active compound. Antibiotics are only partially eliminated in sewage treatment plants. If they are not eliminated during the purification process, they pass through the sewage system and may end up in the environment, mainly in the water compartment. Residual amounts can reach surface waters, groundwater, or sediments. Active substances discharged with liquid manure that is applied as fertilizer in agriculture can be washed off from the topsoil after rain. Furthermore, direct discharge, especially from poultry processing, meat processing, and aquaculture, as well as from pets (e.g., aquariums) is also possible and can contribute toward an increase in the total concentration of antibiotics in sewage and surface water. It has been found that the concentrations of antibiotics measured in different countries are in the same range of concentrations in the different compartments such as sewage and surface water, respectively. In general, concentrations were in the higher microgram-per-liter range in hospital effluent, in the lower microgram-per-liter range in municipal wastewater, and in the higher and lower nanogram-per-liter range in different surface waters, groundwater, and seawater.

The occurrence of β-lactams (including penicillins, cephalosporins, carbapenems, monobactams, and β-lactamase inhibitors) has not been covered frequently, despite the fact that β-lactams account for by far the highest proportion of consumption. It is not clear whether they are not present in the aquatic environment because of the possible cleavage of the β-lactam-ring, whether this finding is due to the fact that they have not been analyzed, or whether it is due to possible analytical shortcomings and difficulties. Antibiotics have also rarely been found in drinking water (Ye et al., 2007).

Human and veterinary antibiotics are present in sediments. Kim and Carlson (2007) detected tetracyclines, sulfonamides, and macrolides. The sediment concentration measured in an agriculture-influenced river was much higher than in the overlying water matrix, indicating the input of antibiotics with surface run-off from agricultural fields. In intensive fish farming, infections are treated by feeding antimicrobial agents directly into the water. The substances used in fish farming can enter the sediments directly from the water without undergoing any kind of purification process. This results in high local concentrations in the water compartment and in the adjoining sediments. This phenomenon had already been investigated more than 20 years ago, where results had demonstrated the presence of antibiotics applied extensively in fish farming in sediments beneath fish farms (Samuelson, 1989).

18.4 FATE IN THE AQUATIC ENVIRONMENT

Elimination only means that the (parent) compound of interest is not detectable anymore by compound-specific analysis in the compartment or phase of sampling. It has been removed from the compartment of interest, for example, the water phase. Therefore, removal is an adequate expression for this situation too. The elimination of only the parent compound is also called primary elimination. Primary elimination is normally reported if specific analytical methods such as liquid chromatography–mass spectrometry (LC–MS) are applied in fate studies. Sum parameters such as dissolved organic carbon (DOC) loss give a measure of the degree of total elimination. If the compound is fully converted into inorganic salts, full mineralization took place. Only the measurement of carbon dioxide production can give a measure of the degree of mineralization that results in the complete breakdown of a molecule, its metabolites, and transformation products into carbon dioxide, water, and inorganic salts such as sulfate, phosphate, ammonium, and nitrate. Since antimicrobials in the environment may have a severe impact on aquatic and terrestrial ecosystems, knowledge about their elimination is of predominant importance. Elimination of organic compounds in the environment is the result of different processes. These processes can be biotic ones, that is, bio-degradation by bacteria and fungi. Nonbiotic elimination processes are sorption, hydrolysis, photolysis, oxidation, and reduction. It has to be noted that the results of bio or photodegradation studies depend on conditions such as temperature, composition of matrix, latitude, and the like.

Binding to particles or the formation of complexes may interfere with detection, as well as a loss in antibacterial activity. The loss of antibacterial activity, for example, was demonstrated for an aquaculture antimicrobial in seawater driven by the formation of complexes with the magnesium and calcium naturally present in marine water. Tetracyclines are able to form complexes with double cations, such as calcium or magnesium (Christian et al., 2003). Humic substances may alter the surface properties and sites available for sorption and reactions. They can either suppress or promote sorption of organic compounds to mineral surfaces. Limited evidence in the literature points to the reduction in sorption of the antibiotic tetracycline to clay minerals in the presence of humic substances.

Antibiotics applied in human medicine can reach the terrestrial environment through sewage sludge (Golet et al., 2002). Fluoroquinolones (FQs) become highly enriched in sewage sludge (concentrations ranging from 1.4 to 2.42 mg/kg of dry matter). These results indicate the importance of sludge management strategies to prevent whether human-excreted antibiotics enter the environment.

Some antibiotics are light sensitive (e.g. quinolones, tetracyclines, sulfonamides, tylosin, and nitofuran antibiotics). However, not all compounds are photodegradable (Turiel et al., 2005). If a substance is light sensitive, photodecomposition may be of major significance in the elimination process. In general, data on the sensitivity of antibiotics against light, moisture, and temperature can be found in the medical and pharmaceutical literature. Data from the drug registration procedure may provide guidance on compounds where photodecomposition can be expected to play a role. Photodecomposition takes place mainly in clear surface water. Photochemical decomposition can play an important role in surface water as an additional elimination pathway or for effluent treatment (Lorenzo et al., 2008; Lamshöft

et al., 2008; Vasconcelos et al., 2009). The effectiveness of the process depends on light intensity and frequency. Photodecomposition may not occur when the compounds are present in turbid water, if the creek, river, or lake is shadowed by trees, or if the compounds are in soil, sewage, and sewage pipes since they have low light exposure. Frequency relates to the absorption spectrum of a compound, and the absorption spectrum may be affected by sorption and complexation. Therefore, the effectiveness of phototransformation in the environment cannot always be derived in a straightforward way from results obtained in laboratory tests. It can also vary with the season and the latitude. The effectiveness of depletion processes differs under environmental conditions such as pH or water hardness and depends on the type of matrix, location, season, and latitude. It should be noted that incomplete phototransformation and photodegradation can lead to more or less stable or more or less toxic compounds, although this does not necessarily have to happen.

The significance and extent of direct and indirect photolysis of antibiotics in the aquatic environment are different for each compound. Studies taking into account indirect photolysis and interaction with dissolved organic matter (DOM) such as humic and fulvic acids are rare.

Tetracyclines are susceptible to photodegradation. For example, Samuelsen (1989) investigated the sensitivity of oxytetracycline toward light in seawater as well as in sediments. The antibacterial substance proved to be stable in sediments rather than in seawater. No mechanism of decomposition other than photodegradation is known for this antimicrobial. Another important pathway for the nonbiotic elimination of organic substances in the environment is hydrolysis. Some instability in water could be demonstrated for some tetracyclines (Halling-Sørensen, 2000). In general, the hydrolysis rates for oxytetracycline increase as the pH deviates from pH 7 and as temperature increases. The half-lives of oxytetracycline under investigation varied due to differences in temperature, light intensity, and flow rate from one test tank to another. However, sulfonamides and quinolones are resistant to hydrolysis. In laboratory biodegradability testing with sewage sludge, it has been found that β-lactams are rapidly hydrolyzed. This led to the deactivation of antibiotic activity. A subsequent step was decarboxylation. Even if the compounds are structurally closely related, the degree of hydrolysis and decarboxylation, the share of microbial activity in these processes, and their kinetics all differ (Längin et al., 2009). This means that many of the most frequently applied penicillins probably cannot be detected in the environment at the expected concentration level. The β-lactam ring of β-lactam antibiotics, for example, penicillins, can be opened by β-lactamase, an enzyme present in bacteria. Li et al. (2008b) reported thermal decomposition of penicillin G as treatment of the effluent of a production plant.

Effluents from manufacturing of antibiotic drugs are well known for the difficulty of their elimination by traditional biotreatment methods, and their important contribution to environmental pollution is due to their fluctuating and recalcitrant nature. For advanced effluent treatment, oxidation processes are usually applied. However, ozonation will not work well for all types of molecules. The presence of carbon–carbon double bonds, aromatic bonds, or nitrogen is a necessary prerequisite. However, the presence of these structural elements does not guarantee the fast and full degradation or even the mineralization of a molecule.

The effect of ozonation on the degradation of oxytetracycline (OTC) in aqueous solution at different pH values (3, 7, and 11) was investigated by Li et al. (2008c).

The results demonstrate that ozonation as a partial step in a combined treatment concept is a potential technique for biodegradability enhancement for effluents from pharmaceutical industries containing high concentrations of oxytetracycline, provided that the appropriate ozonation period is selected. It has been shown that chemical oxygen demand (COD) removal rates increase with increasing pH as a consequence of enhanced ozone decomposition rates at elevated pH values. The results of bioluminescence data indicate that the initial by-products after partial ozonation (5–30 min) of OTC were more toxic than the parent compound (Li et al., 2008c).

Most antibiotics tested to date have not been biodegradable under aerobic conditions (Al-Ahmad et al., 1999, Ingerslev et al., 2001, Ingerslev and Halling-Sørensen, 2001; Thiele-Bruhn, 2003; Alexy et al., 2004; Gartiser et al., 2007a; Li et al., 2008; Kallenborn et al., 2008). Biodegradability has been poor for most of the compounds investigated in laboratory tests such as the test series (301–303, 308) of the Organization of Economic Cooperation and Development (OECD)–even for some of the β-lactams (Alexy et al., 2004). Out of 16 antibiotics tested, only benzyl penicillin (penicillin G) was completely mineralized in a combination test (combination of the OECD 302 B and OECD 301 B tests (Gartiser at al., 2007a). Trials simulating sewage treatment (OECD 303 A) with radio-labeled compounds revealed that approximately 25% of benzyl penicillin was mineralized within 21 days, whereas ceftriaxone and trimethoprim were not mineralized at all (Junker et al., 2006).

Some antibiotics occurring in soil and sediment proved to be quite persistent in laboratory testing as well as in field studies (Kim et al. 2005). Some do not biodegrade well under anaerobic conditions (Gartiser et al., 2007b). Substances extensively applied in fish farming had long half-lives in soil and sediment, as reported in several investigations (Marengo et al., 1997; Capone et al., 1999; Lai et al., 2008). However, some substances were at least partly degradable (Capone et al., 1999; Thiele-Bruhn, 2003).

18.5 EFFECTS ON BACTERIA IN THE AQUATIC ENVIRONMENT

Nitrification is an important step in wastewater purification, eliminating toxic ammonia. The second step of nitrification, that is, oxidation of nitrite to nitrate, is particularly sensitive. Inhibition of this step under uncontrolled conditions may lead to accumulation of nitrite nitrogen in the plant effluent, a form of nitrogen that is particularly toxic. Several antibiotics proved to have low toxicity in relation to nitrifying bacteria in acute tests. These substances showed no effects upon nitrification in concentrations even higher than what might be environmentally expected (Tomlinson et al., 1966; Gomez et al., 1996). An antimicrobial was found to require high concentrations in order to inhibit the nitrification process in a short-term test (2–4 hours), but a prolonged test period over 5 days showed effects one order of magnitude below the inhibitory concentrations of the acute test (Tomlinson et al., 1966). In a study by Dokianakis et al. (2004) the effects caused by the presence of seven different pharmaceuticals on a culture of nitrite-oxidizing bacteria isolated from activated sludge were reported. For ofloxacin and sulfamathoxazole significant inhibition was observed. In the same study, triclosan presented a substantial inhibitory effect on the substrate (nitrite) reduction rate. Lincomycin showed significant inhibition on nitrification activity in a sequence batch reactor (SBR) test, which is consistent with its antibiotic activity spectrum. Substances that are not or are only

partly eliminated in the sewage treatment plant will reach surface water where they may affect organisms of different trophic levels. In a model aquatic system using synthetic freshwater, nitrifying bacteria were significantly affected by an aquaculture antibiotic. The disruption of the nitrification process already occurred in concentrations likely to be found in fish treatment tanks and sediments (Klaver and Matthews, 1994).

Christensen and co-workers found synergistic mixture effects of antibiotics against sewage sludge bacteria (Christensen et al., 2006). Methanogenes are the most sensitive group of microorganisms participating in the anaerobic digestion process. Tests showed that the pharmaceuticals tested (among them sulfamathoxazole) caused mild inhibition of the methanogenes in most cases, which was in turn directly related to the tendency of the compounds to adsorb on the anaerobic biomass (Fountoulakis et al., 2004). The inhibition of anaerobic bacteria by antibiotics observed in the degradation tests was higher than expected from the results of the inhibition tests. Possible explanations for this are that distinct substrates were used (yeast extract versus sodium benzoate), that the digestion sludge loses activity during the washing steps performed for the degradation tests, and that the exposure time in the degradation tests was eight times longer than in the inhibition test.

The results of toxicity tests with bacteria indicate that adverse toxic effects on natural bacterial communities cannot be excluded. The unwanted effects of microbial growth have long been controlled through the use of antimicrobials such as antibiotics. A vast amount of literature is available on the emergence of resistance and the use of antimicrobials in medicine, veterinary medicine, animal husbandry, and aquaculture. As for an overview on effects on higher aquatic organisms, see elsewhere (Kümmerer, 2009). In general, the emergence of resistance is a highly complex process that is not yet fully understood with respect to the significance of the interaction of bacterial populations and antibiotics.

REFERENCES

Al-Ahmad A, Daschner FD, Kümmerer K (1999). Biodegradability of cefotiam, ciprofloxacin, meropenem, penicillin G and sulfamathoxazole and inhibition of waste water bacteria. *Arch Environ Contam Toxicol* 37:158–163.

Alexy R, Kümpel T, Kümmerer K (2004). Assessment of degradation of 18 antibiotics in the Closed Bottle Test. *Chemosphere* 57:505–512.

Capone DG, Weston DP, Miller V, Shoemaker C (1999). Antibacterial residues in marine sediments and invertebrates following chemotherapy in aquaculture. *Aquaculture* 145:55–75.

Christensen AM, Ingerslev F, Braun A (2006). Ecotoxicity of mixtures of antibiotics used in aquaculture. *Environ Toxicol Chem* 25:2208–2215.

Christian T, Schneider RJ, Färber HA, Skutlarek D, Meyer MT, Goldbach HE (2003). Determination of antibiotic residues in manure, soil, and surface Waters. *Acta Hydrochim Hydrobiol* 31:36–44.

Cunningham V (2008). Special characteristics of pharmaceuticals related to environmental fate. In K Kümmerer (Ed.), *Pharmaceuticals in the Environment. Sources, Fate, Effects and Risk*. Springer, Berlin, Heidelberg, and New York.

De Wirth K, Schröder H, Meyer E, Nink K, Hofman S, Steib-Bauert M, Kämmerer R, Rueß S, Daschner FD, Kern WV (2004). Antibiotic use in Germany and Europe. *Deutsche Medizinischen Wochenschrift* 129:1987–1992.

Dokianakis SN, Kornaros ME, Lyberatos G (2004). On the effect of pharmaceuticals on bacterial nitrite oxidation. *Water Sci Technol* 50:341–346.

European Federation of Animal Health (FEDESA) (2001). Antibiotic use in farm animals does not threaten human health. FEDESA/FEFANA press release. Brussels, July 13, 2001.

Fountoulakis M, Drillia P, Stamatelatou K, Lyberatos G (2004). Toxic effect of pharmaceuticals on methanogenesis. *Water Sci Technol* 50:335–440.

Gartiser S, Urich E, Alexy R, Kümmerer K (2007a). Ultimate biodegradation and elimination of antibiotics in inherent tests. *Chemosphere* 67:604–613.

Gartiser S, Urich E, Alexy R, Kümmerer K (2007b). Anaerobic inhibition and biodegradation of antibiotics in ISO test schemes. *Chemosphere* 66: 1839–1848.

Golet EM, Strehler A, Alder AC, Giger W (2002). Determination of fluoroquinolone antibacterial agents in sewage sludge and sludge-treated soil using accelerated solvent extraction followed by solid-phase extraction. *Analy Chem* 74:5455–5462.

Gomez J, Mendez R, Lema JM (1996). The effect of antibiotics on nitrification processes. *Appl Biochem Biotechnol* 57/58:869–876.

Halling-Sørensen B (2000). Algal toxicity of antibacterial agents used in intensive farming. *Chemosphere* 40:731–739.

Ingerslev F, Halling-Sørensen B (2001). Biodegradability of metronidazole, olaqiondox, and tylosin, and formation of tylosin degradation products in aerobic soil/manure slurries. *Ecotoxicol Environ Saf* 48:311–320.

Ingerslev F, Torång, Loke ML, Halling-Sørensen B, Nyholm N (2001). Primary biodegradation of veterinary antibiotics in aerobic and anaerobic surface water simulation systems. *Chemosphere* 44:865–872.

Junker T, Alexy R, Knacker T, Kümmerer K (2006). Biodegradability of 14C-labelled antibiotics in a modified laboratory scale sewage treatment plant at environmentally relevant concentrations. *Environ Sci Technol* 40:318–326.

Kallenborn R, Fick J, Lindberg R, Moe M, Nielsen KM, Tysklind M, Vasskog T (2008). *Pharmaceutical residues in Northern European Environments: Consequences and perspectives*. In: K Kümmerer (Ed.), *Pharmaceuticals in the Environment. Sources, Fate Effects and Risk*. Springer, Berlin, Heidelberg, and New York.

Kim SC, Carlson K (2007). Temporal and spatial trends in the occurrence of human and veterinary antibiotics in aqueous and river sediment matrices. *Environ Sci Technol* 41:50–57.

Kim S, Eichhorn P, Jensen JN, Weber AS, Aga DS (2005). Removal of antibiotics in wastewater: Effect of hydraulic and solid retention times on the fate of tetracycline in the activated sludge process. *Environ Sci Technol* 39:5816–5823.

Klaver AL, Matthews RA (1994). Effects of oxytetracycline on nitrification in a model aquatic system. *Aquaculture* 123:237–247.

Kümmerer K (2004). Resistance in the environment. *Antimicrob Chemother* 54:311–320.

Kümmerer K (Ed.) (2008). *Pharmaceuticals in the Environment. Sources, Fate, Effects and Risk*. Springer, Berlin, Heidelberg, and New York.

Kümmerer K (2009). Antibiotics in the aquatic environment—A review—Part I. *Chemosphere* 75:417–434.

Kümmerer K, Henninger A (2003). Promoting resistance by the emission of antibiotics from hospitals and households into effluents. *Clin Microb Infect* 9:1203–1214.

Lai HT, Chien, Lin JS (2008). Long-term transformation of oxolinic acid in water from an eel pond. *Aquaculture* 275:96–101.

Lamshöft M, Zühlke S, Spiteller M (2008). Photolysis of (14)C-sulfadiazine in water and manure. *Chemosphere* 71:717–725.

Längin A, Alexy R, König A, Kümmerer K (2009). Deactivation and transformation products in biodegradability testing of β-lactams amoxicillin and piperacillin. *Chemosphere* 75:347–354.

Larsson DG, De Pedro C, Paxeus N (2007). Effluent from drug manufactures contains extremely high levels of pharmaceuticals. *J Hazard Mater* 148:751–755.

Li D, Yang M, Hu J, Ren L, Zhang Y, Chang H, Li K (2008a). Determination and fate of oxytetracycline and related compounds in oxyteracycline production wastewater and the receiving river. *Environ Toxicology Chem* 27:80–86.

Li D, Yang M, Hu J, Ren L, Zhang Y, Chang H, Jin F (2008b). Determination of penicillin G and its degradation products in a penicillin production wastewater treatment plant and the receiving river. *Water Res* 42:307–317.

Li K, Yediler A, Yang M, Schulte-Hostede S, Wong MH (2008c). Ozonation of oxytetracycline and toxicological assessment of its oxidation by-products. *Chemosphere* 72:473–478.

Lorenzo F, Navaratnam S, Edge R, Allen NS (2008). Primary photophysical properties of moxifloxacin—A fuoroquinolone antibiotic. *Photochem Photobiol* 84:1118–1125.

Marengo JR, O' Brian RA, Velagaleti RR, Stamm JM (1997). Aerobic biodegradation of (14C)-sarafloxacin hydrochloride in soil. *Environ Toxicol Chem* 16:462–471.

Phillips PJ, Smith SG, Kolpin DW, Zaugg SD, Buxton , HT Furlong ET, Esposito K, Stinson B (2010). Pharmaceutical formulation facilities as sources of opioids and other pharmaceuticals to wastewater treatment plant effluent. *Environ Sci Technol* 44:4910–4916.

Samuelsen OB (1989). Degradation of oxytetracycline in seawater at two different temperatures and light intensities, and the persistence of oxytetracycline in the sediment from a fish farm. *Aquaculture* 83:7–16.

Sarmah AK, Meyer MT, Boxall ABA (2006). A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs). *Chemosphere* 65:725–759.

Schuster A, Hädrich C, Kümmerer K (2008). Flows of active pharmaceutical ingredients originating from health care practices on a local, regional, and nationwide level in Germany—Is hospital effluent treatment an effective approach for risk reduction? *Water Air Soil Pollut Focus* 8:457–471.

Thiele-Bruhn S (2003). Pharmaceutical antibiotic compounds in soils—A review. *J Plant Nutri Soil Sci* 166:145–167.

Thomas KV (2008). The relevance of different point sources. Lecture given at the First International Conference on Sustainable Pharmacy, Osnabrück, April 24–25, 2008. Available: http://www.dbu.de/550artikel27307_135.html.

Tomlinson TG, Boon AG, Trotman CAN (1966). Inhibition of nitrification in the activated sludge process of sewage disposal. *J Appl Bacteriol* 29:266–291.

Trivedi P, Vasudevan D (2007). Spectroscopic investigation of ciprofloxacin speciation at the goethite–water interface. *Environ Sci Technol* 41:3153–3158.

Turiel E, Bordin G, Rodríguez AR (2005). Study of the evolution and degradation products of ciprofloxacin and oxolinic acid in river water samples by HPLC-UV/MS/MS-MS. *J Environ Monitor* 7:189–195.

Union of Concerned Scientists (2001). 70 Percent of all antibiotics given to healthy livestock. Press release, Cambridge, January 8.

Vasconcelos TG, Henriques DM, König A, Kümmerer K, Martins AF (2009). Photodegradation of the antimicrobial ciprofloxacin: Identification and biodegradability assessment of the primary metabolites. *Chemosphere* 76:487–493.

Wise R (2002). Antimicrobial resistance: Priorities for action. *J Antimicrob Chemother* 49:585–586.

Ye Z, Weinberg HS, Meyer MT (2007). Trace analysis of trimethoprim and sulfonamide, macrolide, quinolone, and tetracycline antibiotics in chlorinated drinking water using liquid chromatography electrospray tandem mass spectrometry. *Anal Chem* 79:1135–1144.

Zuccato E, Castiglioni S, Bagnati R, Melis M, Fanelli R (2010). Source, occurrence and fate of antibiotics in the Italian aquatic environment. *J Hazard Mater* 179:1042–1048.

19

RESIDUES OF VETERINARY DRUGS IN WILD FISH

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19.1 INTRODUCTION

Whenever pharmaceutical compounds are found in fish or fishery products for human consumption, such residues are usually linked to legal or illegal preharvest treatment in aquaculture that is steadily growing. During the last 50 years, worldwide production of food fish from aquaculture (including finfishes, crustaceans, molluscs and other aquatic animals for human consumption) has increased from less than a million tons in the early 1950s to 52.5 million tons in 2008 (FAO, 2011). Meanwhile, aquaculture contributes about half of the fish consumed by the human population worldwide (FAO, 2011). Indeed, veterinary pharmaceuticals are frequently applied in aquaculture, resulting in residues detected in food for human consumption. Therefore, maximum residue levels (MRLs) have been set for those pharmaceuticals authorized for application with specific fish species or other fishery products such as shrimp. The use of nonauthorized pharmaceuticals is illegal, but nevertheless such residues are frequently found in monitoring investigations of food samples. In the European Union (EU), zero tolerance applies to residues of nonauthorized veterinary drugs found in fish or fish products. But can wild fish not intentionally treated with pharmaceuticals also contain such residues? And if yes, where do they come from?

19.2 LEGAL BACKGROUND

In the European Union, residues of veterinary drugs in food are only accepted if the respective compound is registered and listed in Table 1 "allowed substances" of

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the Annex of EU Commission Regulation 37/2010 on pharmacologically active substances and their classification regarding MRLs in foodstuffs of animal origin. Table 2 of the Annex compiles all prohibited substances such as nitrofuranes or chloramphenicol. In general, zero tolerance applies to all substances listed in Table 2 and to those not listed in this Annex. Commission Decision 2002/657/EC allows the establishment of minimum required performance limits (MRPLs), which may be set as "action limits" for internationally traded food consignments (2005/34/EC). Food consignments containing a pharmacologically active substance that exceeds the corresponding MRPL value are to be intercepted at the time of import. The food consignments need to be destroyed or sent back to their countries of origin. A "Gentlemen's Agreement" (Statement to the Minutes of the Standing Veterinary Committee Meeting of 21 September 2004) enabled the EU member states to apply the approach of Decision 2005/34/EC also to intra-EU residue control and trade. The Gentlemen's Agreement has, however, no legal foundation in EU law. In general, MRPLs do not apply to nationally traded food consignments. What applies to all MRPLs is that technical feasibility alone and not the health risk is and was the yardstick for their establishment and that they have never, as a rule, undergone a risk assessment.

According to Article 18 of new EU Regulation 470/2009 so-called reference points for action (RPAs) can be established for residues from pharmacologically active substances, which are no MRL, no provisional MRL, or "nonharmful" substances that do not require the establishment of an MRL. RPAs may be established when "it is deemed necessary in order to ensure the functioning of controls of food of animal origin imported or placed on the market in accordance with Regulation (EC) No 882/2004." The RPA should represent the lowest residue concentration that can be quantified with an analytical method validated in accordance with EU requirements (Article 19). In contrast to the MRPL values, RPAs shall be reviewed regularly in the light of new scientific data relating to food safety. According to Article 19 (3) "the risk assessment shall be based on methodological principles as well as scientific methods to be adopted by the Commission in consultation with EFSA," the European Food Safety Agency. Until today, RPAs have not been established and the conversion of MRPLs into RPAs is intended but has not yet conducted.

19.3 RAPID ALERT SYSTEM FOR FOOD AND FEED

In 1979, the EFSA established the Rapid Alert System for Food and Feed (RASFF) on the legal basis of EU Regulation EC/178/2002. It was intended "to provide the control authorities with an effective tool for exchange of information on measures taken to ensure food safety" (http://ec.europa.eu/food/food/rapidalert/index_en.htm). In the years 2009 and 2010, a total of 122 notifications were reported by the RASFF for residues of veterinary medicinal products in fish and fishery products. All of these notifications imply a violation of European food law by detecting prohibited or unauthorized substances and/or exceeding MRL or MRPL values. Of these notifications, 114 were reported for fish and fishery products from southeast Asia.

Most of the notifications (99 out of 122) concerned findings of nitrofurans primarily found as their metabolites and mainly found in crustaceans and products thereof (98 out of 99). Other notifications were reported for residues of malachite green and its

metabolite leucomalachite green (9), chloramphenicol (7), Victoria pure blue BO (1), trimethoprim (2), neomycin (2), enrofloxacin (1), and oxytetracycline (1).

19.4 UNINTENTIONAL SOURCES FOR PHARMACEUTICAL RESIDUES IN FISH OR FISHERY PRODUCTS

In 2001, the detection of chloramphenicol in giant prawns triggered a discussion about how to deal with foods containing residues of veterinary drugs whose use is prohibited in food-producing animals in the European Union. Chloramphenicol is prohibited as it may cause adverse human health effects, and a threshold value could not be derived because of its potential for the induction of aplastic anemia and due to positive results in genotoxicity testing. However, the destruction of tons of shrimps containing only trace levels of chloramphenicol sparked great controversy, including a discussion about a potential background contamination from other even natural sources that have, however, never been proven.

A few years later, the metabolite semicarbazide (SEM), classified as a weak, nongenotoxic carcinogen, gained some attention. SEM is a degradation product of the veterinary drugs nitrofural (nitrofurazone) and nitrofurantoin. The detection of SEM is used in food control as an indicator for illegal treatment. But, recently, it has been observed that SEM residues may also come from other sources, and even the natural occurrence in marine foods has been described (Gatermann et al., 2004; Hoenicke et al., 2004; Hoenicke and Gatermann, 2006; Saari and Peltonen, 2004).

19.4.1 Residues of Antibiotic and Antiparasitic Residues in Wild Fish Caught Close to Aquacultural Farming Areas

Intensive rearing of fish and fishery products aquaculture often necessitates the application of pharmaceuticals to promote animal health even under the stressful conditions caused by high stocking rates. In aquaculture, pharmaceuticals are usually not individually administered to each organism (e.g., dip application is often not practicable). Thus, high quantities of pharmaceuticals need to be applied due to the effects of dilution and of drift via current of the active ingredients used in sea farming. Fortt et al. (2007) reported the detection of residues of tetracyclines and quinolones in wild fish living around a salmon aquaculture area in Cochamo, Region X, Chile. Residues were found in fish meat of two species of wild fish [robalo (*Scorpaena hystrio*) and cabrilla (*Elginops maclovinus*)] intended for human consumption. The authors stated that their findings suggest "that the antibiotic usage in salmon aquaculture in Chile has environmental implications that may affect human and animal health."

19.4.2 Residues of Antidepressant Drugs in Wild Fish Caught Downstream from Municipal Sewers

Residues of pharmaceuticals administered in human medical care are frequently detected in the aquatic environment (Daughton and Ternes, 1999; Heberer 2002, 2010; Kolpin et al., 2002). Research on the occurrence of such residues in the aquatic

environment began back in the early 1990s. Until today, more than 150 pharmaceutical compounds, including some of their metabolites, have been detected in municipal sewage and surface water (Heberer, 2010). Thus, there is little surprise that such residues discharged into surface water may also turn up in fish living downstream from municipal sewers.

Brooks et al. (2005) reported the occurrence of antidepressant drugs in fish caught from an effluent-dominated stream in north Texas. Fish of different species (*Lepomis macrochirus*, *Ictalurus punctatus*, *Cyprinus carpio*, and *Pomoxis nigromaculatus*) were caught and sacrificed. Liver, brain, and lateral filet tissues were dissected, extracted by solid-phase extraction, and analyzed applying gas chromatography–mass spectrometry with negative chemical ionization. Brooks et al. (2005) detected the selective serotonin reuptake inhibitors fluoxetine and sertraline and the metabolites norfluoxetine and desmethylsertraline at levels greater than 0.1 ng/g in all tissues examined from fish residing in the municipal effluent-dominated stream.

Despite these findings the uptake of sewage-borne pharmaceutical residues by aquatic organisms is limited. Most of the compounds that are discharged by municipal sewage treatment plants are quite polar and are thus not accumulated in fish. On the other hand such compounds may easily leach into the subsoil or sediment, resulting in positive findings of trace residues in groundwater or even drinking water (Heberer, 2010). Nonpolar compounds that might be accumulated in the fatty tissues of fish are often already significantly decreased in concentration inside the sewage treatment plants both by absorption to sewage sludge or even by microbial removal.

19.4.3 Residues of Triphenylmethane Dyes in Wild Fish Downstream of Municipal Sewers

19.4.3.1 Malachite Green—A Multiple-Use Compound Malachite green (4-[(4-dimethylaminophenyl)-phenyl-methyl]-*N*,*N*-dimethyl-aniline) is a triphenylmethane dye used to color materials such as polyacrylonitrile fibers, silk, wool, jute, leather, cotton, and paper (Srivastava, 2004). Malachite green is also used legally (ornamental fish) or illegally (fish for human consumption) as a veterinary drug since the early 1930s. It is applied as topical antiseptic or to treat parasites, fungal infections, and bacterial infections in fish and fish eggs. Other applications are uses as biological stain for the microscopic analysis of cell and tissue samples, as gain medium in dye lasers, for the detection of latent blood in forensic medicine, or as pH indicator compound (pH 0.2–1.8). Tripathi et al. (2007) even reported the illegal coloring of foodstuffs (sweets) with malachite green in India.

In veterinary medicine, malachite green was found to be highly active to fight mycoses in commercial aquaculture caused by the fungus Saprolegnia infecting fish eggs and fish (Olah and Farkas, 1978; Srivastava and Srivastava, 1978). It is also used for the treatment against ciliate protozoa (*Ichthyophthirius* sp.) in freshwater aquaria (Leteux and Meyer, 1972; Schachte 1974). The diseases resulting from this parasitation ("ich" or the "white spot disease") can lead to major animal health problems for aquarists and commercial fish producers worldwide (Francis-Floyd and Reed, 2002). Due to its broad fungicidal and antiparasitical properties, malachite green became and still is very popular among fish farmers. Applied dosage ranges from 100 ppm, used for a few seconds as a dip application, down to 0.1 ppm, when used in prolonged

treatments of fish cultivated in ponds. Malachite green is readily absorbed by fish and easily converted into its reduced and colorless metabolite leucomalachite green (LMG), which is the dominant residue in fat tissues of fish exposed to malachite green.

The application of malachite green has several advantages as it is easily available, provides high efficacy, and is also very cheap due to its very low costs. The most striking advantage of malachite green is, however, that there is no real alternative for its use against the above mentioned fish diseases. In a review article on malachite green Sudova et al. (2007) discussed potential alternatives for its use in aquacultures and emphasized the difficulties in substituting malachite green for the treatment of fish eggs and fish. They concluded that "in spite of partial success in the treatment of some diseases using 'replacement' preparations, no truly adequate substitute for malachite green has been found."

The disadvantage of its application is that malachite green is (worldwide) not registered for use with fish for human consumption for toxicological reasons (see Section 19.4.3.4). In 1983, it was banned in the United States for all food-related applications. In the European Union, zero tolerance applies to all residues of malachite green, including its metabolite leucomalachite green in foodstuffs because it is not listed in Table 1 "allowed substances" of the Annex of EU Commission Regulation 37/2010. In the European Union, an MRPL of 2 μg per kg has been set for residues of malachite green as action limit for internationally traded food consignments (Commission Decision 2003). But despite all prohibitions for the use of malachite green with food-producing animals, consumers are exposed to such residues as demonstrated by frequent findings in fish and fish products most likely resulting from illegal uses (see Section 19.3).

19.4.3.2 Occurrence of Malachite Green Residues in Wild Fish by Unintentional Contamination In Berlin, Germany, Schuetze et al. (2008a, 2008b, 2010) investigated the uptake of different organic contaminants by European eels (*Anguilla anguilla*) caught from surface waters upstream and downstream from the sewers of different municipal STPs.

Residues of malachite green and especially of its corresponding leuco metabolite leucomalachite green (LMG) were found only in wild eels caught downstream from the municipal STPs. The eels were caught from different lakes, a river, and a canal. The appearance of malachite residues in the samples could be directly attributed to the presence of discharges of treated municipal sewage into the receiving surface waters. The highest amount of residues was found in eels residing in a canal carrying the highest loads and portions (up to 40%!) of treated municipal sewage. On the other hand, no residues were found in an upstream lake that was not influenced by municipal sewage discharges. Malachite green residues were detected applying high-performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (MS/MS) with total concentrations up to 0.765 μg/kg fresh weight in the tissues of 25 out of 45 investigated eel samples (Schuetze et al., 2008a).

In none of these cases did the investigated samples exceeded the MRPL value of 2 μg/kg of malachite green residues (sum of malachite green and leucomalachite green) set as the "action limit" for internationally traded food consignments (Commission Decision 2003 and 2005/34/EC) in the European Union. In return this also means that the results obtained for this worst-case scenario (up to 40% of

municipal sewage in the surface water) will not explain any exceedance of the MRPL in (imported) fish by background contaminations only caused by purified municipal sewage effluents (Schuetze et al., 2008a).

As mentioned in Section 19.4.3.1, malachite green is a multiple-use compound also used to color materials. Thus, it appears reasonable that the residues of malachite green found in the eel samples originate from such uses or from legal uses as veterinary drug applied to ornamental fish (private aquaria). The findings by Schuetze et al. (2008a) also supported some earlier assumptions made by Sudova et al. (2007). They also pointed out the persistence of malachite green residues in the environment and supposed that these residues may turn up into untreated fish intended for human consumption. However, Sudova et al. (2007) focused primarily on uses with ornamental fish stating that "every care must be taken when malachite green used for baths of aquarium or ornamental fish is being disposed of" and pointing out that "if enough attention is not paid to the problem, malachite green contained in baths or industrial waste water might penetrate to the aquatic environment and cause serious problems there." This apprehension has now already been proven, but perhaps the main sources for the occurrence of malachite green residues are not uses with ornamental fish but wash-off from clothes and other colored materials drained into the surface water via treated municipal sewage.

19.4.3.3 Crystal (Gentian) Violet Crystal (gentian) violet (4-[4,4-bis(dimethylamino)benzhydrylidene]cyclohexa-2,5-dien-1-yl-idene-dimethylammoniumchloride), another triphenylmethane dye, and its metabolite leucocrystal violet were also investigated and found in the same eel samples (see Section 19.4.3.2) caught in Berlin, Germany (Schuetze et al., 2008b). Residues were found in 35 out of the investigated 45 samples. Again, the leuco metabolite was the dominant residue detected with concentrations up to 6.7 μg/kg fresh weight in tissues, whereas the parent compound was only found at trace levels up to 0.35 μg/kg fresh weight. As already observed with malachite green (see Section 19.4.3.2), the appearance of crystal violet residues in the eel samples could directly be attributed to the presence of discharges of treated municipal sewage in the respective surface waters.

Similar to malachite green, crystal violet can be used as a veterinary drug for the antifungal and antiparasitic treatment of fish. Again the treatment of ornamental fish is legal, whereas the treatment of fish for human consumption is illegal because it is not registered for use with fish for human consumption, worldwide, again for toxicological reasons (see Section 19.4.3.4). Of course, it is also not listed in Table 1 "allowed substances" of the Annex of EU Commission Regulation 37/2010. In this case a MRPL value has also not been established by the European Commission. Crystal violet and other methyl violet derivatives are also multiple-use compounds. They were or still are also used as biological stains, as pH indicator compounds, as hair dyes, for the coloring of textiles, in paints, in carbonless copy papers, in ribbon tapes, in printing inks, and in medicinal products for human use. Again, it seems very likely that the residues of crystal violet found in the eel samples originate from such uses and from (compared to malachite green, which is less popular) legal uses of crystal violet as a veterinary drug for the treatment of ornamental fish.

19.4.3.4 Assessment of Human Health Risks According to Article 6 of EU Regulation 470/2009 the "scientific risk assessment shall consider the metabolism

and depletion of pharmacologically active substances in relevant animal species, the type of residues and the amount thereof, that may be ingested by human beings over a lifetime without an appreciable health risk expressed in terms of acceptable daily intake (ADI)." The use of alternative approaches to ADI is also possible, if they have been laid down by the Commission, which also includes that technical requirements are in accordance with internationally agreed standards. In the case of malachite green it is not possible to apply an ADI approach because current data suggests that malachite green and especially leucomalachite green may be carcinogenic and also provided some evidence that these compounds are in vivo mutagens. There is some evidence of carcinogenic activity of leucomalachite green in female B6C3F1 mice. (NTP, 2005) In general, there is a lack of toxicological data to be derived from mechanistic studies and reprotoxicity and teratogenicity studies. EFSA concluded that both compounds malachite green and leucomalachite green should be viewed as genotoxic and/or carcinogenic (EFSA, 2005b). As a nonthreshold mechanism cannot be excluded for LMG, it is also not possible to establish an ADI either for leucomalachite green or malachite green, which is easily reduced to leucomalachite green (Heberer and Batt, 2007).

Thus, only a "case-related" risk assessment on the basis of a "margin of exposure" (MOE) concept can be applied to evaluate consumer risks by contaminated foodstuffs (Heberer and Batt, 2007). An MOE may be applied as a management tool to evaluate potential health risks for consumers when an ADI cannot be established. However, a MOE should not be regarded as a substitute for a regular risk assessment based on an ADI. To calculate an MOE, the following data are needed: (1) A residue level detected in the individual food consignment (cs), (2) consumption data (cd) to calculate the human exposure (HExp = cd × cs), and (3) toxicological dose–response data to derive the lowest observed effect level (LOEL) -> MOE = LOEL / HExp. With regard to consumers' health protection, a margin of exposure below 10,000 is not regarded as being acceptable for genotoxic carcinogens (EFSA, 2005a), which applies to residues of malachite green.

Table 19.1 demonstrates an MOE calculation applying different models based on different acute and chronic consumption data. The calculations of the intake of malachite green residues are based on individual consumption data, maximum residues found in commercial eel samples [= 4500 μg/kg as reported by Zhang (2005)], and the maximum concentration found in nontreated wild eels [= 0.765 μg/kg as reported by Schuetze et al. (2008a)]. The LOEL (slightly raised incidence of neoplasms at a dose equal to 13 mg LMG per kg body weight) was taken from the studies of the U.S. National Toxicology Program (NTP, 2005).

Table 19.1 compares two scenarios, an intake of malachite green residues from a highly contaminated commercial eel sample (Zhang, 2005) and the wild eel sample unintentionally contaminated with malachite green residues from the study conducted by Schuetze et al. (2008a). In the first case, the resulting MOE value was below 10,000 for all (acute and chronic) scenarios. Especially the relevant and most realistic scenario based on acute consumption data for children aged 2 to 5 years revealed an MOE value of only 300, representing an unacceptable risk for human consumer's health according to EFSA requirements (EFSA, 2005a).

In the second case, the MOE approach was used to evaluate the human health risk associated with the consumption of eels contaminated by effluents of treated sewage (highest residue found). With a calculated MOE of at least 1.8 million

TABLE 19.1 Calculation of the Margin of Exposure (MOE) Using Different Models for Acute and Chronic Consumption[a]

Consumption Model/Data	Calculated Maximum Intake for Commercial Eel ($\mu g/kg_{bw}$ day)	Calculated Maximum Intake for Wild Eel ($\mu g/kg_{bw}$ day)	LOEL (mg/kg bw)	MOE Commercial Eel	MOE Wild Eel
VELS: acute consumption data[b] (Child 2–5 years, 16.15 kg bw, 152.5 g fish/d)	42.49	0.007224	13	306	1.80×10^6
VELS: chronic consumption data[b] (Child 2–5 years, 16.15 kg bw, 5.6 g fish/d)	1.56	0.0002653	13	8331	49.0×10^6
CVMP (EMEA): chronic consumption data[c] (Adult 60 kg bw, 300 g fish/d)	22.5	0.003825	13	577	3.40×10^6

[a] Intake calculations are based on individual consumption data, maximum residues found in commercial eel samples [= 4500 μg/kg as reported by Zang (2005)] and the maximum concentration found in nontreated wild eels [= 0.765 μg/kg as reported by Schuetze et al. (2008)]. bw: body weight, LOEL: lowest observed effect level obtained from the studies of the U.S. National Toxicology Program (NTP, 2005).

[b] Consumption data obtained from the Verzehrsstudie zur Ermittlung der Lebensmittelaufnahme von Saeuglingen und Kleinkindern für die Abschaetzung eines akuten Toxizitaetsrisikos durch Rueckstaende von Pflanzenschutzmitteln (VELS) consumption study for children aged between 2 and 5 years (Banasiak et al., 2005).

[c] Consumption data from the risk analysis approach for residues of veterinary medicinal products in food of animal origin used by the Committee for Medicinal Products for Veterinary Use (2001) of the European Medicines Agency (EMEA).

(see Table 19.1; Schuetze et al., 2008a), the resulting risk can be classified as being very low and the risk of adverse health effects is very low for single or even casual consumption of such eel filets. Although the MOE approach is a useful risk management tool to measure the risk-related parameters that can be considered unmeasurable, any oral exposure to residues of malachite green and leucomalachite green is not desirable and should be avoided because of their potential to act as genotoxic carcinogens. From a toxicological point of view, MRPL values are irrelevant because technical feasibility alone and not the health risk is the yardstick for their establishment. Thus, they have never undergone a risk assessment.

In the case of crystal violet a risk assessment is not possible neither based on an ADI nor based on an MOE approach. Crystal violet has been classified as a suspected carcinogen, which makes it impossible to establish an ADI. The application of an MOE approach is not possible as adequate toxicological data is missing. In this case, with crystal violet being classified as a suspected carcinogen, the application of a zero tolerance approach is inevitable both from a legal and a toxicological point of view (Schuetze et al., 2008b; Heberer, 2009a).

19.4.3.5 Legal implications Exceeding zero tolerance does not, in most cases, imply a consumers' health problem, but it always has legal implications challenging the marketability of the respective food. In the European Union, zero tolerance applies when residues of nonauthorized veterinary drugs are found in fish even if they are caused by unintentional contamination. Existing MRPLs shall help to avoid impeding international trade of food consignments. They are, however, not relevant for the local or national tradability of the food consignments. Thus, within the European Union, zero tolerance generally applies to all residues of crystal violet, including its metabolite, as well as to all residues of malachite green, including leucomalachite green, caused by unintentional contamination in domestic surface waters.

19.5 CONCLUSIONS AND PERSPECTIVES

Residues of pharmaceuticals in fish and fishery products usually originate from intentional therapeutic application of veterinary drugs. In Europe, only very few substances are registered for use in the treatment of fish for human consumption. Thus, the occurrence of nonauthorized or even illegal pharmaceutical residues is continuously reported via the European rapid alert system RASFF. Such residues are unacceptable from a legal point of view but may also pose a sometimes even serious consumer's health risk problem.

The results from a joint Food and Agriculture Organization of the United Nations (FAO)/World Organisation for Animal Health (OIE)/World Health Organization (WHO) expert consultation on "antimicrobial use in aquaculture and antimicrobial resistance" (WHO, 2006) also addressed the issue of antibiotic resistance by trying to rate risks for public health in relation to the use of antimicrobials in aquaculture. It was concluded that "the issue of direct transmission of resistant human pathogenic bacteria represents a low risk as such bacteria are rarely transmitted through aquaculture products" (WHO, 2006). "The greatest risk to public health associated with antimicrobial use in aquaculture is assumed to be the development of a reservoir of transferable resistance genes in bacteria in aquatic environments. Such genes can be disseminated by horizontal gene transfer to other bacteria and ultimately reach human pathogens, and

thereby potentially cause treatment problems due to resistance. Antimicrobial residues represent a low, but still significant public health risk" (WHO, 2006). Consumption of adequately cooked food and good kitchen hygiene reduces human health risks by resistant and/or pathogen bacteria to a minimum, but special food habits such as the consumption of raw fish (e.g., sushi) may be a source for the ingestion of such bacteria (Heberer, 2009b). Recent investigations now also provided some evidence that residues of pharmaceuticals may be found in wild fish not intentionally treated with veterinary drugs. Fish residing close to sea farming areas will, due to drifting (water current) of veterinary drugs applied to aquacultured fish or other fishery products, also be exposed, which results in an uptake of these substances (Fortt et al., 2007).

Pharmaceuticals have also been detected in wild living fish downstream from municipal sewage treatment plants. Such residues may derive from application in human medicine (Brooks et al., 2005), legal treatment of ornamental fish (Schuetze et al., 2008a, 2008b), or discharges of multiple-use compounds via municipal sewers (Schuetze et al., 2008a, 2008b). Usually, they will only occur in fish at very low levels far beyond therapeutically active doses. These trace levels will not imply a consumers' health problem. However, in light of the European zero tolerance approach, such residues always cause legal implications challenging the marketability of the respective food. Apart from the mere detection of such residues in wild fish and apart from the discussed questions of potential human health aspects and legal implications, there are some additional questions arising from these findings and the frequent detection of drug residues in surface waters. Due to their polar structures (low octanol–water coefficients), most of the pharmaceutical residues discharged by municipal STPs into the receiving surface waters at nanogram to microgram per liter concentrations will not be enriched in fish tissues. However, wild fish residing in surface waters downstream from municipal sewers are continuously exposed to a mixture of pharmaceutical compounds, including some antibiotics and their metabolites at subtherapeutic doses. In a review, Heberer (2010) reported that 78 different antibiotic pharmaceuticals and their metabolites have been detected in surface or sewage water, worldwide. The question is: Will such permanent multicompound exposure have any impact on animal health or will it cause or promote antibiotic resistance? However, the knowledge on impacts and sustainability of the extended use of antibiotics in aquaculture or the effects derived from the permanent occurrence of such residues in the aquatic environment is still very limited and require more research.

REFERENCES

Banasiak U, Heseker H, Sieke C, Sommerfeld C, Vohmann C (2005). Abschätzung der Aufnahme von Pflanzenschutzmittel-Rückständen in der Nahrung mit neuen Verzehrsmengen für Kinder. *Bundesgesundheitsbl Gesundheitsforsch Gesundheitsschutz* 48:84–98.

Brooks BW, Chambliss CK, Stanley JK, Ramirez A, Banks KE, Johnson RD, Lewis RJ (2005). Determination of select antidepressants in fish from an effluent-dominated stream. *Environ Toxicol and Chem* 24(2):464–469.

Daughton CG, Ternes TA (1999). Pharmaceuticals and personal care products in the environment: Agents of subtle change? *Environ Health Perspect* 107:907–938.

European Food Safety Agency (EFSA) (2005a). Opinion of the scientific committee on a request from EFSA related to a harmonised approach for risk assessment of substances which are both genotoxic and carcinogenic. *EFSA J* 282:1–31.

European Food Safety Authority (EFSA) (2005b). Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food (AFC) to review the toxicology of a number of dyes illegally present in food in the EU. *EFSA J* 263:1–71.

FAO (2011). The State of World Fisheries and Aquaculture 2010, FAO Fisheries and Aquaculture Department, Food and Agriculture Organization of the United Nations (FAO), Rome, 2011, 218 pp.

Fortt ZA, Cabello FC, Buschmann RA (2007). Residues of tetracycline and quinolones in wild fish living around a salmon aquaculture center in Chile. *Rev Chil Infect* 24:14–18.

Francis-Floyd R, Reed P (2002). Ichthyophthirius multifiliis (White Spot) infections in fish. CIR920 of the Fisheries and Aquatic Sciences Department Series, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida 1991, revised July 2002. Available: http://edis.ifas.ufl.edu/pdffiles/FA/FA00600.pdf. Accessed February 17, 2011.

Gatermann R, Hoenicke K, Mandix M (2004). Formation of semicarbazide (SEM) from natural compounds in food by heat treatment. *Czech J Food Sci* 22:353–354.

Heberer T (2002). Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: A review of recent research data. *Toxicol Lett* 131:5–17.

Heberer T (2010). Chemische und pharmazeutische Rückstände im Wasserkreislauf, Block III: Arzneimittel—Therapie, Sicherheit, Anwendung und Vermarktung. In Koesling, V. and Schuelke, F. (Eds.), *Die chemisch-pharmazeutische Industrie am Beispiel Schering*. DTMB Buchreihe, Berlin, pp. 240–251.

Heberer T (2009a). Zero tolerance of chemical pollutants in food and animal feed: European policies and public health. *J Epidemiol Commun Health* 63(11):865–866.

Heberer T (2009b). Organic compounds used in aquaculture. In L Shore and A Pruden (Eds.), *Hormones and Pharmaceuticals Generated by Concentrated Animal Feeding Operations, Transport in Water and Soil, Series: Emerging Topics in Ecotoxicology: Principles, Approaches and Perspectives,* Vol. 1. Springer Science Publishers, Norwell, MA, pp. 95–114.

Heberer T, Batt N (2007). Risk assessment of malachite green residues—Literature study (prepared by Germany). Agenda Item 9, CRD 9, Joint FAO/WHO Food Standards Programme Codex Committee On Residues of Veterinary Drugs in Foods, 17th session. Breckenridge, CO.

Hoenicke K, Gatermann K (2006). How can zero tolerances be controlled? The case study of Nitrofurans. *Accred Qual Assur* 11:29–32.

Hoenicke K, Gatermann R, Hartig L, Mandix M, Otte S (2004). Formation of semicarbazide (SEM) in food by hypochlorite treatment: Is SEM a specific marker for nitrofurazone abuse? *Food Addit Contam* 21:526–537.

Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999–2000: A national reconnaissance. *Environ Sci Technol* 36:1202–1211.

Leteux F, Meyer F (1972). Mixtures of malachite green and formalin for controlling *Ichthyophthirius* and other protozoan parasites of fish. *Prog Fish Cult* 34:21–26.

National Toxicology Program (NTP) (2005). Toxicology and carcinogengesis studies of malachite green chloride and leucomalachite green (CAS Nos. 569-64-2 and 129-73-7) in F344/N rats and B6C3F1 mice (feed studies). NIH Publication No. 05–4463. U.S. Department of Health and Human Services. Available: http://ntp.niehs.nih.gov/files/527_FINAL.pdf. Accessed February 17, 2011.

Olah J, Farkas J (1978). Effect of temperature, pH, antibiotics, formalin and malachite green on the growth and survival of *Sapprolegnia* and *Achylya* parasitic on fish. *Aquaculture* 13:273–288.

Saari L, Peltonen K (2004). Novel source of semicarbazide: Levels of semicarbazide in cooked crayfish samples determined by LC/MS/MS. *Food Addit Contam* 21:825–832.

Schachte JH (1974). A short term treatment of malachite green and formalin for the control of *Ichthyophtirius multifiliis* on channel catfish in holding tanks. *Prog Fish Cult* 36:103–104.

Schuetze A, Heberer T, Juergensen S (2008a). Occurrence of residues of the veterinary drug malachite green in eels caught downstream from municipal sewage treatment plants. *Chemosphere* 72:1664–1670.

Schuetze A, Heberer T, Juergensen S (2008b). Occurrence of residues of the veterinary drug crystal (gentian) violet in wildlife eels caught downstream from municipal sewage treatment plants. *Environ Chem* 5:194–199.

Schuetze A, Heberer T, Effkemann S, Juergensen S (2010). Occurrence and assessment of perfluorinated chemicals in wildlife fish from northern Germany. *Chemosphere* 78:647–652.

Sudova E, Machova J, Svobodova Z, Vesely T (2007). Negative effects of malachite green and possibilities of its replacement in the treatment of fish eggs and fish: A review. *Vet Med-Czech* 52:527–539.

Srivastava S (2004). Toxicological effects of malachite green. *Aquat Toxicol* 66:319–329.

Srivastava GC, Srivastava RC (1978). A note on potential applicability of malachite green oxalate in combating fish-mycoses. *Mycopathologia* 64:169–171.

Tripathi M, Khanna SK, Das M (2007). Surveillance on use of synthetic colours in eat-ables vis a vis Prevention of Food Adulteration Act of India. *Food Control* 18:211–219.

World Health Organization (WHO) (2006). Antimicrobial use in aquaculture and antimicrobial resistance. Report of a joint FAO/OIE/WHO expert consultation on antimicrobial use in aquaculture and antimicrobial resistance, Seoul, Republic of Korea, Geneva, June 13–16, 2006.

Zhang X (2005). Hong Kong people fear poisonous Chinese fish. *The Epoch Times*. Available: http://en.epochtimes.com/news/5-8-31/31823.html. Accessed February 16, 2011. Concentrations now reported at http://www.reference.com/browse/malachite+green. Also accessed February 16, 2011.

20

ROLE OF QUATERNARY AMMONIUM COMPOUNDS ON ANTIMICROBIAL RESISTANCE IN THE ENVIRONMENT

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The widespread use of biocides worldwide over the last 80 years has led to their extensive presence in the environment. The limited scientific evidence available indicates that bacterial resistance to biocides may contribute to the development and dissemination of antibiotic resistance, which is a serious concern relative to the worldwide anti-infective therapy for humans and animals. Thus, a better understanding of the role biocides play in antibiotic resistance is necessary in order to protect both human and animal health. Two recent scientific reports summarize the relationship between biocides and emerging antibiotic resistance, as well as the mechanisms and conditions leading to the development of cross resistance between biocides and antibiotics (SCENIHR, 2009, 2010).

Quaternary ammonium compounds (QACs) are biocides that have recently come under scrutiny for their role in antibiotic resistance. QACs are cationic bioactive agents that have been extensively used in domestic, agricultural, industrial, and clinical applications since the late 1930s, at about the time antimicrobials, namely, the sulfamides were introduced. QACs are lytic biocides capable of killing over 99% of microorganisms at typical application concentrations. In contrast to other biocides, QACs are not chemically transformed after application; thus, when released into the environment they retain their biocidal properties. QACs are now ubiquitous and have been detected in environmental media such as wastewater, surface water, aquatic sediments, and soil at concentrations below inhibitory levels. Many studies have shown that exposure to QACs at subinhibitory concentrations leads to the

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selection of intrinsically resistant bacteria in a microbial community or results in the development or acquisition of resistance mechanisms such as modification in the cell membrane composition and hydrophobicity, overexpression of efflux pumps, and acquisition of integrons and plasmids. As a result, QAC resistance genes and determinants are abundant in the environment.

Antibiotics are as abundant as QACs in the environment. While some resistance mechanisms are common to both biocides and antibiotics, QACs and most antibiotics currently in use have no common mode of action. As a result, the role of QACs in the development of antibiotic resistance had been assumed to be negligible. However, recent findings have shown that exposure to QACs at subinhibitory concentrations results in the development of cross- and co-resistance mechanisms that confer tolerance to many antibiotics as well as co-selection of antibiotic resistance through mobile genetic elements, such as integrons and plasmids. Multidrug efflux pumps, abundance of QAC resistance genes, along with antibiotic resistance genes in the same mobile genetic elements, as well as the presence of *qac*ΔE genes, which confer resistance to QACs in the conserved region of the clinically important class-1-type integrons, all demonstrate that QACs play a significant role in the development, selection, and dissemination of antibiotic resistance.

In this chapter, the role of QACs on antimicrobial resistance is examined by providing systematic information on (1) the distribution of QACs in engineered and natural systems, (2) the physical, chemical, and biological processes that determine the fate and effect of QACs in engineered and natural systems, (3) the mode of antimicrobial action of QACs, (4) the intrinsic and acquired resistance mechanisms against QACs, and (5) the relationship between QAC resistance and antibiotic resistance.

DEFINITIONS

Antimicrobial Agent: a natural or synthetic chemical substance that inhibits microbial growth.

Resistance: relative tolerance of a microorganism to a particular antimicrobial treatment under a given set of conditions.

Species-Level Resistance: ability of a microorganism to tolerate higher concentrations of an antimicrobial agent than other microorganisms of the same species.

Community-Level Resistance: relative insusceptibility of a microbial community to an antimicrobial agent after exposure to the agent for certain duration.

Cross Resistance: an acquired ability of a microorganism to tolerate a certain antimicrobial agent by a mechanism developed against another antimicrobial agent with the same mode of action.

Co-resistance: an acquired ability of a microorganism to tolerate a certain antimicrobial agent by a mechanism developed against another antimicrobial agent with a totally distinct mode of action.

Co-selection: a process of acquisition of tolerance to multiple antimicrobial agents with distinct mode of actions by a mobile genetic element (i.e., plasmids, integrons, and transposons) acquired by a microorganism during exposure to one of the agents.

Biotransformation: modification of a chemical compound by an enzyme.

Integron: a gene acquisition element able to capture and express promoterless gene cassettes by site-specific recombination.

Gene Cassette: a circular, mobile genetic element that includes at most two genes or open reading frames and a recombination site.

Plasmid: a self-replicating, extra-chromosomal deoxyribonucleic acid (DNA) molecule that can be transferred horizontally by conjugation or mobilization.

Efflux Pump: a transmembrane protein involved in the extrusion of a toxic substance from inside to outside of a cell.

20.1 QAC CHARACTERISTICS, DEMAND, AND CONSUMPTION

QACs are organic compounds that contain four functional groups attached covalently to a central nitrogen atom (R_4N^+) (Fig. 20.1). The functional groups (R) include at least one long-chain alkyl group and the rest are either methyl, benzyl, or ester groups. QACs are among the high production volume chemicals (HPVs, i.e., chemicals manufactured or imported in amounts equal to or greater than one million pounds per year) found on the lists of both the U.S. Environmental Protection Agency (U.S. EPA, 2006) and the Organization for Economic Co-operation and Development (OECD). QACs possess surface-active properties, self-assembly characteristics, detergency, and antimicrobial properties. The unique physical/chemical properties of QACs have resulted in a variety of uses and a high level of popularity in domestic, agricultural, health care, and industrial applications such as surfactants, emulsifiers, fabric softeners, disinfectants, pesticides, corrosion inhibitors, and personal care products (Garcia et al., 1999; Steichen, 2001; Patrauchan and Oriel, 2003). The 2004 worldwide annual consumption of QACs was reported as 500,000 tons and was expected to reach or exceed 700,000 tons (Hauthal, 2004).

QACs can be classified in five major groups depending on the type of functional groups: monoalkonium, dialkonium, benzalkonium, diesteralkonium, and pyridalkonium halides (Fig. 20.2).

About 80% of QACs are used in fabric softeners. The most common fabric softener active ingredients that are commercially viable in today's marketplace are dialkonium and diesteralkonium halides. The first group has the highest demand in the market; however, esteralkonium salts are good substitutes for dialkonium salts because they are readily biodegradable and less toxic than dialkonium salts. QACs are also utilized in laundry detergents. Laundry detergents that also provide fabric

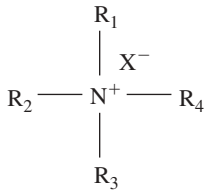


FIGURE 20.1 General structure of quaternary ammonium compounds (R represent functional groups and X is a halide).

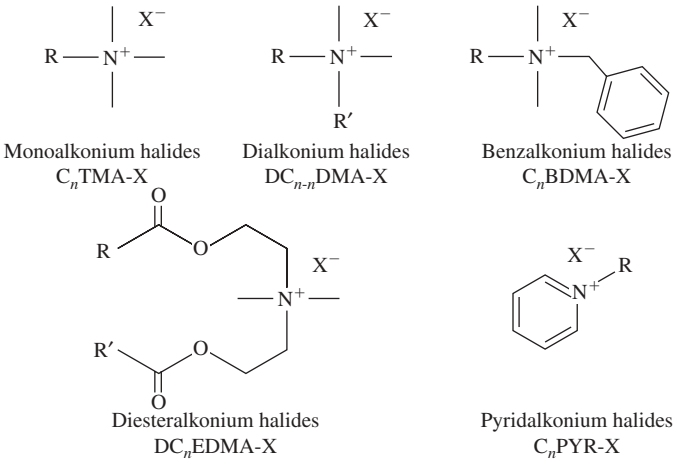


FIGURE 20.2 Representative QAC groups, their general structure and abbreviations used in this study (R and R′, are alkyl groups of various chain length).

softening contain relatively simple QACs such as monoalkonium salts with an alkyl chain length of 12–18 carbons (Zachvieja, 2001).

QACs reduce surface and interfacial tension by sorbing to a surface or an interface such as hair and skin. The adsorption ability of QACs onto organic surfaces makes their use extremely important in the personal care industry. Skin care products and hair conditioners contain mainly alkyl QACs (including mono-, di-, and trialkonium salts) and polymeric QACs (e.g., polyquaternium 7 and 11) in their formulations (Tang, 2001).

QACs are extensively used as bioactive agents. They exhibit a broad spectrum of antimicrobial activity over a wide range of pH and are used in domestic, industrial, agricultural, and medical applications as wood preservatives, pesticides, fungicides, sanitizers/disinfectants, and hard-surface cleansers. They are effective against a variety of bacteria, fungi, and viruses at very low concentrations. When QACs are used as disinfectants, the applied concentration is typically between 400 and 500 ppm and almost always below 1000 ppm (e.g., 0.1% w/v in Lysol). The most commonly used QACs as bioactive agents are monoalkonium (C_{16}), dialkonium ($C_{8–10}$), and benzalkonium ($C_{12–16}$) chlorides (Tiedink, 2001). The same types of QACs are also used in agricultural applications to sanitize animal houses.

Another major use of QACs is in organoclays, which are produced by the displacement of the inorganic cations on a clay mineral (i.e., morillonite or hectorite) by organic cations. The organic cations used in the manufacture of organoclays are QACs such as dialkonium and benzalkonium salts. Organoclays are used in a number of different formulations such as oil-based drilling fluids, printing inks, oil-based paints, latex polymers, and nail polishers (Hoey, 2001). Organoclays are able to adsorb organic molecules from both aqueous systems and air and are used in landfill liners, groundwater remediation (Boyd et al., 1988), and air filters. The annual demand for organophilic clays, which typically contain 40% by weight of QACs, is around 16% of the QAC market.

In summary, QACs are important, ubiquitous chemicals. Because QACs are used in high quantities in domestic, agricultural, industrial, and clinical applications, humans and microorganisms are in constant contact with them. Among the five groups of QACs, monoalkonium, dialkonium, and benzalkonium chlorides, which have 8–16 carbon alkyl chain length, are widely utilized as biocides. These QACs are particularly important relative to the potential development and transfer of antimicrobial resistance within the aforementioned QAC applications. For this reason, these three groups of QACs are the primary focus of this chapter.

20.2 PROPERTIES

QACs are moderately large molecules, with molecular weights typically between 250 and 400 g/mol, composed of two distinctly different moieties: hydrophobic alkyl groups and a hydrophilic, positively charged central N atom, which retains its cationic character at all pH values. QACs have distinct physical/chemical properties, which are conferred by their substituents, mainly the alkyl groups. QACs have high boiling points and low Henry's law constants, resulting in very low volatilization from the surfaces that are applied to or the various environmental media.

QACs are cationic surfactants, and as all surfactants do they form micelles. Micelle formation is triggered by the coexistence of polar and hydrophobic moieties in the same molecule. In a polar solvent such as water, the polar moiety tends to interact with the solvent, whereas the hydrophobic moiety is repelled. When the surfactant concentration increases in a solution, the attraction between the hydrophobic moieties of the surfactant molecules prevails, resulting in the formation of biphasic aggregates called *micelles*. The affinity of a surfactant to form a micelle is defined by a parameter called *critical micelle concentration* (CMC), which is the threshold surfactant concentration at which micellization begins. The CMC is not only a measure of solubility for surfactants, such as QACs but also a criterion that determines the effective QAC application concentration for disinfection because the lytic effectiveness of QACs is the highest at the CMC value.

Tezel (2009) measured the CMC of nine representative QACs belonging to monoalkonium, dialkonium, and benzalkonium chloride groups (Table 20.1). CMC values of QACs ranged from 230 to 5576 mg/L with a median of 852 mg/L ($n = 9$). The lowest CMC in each QAC group was for those QACs with the longest alkyl chain(s), whereas QACs with the highest CMC had the shortest alkyl chain(s) within the homologous group. The dependence of CMC on the alkyl chain length suggests that the hydrophobicity of QACs significantly affects their CMC.

The CMC values of benzalkonium chlorides are significantly lower than those of monoalkonium chlorides with the same alkyl chain length (Table 20.1). Thus, not only the alkyl chain length but also the addition of a benzyl group to the polar head results in the decrease of CMC. The lower CMC values of benzalkonium chlorides make them efficient antimicrobial agents. As a result, benzalkonium chlorides are the most frequent active ingredients present in many commercial disinfectants on the market.

The major driving force of the micellization of QACs is the hydrophobic interaction between their alkyl groups (Kopecky, 1996). The hydrophobicity of a

TABLE 20.1 Selected Physicochemical Properties, Predicted Environmental Distribution and Persistence of QACs[a]

	CMC (mg/L)	log K_{ow}	*Media Distribution (%)*		Persistence (h)
			Water	Sediment	
Monoalkonium chlorides					
C_{12}TMA-Cl	5576	0.36	83.3	16.7	404
C_{14}TMA-Cl	1638	0.70	61.1	38.9	532
C_{16}TMA-Cl	419	1.50	33.9	66.1	873
Dialkonium chlorides					
DC_8DMA-Cl	5043	0.28	44.9	55.1	693
DC_{8-10}DMA-Cl	852	1.54	22.0	78.0	1210
DC_{10}DMA-Cl	543	2.56	10.8	89.2	1910
Benzalkonium chlorides					
C_{12}BDMA-Cl	1292	0.59	14.9	85.1	1570
C_{14}BDMA-Cl	633	1.67	4.2	95.8	5710
C_{16}BDMA-Cl	230	2.97	3.0	97.0	6440

[a]Compound distribution based on a chemical release scenario of 100 kg/h in water calculated based on the level III fugacity model using the EPI Suite v.4.0 (U.S. EPA, 2009)

compound is well defined by its *n*-octanol/water partition coefficient (K_{ow}). K_{ow} values are also widely used in determining partitioning, bioaccumulation, biavailability, and toxicity properties of a variety of organic compounds. The log K_{ow} values of the target QACs ranged from 0.28 to 2.97 with a median of 1.50 ($n = 9$) (Table 20.1). The highest log K_{ow} in each QAC group was for the QACs that have the longest alkyl chain(s) within each homologous group, as follows: 1.50 ± 0.06, C_{16}TMA-Cl; 2.56 ± 0.01, DC_{10}DMA-Cl; and 2.97 ± 0.03, C_{16}BDMA-Cl, which belong to monoalkonium, dialkonium, and benzalkonium chlorides, respectively. On the other hand, the lowest log K_{ow} in each QAC group was for those with the shortest alkyl chain(s) within each homologous group, as follows: 0.36 ± 0.07, C_{12}TMA-Cl; 0.28 ± 0.22, DC_8DMA-Cl; and 0.59 ± 0.04, C_{12}BDMA-Cl, which belong to monoalkonium, dialkonium, and benzalkonium chlorides, respectively.

The combined effect of the QACs' physicochemical properties is reflected on the media distribution estimated by the Estimation Program Interface (EPI) Suite Level III Fugacity Model (Table 20.1). Although a relatively simple approach, the fugacity model helps demonstrate that these compounds have a tendency to partition into solid phases, such as sediment. The effect of alkyl chain length as well as that of the benzyl group on media distribution is also shown. Relative to partition to solid phases, QACs form the following descending series: monoalkonium < dialkonium < benzalkonium chlorides. Within the same homologous series, QACs with longer alkyl chain(s) have a higher tendency to partition to solid phases.

In conclusion, QAC sorption to solid organic media, such as biomass, soil, and sediment, is favored as the alkyl chain length increases (log $K_{ow}\uparrow$, CMC$\downarrow$). On the contrary, as the alkyl chain length decreases (log $K_{ow}\downarrow$, CMC$\uparrow$), QACs become less hydrophobic and tend to stay in the aqueous media. The physical/chemical properties of QACs strongly affect not only their fate but also their toxicity, bioavailability, and biodegradability. QACs with relatively long alkyl chains are more effectively

eliminated during wastewater treatment by adsorption to biomass or by adsorption to organic and inorganic solids once they are released in the environment. As a result, QACs with short alkyl chains are more mobile and bioavailable than QACs with longer alkyl chains. Therefore, exposure of microorganisms to QACs with shorter alkyl chains is more probable.

20.3 DISTRIBUTION IN THE ENVIRONMENT

For over a century, QACs have been extensively used in many industrial, domestic, and agricultural applications. Their production and usage rates are increasing as they are used in new applications. As a result, QACs are inevitably released into the environment at the production stage or after their application and use of QAC-bearing products. About 75% of the QACs utilized annually are released into wastewater treatment systems, whereas the rest are directly discharged into the environment. Occurrence of QACs in sewage (Merino et al., 2003; Clara et al., 2007; Kreuzinger et al., 2007; Martinez-Carballo et al., 2007a), industrial wastewater (Kreuzinger et al., 2007; Martinez-Carballo et al., 2007b), effluents of laundries and hospitals (Kümmerer et al., 1997; Kreuzinger et al., 2007; Martinez-Carballo et al., 2007a), treated wastewater (Ding and Liao, 2001; Clara et al., 2007; Kreuzinger et al., 2007), sewage sludge (Martinez-Carballo et al., 2007b; Sutterlin et al., 2007), surface waters (Ding and Liao, 2001; Ferrer and Furlong, 2001; Merino et al., 2003; Kreuzinger et al., 2007; Martinez-Carballo et al., 2007a), and aquatic sediments (Ferrer and Furlong, 2002; Kreuzinger et al., 2007; Martinez-Carballo et al., 2007b; Li and Brownawell, 2010) at levels that may threaten biological treatment and environmental systems have been reported. These studies show that the mean concentration of QACs in domestic wastewater, treated effluent wastewater, sewage sludge, and surface water is around 0.5 mg/L, 0.05 mg/L, 5000 mg/kg dry weight, and 0.04 mg/L, respectively.

Randomly collected samples of sewage from treatment plants in Switzerland, which had various inputs from metallurgical processes and the textile industry, had QAC levels ranging from 0.04 to 0.45 mg/L (Michelsen, 1978). Huber (1979) and Kupfer (1982) described monitoring studies in Germany, and Wee (1984) determined the levels of dialkonium chlorides in untreated sewage and final effluent from a plant in the United States. The total QAC concentrations in influent and effluent municipal wastewater ranged from 0.05 to 1.3 mg/L and from 10 to 200 μg/L, respectively, whereas the total monoalkonium chloride concentration in the influent and effluent municipal wastewater was 130 and 30 μg/L, respectively. A recent survey study conducted in Austria reported QAC concentrations ranging from 25 to 300 μg/L and from 0.3 to 3.6 μg/L, in influents and effluents of five municipal wastewater treatment plants, respectively (Martinez-Carballo et al., 2007a). For each homologous group of QACs detected, QACs with relatively short alkyl chains were the most abundant. It is noteworthy that recently reported QAC concentrations in untreated and treated municipal wastewater are less than concentrations reported in the past. Such a decrease may be the result of replacement of QACs with more biodegradable ester QACs, especially as fabric softeners, as well as improvement in the efficiency of treatment systems and/or stricter regulations. Among the measured QACs, benzalkonium salts were the most predominant QACs followed by dialkonium and

monoalkonium salts. The predominance of benzalkonium chlorides is related to the fact that they are the main active ingredients of disinfectants and sanitizers widely utilized in domestic, industrial, and medical applications, as well as to their higher persistence in natural and engineered systems (see Table 20.1).

Contrary to municipal wastewater treatment plants, QAC concentrations are higher in the effluents of specific industrial facilities, such as paper processing, textile, and food processing. Kümmerer et al. (1997) analyzed benzalkonium chlorides in highly complex effluent samples from European hospitals and found QAC concentrations between 0.05 and 6.03 mg/L. Similarly, Martinez-Carballo et al. (2007a) detected QACs, predominantly C_{12} BDMA, at 3 and 5 mg/L QACs in laundry and hospital effluents, respectively.

QAC levels in receiving waters are typically in the low microgram per liter range. Huber (1979) reported QAC concentrations from 5 to 20 μg/L in the Main River in Germany. Concentrations of dialkonium chlorides in sewage and surface water samples collected in Germany were 350–480 μg/L and 6–12 μg/L, respectively (OECD, 1994). Likewise, monoalkonium chloride from 5 to 30 μg/L was reported in randomly collected samples from several rivers in the United States (Wee and Kennedy, 1982; Wee, 1984). Lewis and Wee (1983) conducted a study in which samples were collected at various distances downstream from wastewater treatment facilities in the United State. Mean dialkonium chloride levels were <2, 24, 17, and 33 μg/L for Millers River (in Massachusetts), Otter River (in Massachusetts), Blackstone River (in Massachusetts), and Rapid Creek (in South Dakota). The concentration of DC_nDMA-Cl in the samples collected at distances 4.4 to 55 miles downstream from the wastewater treatment plants ranged from 191 to 100 μg/L. Martinez-Carballo et al. (2007a) measured QACs in 22 surface water samples. Monoalkonium, dialkonium, and benzalkonium halides were detected in most surface waters at nanogram per liter concentrations. The highest measured QAC concentrations were for benzalkonium, that is, C_{12}BDMA and C_{14}BDMA and dialkonium halides, that is, DC_{10}DMA, with a maximum of 1.9 μg/L. The above-presented data, spanning three decades (1980–2010), illustrate that QACs have been present in receiving waters for a very long time, reflecting both their persistence and continuous use.

QACs adsorb strongly on suspended solids such as minerals, biomass, and inorganic particles and are transferred to anaerobic media such as digesters and aquatic sediments. For instance, mean concentrations of DC_nDMA-Cl in anaerobically stabilized sludge samples from five municipal sewage treatment plants in Switzerland were 3670, 960, 470, and 210 mg/kg dry solids in 1991, 1992, 1993, and 1994, respectively (Fernandez et al., 1996), whereas, microgram per liter levels were found in the sewage as discussed above. It was also reported that QAC concentrations in municipal anaerobic digesters may range from 4000 to 10,500 mg/kg dry solids. Lewis and Wee (1983) obtained sediment samples from Rapid Creek (South Dakota) at distances from 0.8 to 88 km downstream from a sewage outfall. DC_nDMA-Cl levels averaged 23 mg/kg dry solids over 18 samples. Fernandez et al. (1991) found that DC_nDMA-Cl was a ubiquitous contaminant in coastal sediments collected near Barcelona, Spain, with a concentration ranging between 42.3 and 1140 mg/kg dry solids. Utsunomiya et al. (1989) reported the levels of QACs in river water and sediment samples from Japan. Levels of QACs in influent sewage, river water, and sediment were 0.10–0.15, 0.05 mg/L and 6.2–69 mg/kg dry solids, respectively. Sun

et al. (2003) studied the fate of QACs in a river running through Toyama City, Japan. They found that total influx of QACs into the river was 1.4 g/min, and the concentration was between 10 and 20 μg/L. The QACs in the sediment samples were 500 times higher than that found in the river water and averaged around 10 mg/kg dry solids. In another study, it was reported that DC_nDMA-Cl was present at 0.63 mg/L in surface water and 9.7 mg/kg dry solids (0–0.6 m depth) and 7.4 mg/kg dry solids (0.6–1.2 m depth) in the sediment at a pond that had been receiving untreated wastewater from a laundromat since 1962 (Federle and Schwab, 1992). Martinez-Carballo et al. (2007b) also measured QACs in sewage sludge and aquatic sediments in Austria and found average total QAC concentrations ranging between 22 and 103 mg/kg dry solids, and 0.06 and 6.8 mg/kg dry solids, respectively. Recently, the occurrence of QACs in wastewater-affected, upper-layer sediments collected from the urbanized lower Hudson River Basin (New York) was reported (Li and Brownawell, 2010). The mean, total QAC concentration in the estuarine sediments ranged from 0.98 to 114 mg/kg dry solids. Among a wide array of QACs detected, dialkonium halides with 12–18 alkyl chain carbons were the most abundant QACs, followed by C_{14}–C_{18} benzalkonium halides and C_{16} monoalkonium halides. This study also showed that QACs are the most dominant pollutants among all pollutants analyzed in the same

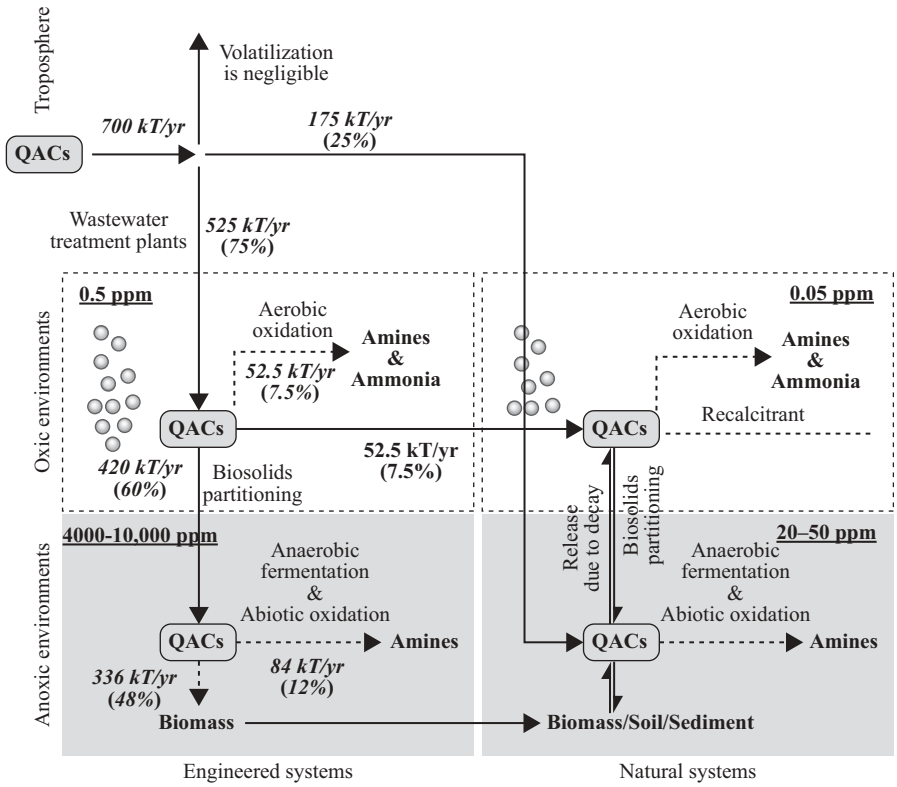


FIGURE 20.3 QAC fluxes and expected concentration levels in different compartments of engineered and natural systems based on reported global QAC consumption and literature data (see text).

sediments, including chlorinated pesticides, polyaromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs).

Based on the above-presented information, gathered by studies conducted between 1980 and 2010 on three continents, America, Europe, and Asia, it can be concluded that QACs are ubiquitous pollutants present in all environmental media. QACs are at milligram per liter levels in the untreated sewage, whereas they are found at microgram per liter and nanogram per liter levels in treated wastewater and surface water, respectively. Benzalkonium halides with a short alkyl chain are the most predominant QACs in aqueous samples. On the contrary, QACs sorb onto solids and accumulate in environmental compartments with high solids concentrations such as sewage sludge, digested sludge, and aquatic sediments at concentrations ranging from 1 to 10,000 mg/kg dry solids. The most abundant QACs found in solids-rich media are dialkonium and benzalkonium halides with long alkyl chains. QAC fluxes and expected QAC concentrations in engineered and natural systems are summarized in Figure 20.3.

20.4 TOXICITY AND MODE OF ACTION

The toxicity and inhibitory effect of QACs on microorganisms at community and species level is evaluated using metabolic assays such as oxygen uptake and substrate utilization assays and dose–response assays such as broth dilution and plate dilution assays, respectively. The toxicant concentration that affects certain portions of the microbial population, such as the concentration that affects 50% of the population (effective concentration, EC_{50}), or results in complete growth inhibition of the whole microbial population, such as the minimum inhibitory concentration (MIC), are used as the endpoints to describe the toxicity of a particular toxicant. Here we summarize the results of studies on the toxicity of QACs at community and species level and describe the possible modes of action of QACs resulting in the inhibition of microbial growth.

20.4.1 Community-Level Toxicity

About 75% of QACs utilized end up in wastewater treatment plants. The EC_{50} values for C_{16}TMA-Br and C_{12}BDMA-Cl obtained from a respirometric assay conducted with activated sludge ranged between 10 and 40 mg/L (Reynolds et al., 1987). The EC_{50} of $C_{14–18}$TMA-Cl for unacclimated activated sludge, determined based on the inhibition of [^{14}C] glucose uptake, was 28 mg/L (Larson and Schaeffer, 1982). Another study showed that DC_{10}DMA-Cl inhibited the chemical oxygen demand (COD) removal in a rotating biological contactor at concentrations above 20 mg/L and the biofilm was totally eliminated at 160 mg/L. The inhibitory effect of benzalkonium chlorides ($C_{12–}C_{16}$) was investigated at a concentration range from 5 to 20 mg/L and different biomass concentrations in an activated sludge system by Zhang et al. (2011). In this study, the inhibition of respiratory enzymes was identified as the mode of action of benzalkonium chlorides and the half-saturation competitive inhibition constant (K_I), which is equivalent to EC_{50}, of benzalkonium chlorides for the activated sludge tested ranged between 0.12 and 3.60 mg/L. The higher EC_{50} values, which represent lower toxicity, were recorded at higher biomass concentrations. Therefore, community-level EC_{50} values should be normalized to

the biomass concentration. Overall, these studies suggest that QACs are unlikely to result in significant toxicity on the microbial community associated with aerobic wastewater treatment systems at the QAC levels normally expected, unless the biomass concentration is very low. However, sudden QAC discharges resulting in transient high QAC levels in treatment plants could upset plant function.

On the other hand, many wastewater treatment plants practice biological nutrient removal, and they use anaerobic, anoxic, and aerobic biological units. A variety of physiologically different microorganisms participate in the wastewater treatment process; therefore, the response of each species to QAC inhibition is expected to be different. For instance, QACs are particularly toxic to nitrifiers. Benzalkonium chloride was inhibitory to a mixed, enriched nitrifying culture at 10–15 mg/L with a noncompetitive inhibition coefficient equal to 1.5 mg/L (Yang, 2007).

QACs have high affinity to adsorb onto biosolids. Generally, adsorption out competes biodegradation in aerobic biological treatment systems; therefore, QACs are transferred to anaerobic digesters as part of the primary and waste activated sludge (Boethling, 1994; Tezel et al., 2006; Ismail et al., 2010). It was reported that QAC concentrations may reach up to 50 mg/L in anaerobic digesters of sewage treatment plants (ECETOC, 1993; Garcia et al., 1999). QAC concentrations may exceed these levels in biological treatment systems of industrial facilities, such as food processing, that extensively use QACs. Under anaerobic conditions, there is no evidence of mineralization of QACs that contain alkyl or benzyl groups (Battersby and Wilson, 1989; Federle and Schwab, 1992; Garcia et al., 1999, 2000; Tezel et al., 2006, 2007), most likely because of the highly reduced nature of these substituent groups. Moreover, QACs are inhibitory to anaerobic microbial processes such as methanogenesis. Tezel et al. (2006) investigated the effect of four QACs—DC_8DMA-Cl, $DC_{8–10}$DMA-Cl, DC_{10}DMA-Cl, and $C_{12–16}$BDMA-Cl—on a mixed mesophilic fermentative/methanogenic culture. All QACs tested in this study had short- or long-term inhibitory effects on the mixed culture at 25 mg/L and above. Methanogenesis was more sensitive to QAC inhibition than acidogenesis. The inhibitory impact of the individual QACs on the methanogenic activity decreased according to the following series: DC_8DMA-Cl > $DC_{8–10}$DMA-Cl > $C_{12–16}$BDMA-Cl > DC_{10}DMA-Cl. Thus, QACs with the shorter alkyl chains are the most inhibitory. Moreover, it was concluded that the inhibitory effect of QACs was inversely proportional to their adsorption affinity on the biomass or their hydrophobicity (Tezel et al., 2006, 2007). Similar results were reported by Garcia et al. (1999).

About 25% of QACs utilized are discharged directly into the environment. The effect of monoalkonium and dialkonium QACs on the aquatic microbial communities in a lake ecosystem was investigated, and the results showed that QACs elicit ecologically significant responses at concentrations even below 1 mg/L and that mainly heterotrophic bacterial activity was affected (Ventullo and Larson, 1986). Tubbing and Admiraal (1991) examined the effect of DC_{18}DMA-Cl on natural populations of bacteria and phytoplankton from the lower River Rhine and reported significant decreases in the growth rate of bacterioplankton and in the photosynthetic rate of phytoplankton at a nominal DC_{18}DMA-Cl concentration between 0.03 and 0.1 mg/L. Nye et al. (1994) investigated the heterotrophic activity in a soil ecosystem treated with C_{16}TMA-Br. The addition of C_{16}TMA-Br to the soil resulted in increased lag periods and decreased rates and extents of mineralization of ^{14}C-labeled organic compounds, as a result of toxicity toward Gram-negative soil microorganisms.

20.4.2 Species-Level Toxicity

A more comprehensive set of toxicity data is available at the species level. Indicator organisms, such as *Vibrio fischeri*, *Daphnia magna*, and *Pseudomonas* sp., are used to test the impact of QACs on microbial biota in environmental media, whereas Gram-negative and Gram-positive pathogenic microorganisms are used to determine the efficacy of QACs in clinical settings.

The toxicity of 15 QACs (with molecular weights ranging between 313.5 and 547.0 g/mol) were investigated in four bioassays, such as Microtox, Spirotox, Protoxkit F, and Artotoxkit M, comprising a bacterium (*V. fischeri*), two ciliated protozoa (*Spirostomum ambiguum* and *Tetrahymena thermophila*), and an anostracean crustacean (*Artemia franciscana*) (Nalecz-Jawecki et al., 2003). The Microtox assay acute toxicity EC_{50} values for the tested QACs ranged between 0.6 and 50 μM (0.24 and 21.5 mg/L at the average QAC molecular weight of 430.25 g/mol). The results indicated that QACs had high toxicity against the bioindicators tested and were toxic not only to bacteria but also to nontarget protozoa and crustacea. It was also stated that the toxicity of QACs decreased as the alkyl chain length increases because the hydrophobicity of QACs with longer alkyl chain length increases, thus resulting in lower bioavailability and higher partitioning with organic or negative charged surfaces (Nalecz-Jawecki et al., 2003).

A detailed study investigating the acute toxicity of nine QAC homologs belonging to the monoalkonium, dialkonium, or benzalkonium chloride group and with different alkyl chain lengths was conducted using the standard Microtox assay (Tezel, 2009). The 5-min EC_{50} values of the target QACs ranged from 0.19 to 1.22 mg/L with a median at 0.38 mg/L ($n = 9$), whereas the 15-min EC_{50} values were between 0.12 and 0.66 mg/L with a median at 0.25 mg/L ($n = 9$) (Table 20.2). These results show that the toxicity of QACs increases as the exposure time increases,

TABLE 20.2 5- and 15-min EC_{50} Values of Monoalkonium, Dialkonium, and Benzalkonium Chlorides Based on the Standard Microtox Acute Toxicity Assay

	EC_{50}[a] (mg/L)	
Compound	5 min	15 min
Monoalkonium chlorides		
C_{12}TMA-Cl	0.26 [0.25–0.33]	0.19 [0.16–0.23]
C_{14}TMA-Cl	0.90 [0.87–0.94]	0.40 [0.38–0.43]
C_{16}TMA-Cl	1.22 [1.08–1.37]	0.56 [0.38–0.83]
Dialkonium chlorides		
DC_8DMA-Cl	0.20 [0.16–0.27]	0.12 [0.08–0.17]
$DC_{8–10}$DMA-Cl	0.28 [0.21–0.36]	0.17 [0.12–0.23]
DC_{10}DMA-Cl	0.49 [0.48–0.51]	0.25 [0.18–0.35]
Benzalkonium chlorides		
C_{12}BDMA-Cl	0.19 [0.14–0.27]	0.14 [0.09–0.21]
C_{14}BDMA-Cl	0.38 [0.31–0.48]	0.27 [0.20–0.36]
C_{16}BDMA-Cl	0.92 [0.73–1.16]	0.66 [0.50–0.86]

[a]Values in brackets are lower and upper 95% estimates.
Source: From Tezel (2009).

meaning that a certain exposure time is necessary for QACs to fully reach the target site(s) and/or for cells to actually react to the QACs. The EC_{50} values obtained in this study are consistent with previously reported values obtained using the same toxicity assay (Leal et al., 1994; Garcia et al., 2001; Nalecz-Jawecki et al., 2003; Sutterlin et al., 2008). The lowest EC_{50}, which indicates the highest toxicity, in each QAC group was for QACs with the shortest alkyl chain within a homologous group (i.e., C_{12}TMA-Cl, DC_8DMA-Cl, and C_{12}BDMA-Cl). On the other hand, the highest EC_{50} value was for QACs that have the longest alkyl chain within a homologous group (i.e., C_{16}TMA-Cl, DC_8DMA-Cl and C_{12}BDMA-Cl) (Table 20.2). According to these results, the toxicity of QACs forms the following series in descending order: C_{12}BDMA-Cl > DC_8DMA-Cl > C_{12}TMA-Cl > DC_{8-10}DMA-Cl > C_{14}BDMA-Cl > DC_{10}DMA-Cl > C_{14}TMA-Cl > C_{16}BDMA-Cl > C_{16}TMA-Cl, at 5-min exposure time. However, the order changed as the exposure time increased as follows: DC_8DMA-Cl > C_{12}BDMA-Cl > DC_{8-10}DMA-Cl > C_{12}TMA-Cl > DC_{10}DMA-Cl > C_{14}BDMA-Cl > C_{14}TMA-Cl > C_{16}TMA-Cl > C_{16}BDMA-Cl. Therefore, QACs with higher CMC values are more toxic (i.e., have lower EC_{50}values) than the ones with lower CMC (Tezel, 2009). These results show that the toxic effect of QACs is more likely not caused by the micelles but rather by the ionic interactions with the cell membrane constituents.

It is widely speculated that anions such as chloride, nitrate, bromide, and anionic organic constituents in the environmental media may decrease the toxicity of QACs by decreasing their bioavailability by physical and ionic interactions. Tezel (2009) tested the effect of various anions and natural organic matter (NOM) on the toxicity of benzalkonium chlorides at environmentally relevant concentrations and above. The results of this study showed that the presence of anions has no significant effect on the QACs toxicity. On the other hand, NOM decreased the toxicity of QACs especially for those with long alkyl chains, that is, the ones with low CMC and high log K_{ow} values.

Sutterlin et al. (2008) elucidated the effect of benzalkonium chlorides on *Pseudomonas putida*, an important Gram-negative soil bacterium. The EC_{50} of benzalkonium chlorides was 6 mg/L, and the toxicity was not significantly affected by the presence of linear alkyl sulfonate, an anionic surfactant and common detergent ingredient.

Among the environmentally significant microbial species, algae represent a group of organisms that are very sensitive to QACs. The EC_{50} values of C_nTMA-Br and C_nTMA-Cl for algae range between 0.03 and 0.38 mg/L (0.1 and 1.2 μM). On the other hand, EC_{50} values for dialkonium QACs range between 0.05 and 18 mg/L (0.1 and 34 μM); therefore the toxicity of dialkonium QACs is equal to or less than the toxicity of monoalkonium QACs on both mass and molar basis (Lewis, 1991; Utsunomiya et al., 1997). Benzalkonium QACs are toxic to aquatic organisms below 1 mg/L.

The MIC of benzalkonium chlorides and dialkonium chlorides for clinically important Gram-negative bacteria range between 45 and 700 mg/L and 4 and 250 mg/L, respectively. The MICs of the same QACs for Gram-positive pathogens are significantly lower than for Gram-negative bacteria, which are around 30 and 10 mg/L, respectively. As a result, clinically important Gram-negative and Gram-positive species are more tolerant of QACs than environmental microorganisms. Moreover, Gram-positive pathogens are more susceptible to QACs than Gram-negative pathogens due to the lack of an outer membrane (Table 20.3).

TABLE 20.3 Minimum Inhibitory Concentration of Benzalkonium Chlorides ($C_{12–16}$) and Dialkonium Chlorides (C_{10}) for Clinically Important Bacteria

	Minimum Inhibitory Concentration (MIC; mg/L)	
Microorganism	$C_{12–16}$BDMA-Cl	DC_{10}DMA-Cl
Gram negative		
Escherichia coli	100	25
Pseudomonas aeruginosa	700	250
Salmonella typhimurium	150	40
Proteus mirabilis	300	200
Campylobacter jejuni	45	4
Legionella pneumophila	80	30
Gram positive		
Enterococcus faecium	30	10
Staphylococcus aureus	40	10
Listeria monocytogenes	25	5

Source: Adapted from McDonnell and Russell (1999).

20.4.3 Mode of Action

QACs are lytic bioactive agents. The main mode of action of QACs against bacterial cells involves perturbation of the lipid bilayer of the bacterial cytoplasmic membrane and the outer membrane of Gram-negative bacteria. Such action leads to a progressive leakage of cytoplasmic components out of the cell and finally cell lysis.

Despite the fact that lysis is the major mode of action of QACs, it is only effective at and above CMC values (Heerklotz, 2008). Concentrations above the CMC kill cells by the disintegration of the membranes and the release of cytoplasmic contents and coagulation of proteins and nucleic acids. At the molecular level, action involves the association of the cationic quaternary nitrogen with the head groups of the acidic phospholipids within the membrane due to ionic interactions. The hydrophobic tail (alkyl groups) then integrates into the lipid core with hydrophobic interactions. Such interactions increase the surface pressure in the exposed layer of the membrane and decrease membrane fluidity. The membrane undergoes a transition from fluid to liquid crystalline state and loses its osmoregulatory and physiological functions. As a result, the fragile cell membrane disintegrates and the cell is ruptured (Maillard, 2002; Gilbert and Moore, 2005). Intermediate levels of QACs inhibit membrane-located processes such as respiration, solute transport, and cell wall biosynthesis. Low concentrations of QACs bind to anionic sites found on the membrane surface, cause the cells to both lose osmotic regulation capability and to leak potassium ions and protons. In addition, inhibition of respiratory enzymes and the dissipation of proton motive force (PMF), which affect the microbial metabolism, active transport, oxidative phosphorilation, and adenosine triphosphate (ATP) synthesis in bacteria, are other modes of action at low QAC concentrations (Knox et al., 1949; McDonnell and Russell, 1999; Maillard, 2002).

In summary, QACs are inhibitory to microorganisms at both the community and species level. QACs do not pose a risk to microbial communities present in conventional aerobic biological treatment units at concentrations present in the wastewater and at typical biomass concentrations. However, the potency of QACs increases in relatively dilute microbial communities. For instance, QACs are more

toxic to microbial communities in natural systems, for example, rivers, lakes, and the like. Therefore, as a result of the effect of the environmental matrix on the fate, behavior, and thus bioavailability of QACs, their toxicity should be described based on models that account for all possible interactions. Studies using standard toxicity assays based on indicator microorganisms such as *V. fischeri*, have reported EC_{50} values less than 1 mg/L for QACs. In view of the above comments relative to matrix effects related to the toxicity of QACs, these reports should include auxiliary and relevant information on the medium composition (e.g., dissolved organic matter, biomass concentration, etc.). Toxicity is well correlated with the physicochemical properties of QACs and increases with increasing CMC. As a result, benzalkonium chlorides are the most toxic QACs tested followed by dialkonium and monoalkonium chlorides. The lowest EC_{50}, which indicates the highest toxicity, was found to be for QACs with the shortest alkyl chain length within a homologous group. QACs with high CMCs are more mobile in engineered and natural systems than the ones with lower CMCs. On the contrary, QACs with a high CMC were more toxic than those with low CMCs. The combination of these two facts brings about a detrimental environmental effect associated with QACs, that is, QACs that are more mobile and bioavailable present a higher risk. As a result, microorganisms present in oxic engineered and natural systems are exposed to the more toxic QACs than the microorganisms present in anoxic systems. In both cases, the exposure concentration is typically less than the CMC and, therefore, the potential mode of action of QACs is related to the loss of membrane osmoregulation, inhibition of respiratory enzymes, and the dissipation of proton motive force.

20.5 RESISTANCE TO QACs

Microorganisms present in QACs-bearing environmental media are exposed to QACs at subinhibitory concentrations. Resistance mechanisms are, therefore, developed mainly to avoid penetration of QACs into the cell by passive diffusion, to expel the QACs from the cell before damage to sensitive cell organelles or genetic material occurs, or to eliminate the QACs at the relatively low concentration levels. The resistance mechanisms to QACs are discussed below.

20.5.1 Intrinsic Resistance

Intrinsic resistance can be described as a relative tolerance of a species or a genus to an antimicrobial agent due to its phenotypic, physiological, or biochemical properties. Phenotypic properties that confer intrinsic resistance to QACs include advanced membrane permeability barriers and chromosomaly transcribed efflux pumps.

At low concentrations, QACs diffuse into the cell through the outer cell layers such as the cell wall (Gram-negative bacteria) and cytoplasmic membrane. The nature and the composition of the outer layers depend on the phenotype, and may act as a permeability barrier to QACs. For instance, presence of an outer membrane surrounding the cell membrane makes Gram-negative bacteria less susceptible to QACs than the Gram-positive bacteria (Table 20.3). In addition, complex lipid-containing cell walls, less acidic outer membrane lipopolysaccharides (LPS), small porins resulting from strong LPS–LPS links, fewer porins, and a slime layer are

physiological traits that confer tolerance to QACs (McDonnell and Russell, 1999; Hegstad et al., 2010).

Intrinsic resistance to QACs is also conferred by the basal level activity of chromosomally encoded, nonspecific efflux pumps. These efflux determinants generally confer resistance to multiple agents, including clinically important antibiotics, and have physiological roles such as conferring resistance to natural substances produced by the host, including bile, hormones and host-defense molecules, as well as colonization and persistence of bacteria in the host. These determinants include mainly resistance nodulation division (RND) family efflux pumps, such as SdeXY, AcrAB-TolC, and MexAB-OprM (Piddock, 2006; Hegstad et al., 2010). These pumps result in the extrusion of QACs from the cell at basal level (Gilbert and McBain, 2003; Daniels and Ramos, 2009). AcrAB-TolC of *Escherichia coli* and MexAB-OprM of *Pseudomonas aeruginosa* are very important multidrug efflux determinants that confer intrinsic resistance to QACs. Mc Cay et al. (2010) reported that growth of *P. aeruginosa* NCIMB 10421 as a continuous culture with a dilution rate equal to 0.04 h^{-1}, fed with a glucose-limited medium amended with benzalkonium chloride (BAC) at 12.5 mg/L over 33 generations, resulted in the development of isolates with increased MIC from 25 to >350 mg BAC/L, that is, a >12-fold decrease in sensitivity to BAC. The increased resistance to BAC by these isolates was contributed to efflux pump activity and attributed to the overexpression of both MexAB-OprM and MexCD-OprJ determinants of the Mex efflux system.

Homology analysis for MexAB-OprM protein within 3399 whole genome sequences revealed that homologs of this protein are present not only in clinically important species but also in environmental microorganisms (www.microbesonline.org) (Fig. 20.4). As a result, efflux-mediated intrinsic resistance is evidently a mechanism to tolerate QACs at environmentally relevant concentrations.

20.5.2 Acquired Resistance

Relative changes in the tolerance to QACs upon sublethal exposure are achieved by temporary modification of the outer membrane, cell membrane, and the density and structure of porins, regulatory hyperexpression of efflux pumps through oxidative stress or (followed by) stress-induced mutagenesis, and acquisition of QAC-specific efflux pumps through mobile recombinational elements, such as plasmids and integrons. In addition, simultaneous development and/or acquisition of two or more resistance mechanisms by a single microorganism has been well documented (Nagai et al., 2003).

Many aerobic and facultative microorganisms acquire resistance to QACs by reducing the permeability of the outer cell layer (Denyer and Maillard, 2002) by changing the composition of the cell membrane fatty acids, phospholipids, and outer membrane lipopolysaccharides (Chaplin, 1952; Guerin-Mechin et al., 1999, 2000; Dubois-Brissonnet et al., 2001; Loughlin et al., 2002; Boeris et al., 2007). With these modifications, the cell surface becomes more anionic and hydrophobic, restricting easy passage of the QACs through the cell surface. Other mechanisms to avoid the intrusion of QACs into the cell are density reduction and composition change of the porins (Ishikawa et al., 2002), as well as change of the outer membrane protein composition (Loughlin et al., 2002; Tabata et al., 2003).

The efflux-mediated QAC resistance has gained significant interest because it has a genetic origin, confers co-resistance to antibiotics, and is transferable among

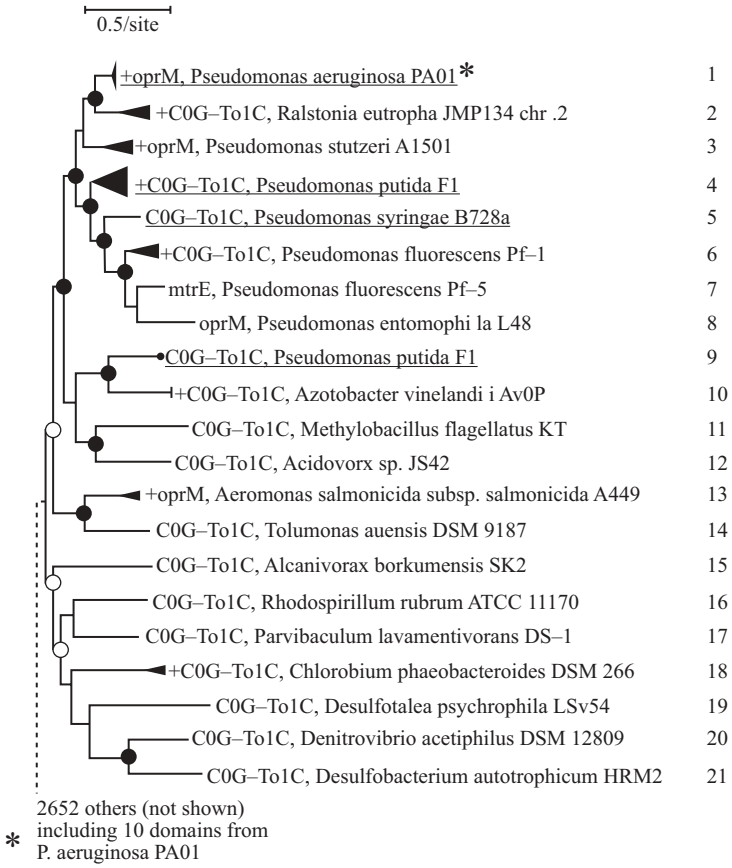


FIGURE 20.4 Gene homology tree for MexAB–OprM of *Pseudomonas aeruginosa* PAO1.

microbial species through horizontal gene transfer. Multidrug efflux pumps mediate the transfer of biocides from the inside to the outside of the cell through an energy or proton-dependent mechanism (Paulsen et al., 1996; Poole, 2007). There are five classes of multidrug efflux systems: small multidrug resistance (SMR) family, drug/metabolite transporter (DMT) superfamily, major facilitator superfamily (MFS), ATP-binding cassette (ABC) family, resistance–nodulation–division (RND) family, and the multidrug and toxic compound extrusion (MATE) family (Putman et al., 2000). Efflux determinants belonging to these families that confer resistance to QACs are given in Table 20.4.

QAC resistance via efflux pumps follows two mechanisms. By the first mechanism, QAC resistance is induced by overexpression of efflux pumps upon exposure to QAC or QAC-induced stress. Such stress either triggers a regulatory system that controls the expression of an efflux determinant or causes a mutation in the genetic element resulting in the overexpression of the efflux determinant or increase in its extrusion efficiency (Grkovic et al., 2002). These efflux pumps are generally chromosomally encoded and act against a wide array of antimicrobial agents. Examples of these efflux pumps include NorA and QacA/B of Gram-positive bacteria and AcrAB-TolC and MexAB-OprM of Gram-negative bacteria. Overexpression of

TABLE 20.4 Efflux Determinants Conferring QAC Resistance[a]

Efflux Family	Efflux Proteins Extruding QACs	Typical Antibiotic Substrate
RND	YhiUV-TolC, AcrAB-TolC, MexAB-OprM, CmeABC, CmeDEF, SdeXY, OqxAB	Aminoglycosides, β-lactams, chloramphenicol, erythromycin, fluoroquinolones, novobiocin, rifampin, tetracyclines, trimethoprim
MFS	*QacA, QacB, NorA, NorB, MdeA*, EmeA, MdfA	Aminoglycosides, chloramphenicol, erythromycin, fluoroquinolones, lincosamides, novobiocin, rifampin, Tetracyclines
MATE	*MepA*, NorM, PmpM	Aminoglycosides, fluoroquinolones
SMR	**QacE, QacEΔ1, QacF, QacG, QacH, QacI, QacJ, smr, EmrE,** SugE	Aminoglycosides, chloramphenicol, erythromycin, tetracyclines

[a]Italic, Gram-positive; roman, Gram-negative; bold, both.
Source: Adapted from Poole (2005).

these efflux pumps results in a two- to eight-fold increased tolerance of the (mutant) microorganism to QACs and other substrates of these pumps (Grkovic et al., 2002; Gilbert and McBain, 2003; Hegstad et al., 2010).

QacA/B of *Staphylococcus aureus*, which belongs to MFS, utilizes the proton motive force to drive the efflux of more than 30 different toxic monovalent or divalent, cationic, lipophilic compounds that belong to 12 distinct chemical classes. Expression of QacA/B is regulated by QacR, a DNA-binding repressor protein. In the absence of QacA/B substrates, QacR binds to the DNA-binding site and downregulates the transcription of QacA/B. The QacA substrates are ligands to QacR. When a substrate is present, it binds to QacR and restricts its binding to the DNA-binding site. As a result, repression of the transcription of QacA/B is inhibited, and, therefore, the QacA/B is overexpressed (Brown and Skurray, 2001; Grkovic et al., 2002; Saidijam et al., 2006;). QacA/B-mediated QAC resistance in clinical *S. aureus* strains has been demonstrated in many studies (Smith et al., 2008; Vali et al., 2008). On the contrary, neither the QacA/B determinant nor the QacR-type regulons have yet been found in environmental media (Gillings et al., 2009a, 2009b; Schluter et al., 2007).

The AcrAB-TolC efflux determinant, which belongs to the RND family, is another multidrug efflux system responsible for resistance to dyes, detergents, fluoroquinolones, and many other lipophilic antibiotics, for example, β-lactams, chloramphenicol, erythromycin, and tetracycline, as well as QACs. This efflux determinant and its homologs are highly abundant in Gram-negative bacteria. The AcrAB-TolC efflux system is regulated by three homologous global activators: MarA, SoxS, and Rob. In particular, SoxS, the effector of the *soxRS* global superoxide response (*sox*) regulon, which regulates overexpression of the AcrAB-TolC efflux system, is of interest to understand the resistance to QACs, as well as many antibiotics, at subinhibitory concentrations (Grkovic et al., 2002). QACs result in oxidative stress at low concentrations. Bore et al. (2007) investigated the changes in *E. coli* K-12, which gradually adapted to a tolerance level of 7–8 times the initial MIC at the transcriptomic and proteomic levels. They found that benzalkonium chloride (BAC) treatment might result in superoxide stress in *E. coli*. Therefore, *E. coli* K-12

acquired higher tolerance to BAC mainly by overexpression of efflux determinants regulated by Mar and SoxS activators. In addition, superoxide stress also facilitates prompt mutagenesis in multidrug efflux pumps or pump regulation systems of microorganisms exposed to subinhibitory levels of antimicrobial agents. The reactive oxygen species (ROS)-related mutation induced by antimicrobial agents results in the overexpression of multidrug efflux pumps and active excretion of the agent(s) (Kohanski et al., 2010a). AcrAB-TolC and its regulons, as well as the homologs of these determinants, conferring multidrug resistance are mainly associated with plasmids and widespread in the environment (Schluter et al., 2007).

In addition to the MFS and RND family multidrug efflux pumps, there are other efflux pumps belonging to the SMR family that confer resistance to QACs. Among them, EmrE and smr are multidrug efflux pumps, whereas QacE, QacΔE, QacF, QacG, QacH, QacI, and QacJ are QAC-specific efflux determinants. The genes of these efflux proteins are mainly associated with mobile genetic elements such as plasmids and integrons. As a result, they can be horizontally transferred between microorganisms of the same or different genera; therefore, these genes are very abundant in the environment (Gaze et al., 2005; Schluter et al., 2007; Gillings et al., 2009a, 2009b).

Gillings et al. (2009b) recently suggested that QACs play a significant role in the evolution and stabilization of clinically important class 1 integrons, which are mobile recombinational elements that acquire and express antimicrobial resistance genes. Class 1 integrons (Fig. 20.5) are composed of a 5′ conserved region consisting of an integrase gene, *int1*, encoding a site-specific recombinase, an *attI* site where cassettes are integrated, and a promoter, P_{ant}, that regulates the expression of gene cassettes. The antibiotic resistance genes that integrons capture are located on gene cassettes. Gene cassettes contain a protein-coding region and a recombination site known as a 59-be site, *attC*, which is responsible for the orientation of integration. The cassettes exist as free, circular DNA but cannot be replicated or transcribed in this form. A recombination event occurs between *attI* and *attC*, integrating the cassette into the integron (Fig. 20.5). The gene on the cassette is then

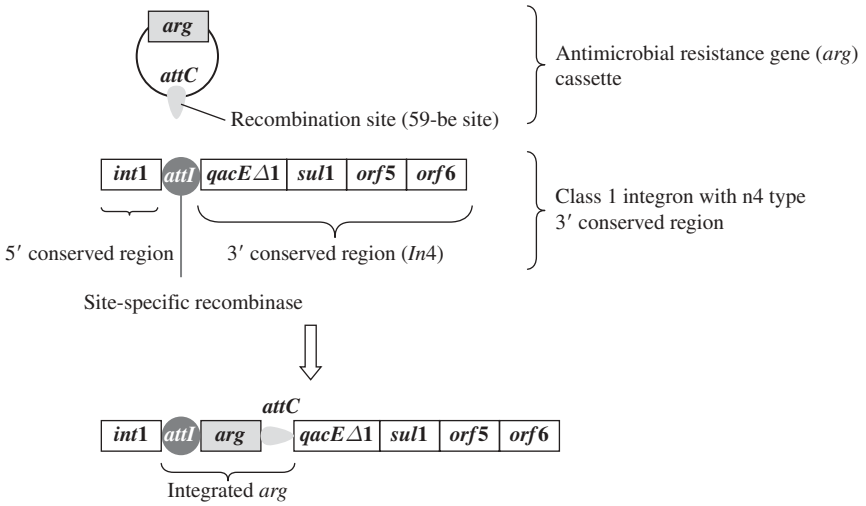


FIGURE 20.5 General structure of a class 1 integron and a gene cassette and the mechanism of antibiotic resistance gene insertion into a class 1 integron.

bound by the *attI* site on the 5′ conserved region and by *attC* on the 3′ conserved region. The 3′ conserved region of an integron may have one of three backbone structures. The first backbone type consists of a Tn*402* (In*16*)-like arrangement consisting of a *tni* module containing three transposition genes and a resolvase gene. The second, In*5* type consists of *qacEΔ1, sul1* (sulfonamide resistance gene), *orf5, orf6* (open reading frames), and a partial *tni* module, *tni*Δ, consisting of two transposition genes. The third, In*4* type carries just *qacEΔ1, sul1, orf5*, and *orf6* (Partridge et al., 2002; Gaze et al., 2005). The presence of genes that confer resistance to QACs and sulfonamides, which were introduced into the market at the same time and before any other antimicrobial agent, in the 3′ conserved region of the class 1 integrons may confirm the aforementioned hypothesis of Gilling et al. (2009b). Presence of *qac* genes and class 1 integrons in a microorganism usually confers resistance to QACs for both Gram-negative and Gram-positive bacteria (Kazama et al., 1998a, and 1998b; Gilbert and McBain, 2003; Smith et al., 2008; Hegstad et al., 2010).

20.5.3 Biotransformation as a Quasi-Resistance Mechanism

A recent discovery by Dantas et al. (2008) revealed that almost all of the clinically important antibiotics can be biotransformed by microorganisms present in the environment. This discovery elicits several questions: Is biotransformation an antimicrobial resistance mechanism? and What is the role of biotransformation in the dissemination of antimicrobial resistance in the environment?

The first question has two conditional answers. The answer is "Yes" if the biotransformation mechanism is controlled by a regulation system induced against the antimicrobial agent, for instance, β-lactamase. The answer is "No" if a species needs an additional resistance mechanism to survive while utilizing the antimicrobial agent. In this case, the highest concentration at which antimicrobial biotransformation stops generally coincides with the MIC.

The "No" answer can be "Yes" under two conditions. If the presence of an antimicrobial agent results in a rapid mutation evolving a resistant mutant of the test species, which is capable of biotransformation of the particular antimicrobial agent, the mutant present in low quantities in the population would utilize the antimicrobial agent and reduce its concentration to a level that the nonmutant species can tolerate. As a result, the species tolerance to the particular agent would be recorded higher than its actual tolerance. Such an event would result in misinterpretation of the susceptibility of a species to a particular antimicrobial agent. Thus, how often is the concentration of a test antimicrobial agent at the end of the incubation of a broth dilution test for determining the MIC measured and reported?

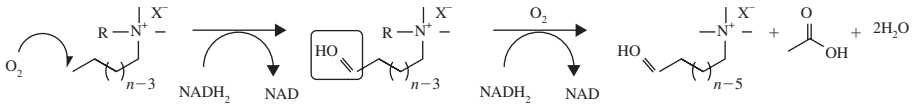
The second condition is more relevant to the microbial communities in environmental media. A microbial community may contain various species with different levels of susceptibility to a certain antimicrobial agent. Presence of a species with a moderate/high tolerance to that antimicrobial agent and also capable of biotransformation of the antimicrobial agent in a microbial community would drastically reduce the inhibitory effect of the antimicrobial agent on the community as a whole. In addition, a reduced antimicrobial concentration due to biotransformation in a community may result in subinhibitory level exposure of susceptible species to the antimicrobial agents, which may facilitate the development of resistance by those species through the above-described mechanisms.

Biotransformation, whether acting as a resistance mechanism or not, plays a significant role in the fate of antimicrobial agents and, therefore, affects the dynamics of antimicrobial resistance in the environment. In this section, we compile the very limited information available on the biotransformation of QACs and examine the role of QAC biotransformation on QAC resistance in the environment.

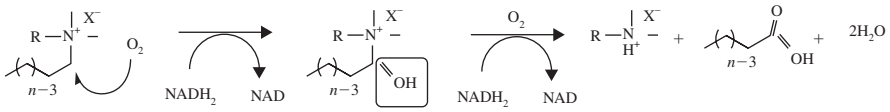
Most uses of QACs lead to their release into wastewater treatment systems or directly into the environment. The fate of QACs in aerobic biological treatment systems and receiving waters has been studied, and the results of these studies have been reviewed extensively (Boethling, 1994; van Ginkel, 1996). These studies indicate that QACs are degraded under aerobic conditions, and up to 90% of QAC removal by means of biodegradation is reported in engineered and natural systems. The half-lives for aerobic ultimate degradation of QACs vary extensively from hours to months, depending on the QAC concentration and structure, microbial acclimation, and presence of QAC resistant/degrading microorganisms. The alkyl chain length not only determines the physical/chemical properties of the QACs, but also controls the fate and effect of these compounds in the environment. Under aerobic conditions, the biodegradability of QACs generally decreases with the number of alkyl groups as $R_4N^+ < R_3MeN^+ < R_2Me_2N^+ < RMe_3N^+ < Me_4N^+$. Moreover, substitution of a methyl with a benzyl group can decrease biodegradability further (Ying, 2006). A comparison of the degradation rates of benzalkonium chlorides and monoalkonium bromides under aerobic conditions was undertaken. The rate of degradation of C_{12}BDMA-Cl, C_{14}BDMA-Cl and C_{16}BDMA-Cl, and C_{12}TMA-Br, C_{14}TMA-Br, and C_{16}TMA-Br was inversely related to the length of the alkyl group (C_n) and the presence of a benzyl group decreased the rate as well. Indeed, C_{16}BDMA-Cl was the most recalcitrant of the tested compounds, with an extent of only 30% degradation after 10 days. Likewise, it was reported that the aerobic degradation of QACs was dependent on the length of the alkyl group; however, the number of alkyl groups had a more pronounced effect on biodegradability. For example, dialkonium QACs were degraded five times slower than monoalkonium QACs (van Ginkel and Kolvenbach, 1991).

Certain microorganisms that are capable of QAC degradation have been isolated, such as *Xanthomonas* (Dean-Raymond and Alexander, 1977), *Pseudomonas* B1 (van Ginkel et al., 1992), *Pseudomonas fluorescens* TN4 (Nishihara et al., 2000), *P. fluorescens* F7 and F2 (Nishiyama and Nishihara, 2002), *Aeromonas hydrophila* sp. K (Patrauchan and Oriel, 2003), *Pseudomonas* spp. strain 7-6 (Takenaka et al., 2007), and *P. putida* A ATCC 12633 (Liffourrena et al., 2008, 2009), which were isolated from either sewage or soil.

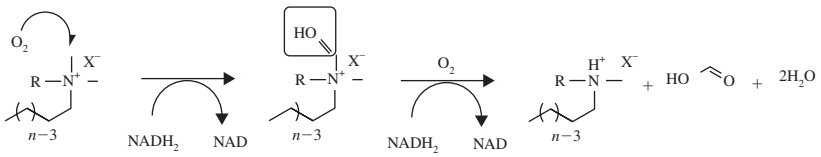
Three biotransformation pathways, which differ in terms of the location of the initial attack on the molecule, have been observed for monoalkonium, dialkonium, and benzalkonium chlorides (Fig. 20.6): (a) hydroxylation of terminal C (ω-hydroxylation), which is not adjacent to the central N, followed by multiple β-oxidation cycles, progressing toward the hydrophilic moiety, resulting in the release of two carbons in each β-oxidation cycle from the alkyl chain of a QAC; (b) hydroxylation of C that is adjacent to the central N (α-hydroxylation), followed by the central fission of the molecule resulting in the separation of the hydrophobic from the hydrophilic moiety; and (c) hydroxylation of the methyl-C attached to the central N, followed by the fission of the methyl group from the molecule. Activation of the C–H bond in the alkyl/methyl group of a QAC commences possibly with nicotinamide adenine dinucleotide H



A. ω-hydroxylation of terminal C of alkyl group



B. α-hydroxylation of C adjacent to N of alkyl group



C. α-hydroxylation of C of methyl group

FIGURE 20.6 Initial steps in the aerobic biotransformation of QACs.

(NADH)-dependent hydroxylation by a monooxygenase enzyme in the presence of oxygen. Activation of the alkyl/methyl group of a QAC is very similar to the activation of alkanes under aerobic conditions, which is predominantly achieved by the *Pseudomonas* genus of Bacteria (van Beilen and Funhoff, 2005).

A bacterium, *Xanthomonas* sp., capable of utilizing the monoalkonium QAC C_{10}TMA-Br as the sole carbon and energy source was isolated (Dean-Raymond and Alexander, 1977). The products of C_{10}TMA-Br biotransformation were identified as 9-carboxynonyl and 7-carboxyheptyl trimethyl ammonium. Identification of these carboxyalkyl trimethyl ammonium salts confirms that C_{10}TMA-Br biotranformation proceeds by ω-hydroxylation followed by β-oxidation of the alkyl moiety [pathway (A), Fig. 20.6). Pathway (A) is the first QAC degradation pathway discovered. In another study, a bacterium, tentatively identified as *Pseudomonas sp*., which grew on monoalkonium chlorides with alkyl chain lengths ranging from 12 to 18 carbons, was obtained through enrichment in a continuous culture inoculated with activated sludge. Growth on monoalkonium chlorides resulted in the production of trimethylamine. The transformation of monoalkonium chlorides was therefore proposed to follow NADH-dependent, α-hydroxylation of the alkyl moiety, followed by a central fission of the C_{alkyl}–N bond (dealkylation) [pathway (B), Fig. 20.6) (van Ginkel et al., 1992). The aldehyde formed as a result of the chemical hydrolysis of the C_{alkyl}–N bond was converted into an alkanoate, which underwent successive β oxidation resulting in the complete oxidation of the alkyl chain. However, the biotransformation of trimethylamine was not achieved with this strain. C_{alkyl}–N fission as the first degradation step of monoalkonium QACs by a mixed culture of microorganisms, as well as various species of the genus *Pseudomonas* was demonstrated in many studies (Nishiyama et al., 1995; Nishiyama and Nishihara, 2002; Liffourrena et al., 2008,

2009). In contrast to the previous findings, however, a recent study proposed the degradation of monoalkonium QACs, that is, C_{12}TMA-Br, by *Pseudomonas sp*. strain 7-6, isolated from a wastewater treatment plant, via dual pathways. Besides the fission of the C_{alkyl}–N bond, *Pseudomonas sp*. strain 7-6 initiates degradation via hydroxylation of the methyl group and cleavage of the C_{methyl}–N bond [demethylation, pathway (C), Fig. 20.6) (Takenaka et al., 2007).

The biotransformation of dialkonium QACs by *P. fluorescens* TN4 (Nishihara et al., 2000) and *Achromobacter sp*. (van Ginkel, 2004) was found to occur in a similar fashion, by two consecutive dealkylations, resulting in the formation of dimethyl amine as the end product.

None of the isolates described above was able to grow on the non-alkyl-containing amines, such as trimethyl amine and dimethyl amine, after dealkylation, most probably due to the lack of methylmonooxygenases. In fact, symbiosis of at least two species is necessary for a complete mineralization of monoalkonium and dialkonium QACs to ammonia and carbon dioxide under aerobic conditions (Kim et al., 2001; Kroon and van Ginkel, 2001).

A biotransformation pathway of benzalkonium chlorides by *A. hydrophila* sp. K was recently reported (Patrauchan and Oriel, 2003). This pathway is similar to that of mono- and dialkonium chlorides and commences with a dealkylation step resulting in the formation of benzyl dimethyl amine (BDMA) as the first intermediate. This bacterium is also capable of growing on BDMA as the sole carbon and energy source and converts it to benzyl methyl amine, benzyl amine, and ammonium by following two demethylations and a debenzylation, respectively. On the other hand, van Ginkel (2004) demonstrated an alternative benzalkonium chloride biotransformation pathway in a mixed culture: Benzalkonium chlorides are transformed into benzyl dimethyl amine, dimethyl amine, and ammonia following consecutive dealkylation, debenzylation, and demethylation steps, and three microorganisms are involved that utilize the alkyl chain, the aromatic moiety, and the dimethyl amine, respectively (van Ginkel 2004). The same pathway of benzalkonium chloride degradation was demonstrated in a microbial community growing on benzalkonium chloride, which was composed mainly of *Pseudomonas* species by Tezel (2009). The most dominant phylotype, BAC54, was similar to *Pseudomonas nitroreducens* (99% similarity) and other phylotypes were similar to *P. putida* (98–99% similarity).

In spite of the fact that the energetic burden of the above-discussed QAC biotransformation pathways (initial attack) is the same, pathway (B) starting with the cleavage of the C_{alkyl}–N bond is the most predominant one. Although, pathway (A) was the first QAC biotransformation pathway identified, it could not be demonstrated in later studies for similar QACs. Moreover, the product of pathway (B), a tertiary amine, is less toxic than the products of pathways (A) and -(C) (Tezel, 2009). The combination of these facts suggests that pathway (B) is naturally selected as a mechanism for QAC biotransformation to cope with the toxic effects of QACs and to eliminate the detrimental consequences of other biotransformation mechanisms. As a result, QAC biotransformation may have evolved as a QAC resistance mechanism in the environment.

The enzymes catalyzing the biotransformation of different QACs may be identical in spite of the different QAC structures they attack and are inducible. Tezel (2009) showed that the biotransformation of C_{14}BDMA-Cl and C_{14}TMA-Cl in resting cells obtained from a mixed community growing on benzalkonium chloride in a fed-batch

mode accelerated upon second amendment of the QACs at the same initial molar concentration. As soon as the QAC was totally removed and the cells were in the resting state, QAC degradation was slower at the first amendment of QACs to the community than the second QAC amendment to the same community (Fig. 20.7).

If biotransformation is a QAC resistance mechanism and is depressed in the absence of QACs, a species or a community growing in the presence of a QAC would be more tolerant to QACs when QAC is still present in the media than when the QAC is completely utilized. The MIC for benzalkonium chloride (BAC) of a BAC-degrading mixed microbial community after BAC was eliminated from the culture media decreased substantially from 198 to 92 mg/L after the BAC was removed from the microbial community from which the inoculum was taken (Table 20.5) (unpublished data). Similarly, Langsrud and Sundheim (1997) showed that *Pseudomonas* isolates retrieved from poultry carcasses that were able to grow at a BAC concentration of 200 mg/L lost their resistance to BAC within 4–8 hours after the removal of BAC. On the contrary, Gilbert and McBain (2003) attributed the resistance observed in *Pseudomonas* isolates to the action of efflux pumps regulated by the presence of BAC.

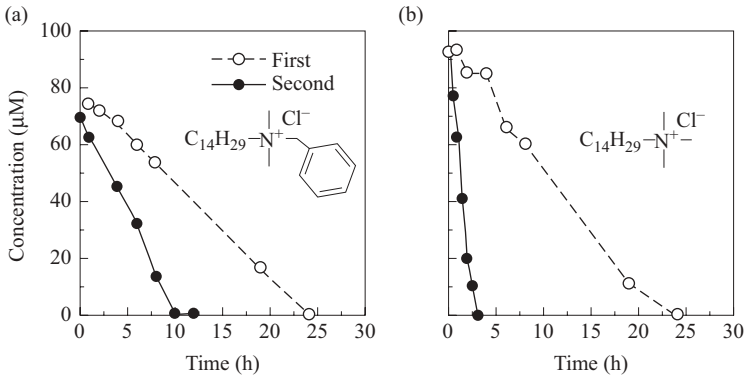


FIGURE 20.7 Profiles of (a) C_{14}BDMA-Cl and (b) C_{14}TMA-Cl utilization by resting cells taken from a mixed community growing on benzalkonium chloride in a fed-batch reactor after first and second QAC amendment.

TABLE 20.5 Mixed Microbial Community Conditions and BAC MICs Measured at Different BAC Concentrations over Time

Culture Age (months)[a]	Dry Organic Matter (g/L)	BAC (mg/L)	MIC mg/L	IC_{50}mg/L
0	2.4 ± 0.1	144 ± 3	198	100
0.5	1.2 ± 0.1	25 ± 0.3	169	84
1.0	0.7 ± 0.1	ND[c]	92	46
3.0	2.2 ± 0.02	ND	100	31
36[b]	2.9 ± 0.05	ND	100	30

[a]Time after a dextrin/peptone and BAC-degrading microbial community was maintained fed every 3.5 days with a dextrin/peptone mixture and no BAC.
[b]Control culture with the same origin, fed every 3.5 days with a dextrin/peptone mixture and no BAC.
[c]ND, not detected.

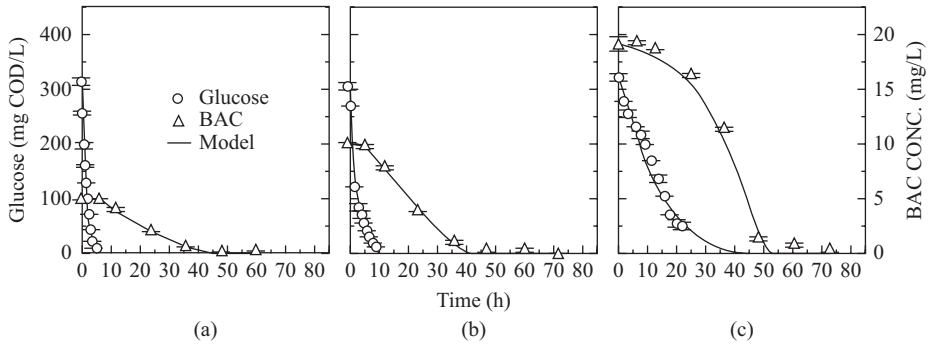


FIGURE 20.8 Profiles of glucose and BAC utilization by a microbial community at (a) 5 mg/L, (b) 10 mg/L, and (c) 20 mg/L initial BAC concentrations.

Many physical and chemical factors affect the biotransformation of QACs in the environment. These factors include the presence of readily biodegradable substrates, anionic organic compounds, and adsorption. Zhang et al. (2011) investigated the inhibitory effect and biodegradation of BAC in a microbial community derived from activated sludge. They showed that the presence of glucose, a readily biodegradable substrate, delayed the utilization of BAC (Fig. 20.8). Degradation of BAC could only begin after a major fraction of glucose was depleted. At high concentrations, BAC resulted in the inhibition of glucose utilization, which in turn resulted in an extended delay in BAC biodegradation. As a result, QAC degradation did not begin as long as a readily biodegradable substrate was available. In a similar study investigating the factors affecting the biotransformation of a monoalkonium chloride, C_{12}TMA-Br, by *P. fluorescens* F7 revealed that glucose halted the biodegradation of the QAC, whereas the presence of anionic organic compounds, such as fatty acids and sodium dodecyl sulfate, enhanced its biodegradation (Nishiyama and Nishihara, 2002).

QACs rapidly and strongly sorb onto a wide variety of materials of environmental relevance such as biomass, sediment, clay, and minerals. Zhang et al. (2011) showed that biomass-associated BAC was degraded 20 times slower than the BAC in the liquid phase (bioavailable fraction). Sorption to sediment, clay, and inorganic minerals was observed to retard and/or even prevent the biotransformation of QACs (Boethling, 1984).

In natural and engineered biological systems, sorption generally out competes biodegradation in aerobic environments, and, therefore, QACs are transferred to anoxic/anaerobic compartments such as anaerobic digesters, as part of the primary and waste activated sludge, and aquatic sediments (Boethling, 1994). Under anaerobic conditions, there is no evidence of mineralization of QACs that contain alkyl or benzyl groups (Battersby and Wilson, 1989; Federle and Schwab, 1992; Garcia et al., 1999, 2000), most likely because of the highly reduced nature of these substituent groups. Moreover, QACs are inhibitory to anaerobic microbial processes such as methanogenesis (Battersby and Wilson, 1989; Garcia et al., 1999, 2000; Tezel et al., 2006, 2007).

On the other hand, esteralkonium chlorides, recently developed analogs of dialkonium chlorides, were reported to be completely degraded by anaerobic digester sludge in a standard test based on biogas formation (Giolando et al., 1995).

Esteralkonium chlorides differ structurally from dialkonium chlorides by the inclusion of two ester linkages between the ethyl and alkyl chains. These ester linkages allow esteralkonium chlorides to be rapidly and completely degraded in standard laboratory screening tests and a range of environmental media such as sludge, soil, and river water with half-lives ranging from 0.8 to 18 days. Likewise, it is known that natural QACs, such as choline and betaine, can be ultimately degraded under anoxic/anaerobic conditions (Neill et al., 1978; King, 1984). As a result, QACs in which the hydrophobic moieties are linked to the head group with ester bonds, choline, betaine (natural QACs), and those in which alkyl chains are linked directly to N^+ have a different fate under anoxic/anaerobic conditions. The latter are recalcitrant under these conditions.

Recently, Tezel and Pavlostathis (2009) showed the transformation of benzalkonium chlorides under nitrate-reducing conditions. In an assay conducted to investigate the effect and transformation potential of BACs under nitrate-reducing conditions at BAC concentrations up to 100 mg/L using a mixed microbial community, BAC inhibited nitrate reduction at and above 50 mg/L, resulted in transient accumulation of nitrite, and inhibited nitrous oxide to dinitrogen conversion. Nitrite, which transiently accumulated in the media because of the BAC inhibition, resulted in the transformation of BAC via a mechanism called *modified Hofmann degradation* (Fig. 20.9). The transformation of BAC proceeded through the cleavage of the C–N bond between the benzyl and alkyl dimethyl amine groups. The nucleophile nitrite attacked the benzyl carbon adjacent to the quaternary nitrogen atom resulting in the cleavage of the C–N bond and the formation of alkyl dimethyl amines. Gas chromatography–mass spectroscopy (GC–MS) analysis of samples obtained from systems used in this study, and in which BAC was transformed, showed the presence of benzonitrile, indicating the potential of nitromethyl benzene formation as an intermediate during the nucleophilic substitution reaction. Benzonitrile was not detected in samples where BAC was not transformed. In a similar assay conducted with a dialkonium chloride, transformation of $DC_{10}DMA$-Cl could not be achieved. This suggests that the above-described mechanism is structure specific, and most probably the presence of a benzyl group is a prerequisite. This transformation mechanism can positively affect the fate of QACs in the environment under anoxic/anaerobic conditions. Nitrite catalytically transformed BACs to their corresponding, less toxic, tertiary amine, thus removing QAC stress. In the above-described study, as soon as the BAC stress was removed by the nitrite-mediated abiotic transformation, reduction of nitrate to dinitrogen was recovered. Can microbes facilitate the formation of reactive species to tolerate antimicrobials by transforming them in the environment? is a relevant question.

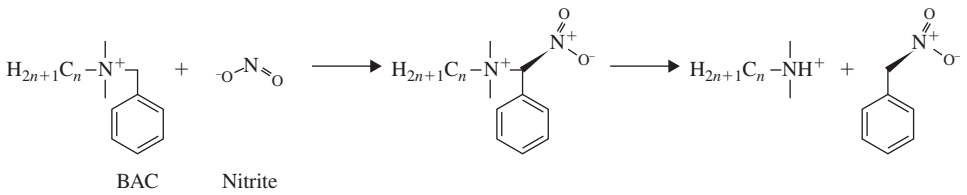


FIGURE 20.9 BAC transformation via the *modified Hofmann degradation* with nitrite acting as the nucleophile (Tezel and Pavlostathis 2009).

QACs possess alkyl, benzyl, and/or methyl functional groups. As discussed above, the initial step in QAC biotransformation is very similar to that of the hydrocarbon degradation under aerobic conditions. Moreover, similar types of microorganisms participate in the biotransformation of both types of compounds under aerobic conditions. Relatively recently, it was discovered that both aliphatic and aromatic hydrocarbons, as well as linear alkylbenzene sulfonate (LAS) detergents, can be degraded under anoxic/anaerobic conditions by the fumarate addition mechanism (Heider et al., 1998; Spormann and Widdel, 2000; van Hamme et al., 2003; Suflita et al., 2004; Davidova et al., 2005; Callaghan et al., 2006, 2008, 2010; Heider, 2007; Washer and Edwards, 2007; Winderl et al., 2007; Grundmann et al., 2008; Lara-Martin et al., 2010). Under this pathway, biotransformation of hydrocarbons and LAS involves the integration of a fumarate molecule to the alkyl group of alkylbenzenes or to the subterminal C atom of alkanes. The enzymes that catalyze fumarate addition to hydrocarbons are benzylsuccinate synthase (Bss) and alkylsuccinate synthase (Ass). These enzymes are glycyl radical enzymes that are grouped in the pyruvate-formate lyase (Pfl) superfamily and have catalytic subunits with a strong homology to Pfl (Coschigano et al., 1998; Krieger et al., 2001; Callaghan et al., 2008; Grundmann et al., 2008). Following this unusual addition reaction, the C-skeleton of the hydrocarbons and LAS is reorganized and then biotransformation proceeds via β-oxidation metabolism. Such biotransformation pathways have been observed to take place under Fe(III)-reducing, denitrifying, sulfate-reducing, and methanogenic conditions. Other terminal electron acceptors shown to be involved during anaerobic hydrocarbon metabolism include manganese oxides, soil humic acids, anthraquinone-2,6-disulfonate, and fumarate.

Tezel (2009) showed that a benzalkonium chloride, C_{14}BDMA-Cl, was transformed following a fumarate addition mechanism with both fumarate and glucose fermentation in a BAC-grown, mixed microbial community under nitrate-reducing conditions. By using the metabolites tentatively identified in the culture media, biotransformation of BAC was successfully simulated using the University of Minnesota Biocatalysis/Biodegradation Database-Pathway Prediction System (UM-BBD-PPS) (http://umbbd.msi.umn.edu/predict/) (Wackett and Ellis, 1999; Ellis et al., 2000; Hou et al., 2003) (Fig. 20.10). According to this pathway, fumarate is added to a methylene carbon that connects the benzene ring to the quaternary nitrogen of BAC. The resulting product, which is fumarate added BAC or BAC*, is activated by coenzyme A (CoA), followed by rearrangement of the BAC* skeleton. The rearrangement results in the separation of the benzyl group from the BAC*, yielding a tertiary alkyl amine and benzoylsuccinate. Then, the tertiary alkyl amine and benzoylsuccinate are utilized as carbon and energy sources via β oxidation

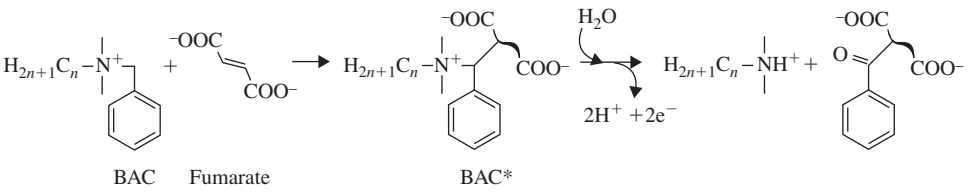


FIGURE 20.10 Initial BAC transformation via fumarate addition.

after being further metabolized through fumarate addition and benzoyl-CoA pathways, respectively (Heider et al., 1998; Boll et al., 2002; Gibson and Harwood, 2002; Davidova et al., 2005). Given the fact that QACs accumulated at relatively high concentrations pose a high risk of developing and disseminating QAC-related antimicrobial resistance in anaerobic/anoxic compartments of the environment, the fumarate addition mechanism is a promising pathway of QAC degradation for the control of QAC-related antimicrobial resistance in anoxic/anaerobic environments.

20.6 QAC-RELATED ANTIBIOTIC RESISTANCE

Antibiotics are ubiquitous and coexist with QACs in the environment. Antibiotic concentrations vary in the environmental media, but are less than those of QACs. The major concerns relative to the presence of QAC-like general-use antimicrobial agents in the environment together with the antibiotics are: (i) commonality of mode of action between QAC and antibiotics might lead to selection of mutants altered in such targets by either agent and the emergence of *cross resistance*; (ii) subtle differences in the QAC susceptibility of a microorganism conferred by an acquisition of a mobile genetic element might facilitate *co-selection* of antibiotic resistance and its persistence in the environment by low, subinhibitory concentrations of QACs as well as antibiotics; and (iii) indiscriminate QAC application

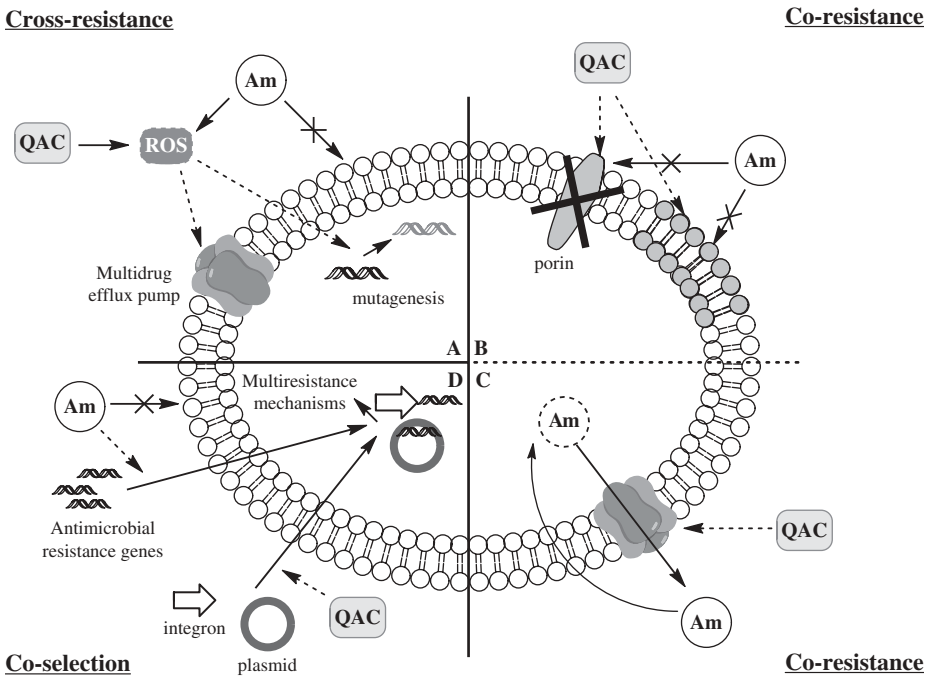


FIGURE 20.11 QAC-induced or selected antibiotic/antimicrobial resistance mechanisms (see text below for the explanation of A, B, C, and D; Am = antimicrobial agent). *(See color insert.)*

might cause the evolution and selection of multidrug-resistant microorganisms through polygamous mechanisms (*co-resistance*), such as efflux pumps (Gilbert and McBain, 2003). Genetic and phenotypic mechanisms acting against QACs and conferring cross and co-resistance to antibiotics or resulting in co-selection of antibiotic resistance upon QAC exposure are presented in Figure 20.11.

QACs do not share a common mode of action with any of the most clinically important antibiotics (Table 20.6); therefore, a true cross-resistance mechanism between QACs and antibiotics is not present. Antibiotics have more specific attack target sites as opposed to QACs' lytic action. On the other hand, both QACs and antibiotics can stimulate microorganisms to produce reactive oxygen species (ROS). ROS can facilitate DNA damage, which activates an error-prone superoxide scavenging (SOS) response leading to increased mutations or inducing the superoxide responsive SoxRS system at subinhibitory concentrations. The former confers resistance to QACs and antibiotics by a mutation on a gene of a multidrug efflux pump, such as Acr-TolC, an efflux system with a broad substrate range (Aleksun and Levy, 2007), which increases pump efficiency, or by a mutation in the specific target site of the particular antibiotic (Kohanski et al., 2010b; Davies and Davies, 2010). On the other hand, the latter one increases the tolerance of a microorganism to QACs and antibiotics by activating the regulatory system of the multidrug efflux pump Acr-TolC leading to the overexpression of the pump as discussed above. Therefore, selection of QAC-resistant mutants, having the oxidative stress-induced resistance mechanisms, may facilitate the development of cross resistance to clinically important antibiotics in the environment (Fig. 20.11A). A number of multidrug efflux pumps sustaining tolerance to QACs have been shown to confer cross resistance to many other antimicrobials in clinically important microorganisms (Table 20.4., with the exception of the SMR family) (Hegstad et al., 2010).

The physiological reaction of a microorganism to QAC exposure at a subinhibitory concentration includes reduction of the number of porins on the cell surface, altering the porin structure and changing the cell surface composition and polarity. These modifications result in a less permeable cell surface and prevent the passage of QACs into the cell. Decreased permeability of the cell surface concomitantly restricts the passage of antibiotics into the cell. As a result, antibiotics cannot reach their target sites. QAC-facilitated modification of the cell surface leads to decreased permeability or selection of microorganisms with such cell surface properties, which confer co-resistance to antibiotics (Fig. 20.11B) (Perichon and Courvalin, 2009; Hegstad et al., 2010).

Unlike RND- and MFS-type efflux pumps, the SMR family efflux pumps, which confer resistance to QACs, do not have a regulatory system and a broad range of substrates. As a result, resistance to antimicrobials other than QACs by the SMR family efflux systems conferring resistance to QACs is a co-resistance mechanism (Fig. 20.11C). Among these efflux pumps, QacE-QacJ and smr are specific to QACs and other monovalent cations, whereas EmrE confers co-resistance to erythromycin, sulfadiazine, and tetracycline (Paulsen et al., 1996).

Mobile genetic elements play a significant role in the acquisition of antimicrobial resistance. The resistance carried by these elements can be horizontally transferred between the microorganisms and disseminated in the environment (Martinez, 2008). The last mechanism of QAC-related antimicrobial resistance is

TABLE 20.6 Antibiotic Types Grouped with Respect to Their Mode of Action, Target Sites, and Corresponding Resistance Mechanisms

Mode of Action Antibiotic Type	Target Site	Resistance Mechanism
DNA/RNA Synthesis		
Fluoroquinolones	Topoisomerase II (DNA gyrase), topoisomerase IV	Acetylation, efflux, altered target
Trimethoprim–sulfamethoxazole	Tetrahydrofolic acid synthesis inhibitors	Efflux, altered target
Rifamycine	DNA-dependent RNA polymerase	ADP-ribosylation, efflux, altered target
Cell Wall Synthesis		
β-Lactams	Penicillin-binding proteins	Hydrolysis, efflux, altered target
Glycopeptides and glycolipopeptides	Peptidoglycan units	Reprogramming peptidoglycan biosynthesis
Lipopeptides	Cell membrane	Altered target
Protein Synthesis		
Aminoglycosides	30S ribosome	Phosphorylation, acetylation, nucleotidylation, efflux, altered target
Tetracyclines	30S ribosome	Monooxygenation, efflux, altered target
Macrolides	50S ribosome	Hydrolysis, glycosylation, phosphorylation, efflux, altered target
Streptogramins	50S ribosome	C-O lyase, acetylation, efflux, altered target
Phenicols	50S ribosome	Acetylation, efflux, altered target

Source: Adapted from Davies and Davies (2010) and Kohanski et al., 2010b.

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co-selection. Many QAC resistance genes associated with plasmids and integrons have been detected at high abundance in wastewater treatment plants and in the environment. These mobile genetic elements also contain genes expressing resistance to all kinds of antibiotics (Schluter et al., 2007; Zhang et al., 2009). Particularly, class 1 integrons are the most important mobile genetic elements of all because they are ubiquitous both in environmental and clinical settings and can carry both QAC and antibiotic resistance. Given the relatively high concentrations of QACs in the environment compared to antibiotics, QAC exposure facilitates the acquisition of class 1 integrons (Gaze et al., 2005; Gillings et al., 2009a). The class 1 integron is maintained by the microorganism in the presence of QACs and enables it to possess gene cassettes conferring resistance to other antimicrobials if needed (Fig. 20.11D) (Partridge et al., 2009).

In summary, the indiscriminate use and release of QACs into the environment pose a major risk by developing and spreading of antimicrobial, including antibiotic, resistance, with far-reaching consequences to both human and environmental health. The development of more environmentally friendly QAC substitutes, as well as the proper handling and sounder management of QAC-bearing waste streams, coupled with new biotechnologies based on recent findings related to the biodegradation of QACs, are all promising means of reducing, if not eliminating, the release of QACs into an increasingly stressed natural environment.

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REFERENCES

Alekshun MN, Levy SB (2007). Molecular mechanisms of antibacterial multidrug resistance. *Cell* 128(6):1037–1050.

Battersby NS, Wilson V (1989). Survey of the anaerobic biodegradation potential of organic chemicals in digesting sludge. *Appl Environ Microbiol* 55(2):433–439.

Boeris PS, Domenech CE, Lucchesi GI (2007). Modification of phospholipid composition in *Pseudomonas putida* A ATCC 12633 induced by contact with tetradecyltrimethylammonium. *J Appl Microbiol* 103(4):1048–1054.

Boethling RS (1984). Environmental fate and toxicity in wastewater treatment of quaternary ammonium surfactants. *Water Res* 18(9):1061–1076.

Boethling RS (1994). Environmental aspects of cationic surfactants. In J Cross and EJ Singer (Eds.), *Cationic Surfactants: Analytical and Biological Evaluation*, Vol. 53. Marcel Dekker, New York.

Boll M, Fuchs G, Heider J (2002). Anaerobic oxidation of aromatic compounds and hydrocarbons. *Curr Opin Chem Biol* 6(5):604–611.

Bore E, Hebraud M, Chafsey I, Chambon C, Skjaeret C, Moen B, Moretro T, Langsrud O, Rudi K, Langsrud S (2007). Adapted tolerance to benzalkonium chloride in *Escherichia*

coli K-12 studied by transcriptome and proteome analyses. *Microbiology-Sgm* 153: 935–946.

Boyd SA, Lee JF, Mortland MM (1988). Attenuating organic contaminant mobility by soil modification. *Nature* 333(6171):345–347.

Brown MH, Skurray RA (2001). Staphylococcal multidrug efflux protein QacA. *J Mol Microbiol Biotechnol* 3(2):163–170.

Callaghan AV, Gieg LM, Kropp KG, Suflita JM, Young LY (2006). Comparison of mechanisms of alkane metabolism under sulfate-reducing conditions among two bacterial isolates and a bacterial consortium. *Appl Environ Microbiol* 72(6):4274–4282.

Callaghan AV, Wawrik B, Chadhain SMN, Young LY, Zylstra GJ (2008). Anaerobic alkane-degrading strain AK-01 contains two alkylsuccinate synthase genes. *Biochem Biophys Res Commun* 366(1):142–148.

Callaghan AV, Davidova IA, Savage-Ashlock K, Parisi VA, Gieg LM, Suflita JM, Kukor JJ, Wawrik B (2010). Diversity of benzyl- and alkylsuccinate synthase genes in hydrocarbon impacted environments and enrichment cultures. *Environ Sci Technol* 44(19):7287–7294.

Chaplin CE (1952). Bacterial resistance to quaternary ammonium disinfectants. *J Bacteriol* 63(4):453–458.

Clara M, Scharf S, Scheffknecht C, Gans O (2007). Occurrence of selected surfactants in untreated and treated sewage. *Water Res* 41(19):4339–4348.

Coschigano PW, Wehrman TS, Young LY (1998). Identification and analysis of genes involved in anaerobic toluene metabolism by strain T1: Putative role of a glycine free radical. *Appl Environ Microbiol* 64(5):1650–1656.

Daniels C, Ramos JL (2009). Adaptive drug resistance mediated by root-nodulation-cell division efflux pumps. *Clin Microbiol Infect* 15:32–36.

Dantas G, Sommer MOA, Oluwasegun RD, Church GM (2008). Bacteria subsisting on antibiotics. *Science* 320(5872):100–103.

Davidova IA, Gieg LM, Nanny M, Kropp KG, Suflita JM (2005). Stable isotopic studies of *n*-alkane metabolism by a sulfate-reducing bacterial enrichment culture. *Appl Environ Microbiol* 71(12):8174–8182.

Davies J, Davies D (2010). Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev* 74(3):417–433.

Dean-Raymond D, Alexander M (1977). Bacterial metabolism of quaternary ammonium compounds. *Appl Environ Microbiol* 33(5):1037–1041.

Denyer SP, Maillard JY (2002). Cellular impermeability and uptake of biocides and antibiotics in gram-negative bacteria. *J Appl Microbiol* 92:35S–45S.

Ding WH, Liao YH (2001). Determination of alkylbenzyldimethylammonium chlorides in river water and sewage effluent by solid phase extraction and gas chromatography mass spectrometry. *Anal Chem* 73(1):36–40.

Dubois-Brissonnet F, Malgrange C, Guerin-Mechin L, Heyd B, Leveau JY (2001). Changes in fatty acid composition of *Pseudomonas aeruginosa* ATCC 15442 induced by growth conditions: Consequences of resistance to quaternary ammonium compounds. *Microbios* 106(414):97–110.

Ellis LBM, Hershberger CD, Wackett LP (2000). The University of Minnesota Biocatalysis/Biodegradation Database: Microorganisms, genomics and prediction. *Nucleic Acids Res* 28(1):377–379.

European Center for Ecotoxicology and Toxicology of Chemicals (ECETOC) (1993). *DHTDMC: Aquatic and Terrestrial Hazard Assessment*. European Center for Ecotoxicological and Toxicological Safety Assessment of Chemicals, Brussels, Belgium.

Federle TW, Schwab BS (1992). Mineralization of surfactants in anaerobic sediments of a laundromat wastewater pond. *Water Res* 26(1):123–127.

Fernandez P, Valls M, Bayona JM, Albaiges J (1991). Occurrence of cationic surfactants and related products in urban coastal environments. *Environ Sci Technol* 25(3):547–550.

Fernandez P, Alder AC, Suter MJF, Giger W (1996). Determination of the quaternary ammonium surfactant ditallowdimethylammonium in digested sludges and marine sediments by supercritical fluid extraction and liquid chromatography with postcolumn ion-pair formation. *Anal Chem* 68(5):921–929.

Ferrer I, Furlong ET (2001). Identification of alkyl dimethylbenzylammonium surfactants in water samples by solid-phase extraction followed by ion trap LC/MS and LC/MS/MS. *Environ Sci Technol* 35(12):2583–2588.

Ferrer I, Furlong ET (2002). Accelerated solvent extraction followed by on-line solid-phase extraction coupled to ion trap LC/MS/MS for analysis of benzalkonium chlorides in sediment samples. *Anal Chem* 74(6):1275–1280.

Garcia MT, Campos E, Sanchez-Leal J, Ribosa I (1999). Effect of the alkyl chain length on the anaerobic biodegradability and toxicity of quaternary ammonium based surfactants. *Chemosphere* 38(15):3473–3483.

Garcia MT, Campos E, Sanchez-Leal J, Ribosa I (2000). Anaerobic degradation and toxicity of commercial cationic surfactants in anaerobic screening tests. *Chemosphere* 41(5):705–710.

Garcia MT, Ribosa I, Guindulain T, Sanchez-Leal J, Vives-Rego J (2001). Fate and effect of monoalkyl quaternary ammonium surfactants in the aquatic environment. *Environ Pollut* 111(1):169–175.

Gaze WH, Abdouslam N, Hawkey PM, Wellington EMH (2005). Incidence of class 1 integrons in a quaternary ammonium compound-polluted environment. *Antimicrob Agents Chemother* 49(5):1802–1807.

Gibson J, Harwood CS (2002). Metabolic diversity in aromatic compound utilization by anaerobic microbes. *Annu Rev Microbiol* 56:345–369.

Gilbert P, McBain AJ (2003). Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. *Clini Microbiol Rev* 16(2):189–208.

Gilbert P, Moore LE (2005). Cationic antiseptics: Diversity of action under a common epithet. *J Appl Microbiol* 99(4):703–715.

Gillings MR, Duan XJ, Hardwick SA, Holley MP, Stokes HW (2009a). Gene cassettes encoding resistance to quaternary ammonium compounds: A role in the origin of clinical class 1 integrons? *ISME J* 3(2):209–215.

Gillings MR, Holley MP, Stokes HW (2009b). Evidence for dynamic exchange of qac gene cassettes between class 1 integrons and other integrons in freshwater biofilms. *FEMS Microbiol Lett* 296(2):282–288.

Giolando ST, Rapaport RA, Larson RJ, Federle TW, Stalmans M, Masscheleyn P (1995). Environmental fate and effects of DEEDMAC—A new rapidly biodegradable cationic surfactant for use in fabric softeners. *Chemosphere* 30(6):1067–1083.

Grkovic S, Brown MH, Skurray RA (2002). Regulation of bacterial drug export systems. *Microbiol Mol Biol Rev* 66(4):671–701.

Grundmann O, Behrends A, Rabus R, Amann J, Halder T, Heider J, Widdel F (2008). Genes encoding the candidate enzyme for anaerobic activation of *n*-alkanes in the denitrifying bacterium, strain HxN1. *Environ Microbiol* 10(2):376–385.

Guerin-Mechin L, Dubois-Brissonnet F, Heyd B, Leveau JY (1999). Specific variations of fatty acid composition of *Pseudomonas aeruginosa* ATCC 15442 induced by quaternary ammonium compounds and relation with resistance to bactericidal activity. *J Appl Microbiol* 87 (5):735–742.

Guerin-Mechin L, Dubois-Brissonnet F, Heyd B, Leveau JY (2000). Quaternary ammonium compound stresses induce specific variations in fatty acid composition of *Pseudomonas aeruginosa*. *Int J Food Microbiol* 55(1–3):157–159.

Hauthal HG (2004). CESIO 2004 - Dynamic surfactants and nanostructured surfaces for an innovative industry. *SÖFW-Journal* 130(10):3–17.

Heerklotz H (2008). Interactions of surfactants with lipid membranes. *Q Rev Biophys* 41(3–4): 205–264.

Hegstad K, Langsrud S, Lunestad BT, Scheie AA, Sunde M, Yazdankhah SP (2010). Does the wide use of quaternary ammonium compounds enhance the selection and spread of antimicrobial resistance and thus threaten our health? *Microb Drug Resis* 16(2):91–104.

Heider J (2007). Adding handles to unhandy substrates: Anaerobic hydrocarbon activation mechanisms. *Curr Opin Chem Biol* 11(2):188–194.

Heider J, Spormann AM, Beller HR, Widdel F (1998). Anaerobic bacterial metabolism of hydrocarbons. *FEMS Microbiol Rev* 22(5):459–473.

Hoey M (2001). Cationic surfactants: Cationic surfactants in organoclays. In K Holmberg (Ed.), *Handbook of Applied Surface and Colloid Chemistry*, Vol. 1. J Wiley, West Sussex, England.

Hou BK, Wackett LP, Ellis LBM (2003). Microbial pathway prediction: A functional group approach. *J Chem Inform Computat Sci* 43(3):1051–1057.

Huber L (1979). The behavior of cation-active surfactants from laundry softeners in water and wastewater. *Muenchener Beitraege zur Abwasser—Fischerei—und Flussbiologie* 31(1): 203–215.

Ishikawa S, Matsumura Y, Yoshizako F, Tsuchido T (2002). Characterization of a cationic surfactant-resistant mutant isolated spontaneously from *Escherichia coli*. *J Appl Microbiol* 92(2):261–268.

Ismail ZZ, Tezel U, Pavlostathis SG (2010). Sorption of quaternary ammonium compounds to municipal sludge. *Water Res* 44(7):2303–2313.

Kazama H, Hamashima H, Sasatsu M, Arai T (1998a). Distribution of the antiseptic-resistance genes qacE and qacE delta 1 in gram-negative bacteria. *FEMS Microbiol Lett* 159(2):173–178.

Kazama H, Hamashima H, Sasatsu M, Arai T (1998b). Distribution of the antiseptic-resistance gene qacE delta 1 in gram-positive bacteria. *FEMS Microbiol Lett* 165(2): 295–299.

King GM (1984). Metabolism of trimethylamine, choline, and glycine betaine by sulfate-reducing and methanogenic bacteria in marine sediments. *Appl Environ Microbiol* 48(4): 719–725.

Kim SG, Bae HS, Lee ST (2001). A novel denitrifying bacterial isolate that degrades trimethylamine both aerobically and anaerobically via two different pathways. *Arch Microbiol* 176(4):271–277.

Knox WE, Auerbach VH, Zarudnaya K, Spirtes M (1949). The action of cationic detergents on bacteria and bacterial enzymes. *J Bacteriol* 58(4):443–452.

Kohanski MA, DePristo MA, Collins JJ (2010a). Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. *Mol Cell* 37(3):311–320.

Kohanski MA, Dwyer DJ, Collins JJ (2010b). How antibiotics kill bacteria: From targets to networks. *Nat Rev Microbiol* 8(6):423–435.

Kopecky F (1996). Micellization and other associations of amphiphilic antimicrobial quaternary ammonium salts in aqueous solutions. *Pharmazie* 51(3):135–144.

Kreuzinger N, Fuerhacker M, Scharf S, Uhl M, Gans O, Grillitsch B (2007). Methodological approach towards the environmental significance of uncharacterized substances—Quaternary ammonium compounds as an example. *Desalination* 215(1–3):209–222.

Krieger CJ, Roseboom W, Albracht SPJ, Spormann AM (2001). A stable organic free radical in anaerobic benzylsuccinate synthase of *Azoarcus* sp strain T. *J Biol Chem* 276(16):12924–12927.

Kroon AGM, van Ginkel CG (2001). Complete mineralization of dodecyldimethylamine using a two-membered bacterial culture. *Environ Microbiol* 3(2):131–136.

Kümmerer K, Eitel A, Braun U, Hubner P, Daschner F, Mascart G, Milandri M, Reinthaler F, Verhoef J (1997). Analysis of benzalkonium chloride in the effluent from European hospitals by solid-phase extraction and high-performance liquid chromatography with post-column ion-pairing and fluorescence detection. *J Chromatogr A* 774(1–2):281–286.

Kupfer W (1982). Determination of cationic surfactants in sewage. *Tenside Surfactant Detergents* 19(1):158–161.

Langsrud S, Sundheim G (1997). Factors contributing to the survival of poultry associated *Pseudomonas* spp. exposed to a quaternary ammonium compound. *J Appl Microbiol* 82(6):705–712.

Lara-Martin PA, Gomez-Parra A, Sanz JL, Gonzalez-Mazo E (2010). Anaerobic degradation pathway of linear alkylbenzene sulfonates (LAS) in sulfate-reducing marine sediments. *Environ Sci Technol* 44(5):1670–1676.

Larson RJ, Schaeffer SL (1982). A rapid method for determining the toxicity of chemicals to activated sludge. *Water Res* 16(5):675–680.

Leal JS, Gonzalez JJ, Kaiser KLE, Palabrica VS, Comelles F, Garcia MT (1994). On the toxicity and biodegradation of cationic surfactants. *Acta Hydrochim Hydrobiol* 22(1):13–18.

Lewis MA (1991). Chronic and sublethal toxicities of surfactants to aquatic animals—A review and risk assessment. *Water Res* 25(1):101–113.

Lewis MA, Wee VT (1983). Aquatic safety assessment for cationic surfactants. *Environ Toxicol Chem* 2(1):105–118.

Li X, Brownawell BJ (2010). Quaternary ammonium compounds in urban estuarine sediment environments—A class of contaminants in need of increased attention? *Environ Sci Technol* 44(19):7561–7568.

Liffourrena AS, Lopez FG, Salvano MA, Domenech CE, Lucchesi GI (2008). Degradation of tetradecyltrimethylammonium by *Pseudomonas putida* A ATCC 12633 restricted by accumulation of trimethylamine is alleviated by addition of Al^{3+} ions. *J Appl Microbiol* 104(2):396–402.

Liffourrena AS, Boeris PS, Salvano MA, Lucchesi GI (2009). A fluorescence assay for tetradecyltrimethylammonium mono-oxygenase activity that catalyzes the cleavage of the C-N bond with the production of trimethylamine. *Anal Biochem* 384(2):343–347.

Loughlin MF, Jones MV, Lambert PA (2002). *Pseudomonas aeruginosa* cells adapted to benzalkonium chloride show resistance to other membrane-active agents but not to clinically relevant antibiotics. *J Antimicrob Chemother* 49(4):631–639.

Maillard JY (2002). Bacterial target sites for biocide action. *J Appl Microbiol* 92:16S–27S.

Martinez JL (2008). Antibiotics and antibiotic resistance genes in natural environments. *Science* 321(5887):365–367.

Martinez-Carballo E, Sitka A, Gonzalez-Barreiro C, Kreuzinger N, Furhacker M, Scharf S, Gans O (2007a). Determination of selected quaternary ammonium compounds by liquid chromatography with mass spectrometry. Part I. Application to surface, waste and indirect discharge water samples in Austria. *Environ Pollut* 145(2):489–496.

Martinez-Carballo E, Gonzalez-Barreiro C, Sitka A, Kreuzinger N, Scharf S, Gans O (2007b). Determination of selected quaternary ammonium compounds by liquid chromatography with mass spectrometry. Part II. Application to sediment and sludge samples in Austria. *Environ Pollut* 146(2):543–547.

Mc Cay PH, Ocampo-Sosa AA, Fleming GTA (2010). Effect of subinhibitory concentrations of benzalkonium chloride on the competitiveness of *Pseudomonas aeruginosa* grown in continuous culture. *Microbiology* 156:30–38.

McDonnell G, Russell AD (1999). Antiseptics and disinfectants: Activity, action, and resistance. *Clin Microbiol Rev* 12(1):147–179.

Merino F, Rubio S, Perez-Bendito D (2003). Solid-phase extraction of amphiphiles based on mixed hemimicelle/admicelle formation: Application to the concentration of benzalkonium surfactants in sewage and river water. *Anal Chem* 75(24):6799–6806.

Michelsen ER (1978). Quantitative determination of quaternary ammonium compounds. *Tenside Detergents* 15(1):169–175.

Nagai K, Murata T, Ohta S, Zenda H, Ohnishi M, Hayashi T (2003). Two different mechanisms are involved in the extremely high-level benzalkonium chloride resistance of a *Pseudomonas fluorescens* strain. *Microbiol Immunol* 47(10):709–715.

Nalecz-Jawecki G, Grabinska-Sota E, Narkiewicz P (2003). The toxicity of cationic surfactants in four bioassays. *Ecotoxicol Environ Safety* 54(1):87–91.

Neill AR, Grime DW, Dawson RMC (1978). Conversion of choline methyl groups through trimethylamine into methane in rumen. *Biochem J* 170(3):529–535.

Nishihara T, Okamoto T, Nishiyama N (2000). Biodegradation of didecyldimethylammonium chloride by *Pseudomonas fluorescens* TN4 isolated from activated sludge. *J Appli Microbiol* 88(4):641–647.

Nishiyama N, Nishihara T (2002). Biodegradation of dodecyltrimethylammonium bromide by *Pseudomonas fluorescens* F7 and F2 isolated from activated sludge. *Microb Environ* 17(4): 164–169.

Nishiyama N, Toshima Y, Ikeda Y (1995). Biodegradation of alkyltrimethylammonium salts in activated sludge. *Chemosphere* 30(3):593–603.

Nye JV, Guerin WF, Boyd SA (1994). Heterotrophic activity of microorganisms in soils treated with quaternary ammonium compounds. *Environ Sci Technol* 28(5):944–951.

Organization for Economic Cooperation and Development (OECD) (1994). Dimethyl dioctadecyl ammonium chloride. Screening Information Data Set (SIDS), published by United Nations Environment Programme (UNEP), Division of Technology, Industry and Economics, Chemicals Branch, Geneva, Switzerland.

Partridge SR, Brown HJ, Hall RA (2002). Characterization and movement of the class 1 integron known as Tn2521 and Tn1405. *Antimicrob Agents Chemother* 46(5):1288–1294.

Partridge SR, Tsafnat G, Coiera E, Iredell JR (2009). Gene cassettes and cassette arrays in mobile resistance integrons. *FEMS Microbiol Rev* 33(4):757–784.

Patrauchan MA, Oriel PJ (2003). Degradation of benzyldimethylalkylammonium chloride by *Aeromonas hydrophila* sp K. *J Appl Microbiol* 94(2):266–272.

Paulsen IT, Brown MH, Skurray RA (1996). Proton-dependent multidrug efflux systems. *Microbiol Rev* 60(4):575–608.

Perichon B, Courvalin P (2009). Antibiotic resistance. In M Schaechter (Ed.), *The Desk Encyclopedia of Microbiology*. Elsevier, Oxford.

Piddock LJV (2006). Multidrug-resistance efflux pumps—Not just for resistance. *Nat Rev Microbiol* 4(8):629–636.

Poole K (2005). Efflux-mediated antimicrobial resistance. *J Antimicrob Chemother* 56(1):20–51.

Poole K (2007). Efflux pumps as antimicrobial resistance mechanisms. *Ann Med* 39(3): 162–176.

Putman M, van Veen HW, Konings WN (2000). Molecular properties of bacterial multidrug transporters. *Microbiol Mol Biol Rev* 64(4):672–693.

Reynolds L, Blok J, Demorsier A, Gerike P, Wellens H, Bontinck WJ (1987). Evaluation of the toxicity of substances to be assessed for biodegradability. *Chemosphere* 16(10–12): 2259–2277.

Saidijam M, Benedetti G, Ren QH, Xu ZQ, Hoyle CJ, Palmer SL, Ward A, Bettaney KE, Szakonyi G, Meuller J, Morrison S, Pos MK, Butaye P, Walravens K, Langton K, Herbert RB, Skurray RA, Paulsen IT, O'Reilly J, Rutherford NG, Brown MH, Bill RM, Henderson PJF (2006). Microbial drug efflux proteins of the major facilitator superfamily. *Curr Drug Targets* 7(7):793–811.

Schluter A, Szczepanowski R, Puhler A, Top EM (2007). Genomics of IncP-1 antibiotic resistance plasmids isolated from wastewater treatment plants provides evidence for a widely accessible drug resistance gene pool. *FEMS Microbiol Rev* 31(4):449–477.

Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) (2009). Assessment of the antibiotic resistance effects of biocides. European Commission, Directorate-General for Health & Consumers, Brussels, Belgium.

Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) (2010). Research strategy to address the knowledge gaps on the antimicrobial resistance effects of biocides. European Commission, Directorate-General for Health & Consumers, Brussels, Belgium.

Smith K, Gemmell CG, Hunter IS (2008). The association between biocide tolerance and the presence or absence of qac genes among hospital-acquired and community-acquired MRSA isolates. *J Antimicrob Chemother* 61(1):78–84.

Spormann AM, Widdel F (2000). Metabolism of alkylbenzenes, alkanes, and other hydrocarbons in anaerobic bacteria. *Biodegradation* 11(2–3):85–105.

Steichen DS (2001). *Cationic Surfactants*. In K Holmberg (Ed.), *Handbook of Applied Surface and Colloid Chemistry*, Vol. 1. J Wiley, West Sussex, England.

Suflita JM, Davidova IA, Gieg LM, Nanny M, Prince RC (2004). Anaerobic hydrocarbon biodegradation and the prospects for microbial enhanced energy production. In *Petroleum Biotechnology: Developments and Perspectivas*, Vol. 151. Elsevier, Amsterdam, The Netherlands.

Sun HF, Takata A, Hata N, Kasahara I, Taguchi S (2003). Transportation and fate of cationic surfactant in river water. *J Environ Monitor* 5(6):891–895.

Sutterlin H, Trittler R, Bojanowski S, Stadbauer EA, Kummerer K (2007). Fate of benzalkonium chloride in a sewage sludge low temperature conversion process investigated by LC-LC/ESI-MS/MS. *Clean-Soil Air Water* 35(1):81–87.

Sutterlin H, Alexy R, Kummerer K (2008). The toxicity of the quaternary ammonium compound benzalkonium chloride alone and in mixtures with other anionic compounds to bacteria in test systems with *Vibrio fischeri* and *Pseudomonas putida*. *Ecotoxicol Environ Safety* 71(2):498–505.

Tabata A, Nagamune H, Maeda T, Murakami K, Miyake Y, Kourai H (2003). Correlation between resistance of *Pseudomonas aeruginosa* to quaternary ammonium compounds and expression of outer membrane protein OprR. *Antimicrob Agents Chemother* 47(7):2093–2099.

Takenaka S, Tonoki T, Taira K, Murakami S, Aoki K (2007). Adaptation of *Pseudomonas sp* strain 7-6 to quaternary ammonium compounds and their degradation via dual pathways. *Appl Environ Microbiol* 73(6):1797–1802.

Tang D (2001). Cationic surfactants: Cationic surfactants in personal care. In K Holmberg (Ed.), *Handbook of Applied Surface and Colloid Chemistry*, Vol. 1. J Wiley, West Sussex, England.

Tezel U (2009). Fate and effect of quaternary ammonium compounds in biological systems. Ph.D. thesis. Georgia Institute of Technology, Atlanta, GA.

Tezel U, Pavlostathis SG (2009). Transformation of benzalkonium chloride under nitrate reducing conditions. *Environ Sci Technol* 43(5):1342–1348.

Tezel U, Pierson JA, Pavlostathis SG (2006). Fate and effect of quaternary ammonium compounds on a mixed methanogenic culture. *Water Res* 40(19):3660–3668.

Tezel U, Pierson JA, Pavlostathis SG (2007). Effect of polyelectrolytes and quaternary ammonium compounds on the anaerobic biological treatment of poultry processing wastewater. *Water Res* 41(6):1334–1342.

Tiedink J (2001). Cationic surfactants: Cationic surfactants in biocides. In K Holmberg (Ed.), *Handbook of Applied Surface and Colloid Chemistry*, Vol. 1. J Wiley, West Sussex, England.

Tubbing DMJ, Admiraal WIM (1991). Inhibition of bacterial and phytoplanktonic metabolic activity in the lower River Rhine by ditallowdimethylammonium chloride. *Appl Environ Microbiol* 57(12):3616–3622.

U.S. Environmental Protection Agency (2006). High Production Volume Challenge Program. U.S. EPA Office of Pollution Prevention and Toxics, Washington, DC. Available: http://www.epa.gov/chemrtk/pubs/general/hazchem.htm.

U.S. Environmental Protection Agency (2009). Estimation Program Interface (EPI) Suite™ v. 4.0. http://www.epa.gov/oppt/exposure/pubs/episuite.htm.

Utsunomiya A, Naito S, Tomita I (1989). Studies on sodium linear alkylbenzenesulfonate (LAS) III. Distribution and fate of LAS and quaternary ammonium surfactant in aquatic environment. *Jpn J Toxicol Environ Health* 35(1):152–161.

Utsunomiya A, Watanuki T, Matsushita K, Tomita I (1997). Toxic effects of linear alkylbenzene sulfonate, quaternary alkylammonium chloride and their complexes on *Dunaliella* sp. and *Chlorella pyrenoidosa*. *Environ Toxicol Chem* 16(6):1247–1254.

Vali L, Davies SE, Lai LLG, Dave J, Amyes SGB (2008). Frequency of biocide resistance genes, antibiotic resistance and the effect of chlorhexidine exposure on clinical methicillin-resistant *Staphylococcus aureus* isolates. *J Antimicrob Chemother* 61(3):524–532.

van Beilen JB, Funhoff EG (2005). Expanding the alkane oxygenase toolbox: New enzymes and applications. *Curr Opin Biotechnol* 16(3):308–314.

van Ginkel CG (1996). Complete degradation of xenobiotic surfactants by consortia of aerobic microorganisms. *Biodegradation* 7(2):151–164.

van Ginkel CG (2004). Biodegradation of cationic surfactants. In U Zoller (Ed.), *Handbook of Detergents Part B: Environmental Impact*, Vol. 121. Marcel Dekker, New York.

van Ginkel CG, Kolvenbach M (1991). Relations between the structure of quaternary alkyl ammonium salts and their biodegradability. *Chemosphere* 23(3):281–289.

van Ginkel CG, Vandijk JB, Kroon AGM (1992). Metabolism of hexadecyltrimethylammonium chloride in *Pseudomonas* Strain-B1. *Appl Environ Microbiol* 58(9):3083–3087.

van Hamme JD, Singh A, Ward OP (2003). Recent advances in petroleum microbiology. *Microbiol Mol Biol Rev* 67(4):503–549.

Ventullo RM, Larson RJ (1986). Adaptation of aquatic microbial communities to quaternary ammonium compounds. *Appl Environ Microbiol* 51(2):356–361.

Wackett LP, Ellis LBM (1999). Predicting biodegradation. *Environ Microbiol* 1(2):119–124.

Washer CE, Edwards EA (2007). Identification and expression of benzylsuccinate synthase genes in a toluene-degrading methanogenic consortium. *Appl Environ Microbiol* 73 (4):1367–1369.

Wee VT (1984). Determination of cationic surfactants in wastewater and river water. *Water Res* 18(2):223–225.

Wee VT, Kennedy JM (1982). Determination of trace levels of quaternary ammonium compounds in river water by liquid chromatography with conductometric detection. *Anal Chem* 54(9):1631–1633.

Winderl C, Schaefer S, Lueders T (2007). Detection of anaerobic toluene and hydrocarbon degraders in contaminated aquifers using benzylsuccinate synthase (bssA) genes as a functional marker. *Environ Microbiol* 9(4):1035–1046.

Yang J (2007). Fate and effect of alkyl benzyl dimethyl ammonium chloride in mixed aerobic and nitrifying cultures. MS thesis. Georgia Institute of Technology, Atlanta, GA.

Ying GG (2006). Fate, behavior and effects of surfactants and their degradation products in the environment. *Environ Int* 32(3):417–431.

Zachvieja J (2001). Cationic surfactants: Cationic surfactants in fabric softening. In K Holmberg (Ed.), *Handbook of Applied Surface and Colloid Chemistry*, Vol. 1. J Wiley, West Sussex, England.

Zhang XX, Zhang T, Zhang M, Fang HHP, Cheng SP (2009). Characterization and quantification of class 1 integrons and associated gene cassettes in sewage treatment plants. *Appl Microbiol Biotechnol* 82(6):1169–1177.

Zhang C, Tezel U, Li K, Liu D, Ren R, Du J, Pavlostathis SG (2011). Evaluation and modeling of benzalkonium chloride inhibition and biodegradation in activated sludge. *Water Res* 45(3):1238–1246.

PART IV

EFFECTS AND RISKS

21

HUMAN HEALTH IMPORTANCE OF USE OF ANTIMICROBIALS IN ANIMALS AND ITS SELECTION OF ANTIMICROBIAL RESISTANCE

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Antimicrobials are widely used in animals for therapy, disease prophylaxis, and growth promotion (National Research Council, 1999). Total quantities used in animals exceed those in humans in some countries (WHO, 1997; Mellon et al., 2001). Adverse health consequences include increased frequency, duration, and severity of infection in humans (Barza, 2002; Helms et al., 2005). Antimicrobials are usually administered to companion animals on an individual basis, but in food animals they are often administered to entire groups, in some cases for prolonged periods of time, thereby dramatically increasing selection pressure (McEwen and Fedorka-Cray, 2002). Antimicrobials select for resistance in zoonotic and commensal bacteria of animals that are transmitted to humans, primarily through the food chain, but also by direct contact and through contaminated water or other environmental routes (FAO/WHO/OIE, 2003). Several options are available to reduce adverse human health effects, including improved regulation, reduction in financial incentives, adoption of prudent antimicrobial use programs, and in some cases restrictions and bans on certain types of use, particularly involving antimicrobials of critical importance to human health (Aarestrup et al., 2008). Efforts to reduce resistance selection pressure by decreasing overall antimicrobial use in animals are intensely controversial with industry and veterinarians; nevertheless, some important steps in this direction have been taken in certain countries, and these provide valuable lessons for others (WHO, 2003). Globally, much needs to be done to curb unnecessary antimicrobial use in animals.

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21.1 USE OF ANTIMICROBIALS IN ANIMALS

Nonhuman uses of antimicrobials that are active against bacteria (as opposed to antivirals, antiparasitics, etc.) include administration to food production animals (e.g., aquaculture species, cattle, poultry, swine), companion animals (e.g., dogs, cats, birds, horses), and some plants (e.g., apples and pears). Horticultural use is comparatively minor (estimated at 0.1% that in food animals) relative to use in humans and animals (Vidaver, 2002) and will not be considered further in this chapter. The food animal sector has received by far the greatest research and regulatory attention in terms of potential human health impact, probably in response to the very large volumes of drugs administered to these animals, the controversies associated with antimicrobial growth promoters (AGPs), and the importance of food as a vehicle of exposure of humans to antimicrobial residues and antimicrobial-resistant bacteria. Antimicrobials were first used in animals and plants soon after World War II, in animals particularly as feed additives, and since then, most classes of antimicrobials used in humans have also been approved for use in animals in one form or another, plus some classes used in animals are not used in humans, sometimes because they are too toxic (WHO, 1997; Prescott, 2006). Antimicrobial regulation and use patterns vary enormously around the globe; in this chapter we discuss the general case with specific regional/national examples to illustrate important points.

21.1.1 Food Production Animals

In food animals, antimicrobials are used for therapy, disease prophylaxis, and growth promotion and are administered mainly in feed or by injection (National Research Council, 1999; McEwen and Fedorka-Cray, 2002). In principle, therapeutic treatment is administered to cure bacterial infections, prophylactic treatment is used to prevent infection, and growth promoters are administered to improve feed efficiency (ratio of feed intake to weight gain) and decrease time to market weight. In practice, the motive and effect of treatment often does not fit neatly into any single category, and this has major implications to the control of overuse (Dunlop et al., 1998b). The main contributing factor to this blurring of the lines is the fact that the great majority of food animals are raised and handled in groups, particularly for those reared "intensively" (under conditions of close confinement, sometimes indoors under controlled environmental conditions, often in groups of uniform age), and to a lesser extent those reared "extensively" (typically on open range or pasture, often in mixed age groups) (National Research Council, 1999). Therefore, many antimicrobials are administered to entire groups of animals, for example, tylosin in the feed for a pen of 40 growing/finishing pigs, enrofloxacin in the water for a barn of 30,000 broiler chickens, or chlortetracycline in the feed for a pen of 200 fattening cattle in a feedlot. In some cases, it is possible to isolate individual sick animals for individual treatment of one or more days (e.g., feedlot steer with pneumonia), but in other cases it is normally not practical to identify, isolate, and treat individual sick animals (e.g., broiler chickens, penned salmon). Thus, when some animals in groups are diagnosed with a bacterial infection, therapeutic treatment may be administered to the entire group (via feed, water, injection), but at the individual animal level, such treatment may actually be therapeutic if the animal is infected, but prophylactic if the animal is colonized or at risk of infection by virtue of spread from others in the group or the

local environment (WHO, 1997; Aarestrup et al., 2008). So too with growth promoters; some research indicates that to the extent that they have any effect on production it is mediated through disease prophylaxis (WHO, 2003).

Availability of antimicrobials for food animals varies considerably by country and region. In most parts of the world, including both developed (e.g., Canada and the United States) and underdeveloped countries, many antimicrobials are available over the counter without a veterinary prescription (WHO, 1997; National Research Council, 1999; Health Canada, 2002). Typically, where regulations concerning prescription-only use are in place and enforced, they apply most often to newer compounds and usually those that are administered at the individual-animal level for veterinary use (i.e., not growth promotion). Some countries also permit antimicrobials to be used in an extra-label manner, under conditions for which the drug has not been officially approved by regulatory authorities (FAO/OIE/WHO, 2004). Over-the-counter availability and extra-label use are conditions of availability that favor increased use of antimicrobials, often in ways that contribute to selection and spread of resistant bacteria (FAO/OIE/WHO, 2004; Dutil et al., 2010).

Intensive livestock rearing (including most aquaculture) is important for efficient livestock rearing, but antimicrobial use tends to be much greater under these conditions because stocking densities are higher, and this favors the spread of infectious disease (EMEA, 1999, McEwen and Fedorka-Cray, 2002; FAO/WHO/OIE, 2003; FAO/OIE/WHO, 2008; Carson et al., 2008). With group-level treatment, many uninfected animals are also exposed to antimicrobials, increasing resistance selection pressure on bacteria inhabiting a variety of environmental niches, including the gastrointestinal tract and other body surfaces of animals, the local pen environment, and beyond (Aarestrup et al., 2008; Heuer et al., 2009).

21.1.2 Companion Animals (Pets)

In developed countries, antimicrobial use in companion animals (dogs and cats in particular) is much more similar to humans than food animals, reflecting their tendency to be housed as individuals within households, where they are often treated as members of the family (Prescott et al., 2002; Ogeer-Gyles et al., 2006). Antimicrobial treatments are administered on an individual animal basis usually after veterinary diagnosis and prescription; over-the-counter use of antimicrobials is much less common in companion animals than in food animals. In general, however, companion animals are more likely than food animals to be treated with the same antimicrobials that are used in humans, in part because the higher cost of treatment is not of the same concern to pet owners and concerns about residues of drug in animal-derived food are not at issue in pets, with the exception of horses, which are sometimes sent to slaughter.

21.2 SELECTION AND SPREAD OF BACTERIA RESISTANT TO ANTIMICROBIALS OF HUMAN HEALTH IMPORTANCE FOLLOWING ANTIMICROBIAL USE IN ANIMALS

Antimicrobial use in virtually any setting, including animal production, exerts resistance selection pressure on bacteria. These bacteria include the target pathogens

of animals for which the antimicrobials are administered in the first place (at least for therapy and prophylaxis) and nontarget or bystander organisms, such as commensals that inhabit the gut and bacteria found in the soil, water, and other environmental sites (WHO, 1997; O'Brien, 2002; Kozak et al., 2009). In the broadest sense, selection of resistance in any species of bacteria is potentially of human health significance given the incredible capacity of bacteria to share resistance genes and for these to spread throughout the local and distant ecosystems, eventually globally, given favorable conditions (Witte, 2000; Aarestrup, 2006). The molecular aspects of resistance and spread of determinants through the environment are, however, the subjects of other chapters; we focus here on a few principles and examples that are important in the animal sector.

Factors that favor selection and spread in animals of resistance of importance to human health include those pertaining to the drug and its administration, the animals, the bacteria themselves, and a wide range of environmental factors (FAO/WHO/OIE, 2003, Aarestrup, 2006). Examples of drug-related factors include their relative importance to human health and the ease and rapidity with which resistance develops in bacteria. The World Health Organization and other institutions have classified antimicrobials with respect to importance to human health for the purposes of assessing and managing resistance risks associated with their use in animals (FAO/OIE/WHO, 2008; Collignon et al., 2009). Table 21.1 shows the major classes of antimicrobials used in animals grouped according to the WHO classification, along with some antimicrobials that are not used in humans (so far at least) and therefore not part of the classification (Aarestrup et al., 2008). Other things being equal, it is self-evident that use of a drug classified as critically important to human health (e.g., fluoroquinolone, third-generation cephalosporin) is more likely to select for resistance of human health importance that one classified of lesser importance (e.g., an amphenicol). Furthermore, resistance develops more quickly for some drugs (e.g., fluoroquinolone resistance in *Campylobacter jejuni* requires only a one-step mutation) than others (McDermott et al, 2002; Aarestrup, 2006).

Treatment-related factors are also important, for example, selection pressure is believed to increase with the numbers of animals exposed, duration of exposure, and underdosing, hence the concern with AGPs that are administered in low doses for long periods of time to entire herds or flocks of animals (WHO, 1997; FAO/WHO/OIE, 2003). Examples of animal factors are age and production type; selection of resistant bacteria in very young animals may be less likely to result in human exposure than in animals closer in age to slaughter because the prevalence of resistance often decreases with age and reduced selection pressure (Jiang et al., 2006; Lowrance et al., 2007). Similarly, selection in dairy cows may also be less likely to lead to human exposure when milk is pasteurized prior to consumption (Sischo, 2006). With respect to bacteria-related factors, resistance selection and spread are especially important to human health when the bacteria are human pathogens, when they readily colonize and spread within and between animal populations, when they have an increased propensity to act as reservoirs of resistance genes, and when they flourish outside of the host and contaminate food, water, and other environmental vehicles of human exposure (WHO, 1997; FAO/WHO/OIE, 2003; Heuer et al., 2009).

Resistance to a wide variety of antimicrobials has emerged in some important pathogens of animals, such as strains of *Escherichia coli* that cause diarrhea in pigs, respiratory disease in cattle, and cellulitis and other infections in poultry (White,

TABLE 21.1 Antimicrobial Agents Approved for Use in Human and Veterinary Medicine[a]

Antimicrobial Class/Drug	Examples of Antimicrobials Used in Human Medicine	Examples of Antimicrobials Used in Veterinary Medicine or as Growth Promoters
Antimicrobial Classes Classified as "Critically Important" for Human Health by the WHO		
Aminoglycosides	Amikacin, arbekacin, gentamicin, kanamycin, netilmicin, neomycin, tobramycin, streptomycin	Amikacin, apramycin, gentamicin, neomycin, streptomycin, dihydrostreptomycin, kanamycin, framycetin, paromomycin (aminosidine)
Ansamycins	Rifabutin, rifampin, rifaximin	Rifampicin
Carbapenems and other penems	Ertapenem, faropenem, imipenem, meropenem, doripenem	None approved or known to be used
Cephalosporins, third generation	Cefixime, cefotaxime, cefpodoxime, ceftazidime, ceftizoxime, cefoperazone, cefoperazone/sulbactam, ceftriaxone	Cefpodoxime, ceftiofur, cefoperazone, cefovecin
Cephalosporins, fourth generation	Cefipime, cefpirome, cefoselis	Cefquinome
Lipopeptides	Daptomycin	None approved or known to be used
Glycopeptides	Teicoplanin, vancomycin	Avoparcin[b]
Macrolides, including 14-, 15-, 16-membered compounds, ketolides	Azithromycin, clarithromycin, erythromycin, midecamycin, roxithromycin, spiramycin, telithromycin	Erythromycin, pirlimycin, spiramycin, tylosin, tulathromycin, kitasamycin, oleandomycin, tilmicosin, jasamycin
Oxazolidinones	Linezolid	None approved or known to be used
Penicillins, amino	Ampicillin/amoxicillin, ampicillin/sulbactam, amoxicillin/clavulanate, piperacillin, piperacillin/tazobactam	Ampicillin/amoxicillin, ampicillin/sulbactam, amoxicillin/clavulanate
Penicillins, natural	Penicillin G, penicillin V	Penicillin G, penicillin V
Quinolones	Cinoxacin, nalidixic acid, pipedemic acid, ciprofloxacin, enoxacin, gatifloxacin, gemifloxacin, levofloxacin, lomefloxacin, moxifloxacin, norfloxacin, ofloxacin, sparfloxacin	Nalidixic acid, oxolinic acid, flumequine, pipemidic acid, danofloxacin, difloxacin, enrofloxacin, ibafloxacin, marbofloxacin, sarafloxacin, orbifloxacin, moxifloxacin

(*Continued*)

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TABLE 21.1. (Continued)

Antimicrobial Class/Drug	Examples of Antimicrobials Used in Human Medicine	Examples of Antimicrobials Used in Veterinary Medicine or as Growth Promoters
Streptogramins	Quinupristin/dalfopristin, pristinamycin	Virginiamycin[c]
Drugs used solely to treat tuberculosis or other mycobacterial disease	Cycloserine, ethambutol, ethionamide, isoniazid, paraaminosalicylic acid, pyrazinamide	None approved or known to be used
Antimicrobial Classes Classified as "highly Important" for Human Health by the WHO		
Cephalosporins, first generation	Cefazolin, cephalexin, cephalothin, cephradine	Cephalothin, cephalonium, cephalexin, cefadroxil, cefazolin
Cephalosporins, second generation	Cefaclor, cefamandole, cefuroxime, loracarbef	Cefuroxime
Cephamycins	Cefotetan, cefoxitin	None approved or known to be used
Clofazimine	Clofazidime	None approved or known to be used
Monobactams	Aztreonam	None approved or known to be used
Penicillins, amino	Mecillinam	None approved or known to be used
Penicillins, antipseudomonal	Azlocillin, carbenicillin, mezlocillin, ticarcillin, ticarcillin/clavulanate	None approved or known to be used
Polymyxins	Polymyxin B, colistin	Polymyxin B, colistin
Spectinomycin	Spectinomycin	Spectinomycin
Sulfonamides, dihydrofolate reductase inhibitors, and combinations	Para-aminobenzoic acid, pyrimethamine, sulfadiazine, sulfamethoxazole, sulfapyridine, sulfisoxazole, trimethoprim	Sulfadiazine, sulfadimidime, sulfadimethoxine, trimethoprim, baquiloprim
Sulfones (antileprosy)	Dapsone	None approved or known to be used
Tetracyclines	Chlortetracycline, doxycycline, minocycline, oxytetracycline, tetracycline	Chlortetracycline, doxycycline, oxytetracycline, tetracycline
Antimicrobial Classes Classified as "Important" for Human Health by the WHO		
Amphenicols	Chloramphenicol, thiophenicol	Chloramphenicol, florfenicol, thiamphenicol
Cyclic polypeptides	Bacitracin	Bacitracin
Fosfomycin	Fosfomycin	Fosfomycin
Fusidic acid	Fusidic acid	Fusidic acid
Lincosamides	Clindamycin, lincomycin	Clindamycin, lincomycin

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Mupirocin	Mupirocin	Mupirocin
Nitrofurans	Furazolidone, nitrofurantoin, nitrofurazone	Furazolidone, nitrofurantoin, nitrofurazone
Nitroimidazoles	Metronidazole, tinidazole	Metronidazole, dimetridazole
Penicillins, antistaphylococcal	Cloxacillin, dicloxacillin, flucloxacillin, methicillin, nafcillin, oxacillin	Cloxacillin, nafcillin, methicillin, oxacillin, dicloxacllin
Antimicrobial Classes Not Known to Be Used in Humans		
Bambermycin	None approved or known to be used	Flavomycin
Novobiocin	Might be some use for humans in some countries	Novobiocin
Orthosomycins	None approved or known to be used	Avilamycin
Quinoxaline	None approved or known to be used	Olaquindox, carbadox
Pleuromutilins	None approved or known to be used	Tiamulin, valnemulin
Polyethers	None approved or known to be used	Monensin, salinomycin, lasalocid, narasin[d]

[a]This table is primarily based on antimicrobials approved for use in Australia, Europe, and the United States. Thus, it is not a complete list of antimicrobials used in all countries. In addition, there will also be some off-label use of human drugs in food animal production.
[b]Up until 2000, avoparcin was used extensively as a growth promoter around the world (except in North America).
[c]Up until 2000, virginiamycin was used extensively as a growth promoter in Europe. It is still used extensively in North America, Australia and many other parts of the world.
[d]Used extensively for growth promotion and/or control of coccidiosis around the world.
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2006). While this sometimes necessitates the use of newer and more expensive classes of antimicrobials, including drugs of critical importance to humans (e.g., fluorquinolones, third-generation cephalosporins), resistance in many other purely animal pathogens (e.g., *Pasteurella multocida*) is much less common, depending on the region of the world (Burch et al., 2008). Consequently, within the veterinary community there is generally not perceived to be an antimicrobial resistance crisis, as there has been in the human health community. This has major implications to resistance control efforts, as will be discussed later. Concerns about human health impact, rather than animal health, has in recent decades been the principal driver of extensive research, focusing largely on the zoonotic enteropathogens that can be transmitted from animals to humans and cause disease and the commensals that can act as donors of resistance genes to pathogens and as indicators of resistance selection pressure.

Among the enteric zoonoses, nontyphoidal *Salmonella* and *Campylobacter* are typically among the most common etiologic agents of enteric illness in humans (Newell et al., 2010; Scallan et al., 2011). Some strains of these bacteria are also important animal pathogens (Poppe et al., 1998). Although these pathogens can be found in a wide range of animate and inanimate niches, they appear to thrive and spread readily in food animal populations, particularly pigs and poultry (Council for Agricultural Science and Technology, 2004; Young et al., 2009). In developed countries, foods from animals are believed to be the principal sources of human infection; examples include *Salmonella enterica* serovar Enteritidis from eggs, *Salmonella* Heidelberg from poultry, *C. jejuni* from poultry, and *Salmonella* Typhimurium from cattle (Fey et al., 2000; Swartz 2002; Dutil et al., 2010). In some repects, there is very solid evidence that antimicrobial use in animals has contributed to the selection and spread of antimicrobial resistance in *Salmonella* and *Campylobacter*. In other respects, however, there are large gaps in our understanding of this contribution leaving it open to challenge from an epidemiological perspective. Three examples are briefly described below to illustrate these points: cephalosporin resistance in *Salmonella* Heidelberg, fluoroquinolone resistance in *C. jejuni*, and multidrug resistance in *Salmonella* Typhimurium DT104. These examples also illustrate the importance of integrated surveillance of antimicrobial resistance and antimicrobial use that addresses human, animal, and food sectors.

21.3 TRENDS IN CEPHALOSPORIN RESISTANCE IN *SALMONELLA* HEIDELBERG

Salmonella Heidelberg is an important cause of human illness in many countries and is usually associated with contaminated food, most commonly poultry, but also other foods from other animals (Smith et al., 2008). Routine monitoring conducted by the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) detected a high degree of temporal correlation in trends of resistance to ceftiofur, a third-generation cephalosporin (classified by WHO as Critically Important to Human Health) among *Salmonella* Heidelberg from clinical infections in humans, from poultry samples collected at retail, and in *E. coli* from poultry samples collected at retail (Fig. 21.1) (CIPARS, 2009; Dutil et al., 2010). Ceftiofur is used only in animals, but it exhibits cross resistance to ceftriaxone, another third-generation

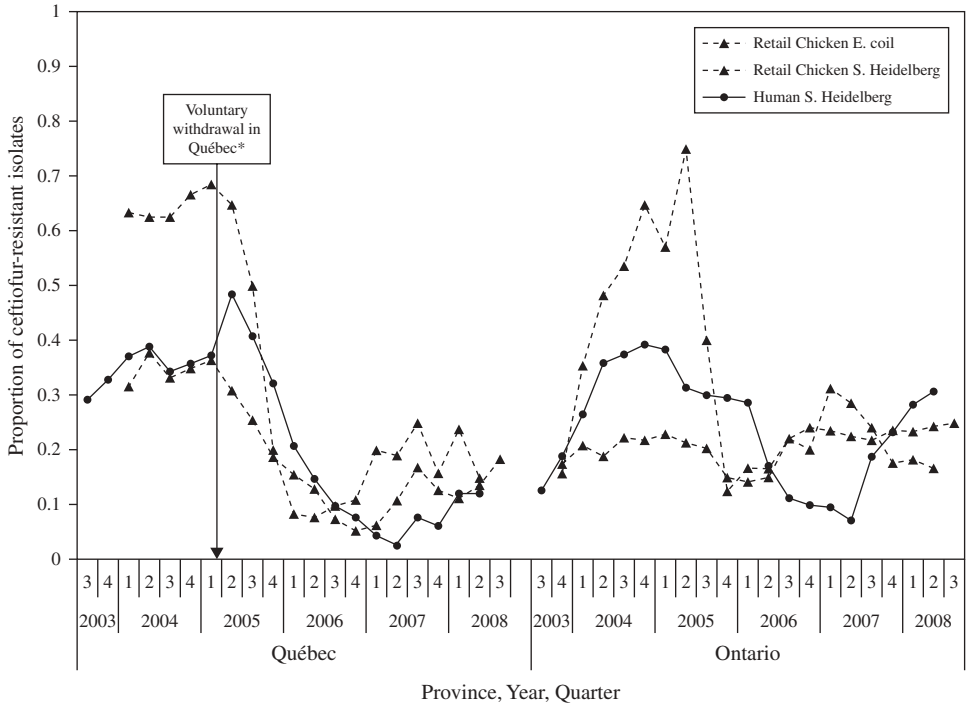

FIGURE 21.1 Proportion (moving average of previous three quarters) of isolates resistant to ceftiofur among retail chicken *E. coli*, and retail chicken and human clinical *Salmonella* Heidelberg isolates from 2003 to 2008 in Québec and Ontario. [Reprinted with permission from CIPARS, (2009)]. Asterisk (*)Indicates voluntary withdrawal of ceftiofur use in hatcheries.

cephalosporin that is used in humans and was a drug of choice for treatment of severe cases of salmonellosis in children (FAO/OIE/WHO, 2008; Collignon et al., 2009). There is strong evidence that these temporal trends were associated with the extra-label, routine injection of low doses of ceftiofur into eggs at the hatchery level (potentially involving exposure to hundreds of thousands of birds) for prophylaxis of *E. coli* infections in broilers. Voluntary withdrawal of this practice was followed by a precipitous drop in the prevalence of resistance to ceftiofur (Fig. 21.1); subsequent reintroduction of its use, apparently in a more limited way, was followed by a return to higher prevalences of resistance (Dutil et al., 2010).

21.4 EMERGENCE OF FLUORQUINOLONE RESISTANCE IN *C. JEJUNI*

Fluoroquinolones are also classified by the WHO as critically important for humans (Collignon et al., 2009; Table 21.1). They are widely used for treatment of human infection and have also been approved for veterinary use in some countries (WHO, 1998). Researchers in several countries have documented temporal changes in the prevalence of resistance to quinolones (e.g., nalidixic acid) and fluoquinolones (e.g., ciprofloxacin) among *C. jejuni*, an important cause of bacterial enteritis in humans (Fig. 21.2) (Endtz et al., 1991, Blaser and Engberg, 2008). Prior to the

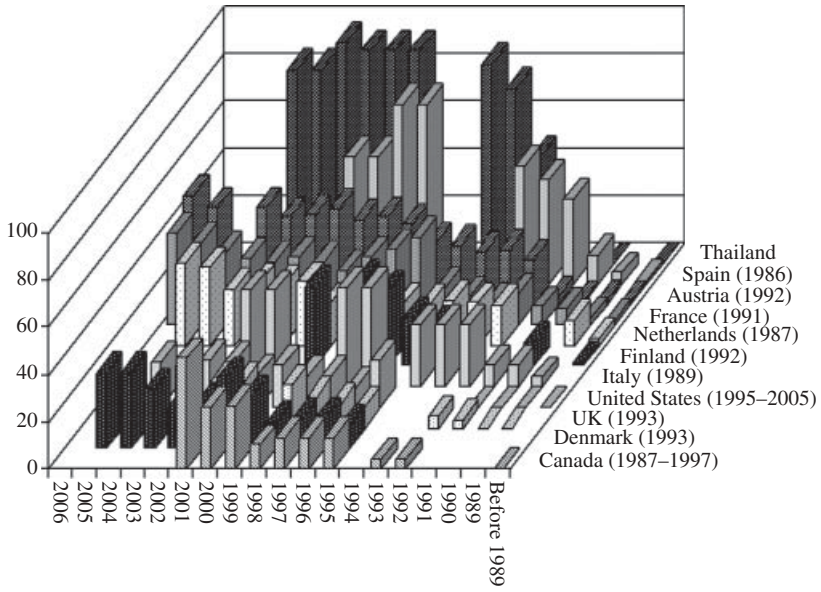

FIGURE 21.2 Trends in quinolone resistance rates among *C. coli* and *C. jejuni* isolates from humans from 11 countries, 1989–2006. The bars represent both nalidixic acid and fluoroquinolone resistance and are based on mean values of resistance from numerous reports. Year in parentheses is year of licensure for use in veterinary medicine in each country. Canada and the United States banned veterinary use of fluoroquinolones in 1997 and 2005, respectively. [Reprinted with permission from Blaser and Engberg (2008); original data sources provided in Blaser and Engberg (2008).]

approval and use of fluoroquinolones for treatment of *E. coli* infection in broilers, resistance to this class of antimicrobials was uncommon among *C. jejuni* from broilers and from human infections; but, following its introduction, the prevalence of fluoroquinolone resistance in *C. jejuni* have tended to increase, in some countries dramatically (Fig.21.2). In Australia, however, where fluoroquinolones have never been approved for use in animals but are commonly used in humans, rates of fluoroquinolone resistance in *C. jejuni* are much lower than in developed countries where the drug is used in animals (Aarestrup et al., 2008).

21.5 EMERGENCE AND GLOBAL SPREAD OF MULTIDRUG-RESISTANT *SALMONELLA* TYPHIMURIUM DT104

Salmonella Typhimurium is an important pathogen of several species of animals, including humans (Threlfall et al., 1978; Poppe et al., 1998; Cloeckaert and Schwarz, 2001; Swartz, 2002). In past decades there have been several multidrug-resistant strains (or clones) that have caused epidemics in one or more countries in food animals and people (Threlfall et al., 1978); one such strain, Salmonella Typhimurium DT104 (definitive type), was for several years a serious problem in many countries of the world, and was one of the reasons for the resurgence of concerns in the mid-1990s about human health effects of antimicrobial use in food animals (WHO, 1997; Besser

et al., 2000). Typically, DT104 displayed a penta-resistant pattern, mediated by genes on a class I integron located on the bacterial chromosome, of resistance to ampicillin, chloramphenicol, streptomycin, sulfonamide, and tetracycline, and while some strains were also resistant to other antimicrobials, it appears that the spread was mostly clonal in nature (Poppe et al., 1998, Davis et al., 1999). Although antimicrobial use in animals is thought to have been an important determinant in the emergence and global spread of DT104, it is not known when, where, how, or in what species (animals and humans) antimicrobial use contributed to the development and assembly of the resistance gene complex in this strain, nor is it very clear what role antimicrobial use played in the remarkable global spread of this pathogen, relative to many other factors (e.g., human and animal travel, ability of the organism to colonize animals and humans, environmental adaptation, etc.). Very likely these factors acted in combination, but better understanding of these phenomena was hampered by the lack of an integrated global antimicrobial resistance and antimicrobial use surveillance system. A second source of uncertainty concerning DT104 relates to the nature of the drugs to which the core penta-resistance determinants encode resistance; they are older antimicrobials that have for decades been widely used in many sectors, including animals (terrestrial food animals, poultry, in some cases aquaculture), humans, and in some cases even plants. So far at least, it has not been possible to attribute development and spread of this particular collection of resistance genes to antimicrobial use in any particular country or species, whether animal or human (Poppe et al., 1998; Davis et al., 1999; Cloeckaert and Schwarz, 2001). This example illustrates the difficulty in attributing a causal role to antimicrobial use in any one sector (and even more so among the various species within the animal sector) unless use of the antimicrobial in question is essentially restricted to that sector and integrated surveillance programs are in place to record the necessary microbiological and epidemiological data.

21.5.1 Enterococci

Enterococcus faecium and *faecalis* are commensal bacteria found in the feces of a wide range of animals including humans (McDonald et al., 1997; Woodford, 1998; Klare et al., 2003). Enterococci are important opportunistic pathogens of humans and resistance to antimicrobials can greatly increase the severity of infection. A notable example of this is resistance to the glycopeptide antimicrobial, vancomycin. Vancomycin-resistant enterococci (VRE) were especially a problem in hospital environments in the 1990s where infections were associated with high mortality rates due to very limited alternative treatment options (Woodford, 1998). Enterococci are frequently included in antimicrobial surveillance programs as Gram-positive indicators of antimicrobial selection pressure (McEwen et al., 2006). These bacteria also figure prominently in the controversy over the human health impacts from resistance arising from antimicrobial use in animals, particularly as it relates to antimicrobial growth promoters (AGPs) (WHO, 1997; Acar et al., 2000).

Several decades ago, a British inquiry (Swann, 1969) into antimicrobial resistance in agriculture issued a recommendation that antimicrobials used for therapy in humans or animals should not be available for use as AGPs, with the intent of reducing the adverse effects of resistance on human health. Soon after, the United Kingdom and other European countries (but not countries in North America and

many other regions) implemented the recommendation, separately classifying veterinary antimicrobials, which were mostly members of classes also used in humans and for which veterinary prescription was generally required, from feed additives (AGPs), which were members of classes not used in humans and for which no prescription was required (Prescott, 2006). One feed additive that was used widely in Europe from the 1970s until the 1990s was avoparcin, a member of the glycopeptides class. At the time of its introduction, another glycopeptide, vancomycin, was available for use in humans, but little used until the 1980s (WHO, 1997; Levine, 2006).

In the mid-1990s, after VRE problems emerged, researchers in Denmark documented an association between the use of avoparcin in poultry and pigs and a relatively high prevalence of fecal carriage of VRE in animals on exposed farms (Bager et al., 1997). European studies also documented VRE in meat and from feces of people in the community setting (Witte, 2000; Klare et al., 2003). The timing of these observations coincided with a growing sense of crisis in the medical community concerning rising trends in antimicrobial resistance among important human pathogens. The problem of VRE contributed significantly to the sense of crisis, and the realization that use of an AGP in livestock may well have played a very important role in this problem led to calls for much greater scrutiny and indeed restrictions on antimicrobial use in animals, particularly AGPs (WHO, 1997). This was a seminal event in the fate of AGPs in Europe; in 1995 Denmark banned the use of avoparcin as a feed additive and shortly after, the pig and poultry industries voluntarily withdrew the use of all AGPs in that country (WHO, 2003). By 2005, all feed additives, most of which were by this time members of classes used in humans, were banned across Europe.

Interestingly, VRE was not as big a problem in Danish and many other European hospitals as was the case in North America, perhaps due to more stringent controls on the use of vancomycin in these settings and/or better hospital infection control (Woodford, 1998; McDonald et al., 1997). Ironically, avoparcin was never used as a growth promoter in North America, and yet as noted previously, VRE was a serious problem in North American hospitals. These points have been interpreted by some as evidence that avoparcin may not actually have had a prominent role in the VRE public health problem (Phillips et al., 2004; Phillips, 2007). In contrast, others have argued that the avoparcin story, to the extent that it is truly understood, is actually a good example of how selection of resistance in one part of the globe can contribute to a serious problem in another (WHO, 1997; FAO/WHO/OIE, 2003). Thus, antimicrobial selection pressure at the farm level can increase the pool of resistance genes/bacteria in the wider community (people, food, animals, environment), which provides "fertile soil" for further selection of pathogenic strains within the hospital environment, given the right conditions of further antimicrobial use, lapses in infection control, and highly vulnerable populations (Woodford, 1998; Witte, 2000). Of course, with widespread international travel and the enormous amount of international trade that takes place in animals and animal-derived food products, it is not difficult to imagine that enterococci and their resistance genes (and likewise other bacteria) could easily have spread undetected from countries using avoparcin to others. Another lesson that should be gleaned from the avoparcin story is that it is unwise to base policy on the principle that some antimicrobials are only used in animals, because this situation can change as pressures to find effective treatments for humans leads to adoption of drugs from within classes previously used very little or

not at all. Moreover, better understanding of the phenomenon of co-selection, whereby use of one antimicrobial class selects for resistance to another, unrelated class due to linkage of the respective genes, also tends to defeat resistance containment efforts that depend on restriction of drug use to animals. For example, in Denmark, continued macrolide (tylosin) use in pigs was shown to sustain VRE prevalence in pigs after the avoparcin ban, while in poultry the VRE prevalence declined markedly after the ban. Linkage of genes encoding resistance to macrolides and glycopeptides was demonstrated on enterococci from the pigs (Hammerum et al., 2007).

21.5.2 Other Bacteria

Like enterococci, *E. coli* are both commensals and pathogens of a wide range of animals including humans (Swartz, 2002, White, 2006; Kennedy and Collignon, 2010), and they too have been used widely in antimicrobial resistance surveillance programs as Gram-negative indicators of antimicrobial selection pressure (McEwen et al., 2006). Given their relative ubiquity in animal and human populations, they are also considered to be important reservoirs of resistance genes for human pathogens, for example, *Salmonella* and *Shigella* (Witte, 2000; Kennedy and Collignon, 2010). There is abundant evidence that antimicrobial use in animals selects for resistance in *E. coli* from animals. For example, Dunlop and co-workers showed that use of several types of antimicrobials in the feed of pigs in the postweaning and finishing stages of production was positively associated with resistance to the same as well as unrelated antimicrobials in generic *E. coli* from finisher pigs (Dunlop et al., 1998a). Similar findings have been reported in other animals, for example, poultry, cattle, and companion animals (Levy et al.,1976; Ogeer-Gyles et al., 2006; Alexander et al., 2008).

While there appears to be considerable host specificity among many pathogenic strains of *E. coli*, at least in terms of their association with clinical disease, animals are reservoirs of some zoonotic *E. coli*. For example, *E. coli* O157:H7 is a well-known cause of hemorrhagic colitis and hemolytic uremic syndrome in humans, for which an animal reservoir (ruminants, especially cattle) is important epidemiologically (Cernicchiaro et al., 2009). While antimicrobial resistance is generally not considered to play an important role in the epidemiology of *E. coli* O157:H7 colonization in ruminants or infection in humans, there is growing evidence that resistance may be more important for the zoonotic potential of other strains of *E. coli*. Food, including food of animal origin, may be an important source of antimicrobial-resistant *E. coli* infections of humans, particularly urinary tract infections and possibly also bloodstream infections (Manges et al., 2007; Jakobsen et al., 2010; Kennedy and Collignon, 2010). For example, Johnson and co-workers demonstrated a high degree of similarity between isolates of *E. coli* from urinary tract infections in the United States and from poultry purchased at retail (Johnson et al., 2007).

Methicillin-resistant *Staphylococcus aureus* (MRSA) are very important pathogens of humans in both community and hospital settings, are responsible for considerable morbidity and mortality, and are typically resistant to a wide spectrum of antimicrobials. While *S. aureus* has for many years been a recognized pathogen of animals, particularly in the case of mastitis in cattle, MRSA were until recently relatively rare in animals, and those found were considered distinct from human pathogenic strains (Weese et al., 2010). Recently, however, strains of MRSA

pathogenic to humans have emerged in a variety of animal populations, including pigs and companion animals, such as dogs and horses (Boost et al., 2007; Lewis et al., 2008; Loeffler and Lloyd, 2010; Weese et al., 2010). An aggressive MRSA control program in the Netherlands provided an opportunity to detect an outbreak of MRSA in people from pig farms or who had contact with pigs (van Belkum et al., 2008). Subsequent investigations showed high prevalence of MRSA in pigs on contact farms and pigs from the wider population of pig farms in the Netherlands; moreover, farmers and veterinarians who had contact with pigs were also shown to have higher rates of carriage of MRSA than people in the general population (Voss et al., 2005; Wulf et al., 2006). Surveys conducted in other European countries, Canada, and the United States have also found MRSA carriage in pigs, and contamination of pork (Weese et al., 2010), although the importance of the food-borne route of transmission is unknown; available epidemiological data point toward direct transmission from pigs to humans. The predominant strain isolated from pigs and humans whose infections were epidemiologically linked to pigs has been sequence type (ST) 398, which has previously been associated with human disease but is not typically a major epidemic strain (Wulf et al., 2006; Weese et al., 2010). To date, there is little evidence that antimicrobial use in animals did or did not play a role in the emergence of MRSA in pigs, but such a role is possible. It has been suggested that the epidemic strain ST 398 emerged from within the pig population and probably spread via international trade in animals, food, or other products or international human travel (Weese et al., 2010).

In contrast to the situation with ST 398 in pigs, MRSA infections in companion animals (dogs and horses) appear to have been more localized, have involved different strains, and have been associated epidemiologically with infections in people in the household (dogs) or in contact (horses), although subsequent spread has been reported in horses and in some populations infection may be endemic in that species (Weese and Lefebvre, 2007; Weese et al., 2010). The available evidence seems to favor the hypothesis that human carriers of MRSA initially transmitted infection to the dogs; in the case of horses, strains may have originated with humans but became horse adapted (Weese and Lefebvre, 2007; Weese et al., 2010). Again, there is little evidence as yet that antimicrobial use in animals had a role in the development and spread of MRSA in dogs. In horses, prior antimicrobial use has been a risk factor for infection (Weese et al., 2010).

21.6 INTEGRATED SURVEILLANCE OF ANTIMICROBIAL USE AND RESISTANCE IN ANIMALS, FOOD, AND HUMANS

National antimicrobial resistance surveillance programs, and to a lesser extent, those for antimicrobial use, have provided much of the data that has furthered our current understanding of antimicrobial resistance trends in bacteria from animals and humans, quantities of antimicrobials used in various sectors, and relationships among use and resistance, especially at national levels. Globally, the terms "surveillance" and "monitoring" are used more or less interchangeably in program titles and associated literature, and while technically some differences exist [surveillance involves monitoring plus the taking of appropriate action(s) based on the results of such monitoring], the essential goals are similar (McEwen et al., 2006). In short,

these goals are to gather, analyze, interpret, and report representative data on antimicrobial resistance and antimicrobial use in order to monitor temporal and spatial trends, identify emerging problems and opportunities for intervention, and to communicate the relevant information to those in a position to make use of it for the benefit of human and animal health (WHO, 1997; FAO/OIE/WHO, 2004; Crump et al., 2011). Integrated surveillance programs are those that bring together, ideally in a proactive and comprehensive manner, the human, animal, food, and environmental sectors, as well as data concerning both resistance and use (WHO, 2009).

Existing programs vary enormously in scope, depth, and resource base and emerged from a period of heightened global concern, dialog, and activity with regard to antimicrobial resistance, roughly 1995–2005. During this period, numerous expert working groups and stakeholder panels, some national, others international, published reviews and reports describing the problem of antimicrobial resistance and providing recommendations for its remediation (WHO,1997; JETACAR, 1999; National Research Council,1999; Health Canada, 2002; FAO/WHO/OIE, 2003). Virtually all pointed to the numerous data gaps that were barriers to sound policy development, and in addition to recommending more research, essentially all of these reports supported improved surveillance of antimicrobial resistance and antimicrobial use. Among the earliest models on the scene were DANMAP, the Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP, 2010), and NARMS, the American National Antimicrobial Resistance Monitoring System (Crump et al., 2011). Subsequently, numerous other national programs developed; many adopting components of DANMAP or NARMS and adding their own features of relevance to their respective countries, resources, and sponsoring agencies. Examples include CIPARS in Canada, NORMVET in Norway, SVARM in Sweden, MARAN in the Netherlands, and programs in France, Japan, Korea, New Zealand, and other countries (McEwen et al., 2006). While these examples are all national in scope, there is a recognized need for surveillance at the international level, given the global nature of antimicrobial resistance and the need for sharing knowledge, expertise, and resources among developed and underdeveloped countries. To this end, the World Health Organization has recently taken the initiative of developing guidelines and tools to support integrated surveillance globally (WHO, 2009).

The core elements of these programs include collection of representative isolates of key species of bacteria (typically nontyphoidal *Salmonella*, *E. coli*, and in many cases also *Campylobacter* spp. and *Enterococcus* spp.) from clinical cases in humans and healthy animals/carcasses at slaughter, and conducting susceptibility testing using standardized methods and then reporting the results on an annual basis. Some programs also sample from a wider range of food animal species at slaughter, retail foods, healthy animals on farms, and aquaculture. Antimicrobial use monitoring has been much slower to develop outside of Scandinavia (Grave et al., 2006). The Danish veterinary registry of antimicrobial use information, VETSTAT, is the most comprehensive and well known; it involves routine collection, analysis, and interpretation of antimicrobial use data collected from both human and nonhuman sectors, including pharmacies, hospitals, and veterinarians. The data are presented in an integrated fashion with resistance monitoring data in DANMAP reports (DANMAP, 2010). Use data are reported quantitatively as overall weight of antimicrobials

used (e.g., national consumption, kilograms of active ingredient) by species (humans, pigs, cattle, etc.), and in standardized units (e.g., defined daily doses in humans, animal daily doses). Several other countries, especially within Europe, also provide national antimicrobial consumption data in humans and animals (Grave et al., 2006). Resistance monitoring in bacteria from other types of environmental samples is very limited to nonexistent in these programs; the focus is on humans, animals, and food.

Among the important functions that these surveillance systems provide is documentation of the effects of interventions (e.g., ban on AGPs as discussed below), identification of problems warranting further investigation (e.g., MRSA surveillance in the Netherlands as discussed previously), and providing data to support regulatory initiatives (e.g., withdrawal of approval for fluoroquinolones in poultry in the United States (Nelson et al., 2007)). For example, DANMAP and VETSTAT data formed the basis for the World Health Organization's review of the effects of termination (ban) of the use of AGPs in food animals in Denmark (WHO, 2003). This review also used comprehensive Danish monitoring data on zoonoses, animal and food production, as well as the results of extensive Danish research into effects on animal health and alternatives for AGPs. The review determined the effects of the ban on antimicrobial use, antimicrobial resistance, animal health, human health, zoonoses (e.g., *Salmonella*, *Campylobacter*, *Yersinia*, and others in poultry, pigs, humans), animal production (e.g., morbidity, mortality, feed efficiency), economics, and exports. In brief, the results showed that the ban had the expected result of dramatically reducing the quantities of AGPs (e.g., avoparcin, tylosin, spiramycin) used, as the various drugs were banned at intervals in various species and production types (1995–2001). There was also a modest increase in the use of certain therapeutic antimicrobials (e.g., tetracycline), notably in postweaning pigs, although there was a net reduction in the total antimicrobial consumption in food animals. No effects on zoonoses were detected but increased problems with postweaning diarrhea were observed in pigs, accompanied by some production losses in weaned pigs; however, finisher pig health and production were unaffected as was poultry health; the minor losses observed in poultry feed efficiency were essentially offset by the savings to farmers from discontinuation of AGPs in feed (WHO, 2003). Other economic indicators and exports were unaffected. Research on alternatives to AGPs yielded some promising results, to be discussed later. Subsequent to publication of the WHO review, analysis of longer term monitoring data, including several years after the review, showed that the production losses in weaned pigs were not sustained (Aarestrup et al., 2010). Thus, DANMAP and VETSTAT provided essential data for evaluation of the effects of AGP termination on antimicrobial resistance and use, as well as other outcomes. This information is highly relevant to antimicrobial use policy deliberations in countries other than Denmark. As might be expected, the data have been reviewed and interpreted differently by groups with different interests in the use of antimicrobials in animals. Some have pointed to the production losses and diarrhea in weaned pigs, as well as the associated increases in therapeutic antimicrobials, as evidence of negative effects of the AGP ban and have questioned the benefits that were gained in public health (Phillips et al., 2004; Phillips, 2007). Overall the data show that AGPs provided minimal benefits to animal production, and other countries could stop using AGPs and reduce overall antimicrobial use without substantial loss or hardship to their food animal industries.

21.7 ASSESSMENT OF HUMAN HEALTH RISKS

Among the many stumbling blocks in improving use of antimicrobials is that collectively, there is little agreement among the various interest groups (e.g., scientists of various persuasions, public health, animal health, pharmaceutical industry, farming industries) about whether a problem even exists, and if it does, how big it is, what actions should be taken to deal with it, how much benefit, if any, would accrue from such actions, and what would be their unintended consequences (Barza, 2002; FAO/WHO/OIE, 2003; Cox and Popken, 2006; McEwen, 2006; Phillips, 2007; Heuer et al., 2009). Essentially, these are matters of risk and benefit and how the former should be managed; thus far, efforts to do so have been unsatisfactory to many, and much of this can be attributed to an overall poor understanding of the risks and benefits involved.

Risk assessment is familiar to many working in the environmental sciences, indeed the principles of risk assessment, as classically described in 1983 in the "Red Book" by the National Academy of Sciences, were largely developed to address human health concerns about other environmental hazards, particularly those of a toxicological nature (National Research Council, 1983). These principles, and more broadly those of risk analysis (risk assessment, risk management, and risk communication), have been taken up by the food safety community and figure prominently in the workings of many national food safety authorities (e.g., Health Canada, U.S. Food and Drug Administration) and international agencies (e.g., Food and Agriculture Organization, World Health Organization). From an international trade perspective, risk analysis is officially used by Codex Alimentarius, the international food standard setting agency (WHO, 1995; Health Canada, 2002; Tollefson et al., 2006; Codex Alimentarius Commission, 2009). From the perspective of antimicrobial use in animals, there are well-established procedures that are used by these national and international agencies for assessment, management, and communication of risks to human health arising from antimicrobial residues that occur in animal-derived foods (e.g., meat, fish, eggs, milk), particularly as it relates to human health safety evaluation of new antimicrobial submissions in the drug approval/licensure process (Tollefson et al., 2006). For the most part, these concern toxicological issues (tissue damage, carcinogenicity, and allergy) but also the microbiological effects of residues on the enteric flora of humans following consumption of residues in foods (WHO, 1995). Risk management usually takes the form of imposing restrictions on times from administration of specific drugs to animals and marketing of their food products (withdrawal times), restriction of extra-label use, and frequently limiting availability to veterinary prescription (FAO/OIE/WHO, 2004). In most jurisdictions, antimicrobials with carcinogenic properties are not approved for use in food animals (e.g., nitrofurazones, carbadox) (National Research Council, 1999). These antimicrobial residue risk management procedures have been in wide use for decades, are generally well accepted by stakeholders, and work quite well, judging by the infrequency with which hazardous concentrations of antimicrobial residues are detected in monitoring programs, and the extreme rarity with which adverse human health outcomes are attributed to these residues (Waltner-Toews and McEwen, 1994).

In stark contrast, procedures for assessment, management, and communication of antimicrobial resistance risks to human health arising from antimicrobial use in animals are not well developed, are not widely used by national and international

agencies, and are not widely accepted by stakeholder groups (FAO/OIE/WHO, 2004; McEwen, 2006; Snary and McEwen, 2008). The main reason for this is the incredible complexity of human exposure pathways that is imparted by the microbiological nature of resistance hazards, but this is compounded by other factors, including the fact that serious attempts to characterize resistance risks have only recently emerged, and many of the interest groups involved are not familiar with, or indeed are very wary of, risk assessment (FAO/OIE/WHO, 2004). Moreover, many of the risk management options that are proposed to curtail resistance are very threatening to some stakeholders because they involve, in one form or another, reductions in overall antimicrobial use, for example, banning of certain drugs and other serious restrictions.

Bacteria and their toxins are important causes of illness, and people are exposed to them by direct contact with other people in the case of communicable disease (e.g., tuberculosis, MRSA) but also indirectly in the case of bacteria of environmental origin through food, water, air, and sometimes direct contact with animals (Holmberg et al., 1984; Helms et al., 2004; Koningstein et al., 2010). Conceptually (and in contrast to industrial chemicals and drugs), these bacteria of environmental origin are naturally occurring and an inherent part of our ecosystem rather than direct products of technology or industry. Their presence as contaminants in food and water is typically a consequence of inadvertent contamination by human activities (e.g., agriculture, improper sewage treatment, food processing) (FAO/WHO/OIE, 2003). Risk management of food and waterborne bacteria has tended to focus on steps that prevent, kill, or reduce contamination with these naturally occurring microbiological hazards, to the extent practicable with available technologies (Council for Agricultural Science and Technology, 2004; FAO/WHO, 2006). Formal assessment of microbiological risks has not been as widely practiced as is the case for chemical risks, nor for as long, but it has been used to study dose–response relationships and model exposure pathways in order to identify best options for intervention and, to a lesser extent, to support standard setting (WHO, 1995; Feingold et al., 2010). Important progress has been made in developing risk assessment models for some of the more common bacterial contaminants of foods from animals, in particular, *Campylobacter*, *Listeria*, and *Salmonella* (FAO/WHO, 2006). It is noteworthy that the scientists, engineers, and risk managers that engage in or are users of microbiological risk assessment tend to be based in the environmental and food sciences, food industry, and water resource protection agencies and, therefore, focus on the environmental bacteria that fall within their remit. In contrast, infectious disease scientists, physicians, and infection control specialists in clinical medicine and public health have tended not to use the formal "Red Book" risk assessment methods for bacterial infection, probably because the need has not been there—the clinical outcomes of bacterial infection are apparent to the attending physician, and there are well-established surveillance programs in place to enumerate incidence (risk) of infection.

Assessment of risks from antimicrobial-resistant bacteria arising from antimicrobial use in animals combines elements of the above three risk assessment approaches (chemicals, bacteria in food, and clinical infectious disease). Similar to chemical risk assessment, there is a focus on hazards that are not entirely naturally occurring but are related to intentionally used chemical compounds in animal husbandry. Similar to microbiological risk assessment, the resistant bacteria follow complex exposure

pathways through animals, food and water, and similar to infectious disease "risk assessment," the actual incidence of clinical infections can be identified and measured by physicians, hospitals, and public health agency surveillance programs. Not surprisingly, therefore, the antimicrobial resistance risk assessments that have been conducted to date have been hybrids of one or more of these three approaches (Institute of Medicine, 1989; Anderson et al., 2001; Hurd et al., 2004; Cox and Popken, 2006). Most of these assessments have been undertaken to support or lobby for risk management of antimicrobials already on the market, and, in this sense, they are "postapproval" risk assessments. There is also a need for models for "preapproval" assessment as part of the new drug approval process. The U.S. Food and Drug administration (FDA) has developed one such model that is qualitative in nature (Tollefson et al., 2006).

The approaches that have been used to assess the human health resistance risks from antimicrobial use in animals are discussed in more depth elsewhere (Bailar and Travers, 2002, Snary and McEwen, 2008). Some of these assessments have been "top-down" approaches to build on the results of resistance surveillance programs in order to identify the increased burden of infectious disease attributable to antimicrobial use in animals (Institute of Medicine, 1989; Nelson et al., 2007). Others have taken a "bottom-up" approach to model use of antimicrobials in animals, selection of resistance in bacteria, contamination of the food chain, exposure of humans, and resultant disease (Anderson et al., 2001; Hurd et al., 2004). In general, these models/approaches have focused on one "bug/drug" combination and have been motivated to either support or refute risk management efforts to control resistance (e.g., withdrawal of approval) (Bailar and Travers, 2002). Essentially, these efforts and their supporting research have shown that antimicrobial resistance arising from the use of antimicrobials in animals can increase the burden of disease in humans above and beyond that from susceptible infections by increasing the frequency, severity, and duration of infections. They differ substantially, however, in their estimates of the magnitude of the increased burden, consequently there is still much uncertainty and lack of agreement on this point.

Antimicrobial resistance risk assessment methods and applications are likely to improve in the near future. The Codex Alimentarius Commission has recently formed an Ad Hoc Intergovernmental Task Force on Antimicrobial Resistance with a mandate to propose guidelines for risk analysis of food-borne antimicrobial resistance (Codex Alimentarius Commission, 2009). Although there is considerable work remaining to be done, this initiative is likely to be a very important step in achieving international consensus on methods and tools for antimicrobial resistance risk assessment as it pertains at least to food, the most important environmental exposure vehicle for humans, and the only one that is addressed by Codex. Hopefully, this will help the international scientific community to soon reach the milestone achieved several years ago by antimicrobial residue risk assessment, and that is a provision of a more uniform and transparent scientific basis for risk management and communication. Codex is an important standard-setting body for international trade in food; thus there are trade and economic implications to this process that may be beneficial in providing an economic incentive, thus far largely absent, for countries to pay more attention to antimicrobial resistance issues. It will, however, need to somehow be expanded beyond just food to more fully reflect the diversity of exposure pathways for which antimicrobial-resistant bacteria and

their genes reach humans following their selection by antimicrobial use in animal populations.

21.8 INTERVENTIONS TO LIMIT IMPACT ON HUMAN HEALTH

For decades, the veterinary community has enjoyed a fairly regular supply of new antimicrobials with which to treat and prevent bacterial infections and to promote growth and improve feed efficiency. The "writing is on the wall," however, that the dwindling supply of new drug classes in human medicine foreshadows a similar trend in veterinary medicine (Shryock and Richwine, 2010). Moreover, many expert panels, public health agencies, and interest groups have been pressing for reductions in antimicrobial use in all sectors to stem the increasing trends in resistance that have been noted in many important pathogens and commensals (WHO, 1997; JETACAR, 1999; FAO/OIE/WHO, 2004). To this end, a number of risk management options, or interventions, for reducing antimicrobial resistance in the animal sector have been proposed, and in some cases, implemented in one or more regions of the globe. These can be broadly classified into measures that pertain to regulation or licensing of antimicrobial use, bans or other restrictions on use, surveillance of antimicrobial use and resistance, prevention of spread (containment), voluntary programs that encourage prudent or judicious antimicrobial use by farmers and veterinarians, and adoption of alternatives to antimicrobials. These are more fully described elsewhere, and some have already been touched on earlier in this chapter (e.g., surveillance, bans) (WHO, 2000; FAO/OIE/WHO, 2004; Aarestrup et al., 2008).

Regulation of veterinary antimicrobials and feed additives would improve with general integration of good antimicrobial resistance risk analysis methods into regulatory decision making and national antimicrobial use policies. As mentioned earlier, formal assessment of resistance risks has only recently been introduced into the new drug approval process in some counties and is far from uniformly adopted globally. The lack of this type of assessment means that new drugs are released on the market without reasonable assurance that use under the conditions of approval is safe from the human health perspective. Although the risk assessment methods and tools to accomplish this are far from perfect, approval decisions need to be taken in timely fashion, therefore drug regulators (risk managers) need to use the available knowledge and tools to make wise licensing decisions that protect public health. In the absence of certainty, this requires the use of a precautionary approach, which is of course a very controversial area, particularly when expressed in terms of the "precautionary principle" (Phillips et al., 2004; Phillips, 2007).

Some countries (e.g., Canada) explicitly require that national regulations balance the need to protect the public from risks posed by new technologies, with any benefits to society that accrue from these technologies (Health Canada, 2002). In the case of antimicrobials for use in animals, some societal benefits arise from the treatment and/or prevention of clinical bacterial infections in animals; examples include improved animal welfare, reduction of losses to farmers from morbidity and mortality in livestock, and treatment of sick companion animals, which in many households are considered members of the family (National Research Council, 1999). It is difficult to quantify these benefits, but there has been little debate over the legitimacy of treating clinical infections in individual animals, so for this alone, it is important for

regulators to ensure the availability to veterinarians and farmers of at least some efficacious antimicrobials. It is more difficult to reach agreement on the right balance of risk and benefit with respect to therapeutic and prophylactic group treatments in animals, particularly for infections that could be prevented by other means (McEwen, 2006). In the case of AGPs, it will be necessary for national authorities to eventually determine (if they have not already done so as in Europe) whether societal benefits (e.g., cheaper food) outweigh the risks from resistance. The available evidence suggests that this probably does apply to the polyether ionophore class of antimicrobials, which are generally accepted to improve production efficiency and (at least so far) have not been shown to select for resistance to other classes of antimicrobials (WHO, 1997; Health Canada, 2002) and are widely used to prevent coccidiosis in poultry, an important production-limiting disease (Table 21.1). It has been argued that growth promoters may under some conditions provide another type of benefit: a reduction in food contamination with pathogenic bacteria; however, the evidence supporting this claim is thus far sketchy (Phillips, 2007; Singer et al., 2007).

Other regulatory interventions that are available include restrictions on the extra-label use of antimicrobials, restrictions on use of critically important antimicrobials for humans, prescription-only availability of all antimicrobials for animals, limitations on distribution and sale of antimicrobials by veterinarians, and improved product labeling (WHO, 2000; FAO/OIE/WHO, 2004). In Denmark, for example, veterinarians are allowed to prescribe fluoroquinolones for use in food animals only when laboratory testing demonstrates that the bacteria causing the infection are not susceptible to any other approved antimicrobial, and then treatment is limited to 5 days (Aaresrup et al., 2008). As described above in the example of ceftiofur resistance in *Salmonella* Heidelberg in Canada, extra-label use of antimicrobials can adversely affect human health, particularly when it leads to routine treatment of large numbers of animals or entire populations (Dutil et al., 2010). Regulatory authorities should not allow such hazardous use practices when there are significant risks to human health (Swartz, 2002). This is particularly likely when the drugs are members of antimicrobial classes of greater importance to human health, as is the case with third-generation cephalosporins (Helms et al., 2004; Nelson et al., 2007; FAO/OIE/WHO, 2008).

Several national and international agencies have classified antimicrobials with respect to their importance to human health for the purposes of assessing and managing resistance risks to humans. For example, WHO has classified, for risk management strategies of nonhuman use, antimicrobials used in humans into three categories: critically important, highly important, and important (FAO/OIE/WHO, 2008; Collignon et al., 2009). Examples in the critically important category include fluoroquinolones, third- and fourth- generation cephalosprins, and macrolides; in the highly important category: first-and second-generation cephalosporins and sulfonamides; and in the important category: lincosamides, nitrofurans, and amphenicols. Similarly, the World Association for Animal Health (OIE) has conducted a parallel classification of antimicrobials for importance to animal health. As might be expected, there is considerable overlap of the two lists, and much remains to be done by way of reconciling the overlap and developing strategies to best utilize these rankings in risk management (FAO/OIE/WHO, 2008).

Veterinarians and farmers are well aware of the need to use antimicrobials carefully in order to prevent violative residues in meat, milk, and eggs of treated animals, and structured residue avoidance programs have been developed for the

major food animal species. Coupled with robust government and industry surveillance and regulatory compliance programs, these efforts have reduced the incidence of residue violations considerably in recent decades (National Research Council, 1999). This important achievement is made possible by the relative ease and certainty with which the source of antimicrobial residue in food can be attributed to use on a specific farm, therefore, the responsible farmer or veterinarian can be identified with sufficient confidence to withstand legal challenge, enabling the enforcement of regulations (Waltner-Toews and McEwen, 1994). This situation does not exist in the case of antimicrobial resistance. Thus far, it is not possible, with certainty, to specifically link the existence of a resistance determinant in a bacterium found within a human or food to a specific treatment event on a farm. For this and other reasons, regulatory programs for the control of antimicrobial resistance in food have been slow to develop in most countries and are nonexistent in others.

There are, however, some voluntary schemes to promote prudent antimicrobial use in animals with a view to containment of antimicrobial resistance. For the most part, these have emerged subsequent to the heightened scrutiny of antimicrobial use in all sectors that has been brought about by the serious antimicrobial resistance problems in human health. This scrutiny was very unwelcome in much of the veterinary community, which felt threatened by the possibility of further restrictions on the veterinarian's right to prescribe or by outright bans. Consequently, veterinary groups and some food animal production organizations developed sets of prudent (or judicious) guidelines with a view to limiting selection and spread of antimicrobial resistance while enabling the continued use of antimicrobials by veterinarians (Health Canada, 2002; Aarestrup et al., 2008). Some of these guidelines are very general in nature, for example, emphasizing the need for a valid veterinary–client–patient relationship, preference for use of narrow-spectrum instead of broad-spectrum antimicrobials, and use of culture and susceptibility testing to guide appropriate antimicrobial selection (CVMA, 2000; AAFP, 2001). Since the guidelines were developed by veterinary organizations, they tend to leave wide latitude to veterinarians for making decisions concerning use of particular antimicrobials under given conditions. There are also examples of more specific and even prescriptive antimicrobial treatment guidelines in veterinary medicine, but not many (Weese, 2006). For example, Burch et al. (2008) have proposed first, second, and last resort choices of antimicrobials for a wide range of bacterial infections of pigs. These choices reflect clinical efficacy and a desire to minimize selection of resistance to antimicrobials of importance to human health, thus first and second choice antimicrobials are of lesser importance to human health than the choices of last resort. Unfortunately, there is little published evidence of either the effectiveness, or lack thereof, of these voluntary efforts to improve prudent antimicrobial use. Denmark has a well-articulated treatment formulary that is designed to improve prudent use, but that country also exerts considerable control over the availability of antimicrobials in other ways; for example, it limits the profit that veterinarians can realize from the sale of antimicrobials and has banned AGPs (Aarestrup et al., 2008). It is difficult when multiple interventions are applied more or less simultaneously to ascertain their individual effects, but there are temporal data to suggest the step of limiting drug sales profits by veterinarians resulted in reduced overall drug consumption (WHO, 2003; DANMAP, 2010). In many other countries, for example, Canada, the lack of antimicrobial use surveillance programs is a barrier to tracking the

effectiveness of voluntary, or for that matter, involuntary, control programs. Targeted research projects are needed to address this issue of the effectiveness of voluntary prudent use programs before they can be recommended over regulatory controls.

The success of such voluntary programs is dependent on sufficient incentive to motivate changes in antimicrobial prescribing and use behavior. Unfortunately, there are often few incentives for veterinarians and farmers to voluntarily reduce antimicrobial use in animals, in fact there are some formidable disincentives to do so (Health Canada, 2002). In the case of food animals, the cost of antimicrobials is a consideration, but many of the older antimicrobials are off-patent, relatively inexpensive, and considered to provide a positive return on investment from reduced morbidity and mortality in livestock. In some markets, there is a premium available to farmers for livestock produced under "organic" or "antibiotic-free" conditions, and there is some evidence that these methods result in lower prevalences of resistance in commensal bacteria (Young et al., 2009), but in most countries these are still niche markets. Otherwise, veterinarians and farmers are not directly rewarded for reducing use of antimicrobials generally or those of critical importance to humans in particular. An important disincentive to behavioral change is the prospect of increased losses from bacterial infection if antimicrobials are not used. In the case of companion animals, the cost of treatment is often not an important factor limiting use.

An important means of reducing antimicrobial selection pressure is to reduce the incidence of bacterial disease in animals and therefore the need for treatment. This can be achieved to a greater or lesser extent through infection control, disease prevention strategies, and in the case of AGPs, through use of nonantimicrobial methods to improve feed efficiency and rate of gain (WHO, 2000). Numerous strategies have been developed for infection control, often called "biosecurity," in food animal production. Examples include all-in-all-out production, improved cleaning and sanitation of facilities between production lots, restriction of entry to premises of nonessential people, vehicles and equipment, and exclusion of rodents, insects, birds, and other vectors of bacteria (FAO/OIE/WHO, 2004; Aarestrup et al., 2008). These measures have mostly been designed to prevent and control important pathogens of animals, especially production-limiting viruses (e.g., Newcastle disease in poultry) but also some bacteria (e.g., *Salmonella enteritidis* in poultry). Some biosecurity measures are applied at the national level and are aimed at excluding exotic disease introduction via imported animals or animal-derived products. Thus far, there have been limited attempts to address entry of antimicrobial resistance at the national level, although Denmark has made some effort in this regard (DANMAP, 2010). At the farm level, biosecurity can successfully exclude some important production-limiting diseases of livestock and poultry; however, its effectiveness in limiting spread of antimicrobial resistance in enteric commensal or environmental bacteria is unknown (FAO/WHO/OIE, 2003). Infection control is not as frequently applied in companion animals, although there are recommendations to do so, particularly to prevent nosocomial (hospital acquired) infections in veterinary hospitals (Benedict et al., 2008, Murphy et al., 2010).

Vaccination is another important nonantimicrobial disease prevention strategy. Increasingly, efficacious vaccines are becoming available for prevention of important bacterial and viral infections of animals. The latter can reduce antimicrobial use

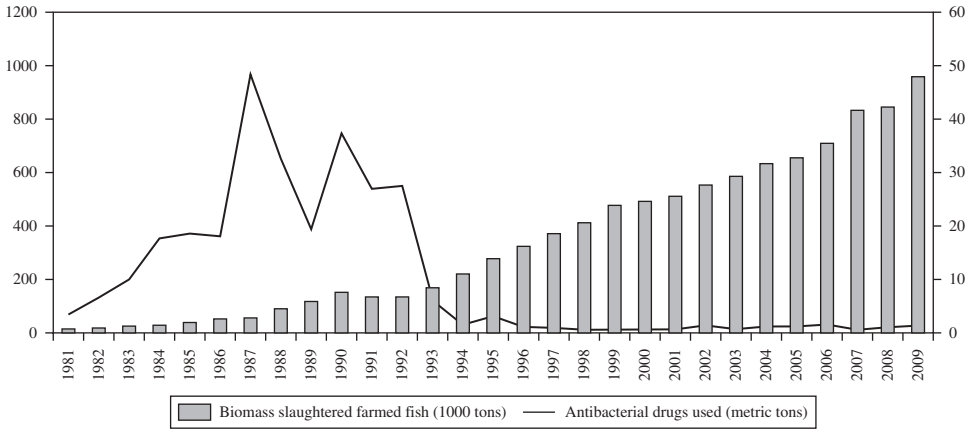

FIGURE 21.3 Annual sales of veterinary antibacterial drugs, in metric tons, for use in farmed fish in Norway versus biomass slaughtered fish. (Figure kindly developed and provided by Dr. K. Grave.)

through reduction of secondary bacterial infections and reducing the inclination to mistakenly administer antimicrobials for treatment and prevention of viral infections. Occasionally, vaccines can dramatically reduce the use of antimicrobials. As shown in Figure 21.3, use of antimicrobials in salmon and trout production dropped precipitously following introduction of vaccines and better management into salmon and trout farming in Norway (Sorum, 2000; FAO/OIE/WHO, 2006). Considerable research has been conducted to develop better vaccines for other animal infections for which prophylactic antimicrobials are often routinely administered, for example, necrotic enteritis in broilers, a condition associated with *Clostridium perfringens* (WHO, 2003; FAO/WHO/OIE, 2003).

As previously mentioned, among the various applications of antimicrobial use in animals, AGPs have been the subject of greatest scrutiny and debate. Not surprisingly, considerable efforts have been made to identify suitable alternative feed supplements that will enhance feed efficiency and rate of growth; those aimed at preventing subclinical disease (another proposed mechanism of AGPs) are addressed above. Examples of alternatives include prebiotics, probiotics, organic acids, fermented liquid feeds; the latter showed some promise in enhancing production efficiency (WHO, 2003).

In general, care is needed to ensure that adoption of an intervention for reduction of antimicrobial use (and therefore resistance selection pressure) does not itself cause harm, that is, the solution should not create a greater new problem. Increased diarrhea in weaned pigs was noted in Denmark following its AGP ban, and similar effects were also seen a decade earlier in Sweden. Swedish farmers attempted to deal with these problems by administering zinc oxide in feed, and concerns were expressed about the possible environmental effects of this treatment, requiring the adoption of other measures (WHO, 1997). Prior to implementation of its own ban, Denmark attempted to anticipate other possible adverse effects of the ban and set in place measures to mitigate their impact, including funds to compensate farmers for losses due to necrotic enteritis (which were less than expected), and funds for an extensive

research program into alternatives for growth promotion and disease prevention. Although this research provided many new insights and promising alternative products, some additional adverse effects (e.g., increased use of therapeutic antimicrobials, as mentioned above) were detected. On balance, however, the overall societal benefits from reduced antimicrobial selection pressure appeared to outweigh the adverse impacts (WHO, 2003; Aarestrup et al., 2008).

21.9 CONCLUSIONS

The quantities of antimicrobials used in animals are large; sometimes greater than those used in humans, sometimes less, depending on the country and survey. Many, but not all, antimicrobials used in animals are members of the same classes as used in humans. Some antimicrobial use in animals is necessary for treating clinical bacterial infections in animals. There is also in some cases excessive use (even abuse), especially when they are administered indiscriminately to groups of animals for prolonged periods of time, and when they are used to compensate for poor animal husbandry practices and without regard for the potential impact on human health or the environment. These antimicrobials select for resistance in *Salmonella*, *Campylobacter*, *E. coli* and many other bacteria, and this resistance can increase the frequency, duration, and severity of illness in humans. Although the food production, processing, and distribution system is believed to be the major route of environmental exposure to humans of resistant bacteria from animals, they may also be transmitted directly through animal contact and through water and other routes. While clear examples of resistance selection in animals and resulting infection in humans have been described, the magnitude of the adverse effects on human health posed by antimicrobial use in animals is poorly understood, largely due to the enormous complexities and uncertainties inherent in the interactions among populations of animals, humans, and bacteria. Some steps in a few countries have been taken to reduce antimicrobial resistance problems in animals through reduction of overall antimicrobial use, but in most countries antimicrobials are still widely available, at low cost, often over the counter, and without veterinary prescription. Until this unhappy situation is dramatically improved, rates of resistance in bacterial populations in animals, humans, and the environment are likely to continue to rise.

REFERENCES

Aarestrup FM (2006). *The origin, evolution and local and global dissemination of antimicrobial resistance*. In FM Aarestrup (Eds.), *Antimicrobial Resistance in Bacteria of Animal Origin. Veterinary and Public Health Aspects*. ASM Press, Washington, DC, p. 339–360.

Aarestrup FM, Wegener HC, Collignon P (2008). Resistance in bacteria of the food chain: Epidemiology and control strategies. *Expert Rev Anti-Infect Ther* 6:733–50.

Aarestrup FM, Jensen VF, Emborg HD, Jacobsen E, Wegener HC (2010). Changes in the use of antimicrobials and the effects on productivity of swine farms in Denmark. *Am J Vet Res* 71:726–733.

Acar J, Casewell M, Freeman J, Friis C, Goossens H (2000). Avoparcin and virginiamycin as animal growth promoters: A plea for science in decision-making. *Clin Microbiol Infect* 6:477–482.

Alexander TW, Yanke LJ, Topp E, Olson ME, Read RR, Morck DW, McAllister TA (2008). Effect of subtherapeutic administration of antibiotics on the prevalence of antibiotic-resistant *Escherichia coli* bacteria in feedlot cattle. *Appl Environ Microbiol* 74:4405–4416.

American Association of Feline Practitioners (AAFP) (2001). Basic guidelines of judicious therapeutic use of antimicrobials in cats. Available: http://www.catvets.com/professionals/guidelines/publications/?Id = 179. Accessed January 2011.

Anderson S, Yeaton Woo R, Crawford L (2001). Risk assessment of the impact on human health of resistant *Campylobacter jejuni* from fluoroquinolone use in beef cattle. *Food Control* 12:13–25.

Bager F, Madsen M, Christensen J, Aarestrup FM (1997). Avoparcin used as a growth promoter is associated with the occurrence of vancomycin-resistant *Enterococcus faecium* on Danish poultry and pig farms. *Prevent Vet Med* 31:95–112.

Bailar III JC, Travers K (2002). Review of assessments of the human health risk associated with the use of antimicrobial agents in agriculture. *Clin Infect Dis* 34(Suppl 3):S135–143.

Barza M (2002). Potential mechanisms of increased disease in humans from antimicrobial resistance in food animals. *Clin Infect Dis* 34(Suppl 3):S123–125.

Benedict KM, Morley PS, van Metre DC (2008). Characteristics of biosecurity and infection control programs at veterinary teaching hospitals. *JAMA* 233:767–773.

Besser TE, Goldoft M, Pritchett LC, Khakhria R, Hancock DD, Rice DH, Gay JM, Johnson W, Gay CC (2000). Multiresistant *Salmonella typhimurium* DT104 infections of humans and domestic animals in the Pacific Northwest of the United States. *Epidemiol Infect* 124:193–200.

Blaser MJ, Engberg J (2008). *Clinical aspects of Campylobacter jejuni* and *Campylobacter coli* infections. In: I, Nachamkin CM Szymanski, and MJ Blaser (Eds.), *Campylobacter*, 3rd ed. ASM Press, Washington, DC, Chapter 6.

Boost MV, O'Donoghue MM, Siu KH (2007). Characterisation of methicillin-resistant *Staphylococcus aureus* isolates from dogs and their owners. *Clin Microbiol Infect* 13:731–733.

Burch, DGS, Duran CO, Aarestrup FM, Guardabassi L, Jensen LB, Kruse H (2008). In *Guide to Antimicrobial Use in Animals*. Blackwell Scientific Publications, Oxford, pp. 102–125.

Canadian Veterinary Medical Association (CVMA) (2000). Guidelines on the prudent use of antimicrobial drugs in animals. Available: http://canadianveterinarians.net/Documents/Resources/Files/85_Resources_Prudent-Use-of-Antimicrobial-Drugs-in-Animals.pdf.

Carson CA, Reid-Smith R, Irwin RJ, Martin WS, McEwen SA (2008). Antimicrobial use on 24 beef farms in Ontario. *Can J Vet Res* 72(2):109–118.

Cernicchiaro N, Pearl DL, Ghimire S, Gyles CL, Johnson RP, Lejeune JT, Ziebell K, McEwen SA (2009). Risk factors associated with *Escherichia coli* O157:H7 in Ontario beef cow-calf operations. *Prev Vet Med* 92:106–115.

CIPARS (2009). Update—*Salmonella* Heidelberg ceftiofur-related resistance in human and retail chicken isolates—2006 to 2008. Public Health Agency of Canada. Available: http://www.phac-aspc.gc.ca/cipars-picra/heidelberg/heidelberg_090326-eng.php.

Cloeckaert A, Schwarz S (2001). Molecular characterization, spread and evolution of multidrug resistance in *Salmonella enterica* typhimurium DT104. *Vet Res* 32(3–4): 301–310.

Codex Alimentarius Commission (2009). Report of the 3rd Session of the Codex ad hoc Intergovernmental Task Force on Antimicrobial Resistance. Jeju, Republic of Korea, October 12–16, 2009. Available: http://www.who.int/foodborne_disease/resistance/codextf_Oct09/en/index.html.

Collignon P, Powers JH, Chiller TM, Aidara-Kane A, Aarestrup FM (2009). World Health Organization ranking of antimicrobials according to their importance in human medicine:

A critical step for developing risk management strategies for the use of antimicrobials in food production animals. *Clin Infect Dis* 49:132–141.

Council for Agricultural Science and Technology (2004). Intervention strategies for the microbiological safety of foods of animal origin. *CAST Issue Paper* 25:1–24.

Cox LA Jr, Popken DA (2006). Quantifying potential human health impacts of animal antibiotic use: Enrofloxacin and macrolides in chickens. *Risk Anal* 26:135–146.

Crump JA, Medalla FM, Joyce KW, Krueger AL, Hoekstra RM, Whichard JM, Barzilay EJ (2011). Emerging Infections Program NARMS Working Group. Antimicrobial resistance among invasive nontyphoidal *Salmonella enterica* in the United States, National Antimicrobial Resistance Monitoring System, 1996–2007. *Antimicrob Agents Chemother*. Mar; 55(3):1148–54.

DANMAP (2010). Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, foods and humans in Denmark. 2010. Available: http://www.danmap.org. Accessed January 2011.

Davis MA, Hancock DD, Besser TE, Rice DH, Gay JM, Gay C, Gearhart L, DiGiacomo R (1999). Changes in antimicrobial resistance among *Salmonella enterica* serovar typhimurium isolates from humans and cattle in the Northwestern United States, 1982–1997. *Emerg Infect Dis* 5:802–806.

Dunlop RH, McEwen SA, Meek AH, Clarke RC, Black WD, Friendship RM (1998a). Associations among antimicrobial drug treatments and antimicrobial resistance of fecal *Escherichia coli* of swine on 34 farrow-to-finish farms in Ontario, Canada. *Prev Vet Med* 34:283–305.

Dunlop RH, McEwen SA, Meek AH, Friendship RM, Black WD, Clarke RC (1998b). Antimicrobial drug use and related management practices among Ontario swine producers. *Can Vet J* 39:87–96.

Dutil L, Irwin R, Finley R, Ng LK, Avery B, Boerlin P, Bourgault A-M, Cole L, Daignault D, Desruisseau A, Demczuk W, Hoang L, Horsman GB, Ismail J, Jamieson F, Maki A, Pacagnella A, Pillai DR (2010). Ceftiofur resistance in *Salmonella enterica* serovar Heidelberg from chicken meat and humans, Canada. *Emerg Infect Dis* 16:48–54.

Endtz HP, Ruijs GJ, Vanklingeren B, Jansen WH, Vanderreyden T, Mouton RP (1991). Quinolone resistance in campylobacter isolated from man and poultry following the introduction of fluoroquinolones in veterinary medicine. *J Antimicrob Chemother* 27:199–208.

European Agency for the Evaluation of Medicinal Products (EMEA) (1999). Antibiotic resistance in the European Union associated with therapeutic use of veterinary medicines. Report and qualitative risk assessment by the committee for veterinary medicinal products. EMEA. London, UK.

Feingold BJ, Vegosen L, Davis M, Leibler J, Peterson A, Silbergeld EK (2010). A niche for infectious disease in environmental health: Rethinking the toxicological paradigm. *Environ Health Perspect* 118:1165–1172.

Fey PD, Safranek TJ, Rupp ME, Dunne EF, Ribot E, Iwen PC, Bradford PA, Angulo FJ, Hinrichs SH (2000). Ceftriaxone-resistant *Salmonella* infection acquired by a child from cattle. *N Engl J Med* 342:1242–1249.

Food and Agriculture Organization (FAO)/WHO (2006). Food safety risk analysis: A guide for national safety authorities. (FAO Food and Nutrition Paper 87). http://www.who.int/foodsafety/publications/micro/riskanalysis06.pdf (last accessed Sept 19, 2011).

Food and Agriculture Organization (FAO)/WHO/OIE (2003). Joint FAO/WHO/OIE Expert Workshop on Non-Human Antimicrobial Usage and Antimicrobial Resistance: Scientific Assessment, Geneva, Switzerland, December 1–5, 2003. http://www.who.int/foodborne_disease/resistance/publications/en/index.html (last accessed Sept 19, 2011).

Food and Agriculture Organization (FAO)/OIE/WHO (2004). Second Joint FAO/OIE/WHO Expert Workshop on Non-Human Antimicrobial Usage and Antimicrobial Resistance: Management Options, Oslo, Norway, March 15–18, 2004. http://www.who.int/foodborne_disease/resistance/publications/en/index.html (last accessed Sept 19, 2011).

Food and Agriculture Organization (FAO)/OIE/WHO (2006). Joint FAO/WHO/OIE Expert Consultation on Antimicrobial Use in Aquaculture and Antimicrobial Resistance, Seoul, Republic of Korea, June 13–16, 2006. http://www.who.int/foodborne_disease/resistance/publications/en/index.html (last accessed Sept 19, 2011).

Food and Agriculture Organization (FAO)/OIE/WHO (2008). Joint FAO/WHO/OIE Expert Meeting on Critically Important Antimicrobials Report of the FAO/WHO/OIE Expert Meeting, FAO, Rome, Italy, November 26–30, 2007. http://www.who.int/foodborne_disease/resistance/publications/en/index.html (last accessed Sept 19, 2011).

Grave K, Jensen VK, McEwen S, Kruse H (2006). Monitoring of antimicrobial drug usage in animals: methods and applications. 22. In FM Aarestrup (Ed.), *Antimicrobial resistance in Bacteria of Animal Origin. Veterinary and Public Health Aspects*. ASM Press, Washington, DC, pp. 375–395.

Hammerum AM, Heuer OE, Emborg HD, Bagger-Skjøt L, Jensen VF, Rogues AM, Skov RL, Agersø Y, Brandt CT, Seyfarth AM, Muller A, Hovgaard K, Ajufo J, Bager F, Aarestrup FM, Frimodt-Møller N, Wegener HC, Monnet DL (2007). Danish integrated antimicrobial resistance monitoring and research program. *Emerg Infect Dis* 13:1632–1639.

Health Canada (2002). Uses of antimicrobials in food animals in Canada: Impact on resistance and human health. Advisory Committee on Animal Uses of Antimicrobials and Impact on Resistance and Human Health. Report Prepared for the Veterinary Drugs Directorate, Health Canada. Available: http://www.hc-sc.gc.ca/dhp-mps/pubs/vet/amr-ram_final_report-rapport_06–27_cp-pc_e.html.

Helms M, Simonsen J, Mølbak K (2004). Quinolone resistance is associated with increased risk of invasive illness or death during infection with *Salmonella* serovar typhimurium. *J Infect Dis* 190:1652–1654.

Helms M, Simonsen J, Olsen KEP, Mølbak K (2005). Adverse health events associated with antimicrobial drug resistance in campylobacter species: A registry-based cohort study. *J Infect Dis* 191:1050–1055.

Heuer OE, Kruse H, Grave K, Collignon P, Karunasagar I, Angulo FJ (2009). Human health consequences of use of antimicrobial agents in aquaculture. *Clin Infect Dis* 49: 1248–1253.

Holmberg SD, Wells JG, Cohen ML (1984). Animal-to-man transmission of antimicrobial-resistant *Salmonella*: Investigations of U.S. outbreaks, 1971–1983. *Science* 225:833–835.

Hurd HS, Doores S, Hayes D, Mathew A, Maurer J, Silley P, Singer RS, Jones RN (2004). Public health consequences of macrolide use in food animals: A deterministic risk assessment. *J Food Prot* 67:980–992.

Institute of Medicine (1989). *Human Health Risks with the Subtherapeutic Use of Penicillin or Tetracyclines in Animal Feed*. National Academy Press, Washington, DC.

Jakobsen L, Kurbasic A, Skjøt-Rasmussen L, Ejrnaes K, Porsbo LJ, Pedersen K, Jensen LB, Emborg HD, Agersø Y, Olsen KE, Aarestrup FM, Frimodt-Møller N, Hammerum AM (2010). *Escherichia coli* isolates from broiler chicken meat, broiler chickens, pork, and pigs share phylogroups and antimicrobial resistance with community-dwelling humans and patients with urinary tract infection. *Foodborne Pathog Dis* 7:537–547.

Jiang X, Yang H, Dettman B, Doyle MP (2006). Analysis of fecal microbial flora for antibiotic resistance in ceftiofur-treated calves. *Foodborne Pathog Dis* 3:355–365.

Johnson JR, Sannes MR, Croy C, Johnston B, Clabots C, Kuskowski MA, Bender J, Smith KE, Winokur PL, Belongia EA (2007). Antimicrobial drug–resistant *Escherichia coli* from

humans and poultry products, Minnesota and Wisconsin, 2002–2004. *Emerg Infect Dis* 13:838–846.

Joint Expert Advisory Committee on Antibiotic Resistance (JETACAR) (1999). The use of antibiotic in food-producing animals: Antibiotic-resistant bacteria in animals and humans. Commonwealth of Australia. Canberra, Australia.

Kennedy K, Collignon P (2010). Colonisation with *Escherichia coli* resistant to "critically important" antibiotics: A high risk for international travellers. *Eur J Clin Microbiol Infect Dis* 29:1501–1506.

Klare I, Konstabel C, Badstübner D, Werner G, Witte W (2003). Occurrence and spread of antibiotic resistances in *Enterococcus faecium*. *Int J Food Microbiol* 88:269–290.

Koningstein M, Simonsen J, Helms M, Mølbak K (2010). The interaction between prior antimicrobial drug exposure and resistance in human *Salmonella* infections. *J Antimicrob Chemother* 65:1819–1825.

Kozak GK, Boerlin P, Janecko N, Reid-Smith RJ, Jardine C (2009). Antimicrobial resistance in *Escherichia coli* isolates from swine and wild small mammals in the proximity of swine farms and in natural environments in Ontario, Canada. *Appl Environ Microbiol* 75:559–566.

Levine DP (2006). Vancomycin: A history. *Clin Infect Dis* 42(Suppl 1):S5–12.

Levy SB, FitzGerald GB, Macone AB (1976). Changes in intestinal flora of farm personnel after introduction of a tetracycline-supplemented feed on a farm. *N Engl J Med* 295:583–588.

Lewis HC, Mølbak K, Reese C, Aarestrup FM, Selchau M, Sørum M, Skov RL (2008). Pigs as source of methicillin-resistant *Staphylococcus aureus* CC398 infections in humans, Denmark. *Emerg Infect Dis* 14:1383–1389.

Loeffler A, Lloyd DH (2010). Companion animals: A reservoir for methicillin-resistant *Staphylococcus aureus* in the community? *Epidemiol Infect* 138:595–605.

Lowrance TC, Loneragan GH, Kunze DJ, Platt TM, Ives SE, Scott HM, Norby B, Echeverry A, Brashears MM (2007). Changes in antimicrobial susceptibility in a population of *Escherichia coli* isolated from feedlot cattle administered ceftiofur crystalline-free acid. *Am J Vet Res* 68:501–507.

Manges AR, Smith SP, Lau BJ, Nuval CJ, Eisenberg JN, Dietrich PS, Riley LW (2007). Retail meat consumption and the acquisition of antimicrobial resistant *Escherichia coli* causing urinary tract infections: A case-control study. *Foodborne Pathog Dis* 4:419–431.

McDermott PF, Bodeis SM, English LL, White DG, Walker RD, Zhao S, Simjee S, Wagner DD (2002). Ciprofloxacin resistance in *Campylobacter jejuni* evolves rapidly in chickens treated with fluoroquinolones. *J Roy Stat Soc* 185:837–840.

McDonald LC, Kuehnert MJ, Tenover FC, Jarvis WR (1997). Vancomycin-resistant enterococci outside the health-care setting: Prevalence, sources, and public health implications. *Emerg Infect Dis* 3:311–317.

McEwen SA (2006). Antibiotic use in animal agriculture: What have we learned and where are we going? *Anim Biotechnol* 17:239–250.

McEwen SA, Fedorka-Cray P (2002). Antimicrobial use and resistance in animals. *Clin Infect Dis* 34(Suppl):S93–106.

McEwen SA, Aarestrup FM, Jordan D (2006). Monitoring of antimicrobial resistance in animals: principles and practices. In FM Aarestrup (Ed)., *Antimicrobial Resistance in Bacteria of Animal Origin. Veterinary and Public Health Aspects*. ASM Press, Washington, DC, pp. 397–413.

Mellon M, Benbrook C, Benbrook K (2001). *Hogging It! Estimates of Antimicrobial Abuse in Livestock*. UCS Publications, Cambridge, MA.

Murphy CP, Reid-Smith RJ, Weese JS, McEwen SA (2010). Evaluation of specific infection control practices used by companion animal veterinarians in community veterinary practices in southern Ontario. *Zoonoses Public Health* 57:429–438.

National Research Council (1983). *Risk Assessment in the Federal Government: Managing the Process*. National Academy Press. Washington, DC.

National Research Council (1999). *The Use of Drugs in Food Animals, Benefits and Risks*. National Academy Press, Washington, D.C.

Nelson JM, Chiller TM, Powers JH, Angulo FJ (2007). Fluoroquinolone-resistant *Campylobacter species* and the withdrawal of fluoroquinolones from use in poultry: A public health success story. *Clin Infect Dis* 44:977–980.

Newell DG, Koopmans M, Verhoef L, Duizer E, Aidara-Kane A, Sprong H, Opsteegh M, Langelaar M, Threfall J, Scheutz F, van der Giessen J, Kruse H (2010). Food-borne diseases—the challenges of 20 years ago still persist while new ones continue to emerge. *Int J Food Microbiol* 139(Suppl 1):S3–15.

O'Brien TF (2002). Emergence, spread, and environmental effect of antimicrobial resistance: How use of an antimicrobial anywhere can increase resistance to any antimicrobial anywhere else. *Clin Infect Dis* 34 (Suppl 3):78–84.

Ogeer-Gyles J, Mathews KA, Sears W, Prescott JF, Weese JS, Boerlin P (2006). Development of antimicrobial drug resistance in rectal *Escherichia coli* isolates from dogs hospitalized in an intensive care unit. *J Am Vet Med Assoc* 229:694–699.

Phillips I (2007). Withdrawal of growth-promoting antibiotics in Europe and its effects in relation to human health. *Int J Antimicrob Agents* 30:101–107.

Phillips I, Casewell M, Cox T, De Groot B, Friis C, Jones R, Nightingale C, Preston R, Waddell J (2004). Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *J Antimicrob Chemother* 53:28–52.

Poppe C, Smart N, Khakhria R, Johnson W, Spika J, Prescott J (1998). *Salmonella* typhimurium DT 104: A virulent and drug-resistant pathogen. *Can Vet J* 39:559–565.

Prescott JF (2006). *History of antimicrobial usage in agriculture: An overview*. In FM Aarestrup (Ed.), *Antimicrobial Resistance in Bacteria of Animal Origin. Veterinary and Public Health Aspects*. Washington, ASM Press, pp. 19–28.

Prescott JF, Hanna WJ, Reid-Smith R, Drost K (2002). Antimicrobial drug use and resistance in dogs. *Can Vet J* 43:107–116.

Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM (2011). Foodborne illness acquired in the United States—Major pathogens. *Emerg Infect Dis* 17:7–15.

Shryock TR, Richwine A (2010). The interface between veterinary and human antibiotic use. *Ann NY Acad Sci* 1213:92–105.

Singer RS, Cox LA Jr, Dickson JS, Hurd HS, Phillips I, Miller GY (2007). Modeling the relationship between food animal health and human foodborne illness. *Prev Vet Med* 79:186–203.

Sischo WM (2006). Stakeholder position paper: Dairy producer. *Prev Vet Med* 73:203–208.

Smith KE, Medus C, Meyer SD, Boxrud DJ, Leano F, Hedberg CW, Elfering K, Braymen C, Bender JB, Danila RN (2008). Outbreaks of salmonellosis in Minnesota (1998 through 2006) associated with frozen, microwaveable, breaded, stuffed chicken products. *J Food Prot* 71:2153–2160.

Snary E, McEwen SA, Guardabassi L, Jensen LB, Kruse H (2008). Antimicrobial resistance risk assessment. In *Guide to Antimicrobial Use in Animals*. Blackwell Scientific Publications, Oxford, UK.

Sorum H (2000). Farming of Atlantic salmon—An experience from Norway. *Acta Vet Scand* 93:129–134.

Swann MM (1969). *The Use of Antibiotics in Animal Husbandry and Veterinary Medicine*. Her Majesty's Stationery Office, London.

Swartz MN (2002). Human diseases caused by foodborne pathogens of animal origin. *Clin Infect* Dis 34(Suppl 3):S111–122.

Threlfall EJ, Ward LR, Rowe B (1978). Epidemic spread of a chloramphenicol-resistant strain of *Salmonella typhimurium* phage type 204 in bovine animals in Britain. *Vet Rec* 103: 438–440.

Tollefson L, Morris D, Boland C, Kay J. (2006). Licensing and approval of antimicrobials for use in animals. In FM Aarestrup (Ed.), *Antimicrobial Resistance in Bacteria of Animal Origin. Veterinary and Public Health Aspects*. ASM Press, Washington DC, pp. 361–374.

van Belkum A, Melles DC, Peeters JK, van Leeuwen WB, van Duijkeren E, Huijsdens XW, Spalburg E, de Neeling AJ, Verbrugh HA; Dutch Working Party on Surveillance and Research of MRSA-SOM (2008). Methicillin-resistant and -susceptible *Staphylococcus aureus* sequence type 398 in pigs and humans. *Emerg Infect Dis* 14:479–483.

Vidaver AK (2002). Uses of antimicrobials in plant agriculture. *Clin Infect Dis* 34(Suppl 3): S107–110.

Voss A, Loeffen F, Bakker J, Klaassen C, Wulf M (2005). Methicillin-resistant *Staphylococcus aureus* in pig farming. *Emerg Infect Dis* 11:1965–1966.

Waltner-Toews D, McEwen SA (1994). Antibacterial residues in foods of animal origin: A risk assessment. *Prev Vet Med* 20:219–234.

Weese JS, Reid-Smith R, Rousseau J, Avery B (2010). Methicillin-resistant *Staphylococcus aureus* (MRSA) contamination of retail pork. *Can Vet* J 51:749–752.

Weese JS, van Duijkeren E (2010). Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus pseudintermedius* in veterinary medicine. *Vet Microbiol* 140:418–429.

Weese JS, Lefebvre SL (2007). Risk factors for methicillin-resistant *Staphylococcus aureus* colonization in horses admitted to a veterinary teaching hospital. *Can Vet J* 48: 921–926.

Weese JS (2006). Investigation of antimicrobial use and the impact of antimicrobial use guidelines in a small animal veterinary teaching hospital 1995–2004. *J Am Vet Med Assoc* 228:553–558.

White DG (2006). Antimicrobial resistance in pathogenic *Escherichia coli* from animals. In: FM Aarestrup (Ed.), *Antimicrobial Resistance in Bacteria of Animal Origin. Veterinary and Public Health Aspects*. ASM Press, Washington, DC, pp. 145–166.

Witte W (2000). Ecological impact of antibiotic use in animals on different complex microflora: Environment. *Int J Antimicrob Agents* 14:321–325.

Woodford N (1998). Glycopeptide-resistant enterococci: A decade of experience. *J Med Microbiol* 47:849–862.

World Health Organization (WHO) (1995). Application of risk analysis to food standards issues, a Joint FAO/WHO Expert Consultation. WHO, Geneva, Switzerland.

World Health Organization (WHO) (1997). The medical impact of the use of antimicrobials in food animals. WHO, Berlin, Germany.

World Health Organization (WHO) (1998). Use of quinolones in food animals and potential impact on human health. WHO, Geneva, Switzerland.

World Health Organization (WHO) (2000). Global principles for the containment of antimicrobial resistance in animals for food. Report of a WHO Consultation with the participation of the Food and Agriculture Organization of the United Nations and the Office International des Epizooties, Geneva, Switzerland.

World Health Organization (WHO) (2003). Impacts of antimicrobial growth promoter termination in Denmark. The WHO international review panel's evaluation of the termination of the use of antimicrobial growth promoters in Denmark, November 6–9, 2002. WHO, Geneva, Switzerland.

World Health Organization (WHO) (2009). First Meeting of the WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR). WHO, Geneva, Switzerland.

Wulf M, van Nes A, Eikelenboom-Boskamp A, de Vries J, Melchers W, Klaassen C, Voss A (2006). Methicillin-resistant *Staphylococcus aureus* in veterinary doctors and students, the Netherlands. *Emerg Infect Dis* 12:1939–1941.

Young I, Rajić A, Wilhelm BJ, Waddell L, Parker S, McEwen SA (2009). Comparison of the prevalence of bacterial enteropathogens, potentially zoonotic bacteria and bacterial resistance to antimicrobials in organic and conventional poultry, swine and beef production: A systematic review and meta-analysis. *Epidemiol Infect* 137:1217–1232.

22

ANTIMICROBIAL RESISTANCE ASSOCIATED WITH SALMONID FARMING

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22.1 INTRODUCTION

Salmonid farming is a growing industry throughout the world, especially in developed countries such as Norway, Chile, United Kingdom, and Canada, where the rapid expansion of salmonid farming has created a number of ecological and public health concerns. Intensive salmonid culture conditions have resulted in growing problems of bacterial diseases, which, in turn, have led to a widespread use of antibacterial agents.

The antibacterials mainly used in salmonid farming to control bacterial pathologies are oxytetracycline, florfenicol, amoxicillin, erythromycin, quinolones (oxolinic acid, flumequine, and enrofloxacin), and potentiated sulfonamides (ormetoprim/sulfadimethoxine and trimethoprim/sulfadiazine). The environmental effects of administering antibacterial agents in aquaculture are of great concern and include antibiotic resistance, residues in organisms and persistence in aquatic environments near salmonid farms (Bruno, 1989; Bernoth, 1991).

Various authors have emphasized the putative negative impact derived from the use of antimicrobial agents in fish farms (Aoki, 1992; Hansen et al., 1992; Alderman and Hastings, 1998; MacMillan, 2001; Teuber, 2001; Cabello, 2006). The use of antibacterial agents is directly related to the emergence and spread of resistant bacteria, producing a public health risk and problems of recurrent infections and antibacterial therapy failure (Aoki, 1992; Smith et al., 1994b). However, various reports identify that several other aquaculture conditions, such as fish feed accumulation and organic

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matter enrichment, usually coincides with an increased frequency of resistant bacterial microbiota in the absence of antibacterial agents.

Another aspect to be mentioned is the impact of organic matter loads coming from fish-farming cages on the benthic bacterial communities. It is well understood that fish-farming activity produces a higher impact in sediment beneath fish farm cages than in the surrounding water, due to the high deposition of organic loads mainly composed by uneaten food, fecal pellets, and dead fish (Wu, 1995). Changes in the structure of benthic bacterial communities are mainly characterized by an increase in the autofluorescent microbial component and aerobic heterotrophic bacterial density (La Rosa et al., 2004). In addition, various studies have demonstrated significant reductions of total meiofaunal density and richness in sediments located beneath fish cages due to the organic nutrient loading (Ritz et al., 1989; Mazzola et al., 1999, 2000; La Rosa et al., 2001).

Considering that salmonid pathogens are mainly susceptible to antibacterials before the use of these drugs in the fish farm environment, the antibacterial resistance determinants found in some pathogenic strains must necessarily come from the associated nonpathogenic microbiota being mainly acquired by horizontal gene transfer (Aoki and Kitao, 1985; Aoki, 1988). The fact that the majority of antibacterial resistance genes originate in the environmental microbiota (Davies, 1994, 1997) underlines the importance of study antimicrobial resistance in nonpathogenic bacteria associated with salmonid farming. The studies of antibacterial resistance of autochthonous bacteria permit an evaluation of its role in the maintenance and transfer to other bacteria, including those that are potentially pathogenic, in order to understand the gene flux encoding for bacterial resistance in salmon farming.

Despite that selection and spread of antibacterial-resistant bacteria is one of the greatest concerns with regard to the use of antibacterials in salmonid farming, it is critical to advance in the understanding of the farm conditions responsible for the emergence and maintenance of resistant bacteria, as well as the molecular elements involved in antibacterial resistance and transfer. In addition, much more work must be carried out to better understand the resistome (see Chapter 3) of salmonid farms that do not use antibiotics.

This chapter will discuss (1), the main advantages in the knowledge of the fate and persistence of antibacterial agents in environments associated with salmonid farming, (2), the incidence and spread of resistant bacteria in fish farms with and without the occurrence of antibacterial selective pressure, (3), the incidence of antibacterial resistance in the most relevant farmed salmonid bacterial pathogens, and (4), the molecular elements encoding for antibacterial resistance occurring in salmonid farm environments and their feasibility of bacterial transfer.

22.2 FATE AND PERSISTENCE OF ANTIBACTERIAL AGENTS USED IN SALMONID FARMING

Various studies have been developed to advance the understanding of the fate and real impact on the environment of used antibacterials in fish farming. However, the occurrence, fate, effects, and risks associated with the release of antibacterials into the environment associated to salmonid farming is not yet completely elucidated. In addition, standardized methods for monitoring antibacterial agents in aquatic environments are needed (Pouliquen et al., 2009).

The most common route of administration of antimicrobial agents in salmonid farming is the use of medicated feed, and this practice may result in a strong selective pressure not only in the animals but also in the exposed environments. When antibacterials are administered to fish as medicated feed, the drug could be released to the environment via urine and feces and from the uneaten medicated feed. Most of studies consider that antibacterials mainly reach the sediment under the cages, and the majority of studies investigate the effect on the sedimentary microbial community. Only a few are related to the effect on the water and fish microbiota.

Considering the low apparent digestibility of oxytetracycline and oxolinic acid (7.1 and 38.1%, respectively) when administered to salmon in feed at a dose of 20 mg/kg fish (Cravedi et al., 1987), it is expected that an important amount will be released in the environment. It is assumed that 20% of the feed administered to fish is not eaten and passes through the cage and is deposited on the sediment near fish cages (Gowen et al., 1989). Sandaa (1993) suggested that during a disease outbreak this percentage may rise.

The degradability of antibacterials used in salmonid farming should be considered as an important parameter when assessing possible environmental impacts. Among the antibacterial agents currently used in salmonid farming, oxytetracycline and the quinolones oxolinic acid and flumequine are highly persistent in marine fish farm sediments, as demonstrated by various authors (Björklund et al., 1990; Samuelsen et al., 1992a; Hektoen et al., 1995).

The dissipation of oxytetracycline in water is much faster than in the sediments, depending on temperature, pH, air saturation, and light intensity (Samuelsen, 1989; Pouliquen et al., 1992). Lunestad et al. (1995) found that oxytetracycline, oxolinic acid, flumequine, and furazolidone were degraded and lost their antibacterial activity when illuminated by daylight at sea level for 21 days. Various studies evidenced that antibacterial activity of oxytetracycline is strongly reduced due its high potential to chelate with cations, particularly Mg^{2+} and Ca^{2+} (Lunestad and Goksøyr, 1990; Barnes et al., 1995). In addition, other studies demonstrated the reduction in the inhibitory activity of quinolones in the presence of magnesium (Smith, 1989; Barnes et al., 1995), whereas Pursell et al. (1995) evidenced that seawater and seawater ions significantly reduce biological activity of flumequine but not its detection. Pouliquen et al. (2007) studied the hydrolysis and photolysis of the antibacterials oxytetracycline, oxolinic acid, flumequine, and florfenicol under abiotic conditions, determining that hydrolysis was active only on oxytetracycline, observing a 20% degradation after 14 days at 8°C. Photolysis was responsible of 70% of the oxytetracycline degradation and 10% of oxolinic acid and flumequine degradation in seawater, whereas florfenicol was not susceptible to both processes. They also observed a relationship between the absence of oxolinic acid and flumequine photolysis and binding to organic matter. The main amount of oxytetracycline is probably in the water column but at a very low concentration to be detected and to exert a selective pressure, mainly due to its biologically activity that depends greatly of the concentration of divalent cations.

It is interesting to mention that after incubation at limiting physical factors, Maki et al. (2006) demonstrated microbial degradation of oxytetracycline residues in marine sediments by isolates that were resistant to oxytetracycline. Smith et al. (1994a) established that in land-based fish farms, a correct design of the effluent treatment systems can significantly reduce the impact on the aquatic environment of administered antibacterials, such as oxytetracycline due to the fact that most of the antibacterial was removed by a sedimentation trap on the used filter.

Oxytetracycline and oxolinic acid maintained stable after 180 days in an artificial marine aquaculture sediment under laboratory conditions (Samuelsen et al., 1994). Hektoen et al. (1995) reported sediment half-lives of oxytetracycline and oxolinic acid of 151 and >300 days at top (0–1 cm) and deeper (5–7 cm) layers, respectively, flumequine half-lives were 60 and >300 days at top and deeper layers, respectively. In addition, Jacobsen and Berglind (1988) found oxytetracycline concentrations able to cause antimicrobial effects up to 12 weeks after administration, whereas Samuelsen (1989) found that persistence of oxytetracycline is highly dependent on the sedimentation rate, reporting a half-life for oxytetracycline in seawater of 168 and 128 h at 15°C in the dark and light, respectively. Otherwise, Lai et al. (2011a) found that decaying of oxolinic acid under aerobic and light conditions was faster than under anaerobic and dark conditions using eel pond sediment.

On the other hand, Coyne et al. (1994) reported that after oxytetracycline therapy only a low concentration of the antibacterial was detected in sediments near salmon cages. As was discussed by Coyne et al. (1994), the high variation in the reported oxytetracycline half-life results could be due to site-specific or management-specific factors affecting the accumulation and persistence of the antibiotic in the sediments.

In contrast to oxytetracycline, oxolinic acid, and flumequine, other antibacterials exhibit a low persistence in fish farm sediments. Florfenicol showed a half-life of 1.7 and 7.3 days in the top and deeper layer, respectively (Hektoen et al., 1995), whereas furazolidone exhibited a half-life of 18 h at 5°C (Samuelsen et al., 1991). Lai et al. (2011b), using water and sediment collected from a marine shrimp pond, determined that half-lives of various sulfonamides in nonsterile water and sediment were 2.0–15 and 0.7–7.3 days, respectively, whereas after sterilization the observed half-lives were 2.9–62.9 and 6.9–85.6 days, respectively, confirming the role of microbial communities in the antibacterial degradation.

An issue of great importance to be considered in the analysis of environmental impact of using antibacterial agents in salmonid farming is the detection of antibacterial residues in wild fish and shellfish near the farming sites after oxytetracycline medication (Björklund et al., 1991; Samuelsen et al., 1992a; Pouliquen et al. 1993; Capone et al., 1996). Coyne et al. (1997) detected oxytetracycline residues in mussels near salmon farms treated with the antibacterial, but the residues were extremely transient and only were detected in mussels within the salmon farm environment. During and after the medication with oxolinic acid of two salmon farms, Samuelsen et al. (1992a) found important levels of residues of oxolinic acid in wild fish, mussels, and crabs harvested near the farm sites, as well as detected elevated numbers of bacteria resistant to oxolinic acid in blue mussels recovered at one of the salmon farms. These results agree with those obtained by Le Bris and Pouliquen (2004), who contaminated and decontaminated samples of blue mussels, and detected that uptake of oxolinic acid and oxytetracycline was fast, mainly accumulated in gills and viscera, respectively, but oxolinic acid was quickly eliminated, whereas oxytetracycline was released more slowly, with a half-life in viscera of 3.9 days. Likewise, residues of oxytetracycline and oxolinic acid were detected in the meat of two species of wild fish that are consumed by humans and were captured near salmon culture pens in Chile, suggesting potential environmental implications of antibiotic usage in salmon aquaculture that may affect human health (Fortt et al., 2007).

In order to facilitate a comparison of results of oxytetracycline residues in undercage sediments obtained by various studies, Smith (1996) suggested expressing

the drug concentration in the sediment (micrograms per gram) as a function of input of oxytetracycline (kilograms per cage) to a cage during the treatment. This unit (concentration/input) describes the concentration of oxytetracycline (microgram per gram) that was detected for every kilogram of oxytetracycline administered to the fish in a cage for the total duration of the treatment. Using this approach, a higher number of studies determined that oxytetracycline in sediments associated with fish farms is quite low, with a mean concentration of 0.76 ± 0.67 μg/g per kg of administered drug, as was described by Smith (1996). It is therefore argued that deposition on the sediment is neither the normal, nor the necessary, fate of the majority of oxytetracycline administered to fish in marine fish farms.

In spite of the fact that most studies consider sediment under salmonid cages as the most important fate of administered antibacterial, other authors suggest that an important concentration of oxytetracycline detected in water column near fish farms is the result of sediment out-washing (Smith and Samuelsen, 1996). In general terms the real fate of antibacterials used in fish farming is an unsolved issue, mainly due to the studies that report a usual lack of correlation between the administered amount of oxytetracycline and the detected concentration in undercage sediments (Coyne et al., 1994). In this trend, Smith (1996) made an interesting critical analysis of the available data on the environmental fate of oxytetracycline administered in marine fish farming.

Another aspect of great relevance to be considered is the relationship between the antibacterial concentration and the associated biological activity mentioned by O'Reilly and Smith (2001) due to the fact that various antibacterials are inhibited by certain environmental parameters (Smith et al., 1996; Vaughan and Smith, 1996). In addition, several studies evidenced that oxolinic acid activity was significantly reduced in marine environments due to its complexation with divalent cations (Barnes et al., 1995; Lunestad and Samuelsen, 2001). It should be noted that as a selective pressure, the biological activity of an antibacterial is more relevant than the amount of the drug present in a particular compartment of the environment.

22.3 ANTIBACTERIAL RESISTANCE IN THE MICROBIOTA ASSOCIATED WITH SALMONID FARMING

An important number of bacteria resistant to the antibacterials frequently used in salmonid farming has been found in salmonid farms and surrounding environments after medication, but an increase and high persistence of antibacterial resistance in unmedicated fish farm sites and nonimpacted environments has also been reported. So, it is not clear how or to what extent an antimicrobial agent that is released into the aquatic environment promotes the selection of antibacterial resistance or improves the persistence of antibacterial-resistant bacteria or their resistance determinants.

22.3.1 In the Presence of Antibacterial Selective Pressure

It is well stated that selective pressure imposed by the use and presence of antibacterials in fish-farming environments exerts a key role in the emergence and maintenance of antibacterial-resistant bacteria. It is expected that under antibacterial selective pressure, the levels of resistant bacteria in fish farming will increase. This is in agreement with widely documented scientific evidence that antimicrobial use in

any extent in any environment is likely to select for antibiotic-resistant bacteria in that environment, so it is expected that the incidence of antibacterial resistance in bacteria from unpolluted areas will be lower than the incidence of resistance within bacteria isolated from fish-farm-impacted environments. Another aspect to be considered is that metal contamination in natural and impacted environments could have an important role in the maintenance and proliferation of antibacterial resistance considering the feasibility of co-selection (Alonso et al., 2001; Stepanauskas et al., 2006).

It has been extensively reported that the use of oxytetracycline usually coincides with the presence of elevated frequencies of resistant bacteria in sediments under salmon cages (Torsvik et al., 1988; Samuelsen, 1989; Lunestad, 1992; Samuelsen et al., 1992b; Herwig et al., 1997). Likewise, Hansen et al. (1992) demonstrated the ability of oxytetracycline to select resistant bacteria in marine sediments using aquaria.

Several studies of antibacterial resistance of sediment microbiota have been performed using seawater sediment microcosms (Hansen et al., 1993; Herwig and Grey, 1997). Hansen et al. (1993) added oxytetracycline and observed the development of a low-level resistant population within 7 days, followed by a high-level resistant population in their microcosm experiment. Herwig and Gray (1997) evaluated the effect of oxytetracycline treatment and observed an important increase in the proportion of resistance to oxytetracycline and sulfadimethoxine: ormetoprim and to a lesser degree to amoxicillin, but Hansen et al. (1993) did not find an increase of resistance to oxolinic acid or flumequine when oxytetracycline alone was added. These results suggest that oxytetracycline can select for sulfonamide resistance but not for quinolone resistance. In addition, Nygaard et al. (1992) used sediments treated with oxytetracycline and oxolinic acid and placed these on the sea bottom for a period of a year. They observed a percentage of resistance to oxytetracycline and oxolinic acid (16 and 20%, respectively) approximately three times more than the level of resistance before the exposure to the antibacterials (5 and 7%, respectively). They also detected a cross resistance toward oxytetracycline and oxolinic acid both in sediments treated with oxytetracycline and oxolinic acid, but only in oxolinic-acid-treated sediment did they find an important level of cross resistance to furazolidone.

Guardabassi et al. (2000a) used *Acinetobacter* spp. as bacterial indicators to monitor antibacterial resistance in a freshwater trout farm before and after treatment with oxolinic acid, observing a high prevalence of oxolinic acid resistance in pond and water receiving the farm effluent after the medication period, as well as a high association between oxolinic acid and oxytetracycline resistance in *Acinetobacter* strains isolated from the stream receiving the farm effluent (Guardabassi et al., 2000a). In addition, after oxytetracycline treatment, Sandaa et al. (1992) isolated a high number of oxytetracycline-resistant bacteria from undercage sediments in Norwegian salmonid farms, observing that the majority of isolates were resistant to oxytetracycline, kanamycin, and sulfamethoxazole and 7 out of 34 multiple resistant strains were able to transfer their resistance to *Escherichia coli*.

Ervik et al. (1994) found that use of quinolones in fish farms resulted in an important increase in the frequency of bacterial strains with a high level of resistance to oxytetracycline. In addition, Hansen et al. (1993) observed an increase of multiple resistant bacteria in sediments a few days after adding oxytetracycline, oxolinic acid, or flumequine.

Spanggaard et al. (1993) reported that oxolinic acid resistance exhibited by microbiota from freshwater trout farms (27%) was higher than those observed by microbiota from an unpolluted stream (16%), observing that strains from the fish and sediment beneath the salmon cages are more resistant than the isolates from the water. They suggested that the gastrointestinal tract of the fish also serves as a niche for selection of resistant microorganisms since concentration of antibiotics is high during periods of medication.

In contrast, a lack of correlation between use of oxytetracycline and the frequency of resistant bacteria have been extensively reported (Kerry et al., 1996, 1997). Kerry et al. (1994) studied on two occasions a salmon farm treated with oxytetracycline and found in the undercage sediments no significant increase in the frequency of resistant bacteria or only a transient rise in the frequency of drug resistance, whereas Kerry et al. (1995b) observed a rise in the frequency of oxytetracycline-resistant bacteria in the sediment only after 24 days of the administration of the antibiotic. In addition, Husevag et al. (1991) found high levels of oxytetracycline-resistant bacteria in marine sediments associated with abandoned fish farm sites when no oxytetracycline residues were detected. Various authors suggested that antibacterial resistance may arise as a result of the increase in the level of innately resistant bacteria, exhibiting multiple nonspecific resistances (Samuelsen et al., 1992b; Smith et al., 1994b; Smith, 1995).

Accumulation of antibacterial residues in sediments also has the potential to inhibit microbial activity, affecting sediment environmental quality. Ma et al. (2006) reported that high concentrations of chloramphenicol inhibit microbial activities in marine sediments and also increase antibacterial resistance of heterotrophic bacteria. They also suggested that pollutant and organic matter accumulation in sediment could be considered an environmental deterioration as a consequence of the presence of chloramphenicol. Klaver and Matthews (1994) detected that concentrations of 12.5–75 mg/L of oxytetracycline-inhibited nitrification in a model aquatic system using aquaria.

Molecular structures carrying antibacterial resistance determinants may be exchanged between bacteria in water, sediments, or the gastrointestinal tract of salmonids, by conjugation, transformation, or transduction. This is a key problem in this activity and could be highly relevant for the spread of antibacterial resistance in the veterinary and human compartments. Rhodes et al. (2000), using different *Aeromonas* species, have provided evidence of the dissemination of determinants of tetracycline resistance between the human and aquaculture environments, specially *tetA*, whereas Schmidt et al. (2001a) suggested the occurrence of horizontal transfer of antibiotic resistance genes among the aeromonads found in and around the sampled fish farms, observing a correlation between conjugative R-plasmids and *tetA* gene. However, Guardabassi et al. (2000b) concluded that occurrence of important flow of tetracycline resistance genes between clinical and aquatic *Acinetobacter* population is unlikely due to the high differences in the tetracycline resistance genes between clinical and aquatic *Acinetobacter* strains and the inability to transfer tetracycline resistance from *Acinetobacter* strains from clinical origin to aquatic strains.

Among the most commonly used antibacterials in salmonid farming, such as oxytetracycline, florfenicol, and the quinolones oxolinic acid and flumequine, several studies have been developed in salmonid farming to investigate the presence of *tet* and *floR* genes, encoding for oxytetracycline and florfenicol resistance, respectively.

It is known that spread of *tet* and *floR* genes is often facilitated by their location on mobile genetic elements, such as plasmids and transposons (DePaola and Roberts, 1995; Schwarz et al., 2004).

Aeromonas species have been established as normal components of fish intestinal microbiota, being used to evaluate the evolution of antibacterial resistance in salmonid farming (Naviner et al., 2006). Agersø et al. (2007) found that tetracycline resistance determinant *tetE* associated with large horizontally transferable plasmids is common in *Aeromonas* spp. strains isolated from farmed fish, water, and sediment of Danish fish farms. Several studies have shown that *tetE* is frequently present in aquatic environments (Sørum et al., 1992; DePaola and Roberts, 1995; Schmidt et al., 2001a), but an important number of unrecognized tetracycline resistance determinants have been reported in these environments as well (Andersen and Sandaa, 1994; Schmidt et al., 2001a, 2001b; Miranda et al., 2003). These results are of great importance considering that the *tetE* gene has been associated with nonconjugative plasmids (Sørum et al., 1992; DePaola and Roberts, 1995). On the contrary, Furushita et al. (2003) identified *tet* genes in 43 tetracycline-resistant strains isolated from fish farms in Japan, observing a high prevalence of *tetB* (31 strains), whereas the other 12 strains contained *tetC*, *tetD*, *tetG*, or *tetY* genes, and 11 strains could transfer by conjugation their *tet* genes to *E. coli*. On the other hand, the genes of 15 strains were not identified.

Another recent study evaluated the effect of oxytetracycline on intestinal microbiota of Atlantic salmon juveniles, observing that species diversity was significantly reduced, and treated microbiota was mainly composed of *Aeromonas* species showing a remarkable increase in their level of oxytetracycline resistance (Navarrete et al., 2008). They also found that 21 out of 27 *Aeromonas* isolates carried the resistance determinant *tetE*, whereas the other strains carried the *tetD*/*H* determinants. These results agree with Le Bris et al. (2007) who detected a high incidence of oxolinic-acid-resistant Aeromonads in fecal matter of rainbow trout treated with oxolinic acid and with Giraud et al. (2006) who reported an increase of oxolinic-acid-resistant bacteria in the intestinal microbiota of fish under treatment. Likewise, Austin and Al-Zahrani (1988) found that administration of antibacterials as oxytetracycline and oxolinic acid caused an increase in intestinal bacteria throughout the digestive tract of treated rainbow trout that were generally resistant to the used antibiotics. These results support the hypothesis that antibacterial treatment can eradicate normal intestinal microbiota and select for resistant bacteria in the salmonid intestinal compartment.

However, no selection of resistant bacteria within intestinal microbiota of Chinook salmon treated with erythromycin was detected by Moffitt and Mobin (2006), whereas Naviner et al. (2007) observed an increase in the frequency of oxolinic acid resistance among *Aeromonas* spp. recovered from pond effluents but not in isolates from intestines of farmed trout treated with oxolinic acid, suggesting that fish feces and uneaten feed have a potential role as selective factors for antibacterial resistance in fish-farming tanks.

22.3.2 In the Absence of Antibacterial Selective Pressure

Several studies have reported important levels of antibacterial resistance in salmonid farm environments in which the fish have not been subjected to antibacterial therapy. Various microcosm experiments showed that sediments containing high levels of

organic matter exhibit important levels of antibacterial-resistant bacteria when compared to sediments with low levels of organic matter.

Kapetanaki et al. (1995) demonstrated that the presence of oxytetracycline is not a necessary causal condition for the development of resistance, due to the occurrence of elevated frequencies of resistance in tanks containing marine sediment overlaid with large amounts of feed, characterized by slow-growing bacteria and resistant to high levels (>512 μg/mL) of oxytetracycline, but the experimental conditions in the tanks were significantly different from those under marine salmon farm cages, precluding its extrapolation to salmonid farming environments. Otherwise, in tanks containing no feed only a slight increase of resistance to oxytetracycline was observed. Likewise, Vaughan et al. (1996) used antibacterial-free laboratory mesocosms containing river sediment and detected that, in the presence of decomposing fish feed, levels of oxytetracycline-resistant bacteria increased rapidly (1.0–25%) after 15 days of incubation. The authors suggested that high frequencies of oxytetracycline resistance may be detected in environments where fish feed accumulates.

Akinbowale et al. (2007) found important levels of resistance to various antibacterials, including amoxicillin, florfenicol, and streptomycin in *Aeromonas* and *Pseudomonas* strains isolated from fish and sediments of rainbow trout farms in Australia and suggested that the observed resistance is intrinsic considering that no antibacterials are licensed for use in aquaculture in Australia. These results are in agreement with other studies that report multiple antibacterial resistance among bacteria isolated from rainbow trout farm environments (Schmidt et al., 2000). In addition, Saavedra et al. (2004) found a high incidence of *Aeromonas hydrophila* resistant to β-lactams isolated from rainbow trout in Portugal.

Kerry et al. (1997) found no evidence for a selection of resistant strains in intestinal microbiota of Atlantic salmon smolts treated with oxytetracycline. Likewise, Kerry et al. (1995a) concluded that factors different that the presence of oxytetracycline may influence the frequency of resistant bacteria in marine sediments. In addition, Samuelsen et al. (1992b) observed a high persistence of oxytetracycline resistance (10–50% after 18 months) in sediments beneath salmonid cages and suggested that resistance can be promoted and maintained due to factors different from the antibacterial concentration.

Kerry et al. (1995b) and Miranda and Zemelman (2002a) reported that unmedicated fish feed contains important levels of antibacterial-resistant bacteria, and this could be an important source of antibacterial resistance genes in environments associated with fish farming. The high frequency of oxytetracycline-resistant bacteria observed in feed samples is in agreement with data obtained by DePaola (1995), who found high numbers of oxytetracycline-resistant Gram-negative bacteria in both medicated and unmedicated pelletized feeds, a fact that strongly support the hypothesis that the resistant microflora introduced in the feed might be one of the most important sources of the elevated frequency of resistance in these systems.

Despite Chile being the second largest producer of farmed salmonids in the world, only a few studies on the antibacterial resistance associated to salmonid farms have been done. The incidence of oxytetracycline resistance in four freshwater Chilean Atlantic salmon farms, with no history of recent antibacterial therapy (more than 6 months) was determined, observing a high frequency of resistant bacteria in fingerling, rearing tank water, and unmedicated pelletized feed samples as well as a high phenotypic diversity of resistant isolates exhibiting high resistance levels

(Miranda and Zemelman, 2002a). Resistant isolates mainly belonged to the enteric group and *Acinetobacter*, *Pseudomonas*, and *Moraxella* genus exhibiting a high proportion of resistance to amoxicillin, ampicillin, erythromycin, and furazolidone and an important frequency of bacterial resistance to florfenicol, chloramphenicol, cefotaxime, and trimethoprim–sulfamethoxazole, as well as a rather low proportion of bacteria resistant to gentamicin, kanamycin, flumequine, and enrofloxacin. The occurrence of simultaneous resistance to various antibacterials was frequent, with 74 strains resistant to 6–10 antibacterials (Miranda and Zemelman, 2002b). Twenty-five representative tetracycline-resistant Gram-negative isolates recovered from Chilean fish farms (Miranda and Zemelman, 2002a) were examined for the presence of tetracycline resistance *tet* genes. A high gene heterogeneity was detected in which *tetA* was found in 6 isolates, whereas 2 strains carried the *tetB*, *tetH*, or *tetB* and *tet34* genes and one strain carried *tetE*, *tetL*, or *tet35* genes, whereas 10 isolates carried unidentified tetracycline resistance genes (Miranda et al., 2003). Of the strains carrying the *tetB* gene, an *Acinetobacter radioresistens* strain also harbored the Tn5706-associated 1063-bp insertion sequence (IS) element IS1597, while a *Moraxella* sp. isolate also carried a 1026-bp IS-like element whose 293-amino-acid transposase protein exhibited 69% identity and 84% similarity to the transposase protein of IS1597, suggesting the presence of a novel IS element. Seven strains out of 18 resistant strains were able to transfer their tetracycline resistance to *E. coli*. Likewise, Jacobs and Chenia (2007) identified a diversity of resistance genes in the absence of antibiotic selective pressure in *Aeromonas* spp. recovered from aquaculture systems, including trout farms in South Africa.

Clearly, the distribution of *tet* genes from the Chilean freshwater salmonid farms was different than those that have previously been described from other geographical locations. As was suggested by Miranda et al. (2003), surveillance studies in Chilean salmonid farms are necessary to monitor the continuing evolution in the distribution of *tet* genes in this environment, considering that oxytetracycline is one of the most used antibacterials in fish farming.

Considering that florfenicol is currently the most used antibacterial agent in Chilean freshwater salmon farming, Miranda and Rojas (2007) found remarkable proportions of florfenicol-resistant bacteria in both control ($18.55 \pm 2.85\%$) and undercage ($26.40 \pm 1.56\%$) sediments of a salmon farm recently treated with florfenicol, suggesting that usage of florfenicol is not a necessary causal condition for the development of elevated frequencies of florfenicol resistance. Resistant isolates mainly belonged to *Pseudomonas* genus and exhibited important proportions of resistance to ampicillin, erythromycin, furazolidone, and cotrimoxazole and susceptibility to gentamicin, kanamycin, and enrofloxacin.

Resistance to florfenicol in Gram-negative bacteria is mediated primarily by the *floR* gene, which is a specific drug exporter that confers resistance to florfenicol and chloramphenicol (Schwarz et al., 2004). The gene is widely disseminated among Gram-negative bacteria in diverse geographic locations (Schwarz et al., 2004), particularly from Gram-negative organisms from animal agricultural sources (Gordon et al., 2008). Because of the importance of florfenicol in treating bacterial infections in Chilean salmon farming, it is critical to monitor the emergence and spread of resistance to this compound. Fernández-Alarcón et al. (2010) detected the *floR* gene in 26 out of 119 florfenicol-resistant strains isolated from various Chilean salmon farms. Most of the *floR*-carrying strains were also resistant to streptomycin,

chloramphenicol, and oxytetracycline. On the other hand, they found that in the majority of assayed nonfermenting bacteria, florfenicol resistance was at least partially mediated by nonspecific efflux pump systems.

Other authors have evaluated the environmental effect of salmon farming wastewater, observing an increase of the Gram-negative bacterial fraction in sediments beneath salmonid cages, probably as a consequence of an increase of antibacterial-resistant bacteria (Vezzulli et al., 2002). It has been observed that fish farms contribute to the environmental contamination by antimicrobials and resistant bacteria (Gordon et al., 2007). In addition, several studies showed that land-based fish farm effluents contained a higher number of oxytetracycline-resistant bacteria than those observed in the inlets, even during periods without medication and mainly belonging to the genus *Aeromonas* and *Pseudomonas* (Schmidt et al., 2000; Miranda and Zemelman, 2002a).

Chelossi et al. (2003) detected a high number of bacterial strains resistant to antibacterials both in control and impacted sediments, suggesting a widespread antibacterial resistance in surrounding areas of fish farms. The authors suggested that the high level of drug resistance could be related to the production of antimicrobial compounds by some fish farm isolates belonging to the *Bacillus* genus. It was shown that microbial antibiotic producers in the environment have developed a large and versatile arsenal of antibiotic resistance genes, being an important part of the resistome (D'Costa et al., 2007).

As a matter of fact, it is clear that environmental impacts of using antibacterial agents in salmonid farming are still uncertain, and their effects on the emergence and persistence of antibacterial resistance varies substantially depending on factors such as chemical and physical properties of the influenced environment, the real biological activity of drug residues, and the density and characteristics of microbiota as well as the resistome of the impacted environment (Wright, 2007).

In salmonid farm environments, antibacterial-resistant microbiota is composed of bacteria exhibiting intrinsic resistance, which are mainly naturally occurring and those exhibiting acquired resistance mainly associated with transferable resistance determinants. The main factors associated to emergence, persistence, and spread of antibacterial-resistant microbiota in salmonid farm environments are described in Figure 22.1.

Various factors determining a high and persistent selective pressure as well as several environmental conditions are of key importance to determine the emergence, persistence, and spread of resistant strains in fish farm environments, whereas the presence of resistant strains could be mainly due to the pre-existing naturally occurring intrinsic resistome or the introduction of resistant bacteria carrying specific genes that code for resistance mechanisms. The understanding of these factors is of critical importance to develop adequate guidelines to eliminate or minimize salmonid farm practices that could promote development and spread of antibacterial resistance in the surrounding environments.

It is highly necessary to discriminate between bacterial strains that exhibit intrinsic nontransferable resistance and those that have active resistance mechanisms mediated by antibacterial resistance determinants, mainly obtained through genetic exchange, in order to elucidate the real potential public health risk of antibacterial resistance detected in these environments. It is clear that most of the intrinsic resistance must not be considered a major problem of clinical relevance because of their low probability of dissemination.

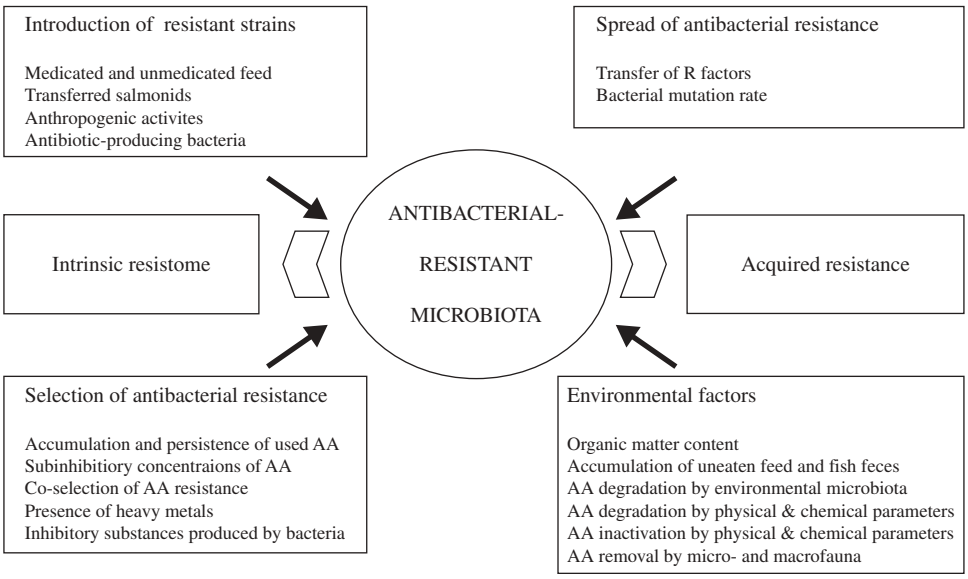

FIGURE 22.1 Main factors determining the occurrence and persistente of antibacterial resistance in salmonid farming environments. AA: Antibacterial Agent.

22.4 ANTIBACTERIAL RESISTANCE IN SALMONID BACTERIAL PATHOGENS

The efficacy of antimicrobial chemotherapy, commonly used to control bacterial disease in salmon farming, is increasingly being compromised by the development of resistance in fish pathogens mainly emerging as a consequence of chromosomal mutation or acquisition of resistance determinants mediated by genetic exchange (Dixon, 1994; Sørum, 2006). Due to this the salmonid industry have been increasingly requiring the use of more expensive alternative antibacterials, against which resistance develops much less readily, thus increasing significantly the production costs of this activity.

Usually resistance to older quinolone antibacterials is due to chromosomal mutations in the *gyrA* gene, the structural gene for the deoxyribonucleic acid (DNA) gyrase A subunit, but these strains are usually still susceptible or only exhibit a low-level resistance to fluoroquinolones. This fact provides evidence that salmonid pathogens such as *Aeromonas salmonicida* (Wood et al., 1986; Hastings and McKay, 1987; Tsoumas et al., 1989), *Flavobacterium psychrophilum* (Izumi and Aranishi, 2004), *Yersinia ruckeri* (Gibello et al., 2004), and *Vibrio anguillarum* (Aoki et al., 1987) are mainly resistant to nalidixic acid and oxolinic acid and sensitive to fluoroquinolones such as flumequine and enrofloxacin. While a single mutational event may lead to complete resistance to older quinolones, full resistance to newer quinolones is mediated by multiple mutations of genes involved in the synthesis of DNA gyrase and changes in the cell envelope (Martínez et al., 1998).

Otherwise, the extensive environmental use of antibacterial agents may result in development and spread of highly transferable resistance plasmids. Plasmid-mediated resistance to chloramphenicol, trimethoprim, sulfonamides, and tetracyclines have

been identified in fish pathogens (Aoki et al., 1971), and the occurrence of plasmids has been detected in marine and freshwater fish pathogens including *V. anguillarum*, *Vibrio salmonicida*, *A. salmonicida*, *A. hydrophila*, *Edwarsiella tarda*, and *Y. ruckeri* (Toranzo et al., 1983; Aoki, 1988).

Most of the studies concerning antibacterial resistance among salmonid bacterial pathogens refer to *A. salmonicida*, the etiological agent of furunculosis, an economically very important disease of wild and farmed salmonid fish (McCarthy, 1975). In *A. salmonicida* low-level quinolone resistance appears to be due to alterations in outer membrane proteins while high-level resistance is probably due to alterations in DNA gyrase (Lewin, 1992).

Inglis et al. (1991) determined the antibacterial susceptibility of 304 strains of *A. salmonicida* isolated from furunculosis outbreaks occurred in salmon farms in Scotland, observing a high incidence of resistance to oxytetracycline (54.9%) and oxolinic acid (36.5%) and a low incidence of resistance to potentiated sulfonamides (10.2%). In addition, other studies demonstrated that a high number of *A. salmonicida* strains isolated from infected salmonids in United States are resistance to potentiated sulfonamides (Starliper and Cooper, 1998), whereas Björklund et al. (1991) found that most of *A. salmonicida* strains isolated from farmed rainbow trout (*Oncorhynchus mykiss*) in Finland were resistant to oxytetracycline. On the contrary, Michel et al. (2003) found that all studied *A. salmonicida* strains were susceptible to florfenicol, agreeing with the observation by Inglis et al. (1993a) who found that all isolates of *A. salmonicida* were sensitive to florfenicol, but exhibited resistance to other antibacterials including the combination of clavulanic acid with amoxicillin.

Enger et al. (1989) reported that *A. salmonicida* was detected in sediment samples several months after an outbreak of the disease and also was detected in sediments from farms not affected by the disease, but could not be detected in sediments not influenced by fish farming. Samuelsen et al. (1992a) found simultaneously *A. salmonicida* strains and oxolinic acid in the gut of wild and farmed salmon treated with this drug and suggested the possibility that *A. salmonicida* develop resistance to this antibacterial that could be easily spread to the environment via salmon feces. Various authors detected reduced susceptibility to oxolinic acid in *A. salmonicida* strains from farmed salmonids in Scotland and Japan (Aoki et al., 1983; Wood et al., 1986; Hastings and McKay, 1987; Barnes et al., 1990, 1992; Inglis et al., 1991). Griffiths and Lynch (1989) demontrated that multiple low-level resistance to oxolinic acid is a result of decreased outer membrane permeability associated with a change from a 38.5- to a 37-kDa outer membrane protein. These results are in agreement with Barnes et al. (1990), who associated an outer membrane protein of approximately 37 kDa with a decreased susceptibility to oxolinic acid among oxytetracycline-resistant *A. salmonicida*. In addition, Wood et al. (1986) detected that *A. salmonicida* mutants exhibiting multiple low-level antibacterial resistance contained a major outer membrane protein of 37 kDa, not significantly present in wild-type strains.

Barnes et al. (1991a) demonstrated that *A. salmonicida* strains developed resistance to oxolinic acid at significantly higher mutation frequencies than they did to fluoroquinolones. This agreed with Barnes et al. (1991b), who observed that resistance to flumequine developed at less than one tenth the frequency of the development of resistance to oxolinic acid, and with Tsoumas et al. (1989), who easily selected in vitro mutant strains of *A. salmonicida* resistant to oxolinic acid. It has been reported that one aminoacidic substitution (serine 83 to isoleucine) in the

GyrA protein of *A. salmonicida* produces resistance to oxolinic acid, whereas an additional aminoacidic substitution (alanine 67 to glycine) increases two- to fourfold the minimum inhibitory concentration (MIC) of enrofloxacin (Oppegaard and Sørum, 1994).

Giraud et al. (2004) investigated the presence of mutations in the quinolone-resistance-determining regions (QRDRs) of the *gyrA* and *parC* genes, encoding for the A subunits of DNA gyrase and topoisomerase IV, respectively, the target enzymes for quinolones (Drlica and Zhao, 1997), as well as the potential participation of efflux mechanisms (Poole, 2000) in 12 strains of *A. salmonicida* with different levels of quinolone susceptibility isolated from diseased farmed fish in France. All strains showed the same mutation at *gyrA* gene (aspartic acid 87 to asparagine) and no mutation in the *parC* gene, but differences in the level of resistance were detected among the isolates probably due to increases in the efflux activity (Giraud et al., 2004). The high homogeneity of the observed mutations in isolates from the same farms at different times suggest that point mutation had spread from a unique source and then evolved differentially by means of the acquisition of other mechanisms such as efflux activity.

Schmidt et al. (2001b) detected class 1 integrons in 26 out of 40 strains of *A. salmonicida* isolated from furunculosis outbreaks in northern Europe, United States, and Canada. Integrons are gene-capture and expression systems characterized by the presence of an *intI* gene encoding for an integrase, a recombination site (*attI*), and a promoter (Ploy et al., 2000). Integrons are able to capture gene cassettes from the environment and incorporate them by using site-specific recombination. The detected integrons harbored inserted gene cassettes encoding for dihydrofolate reductase (*dhfrI, dhfrIIc*, or *dhfrXVI*) and aminoglycoside acetyltransferase [*ant* (*3″*)*1a*], encoding for trimethoprim and aminoglycoside resistance, respectively. In addition, 19 oxytetracycline-resistant strains were able to transfer by conjugation their R-plasmids containing different *tet* genes to an *E. coli* recipient, whereas 15 strains co-transferred their class 1 integrons. The observed gene patterns for strains from different geographical areas suggest the occurrence of different mechanisms of gene acquisition in the emergence of plasmid-borne antibacterial resistance in *A. salmonicida*. Likewise, L'Abée-Lund and Sørum (2001) detected class 1 integrons in 21 out of 38 sulfonamide-resistant *A. salmonicida* strains isolated from various countries, observing the presence of integrated gene cassettes *aadA2* (8 strains), *dfr16* (5 strains), *aadA1* (3 strains), and *dfrIIc* (3 strains), and a high incidence of tetracycline resistance, mainly mediated by *tetA* and *tetE* genes, with the *tetA* gene frequently associated to the Tn1721 transposon, confirming the high importance of this integron in antibacterial resistance occurring in aquatic environments and suggesting that resistance determinants are commonly shared by *A. salmonicida* strains from different environments.

Plasmids carrying resistance determinants to a wide range of antibacterials have been detected in *A. salmonicida* (Aoki et al., 1971, 1972; Aoki and Takahashi, 1986), whereas Sandaa and Enger (1994, 1996), using microcosm experiments, transferred at a high frequency the naturally occurring pRSA1 plasmid, encoding for multiple antibacterial resistance, from an *A. salmonicida* strain to various bacterial strains recovered from fish farm sediments, even in the absence of antibacterial agents. Such transfer is shown to occur in most situations where antibacterial agents are in common use (Levy, 1984). Sandaa et al. (1992) has shown that plasmid transfer can

occur in the environment of fish farms and is favored by the presence of a selective antimicrobial agent, whereas Adams et al. (1998) could transfer R-plasmids encoding for oxytetracycline resistance carrying the *tetA* gene from resistant isolates of *A. salmonicida* to *E. coli*. Inglis et al. (1993b) detected transferable R-plasmids in 11 out of 40 *A. salmonicida* strains in Scotland, observing co-transference with oxytetracycline, streptomycin, and sulfonamides. Casas et al. (2005) studied nine *A. salmonicida* strains isolated from farmed and wild fish in the United States and found that all strains carried a 58-kb tetracycline resistance plasmid, with a high prevalence of *tetA* (8 strains), *sul1* (8 strains), and *dfrA16* (7 strains) resistance genes, with 5 strains transferring their R-plasmids to *E. coli*. Sørum et al. (2003) detected the transferable 45-kb plasmid pRAS1, belonging to the IncU incompatibility group, that confers resistance to tetracycline, sulfonamides, and trimethoprim in an *A. salmonicida* strain, as well as detected the pAr-32 plasmid, carrying a class 1 integron harboring the *aad* cassette and the chloramphenicol resistance gene *catA2* gene in a Japanese strain of *A. salmonicida*. More recently, McIntosh et al. (2008) detected a transferable IncA/C plasmid carried by 13 *A. salmonicida* strains isolated from Atlantic salmon farms in Canada conferring resistance to florfenicol, oxytetracycline, sulfonamides, streptomycin, and mercury.

Florfenicol has been demonstrated to be very effective in treating a number of salmonid pathogens such as *A. salmonicida* and *V. salmonicida* (Fukui et al., 1987; Inglis and Richards, 1991; Nordmo et al., 1998; Samuelson et al., 1998; Brunn et al., 2000; Schmidt et al., 2000) and is thus a drug of great value in salmonid farming.

Flavobacterium psychrophilum is the causative agent of bacterial cold-water disease and rainbow trout fry syndrome and is one of the most important bacterial pathogens affecting salmonid farming worldwide (Nematollahi et al., 2003). Various studies on antibacterial resistance of *F. psychrophilum* have been done. Bruun et al. (2003a) determined the efficacy of oxytetracycline treatment in rainbow trout infected with three *F. psychrophilum* strains exhibiting different MIC values, observing that treatment on trouts infected with strains exhibiting MIC values of 4.0 and 8.0 μg/mL was not successful and only fish infected with strain exhibiting a MIC of 0.25 μg/mL were successfully treated. Schmidt et al. (2000) found high proportions of oxolinic acid resistance among *F. psychrophilum* strains associated to Danish trout farming, concluding that resistance mechanisms were mainly chromosomally determined.

Hesami et al. (2010) studied 72 *F. psychrophilum* strains mainly isolated from Ontario fish farming, observing a decreased susceptibility to potentiated sulfonamides (ormetoprim–sulfadimethoxine and trimethoprim–sulfamethoxazole) in most of the isolates, whereas 61 and 53% of the isolates showed high MIC values for oxytetracycline and florfenicol, respectively.

Bruun et al. (2000) determined the resistance pattern of 387 *F. psychrophilum* strains isolated from diseased rainbow trout in Denmark, observing a reduced susceptibility to amoxicillin and oxolinic acid among the most recent isolates, whereas all isolates were susceptible to florfenicol. Rangdale et al. (1997) observed an important number of *F. psychrophilum* strains resistant to sulfadiazine/trimethoprim and oxytetracycline and susceptibility to florfenicol and enrofloxacin, whereas Kum et al. (2008) found important incidence of resistance to gentamicin, erythromycin, and sulfamethoxazole–trimethoprim and susceptibility to florfenicol and enrofloxacin among *F. psychrophilum* strains isolated from rainbow trout fry in Turkey. Various

reports evidenced that a high number of *F. psychrophilum* strains exhibit a lack of susceptibility to potentiated sulfonamides (Rangdale et al., 1997; Bruun et al., 2000). At this moment it is not known how this occurs, but some authors have suggested an intrinsic resistance mediated by the absence of biochemical pathways on which the sulfonamides act. More recently, Del Cerro et al. (2010) studied 25 strains of *F. psychrophilum* isolated in Spain showing high susceptibility to oxytetracycline and florfenicol. Izumi and Aranishi (2004) demonstrated the presence of 2 amino acid substitutions in the GyrA protein of quinolone-resistant strains of *F. psychrophilum* in Japan (a threonine residue replaced by an alanine or isoleucine residue in position 83 and the aspartic acid replaced by a tyrosine residue in position 87).

Despite the fact that *F. psychrophilum* is one the most important bacterial pathogens in Chilean salmonid farming, no studies on antibacterial susceptibility have been currently published, but the evolution along a period of 3 years of the antibacterial resistance of this species was recently determined in a research project, observing a high susceptibility to florfenicol, oxytetracycline, and fluoroquinolones, whereas a growing incidence of resistance to oxolinic acid and potentiated sulfonamides was detected (INNOVA, 2010).

Most of the infectious diseases in farmed salmonids are caused by *Vibrio* species, as cold-water vibriosis are produced by *V. anguillarum* or *Vibrio ordalii* species, whereas all other species are grouped as *Vibrio*-like bacteria. During the 1980s, tetracycline and sulfa-trimethoprim were extensively used in Norwegian salmon farming to treat cold-water vibriosis caused by *V. salmonicida*, so the emergence of resistant strains was expected (Hjeltnes et al., 1987). Sørum et al. (1990, 1992) described in *V. salmonicida* strains isolated from Norwegian salmonid farms a 170-MDa plasmid carrying the resistance gene *tetE*. They observed an important incidence of resistance to sulfonamides, tetracycline, and trimethoprim. Husevag et al. (1991) detected the simultaneous occurrence of antibacterial-resistant bacteria and *V. salmonicida* in sediments located in abandoned fish-farming sites, suggesting the possibility of transfer of resistance determinants to *V. salmonicida*. On the other hand, Zhao and Aoki (1992) sequenced the *tetG* gene, encoding for resistance to tetracyclines isolated from *V. anguillarum*.

Yersinia ruckeri is the causative agent of enteric redmouth (ERM) disease or yersiniosis, a serious infectious disease of fish that causes high economic losses in the rainbow trout farming industry in many countries (Furones et al., 1993). Several studies on antibacterial susceptibility of *Y. ruckeri* species have been developed. Michel et al. (2003) observed that all studied *Y. ruckeri* isolates were susceptible to florfenicol and chloramphenicol, which is in agreement with Schmidt et al. (2000) who observed that all analyzed *Y. ruckeri* strains were susceptible to all assayed antibacterials, whereas Kirkan et al. (2006) found high levels of susceptibility to ciprofloxacin and resistance to tetracycline and trimethoprim–sulfamethoxazole. Rodgers (2001) determined the MIC values for 124 strains of *Y. ruckeri* and observed an important increase in the MIC values (20-, 16-, or 16-fold for oxolinic acid, oxytetracycline, and the potentiated sulfonamide, respectively), after continuous subculture, suggesting a high potential for antibacterial mutation. Gibello et al. (2004) provided evidence that reduced susceptibility to quinolones of 7 *Y. ruckeri* strains isolated from different diseased rainbow trout during enteric redmouth outbreaks in Spain was due to the substitution of a serine residue by an arginine residue at position 83 of the GyrA protein. DeGrandis and Stevenson (1985) isolated 2 *Y. ruckeri*

strains carrying a 36-MDa plasmid encoding for resistance to tetracyclines and sulfonamides that was transferred to *E. coli* and *Y. ruckeri* strains. In addition, Bruun et al. (2003b) found that 2 motile *Aeromonas* strains were able to transfer their resistance to oxytetracycline to *Y. ruckeri* strains using microcosm experiments.

Renibacterium salmoninarum is the etiological agent of bacterial kidney disease (BKD) in salmonids (Fryer and Lannan, 1993), and only a few studies on its susceptibility to antibacterials have been developed. Rhodes et al. (2008) determined the MIC of erythromycin and azithromycin of 7 strains of *R. salmoninarum* with reduced susceptibility to macrolide antibiotics isolated from fish receiving multiple antibiotic treatments, and observed that 3 isolates exhibited a MIC of 0.008 μg/ mL to either erythromycin or azithromycin, whereas 4 isolates displayed higher MICs, ranging between 0.125 and 0.250 μg/mL for erythromycin and between 0.016 and 0.031 μg/mL for azithromycin. In addition, no mutations for macrolide resistance in the 23S ribosomal DNA (rDNA) gene were found, indicating that reduced susceptibility to macrolide antibiotics was not due to mutations associated with the drug-binding site of 23S ribosomal ribonucleic acid (rRNA). In another study, the complete genome of *R. salmoninarum* ATCC 33209 was sequenced, and various ORFs homologous to antibiotic resistance genes, including genes encoding β-lactamases, efflux proteins, macrolide glycosyltransferases, and rRNA methyltransferases were detected (Wiens et al., 2008). Macrolide resistance is important because erythromycin is the antibacterial of first choice for BKD treatment in many salmonid producer countries such as Chile. Wiens et al. (2008) detected that several classes of macrolide resistance genes are represented in the genome of *R. salmoninarum*, including genes encoding two 23S rRNA methyltransferases (*rlmA* and *spoU*), a macrolide efflux factor (*mefA*), a multidrug resistance efflux pump (*pvsC*), and a 16S rRNA dimethylase (*ksgA*).

Otherwise, no information is available in the scientific literature on antibacterial resistance of other salmonid pathogen species such as *Streptococcus phocae*, the causative agent of systemic disease in farmed salmonids (Romalde et al., 2008), and *Piscirickettsia salmonis* an intracellular pathogen responsible for salmonid rickettsial septicemia (Almendras and Fuentealba, 1997). Mikalsen et al. (2008) performed antibiograms of three strains of *P. salmonis*, using a new formulated agar medium and observed wide inhibition zones (>40 mm) for most of the antibacterials assayed, usually used in salmon farming. Unfortunately, no quality control strains were included in the study, precluding the reliance of the obtained results.

The main issue that precludes the advance in the knowledge of antibacterial resistance of salmonid pathogens is the lack of standardization of media, protocols, and incubation conditions. Other aspects to be solved include the determination of epidemiological cut-off values and the clinical breakpoints for the fish pathogenic species.

22.5 CONCLUSIONS AND PERSPECTIVES

There has been a continuous debate about whether the use of antibacterial agents used in salmonid farms selects for antibacterial resistance and whether resistant bacteria and their resistance genes contribute to resistance development in fish and human bacterial pathogens (MacMillan, 2001).

The environmental and public health significance of the use of antibacterials in salmonid farming is not known because it depends upon a number of factors such as fate, persistence, and biological activity in these environments, as well as the probability that human pathogens might become resistant to the antibacterial or class of antibacterials. Despite concerns expressed in some controversial articles (Cabello, 2006; Heuer et al., 2009), there is currently no solid evidence available to demonstrate a direct link between the use of either antibacterial in fish farming and the occurrence of human pathogens resistant to that antibacterial agent.

To understand the real impact that antibacterial usage in salmonid farming can have on human and animal health, it is necessary to take a more ecological view of resistance. It is necessary to explore the diversity of antibiotic-resistant bacteria and antibiotic resistance genes that exist in this environment, even when no antibiotics are used, and then evaluate how antibiotics affect these microbial populations. However, it is highly necessary to differentiate between antibacterial resistance mediated by chromosomal mutation or intrinsic resistance and antibacterial resistance mediated by genetically mobile determinants such as R-plasmids, integrons, or transposons.

Factors responsible for the occurrence of antibiotic resistance in the absence of antibiotic use are still unclear, but the antibacterial intrinsic resistome (defined as the assemblage of chromosomal genes that are involved in intrinsic resistance and whose presence is independent of previous antibiotic exposure) have a low significance to animal and human health because it cannot be disseminated by horizontal gene transfer. Intrinsic resistance to antibacterials is thought to be a consequence of mutations producing reduced permeability of the bacterial envelope coupled with secondary mechanisms such as multidrug efflux pumps.

The presence of high numbers of antimicrobial-resistant bacteria in salmon farming has ecological and public health implications and emphasizes the need for further studies in relation to the creation of reservoirs of transferable antibacterial resistance in salmon aquaculture systems. Evaluating the role of these environments in the dissemination and evolution of antimicrobial resistance genes and their vectors, as well as on the possibility of the returning of resistance genes to the human population, is highly important. Development of a continuous surveillance of antibacterial resistance in nonpathogenic microbiota is necessary to prevent that fish pathogens can acquire resistance determinants inserted in movable elements. For example, it is highly necessary to develop a continuous monitoring of high-level resistance to fluoroquinolones in order to detect the possibility that *qnr* genes are occurring in salmonid farm environments.

It is a major task for salmonid producer countries worldwide to evaluate the evolution of antibacterial resistance among salmonid pathogens and the emergence and spread of mobile genetic elements that they can harbor. Considering that even in the absence of antibacterial selective pressure R-plasmids could be maintained because a R-plasmid can contain other elements that can confer ecological advantages (Martinez, 2009). Knowledge of the location of the antibacterial resistance genes on mobile genetic elements as well as the conditions for their co-selection and persistence will be valuable for veterinarians and will assist them in selecting the most efficacious antimicrobial agents for the control of each salmonid pathogen.

Strict policies to regulate the use of antibacterials in salmonid farming must be imposed by the producer countries. In this trend, most of salmonid producer countries such as Norway, Chile, United States, Canada, and Denmark stated a

strict regulation of antibacterial use, requiring a veterinarian prescription, and being obligatory to submit copies of the issued prescriptions to the government agencies regulating their use (Grave et al., 2008). It would be highly valuable to integrate relevant information from the prescriptions and to correlate them to gathered resistance data to evaluate the efficacy of stated regulations. While several countries have developed national systems for monitoring antibacterial resistance among salmonid pathogens, no such surveillance exists in most of the salmonid producer countries. Another aspect of great relevance is that antibacterials can never be used in fish farms as prophylactics or growth promoters.

The extra-label use (which is allowed under the veterinary medicines act) of medicated feed in aquaculture is limited to medicated feed products approved for use in aquatic species and no other oral antibiotics can be used (CVM, 2001). This practice, like any activity with direct contact with aquatic environments, is highly questionable since the fate, persistence, and impact on the autochthonous microbiota remains unknown. Thus, it is likely that some of the drug resistance observed in several farms has arisen as a result of extra-label use of antibacterials.

Government agencies of producer countries must develop adequate guidelines to ensure the rational use of antibacterials in aquacultural activities and to establish strategies to predict resistance before it emerges clinically, as well as develop molecular diagnostic techniques to counteract resistance before emergence in pathogens.

Development of cross resistance when several antibacterials are used in fish farming is an important issue because this phenomenon of cross selection contributes to a dramatic increase in the number of multiple-drug-resistant bacteria, thus increasing the risk of transferring plasmids coding for resistance into fish pathogens. This fact prompts the necessity to investigate the potential of the antibacterials used in salmonid farming to co-select any other antibacterial agent avoiding alternating between these antibacterials.

In conclusion, it is unrealistic to abandon the use of antibacterial agents in intensive fish farming. However, further studies are necessary to understand how antibacterial resistance spreads among environmental microbiota and the ecological significance of the occurrence of multidrug-resistant bacteria in fish farm environments, but our current lack of knowledge on elements involved in these resistances emphasizes the necessity of maintaining a strict surveillance of emergence and spread of antibacterial resistance.

REFERENCES

Adams CA, Austin B, Meaden PG, McIntosh D (1998). Molecular characterization of plasmid-mediated oxytetracycline resistance in *Aeromonas salmonicida*. *Appl Environ Microbiol* 64:4194–4201.

Agersø Y, Bruun MS, Dalsgaard I, Larsen JL (2007). The tetracycline resistance gene *tet(E)* is frequently occurring and present on large horizontally transferable plasmids in *Aeromonas* spp. from fish farms. *Aquaculture* 266:47–52.

Akinbowale OL, Peng H, Grant P, Barton MD (2007). Antibiotic and heavy metal resistance in motile aeromonads and pseudomonads from rainbow trout (*Oncorhynchus mykiss*) farms in Australia. *Int J Antimicrob Agents* 30:177–182.

Alderman DJ, Hastings TS (1998). Antibiotic use in aquaculture: Development of antibiotic resistance—Potential for consumer health risks. *Int J Food Sci Technol* 33:139–155.

Almendras FE, Fuentealba IC (1997). Salmonid rickettsial septicemia caused by *Piscirickettsia salmonis*: A review. *Dis Aquat Org* 29:137–144.

Alonso A, Sanchez P, Martinez JL (2001). Environmental selection of antibiotic resistant genes. *Environ Microbiol* 3:1–9.

Andersen SR, Sandaa R-A (1994). Distribution of tetracycline resistance determinants among Gram-negative bacteria isolated from polluted and unpolluted marine sediments. *Appl Environ Microbiol* 60:908–912.

Aoki T, Kitao T (1985). Detection of transferable R plasmids in strains of fish-pathogenic bacterium, *Pasteurella piscicida*. *J Fish Dis* 8:345–350.

Aoki T (1988). Drug-resistant plasmids from fish pathogens. *Microbiol Sci* 5:219–22.

Aoki T (1992). Present and future problems concerning the development of resistance in aquaculture. In C Michel and DJ Alderman (Eds.), *Chemotherapy in Aquaculture: From Theory to Reality*. Office International des Epizooties, Paris, pp. 254–262.

Aoki T, Takahashi A (1986). Tetracycline-resistance gene of a non-transferable R plasmid from fish pathogenic bacteria *Aeromonas salmonicida*. *Bull Jpn Soc Sci Fish* 52:1913–1917.

Aoki T, Egusa S, Kimura T, Watanabe T (1971). Detection of R factors in naturally occurring *Aeromonas salmonicida* strains. *Appl Microbiol* 22:716–717.

Aoki T, Egusa S, Yada C, Watanabe T (1972). Studies of drug resistance and R factors in bacteria from pond cultured salmonids. *Jpn J Microbiol* 16:233–238.

Aoki T, Kitao T, Iemura N, Mitoma Y, Nombra T (1983). The susceptibility of *Aeromonas salmonicida* strains isolated in cultured and wild salmonids to various chemotherapeutics. *Bull Jpn Soc Sci Fish* 49:17–22.

Aoki T, Satoh T, Kitao T (1987). New tetracycline resistance determinant on R plasmids from *Vibrio anguillarum*. *Antimicrob Agents Chemother* 31:1446–1449.

Austin B, Al-Zahrani AMJ (1988). The effect of antimicrobial compounds on the gastrointestinal microflora of rainbow trout, *Salmo gairdneri* Richardson. *J Fish Biol* 33:1–14.

Barnes AC, Lewin CS, Hastings TS, Amyes SGB (1990). Cross resistance between oxytetracycline and oxolinic acid in *Aeromonas salmonicida* associated with alterations in outer membrane proteins. *FEMS Microbiol Lett* 22:337–340.

Barnes AC, Amyes SGB, Hastings TS, Lewin CS (1991a). Fluoroquinolones display rapid bactericidal activity and low mutation frequencies against *Aeromonas salmonicida*. *J Fish Dis* 14:661–667.

Barnes AC, Lewin CS, Hastings TS, Amyes SGB (1991b). In vitro susceptibility of the fish pathogen *Aeromonas salmonicida* to flumequine. *Antimicrob Agents Chemother* 35:2634–2635.

Barnes AC, Lewin CS, Hastings TS, Amyes SGB (1992). Alterations in outer membrane proteins identified in a clinical isolate of *Aeromonas salmonicida* subsp *salmonicida*. *J Fish Dis* 15:279–282.

Barnes AC, Hastings TS, Amyes SGB (1995). Aquaculture antibacterials are antagonized by seawater cations. *J Fish Dis* 18:463–465.

Bernoth EM (1991). Possible hazards due to fish drugs. *Bull Eur Assoc Fish Pathol* 11:17–21.

Björklund H, Bondestam J, Bylund G (1990). Residues of oxytetracycline in wild fish and sediments from fish farms. *Aquaculture* 86:359–367.

Björklund H, Raberg CMI, Bylund G (1991). Residues of oxolinic acid and oxytetracycline in fish and sediments from fish farms. *Aquaculture* 97:85–96.

Bruno DW (1989). An investigation into oxytetracycline residues in Atlantic salmon, *Salmo salar* L. *J Fish Dis* 12:77–86.

Bruun MS, Schmidt AS, Madsen L, Dalsgaard I (2000). Antimicrobial resistance patterns in Danish isolates of *Flavobacterium psychrophilum*. *Aquaculture* 187:201–212.

Bruun MS, Madsen L, Dalsgaard I (2003a). Efficiency of oxytetracycline treatment in rainbow trout experimentally infected with *Flavobacterium psychrophilum* strains having different in vitro antibiotic susceptibilities. *Aquaculture* 215:11–20.

Bruun MS, Schmidt AS, Dalsgaard I, Larsen JL (2003b). Conjugal transfer of large plasmids conferring oxytetracycline (OTC) resistance: Transfer between environmental Aeromonads, fish-pathogenic bacteria, and *Escherichia coli*. *J Aquat Anim Health* 15:69–79.

Cabello FC (2006). Heavy use of prophylactic antibiotics in aquaculture: A growing problem for human and animal health and for the environment. *Environ Microbiol* 8:1137–1144.

Capone DG, Weston DP, Miller V, Shoemaker C (1996). Antibacterial residues in marine sediments and invertebrates following chemotherapy in aquaculture. *Aquaculture* 145:55–75.

Casas C, Anderson EC, Ojo KK, Keith I, Whelan D, Rainnie D, Roberts MC (2005). Characterization of pRAS1-like plasmids from atypical North American psychrophilic *Aeromonas salmonicida*. *FEMS Microbiol Lett* 242:59–63.

Chelossi E, Vezzulli L, Milano A, Branzoni M, Fabiano M, Riccardi G, Banat IM (2003). Antibiotic resistance of benthic bacteria in fish-farm and control sediments of the Western Mediterranean. *Aquaculture* 219:83–97.

Coyne R, Hiney M, O'Connor B, Kerry J, Cazabon D, Smith P (1994). Concentration and persistence of oxytetracycline in sediments under a marine salmon farm. *Aquaculture* 123:31–42.

Coyne R, Hiney M, Smith P (1997). Transient presence of oxytetracycline in blue mussels (*Mytilus edulis*) following its therapeutic use at a marine Atlantic salmon farm. *Aquaculture* 149:175–181.

Cravedi JP, Choubert G, Delous G (1987). Digestibility of chloramphenicol, oxolinic acid and oxytetracycline in rainbow trout and influence of these antibiotics on lipid digestibility. *Aquaculture* 60:133–141.

CVM (Center for Veterinary Medicine) (2001). Extra-label use of medicated feeds for minor species. Compliance Policy Guide Sec 615.115, Food and Drug Administration, 7500 Standish Place, Rockville, MD 20855, United States.

Davies J (1994). Inactivation of antibiotics and the dissemination of resistance genes. *Science* 264:375–382.

Davies JE (1997). Origins, acquisition and dissemination of antibiotic resistance determinants. *Ciba Found Symp* 207:15–27.

D'Costa VM, Griffiths E, Wright GD (2007). Expanding the soil antibiotic resistome: Exploring environmental diversity. *Curr Opin Microbiol* 10:481–489.

DeGrandis SA, Stevenson RMW (1985). Antimicrobial susceptibility patterns and R plasmid-mediated resistance of the fish pathogen *Yersinia ruckeri*. *Antimicrob Agents Chemother* 27:938–942.

Del Cerro, A, Márquez I, Prieto JM (2010). Genetic diversity and antimicrobial resistance of *Flavobacterium psychrophilum* isolated from cultured rainbow trout, *Onchorynchus mykiss* (Walbaum), in Spain. *J Fish Dis* 33(4):285–291.

DePaola A (1995). Tetracycline resistance by bacteria in response to oxytetracycline-contaminated catfish feed. *J Aquat Anim Health* 7:155–160.

DePaola A, Roberts MC (1995). Class D and E tetracycline resistance determinants in Gram-negative from catfish ponds. *Mol Cell Probes* 9:311–313.

Dixon B (1994). Antibiotic resistance of bacterial fish pathogens. *J World Aquaculture Soc* 25:60–63.

Drlica K, Zhao X (1997). DNA gyrase, topoisomerase IV, and the 4-quinolones. *Microbiol Mol Biol Rev* 61:377–392.

Enger O, Husevag E, Goksϕyr J (1989). Presence of the fish pathogen *Vibrio salmonicida* in fish farm sediments. *Appl Environ Microbiol* 55:2815–2818.

Ervik A, Thorsen B, Eriksen V, Lunestad BT, Samuelsen OB (1994). Impact of administering antibacterial agents on wild fish and blue mussels *Mytilus edulis* in the vicinity of fish farms. *Dis Aquat Org* 18:45–51.

Fernández-Alarcón C, Miranda CD, Singer RS, López Y, Rojas R, Bello H, Domínguez M, González-Rocha G (2010). Detection of the *floR* gene in a diversity of florfenicol resistant Gram-negative bacilli from freshwater salmon farms in Chile. *Zoonoses and Public Health* 57:181–188.

Fortt A, Cabello F, Buschmann A (2007). Residues of tetracycline and quinolones in wild fish living around a salmon aquaculture center in Chile. *Rev Chil Infect* 24(1):14–18.

Fryer JL, Lannan CN (1993). The history and current status of *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease in Pacific salmon. *Fish Res* 17:15–33.

Fukui H, Fujihara Y, Kano T (1987). In vitro and in vivo antibacterial activities of florfenicol, a new fluorinated analog of thiamphenicol, against fish pathogens. *Fish Pathol* 22:201–207.

Furones MD, Rodgers CJ, Munn CB (1993). *Yersinia ruckeri*, the causal agent of enteric redmouth disease (ERM) in fish. *Annu Rev Fish Dis* 3:105–125.

Furushita M, Shiba T, Maeda T, Yahata M, Kaneoka A, Takahashi Y, Torii K, Hasegawa T, Ohta M (2003). Similarity of tetracycline resistance genes isolated from fish farm bacteria to those from clinical isolated. *Appl Environ Microbiol* 69:5336–5342.

Gibello A, Porrero MC, Blanco MM, Vela AI, Liébana P, Moreno MA, Fernández-Garayzábal JF, Domínguez L (2004). Analysis of the *gyrA* gene of clinical *Yersinia ruckeri* isolates with reduced susceptibility to quinolones. *Appl Environ Microbiol* 70(1):599–602.

Giraud E, Blanc G, Bouju-Albert A, Weill FX, Donnay-Moreno C (2004). Mechanisms of quinolone resistance and clonal relationship among *Aeromonas salmonicida* strains isolated from reared fish with furunculosis. *J Med Microbiol* 53:895–901.

Giraud E, Douet DG, Bouju-Albert A, Donnay-Moreno C, Thorin C, Pouliquen H (2006). Survey of antibiotic resistance in an integrated marine aquaculture system under oxolinic acid treatment. *FEMS Microbiol Ecol* 55:439–448.

Gordon L, Giraud E, Ganière J-P, Armand F, Bouju-Albert A, de la Cotte N, Mangion C, Le Bris H (2007). Antimicrobial resistance survey in a river receiving effluents from freshwater fish farms. *J Appl Microbiol* 102:1167–1176.

Gordon L, Cloeckaert A, Doublet B, Schwarz S, Bouju-Albert A, Ganiere JP, Le BH, Le Fleche-Mateos A, Giraud E (2008). Complete sequence of the *floR*-carrying multiresistance plasmid pAB5S9 from freshwater *Aeromonas bestiarum*. *J Antimicrob Chemother* 62:65–71.

Gowen RJ, Bradbury NB, Brown JR (1989). The use of simple models in assessing two of the interactions between fish farming and the marine environment. In N de Pauw, E Jaspers, H Ackefors and N Wilkins (Eds.) *Aquaculture, a Biotechnology in Progress*. European Aquaculture Society, Bredene, Belgium, pp. 1071–1080.

Grave K, Hansen MK, Kruse H, Bangen M, Kristoffersen AB (2008). Prescription of antimicrobial drugs in Norwegian aquaculture with an emphasis on "new" fish species. *Prevent Vet Med* 83:156–169.

Griffiths SG, Lynch WH (1989). Characterisation of *Aeromonas salmonicida* mutants with low level resistance to multiple antibiotics. *Antimicrob Agents Chemother* 33:19–26.

Guardabassi L, Dalsgaard A, Raffatellu M, Olsen JE (2000a). Increase in the prevalence of oxolinic acid resistant *Acinetobacter* spp. observed in a stream receiving the effluent from a freshwater trout farm following the treatment with oxolinic acid-medicated feed. *Aquaculture* 188:205–218.

Guardabassi L, Dijkshoorn L, Collard J-M, Olsen JE, Dalsgaard A (2000b). Distribution and in-vitro transfer of tetracycline resistance determinants in clinical and aquatic *Acinetobacter* strains. *J Med Microbiol* 49:929–936.

Hansen PK, Lunestad BT, Samuelsen OB (1992). Ecological effects of antibiotics and chemotherapeutants from fish farming. In CM Michel and DJ Alderman (Eds.), *Chemotherapy in Aquaculture: From Theory to Reality*. Office International des Epizootics, Paris, pp. 174–178.

Hansen PK, Lunestad BT, Samuelsen OB (1993). Effects of oxytetracycline, oxolinic acid, and flumequine on bacteria in an artificial fish farm sediment. *Can J Microbiol* 39:1307–1312.

Hastings TS, McKay A (1987). Resistance of *Aeromonas salmonicida* to oxolinic acid. *Aquaculture* 61:165–171.

Hektoen H, Berge JA, Hormazabal V, Yndestad M (1995). Persistence of antibacterial agents in marine sediments. *Aquaculture* 133:175–184.

Herwig RP, Gray JP (1997). Microbial response to antibacterial treatment in marine microcosms. *Aquaculture* 152:139–154.

Herwig RP, Gray JP, Weston DP (1997). Antibacterial resistant bacteria in surficial sediments near salmon net-cage farms in Puget Sound, Washington. *Aquaculture* 149:263–283.

Hesami S, Parkman J, MacInnes JT, Gray JT, Gyles CL, Lumsden JS (2010). Antimicrobial susceptibility of *Flavobacterium psychrophilum* isolates from Ontario. *J Aquat Anim Health* 22(1):39–49.

Heuer OE, Kruse H, Grave K, Collignon P, Karunasagar I, Angulo FJ (2009). Human health consequences of use of antimicrobial agents in aquaculture. *Clin Infect Dis* 49:1248–1253.

Hjeltnes B, Andersen K, Egidius E (1987). Multiple antibiotic resistance in *Vibrio salmonicida*. *Bull Eur Assoc Fish Pathol* 7:85.

Husevag B, Lunestad BT, Johannessen PJ, Enger O, Samuelsen OB (1991). Simultaneous occurrence of *Vibrio salmonicida* and antibiotic-resistant bacteria in sediments at abandoned aquaculture sites. *J Fish Dis* 14:631–640.

Inglis V, Richards RH (1991). The *in vitro* susceptibility of *Aeromonas salmonicida* and other fish-pathogenic bacteria to 29 antimicrobial agents. *J Fish Dis* 14:641–650.

Inglis V, Frerichs GN, Millar SD, Richards RH (1991). Antibiotic resistance of *Aeromonas salmonicida* isolared from Atlantic salmon, *Salmo salar* L., in Scotland. *J Fish Dis* 14:353–358.

Inglis V, Millar SD, Richards RH (1993a). Resistance of *Aeromonas salmonicida* to amoxycillin. *J Fish Dis* 16:389–395.

Inglis V, Yimer E, Bacon EJ, Ferguson S (1993b). Plasmid-mediated antibiotic resistance in *Aeromonas salmonicida* isolated from Atlantic salmon, *Salmo salar* L., in Scotland. *J Fish Dis* 16:593–599.

INNOVA (2010). Implementación de un sistema de alerta temprana asociado al monitoreo de la resistencia bacteriana en la actividad salmonicultora nacional de la Región de los Lagos y Aysén. Project Report INNOVA CORFO, Chile, Puerto Montt.

Izumi S, Aranishi F (2004). Relationship between *gyrA* mutations and quinolone resistance in *Flavobacterium psychrophilum* isolates. *Appl Environ Microbiol* 70:3968–3972.

Jacobs L, Chenia HY (2007). Characterization of integrons and tetracycline resistance determinants in *Aeromonas* spp. isolated from South African aquaculture systems. *Int J Food Microbiol* 114:295–306.

Jacobsen P, Berglind L (1988). Persistence of oxytetracycline in sediments from fish farms. *Aquaculture* 70:365–370.

Kapetanaki M, Kerry J, Hiney M, O'Brien C, Coyne R, Smith P (1995). Emergence, in oxytetracycline-free marine mesocosms, of microorganisms capable of colony formation on oxytetracycline-containing media. *Aquaculture* 134:227–236.

Kerry J, Hiney M, Coyne R, Cazabon D, NicGabhainn S, Smith P (1994). Frequency and distribution of resistance to oxytetracycline in micro-organisms isolated from marine fish farm sediments following therapeutic use of oxytetracycline. *Aquaculture* 123:43–54.

Kerry J, Gilroy D, Hiney M, Coyne R, Smith P (1995a). The effects of harrowing on oxytetracycline resistance in marine sediment microorganisms beneath a salmon farm. *Bull Eur Assoc Fish Pathol* 15:172–174.

Kerry J, Hiney M, Coyne R, NicGabhainn S, Gilroy D, Cazabon D, Smith P (1995b). Fish feed as a source of oxytetracycline-resistant bacteria in the sediments under fish farms. *Aquaculture* 131:101–113.

Kerry J, Coyne R, Gilroy D, Hiney M, Smith P (1996). Spatial distribution of oxytetracycline and elevated frequencies of oxytetracycline resistance in sediments beneath a marine salmon farm following oxytetracycline therapy. *Aquaculture* 145:31–39.

Kerry J, NicGabhainn S, Smith P (1997). Changes in oxytetracycline resistance of intestinal microflora following oral administration of this agent to Atlantic salmon (*Salmo salar* L.) smolts in a marine environment. *Aquaculture* 157:187–195.

Kirkan S, Göksoy EÖ, Kaya O, Tekbiyik S (2006). In-vitro antimicrobial susceptibility of pathogenic bacteria in rainbow trout (*Oncorhynchus mykiss*, Walbaum). *Turk J Vet Anim Sci* 30:337–341.

Klaver AL, Matthews RA (1994). Effects of oxytetracycline on nitrification in a model aquatic system. *Aquaculture* 123:237–247.

Kum C, Kirkan S, Sekkin S, Akar F, Boyacioglu M (2008). Comparison of in vitro antimicrobial susceptibility in *Flavobacterium psychrophilum* isolated from rainbow trout fry. *J Aquat Anim Health* 20(4):245–251.

L'Abee-Lund TM, Sørum H (2001). Class 1 integrons mediate antibiotic resistance in the fish pathogen *Aeromonas salmonicida* worldwide. *Microb Drug Resist* 7:263–272.

Lai H-T, Lin J-S Chien Y-H (2011a). Effects of light regime and oxygen profile on transformation of oxolinic acid in pond sediment. *Bioresource Technol* 102(9):5425–5430.

Lai H-T, Wang T-S, Chou C-C (2011b). Implication of light sources and microbial activities on degradation of sulfonamides in water and sediment from a marine shrimp pond. *Bioresource Technol* 102(8):5017–5023.

La Rosa T, Mirto S, Mazzola A, Danovaro R (2001). Differential responses of benthic microbes and meiofauna to fish-farm disturbance in coastal sediments. *Environ Pollut* 112:427–434.

La Rosa T, Mirto S, Mazzola A, Maugeri TL (2004). Benthic microbial indicators of fish farm impact in a coastal area of the Tyrrhenian Sea. *Aquaculture* 230:153–167.

Le Bris H, Pouliquen H (2004). Experimental study on the bioaccumulation of oxytetracycline and oxolinic acid by the blue mussel (*Mytilus edulis*). An evaluation of its ability to bio-monitor antibiotics in the marine environment. *Marine Pollut Bull* 48:434–440.

Le Bris H, Dhaouadi R, Naviner M, Giraud E, Mangion C, Armand F, De La Cotte Thorin C, Ganiére J-P, Pouliquen H (2007). Experimental approach on the selection and persistence of antimicrobial-resistant Aeromonads in faecal matter of rainbow trout during and after an oxolinic acid treatment. *Aquaculture* 273:418–422.

Levy SB (1984). Resistance to tetracyclines. In LE Bryan (Ed.), *Antimicrobial Drug Resistance*. Academic, New York, pp. 191–240.

Lewin CS (1992). Mechanisms of resistance development in aquatic microorganisms. In C Michel and DJ Alderman (Eds.), *Chemotherapy in Aquaculture: From Theory to Reality*. Office International des Epizooties, Paris, pp. 288–301.

Lunestad BT (1992). Fate and effects of antimicrobial agents in aquatic environments. In C Michel and DJ Alderman (Eds.), *Chemotherapy in Aquaculture: From Theory to Reality*. Office International des Epizooties, Paris, pp. 152–161.

Lunestad BT, Goksøyr J (1990). Reduction in the antibacterial effect of oxytetracycline in sea water by complex formation with magnesium and calcium. *Dis Aquat Org* 9:67–72.

Lunestad BT, Samuelsen OB (2001). Effects of sea water on the activity of antimicrobial agents used in aquaculture; implications for MIC testing. *Aquaculture* 196:319–323.

Lunestad BT, Samuelsen OB, Fjelde S, Ervik A (1995). Photostability of eight antibacterial agents in seawater. *Aquaculture* 134:217–225.

Ma D, Hu Y, Wang J, Ye S, Li A (2006). Effects of antibacterials use in aquaculture on biogeochemical processes in marine sediment. *Sci Total Environ* 367:273–277.

MacMillan JR (2001). Aquaculture and antibiotic resistance: A negligible public health risk? *World Aquaculture* 32:49–50.

Maki T, Hasegawa H, Kitami H, Fumoto K, Munekage Y, Ueda K (2006). Bacterial degradation of antibiotic residues in marine fish farm sediments of Uranouchi Bay and phylogenetic analysis of antibiotic-degrading bacteria using 16S rDNA sequences. *Fish Sci* 72(4):811–820.

Martinez JL (2009). The role of natural environments in the evolution of resistance traits in pathogenic bacteria. *Proc Roy Soc* B 276:2521–2530.

Martínez JL, Alonso A, Gómez-Gómez JM, Baquero F (1998). Quinolone resistance by mutations in chromosomal gyrase genes. Just the tip of the iceberg? *J Antimicrob Chemother* 42(6):683–688.

Mazzola A, Mirto S, Danovaro R (1999). Initial fish farm impact on meiofaunal assemblages in coastal sediments of the Western Mediterranean. *Marine Pollut Bull* 38:1126–1133.

Mazzola A, Mirto S, La Rosa T, Fabiano M, Danovaro R (2000). Fish farming effects on benthic community structure in coastal sediments: Analysis of the meiofaunal resilience. *ICES J Marine Sci* 57:1454–1461.

McCarthy DH (1975). Fish furunculosis. *Aquaculture Res* 6:13–18.

McIntosh D, Cunningham M, Ji B, Fekete FA, Parry EM, Clark SE, Zalinger ZB, Gilg IC, Danner GR, Johnson KA, Beattie M, Ritchie R (2008). Transferable, multiple antibiotic and mercury resistance in Atlantic Canadian isolates of *Aeromonas salmonicida* subsp. *salmonicida* is associated with carriage of an IncA/C plasmid similar to the *Salmonella enterica* plasmid pSN254. *J Antimicrob Chemother* 61:1221–1228.

Michel C, Kerouault B, Martin C (2003). Chloramphenicol and florfenicol susceptibility of fish-pathogenic bacteria isolated in France: Comparison of minimum inhibitory concentration, using recommended provisory standards for fish bacteria. *J Appl Microbiol* 95:1008–1015.

Mikalsen J, Skjærvik O, Wiik-Nielsen J, Wasmuth MA, Colquhoun DJ (2008). Agar culture of *Piscirickettsia salmonis*, a serious pathogen of farmed salmonid and marine fish. *FEMS Microbiol Lett* 278(1):43–47.

Miranda CD, Rojas R (2007). Occurrence of florfenicol resistance in bacteria associated with two Chilean salmon farms with different history of antibacterial usage. *Aquaculture* 266:39–46.

Miranda CD, Zemelman R (2002a). Bacterial resistance to oxytetracycline in Chilean salmon farming. *Aquaculture* 212:31–47.

Miranda CD, Zemelman R (2002b). Antimicrobial multiresistance in bacteria isolated from freshwater Chilean salmon farms. *Sci Total Environ* 293:207–218.

Miranda CD, Kehrenberg C, Ulep C, Schwarz S, Roberts MC (2003). Diversity of tetracycline resistance genes in bacteria from Chilean salmon farms. *Antimicrob Agents Chemother* 47:883–888.

Moffit CM, Mobin SMA (2006). Profile of microflora of the posterior intestine of Chinook salmon before, during, and after administration of rations with and without erythromycin. *North Am J Aquaculture* 68:176–185.

Navarrete P, Mardones P, Opazo R, Espejo R, Romero J (2008). Oxytetracycline treatment reduces bacterial diversity of intestinal microbiota of Atlantic salmon. *J Aquat Anim Health* 20:177–183.

Naviner M, Giraud E, Le Bris H, Armand F, Mangion C, Ganière JP (2006). Seasonal variability of intestinal microbiota in rainbow trout (*Oncorhynchus mykiss*), with a particular attention to *Aeromonas* spp. as candidate indicator of antimicrobial resistance. *Rev Méd Vét* 157:597–602.

Naviner M, Giraud E, Thorin C, Le Bris H, Pouliquen H, Ganière JP (2007). Effects of three dosages of oral oxolinic acid treatment on the selection of antibiotic-resistant *Aeromonas*: experimental approach in farmed trout. *Aquaculture* 269:31–40.

Nematollahi A, Decostere A, Pasmans F, Haesebrouck F (2003). *Flavobacterium psychrophilum* infections in salmonid fish. *J Fish Dis* 26:563–574.

Nordmo R, Holth Riseth JM, Varma KJ, Sutherland IH, Brokken ES (1998). Evaluation of florfenicol in Atlantic salmon, *Salmo salar* L.: efficacy against furunculosis due to *Aeromonas salmonicida* and cold water vibriosis due to *Vibrio salmonicida*. *J Fish Dis* 21:289–297.

Nygaard K, Lunestad BT, Hektoen H, Berge JA, Hormazabal V (1992). Resistance to oxytetracycline, oxolinic acid and furazolidone in bacteria from marine sediments. *Aquaculture* 104:31–36.

Oppegaard H, Sϕrum H (1994). *gyrA* mutations in quinolone-resistant isolates of the fish pathogen *Aeromonas salmonicida*. *Antimicrob Agents Chemother* 38:2460–2464.

O'Reilly A, Smith P (2001). Use of indirect conductimetry to establish predictive no effect concentrations of oxytetracycline and oxolinic acid in aquatic sediments. *Aquaculture* 196:13–26.

Ploy MC, Lambert T, Couty JP, Denis F (2000). Integrons: An antibiotic resistance gene capture and expression system. *Clin Chem Lab Med* 38:483–487.

Poole K (2000). Efflux-mediated resistance to fluoroquinolones in gram-negative bacteria. *Antimicrob Agents Chemother* 44:2233–2241.

Pouliquen H, Le Bris H, Pinault L (1992). Experimental study of the therapeutic application of oxytetracycline, its attenuation in sediment and sea water, and implications for farm culture of benthic organisms. *Marine Ecol Prog Ser* 89:93–98.

Pouliquen H, Le Bris H, Pinault L (1993). Experimental study of the contamination kinetics of sea water polluted by oxytetracycline contained in effluents released from a fish farm located in salt-marsh. *Aquaculture* 112:113–123.

Pouliquen H, Delépée R, Larhantec-Verdier M, Morvan ML, Le Bris H (2007). Comparative hydrolysis and photolysis of four antibacterial agents (oxytetracycline oxolinic acid, flumequine and florfenicol) in deionised water, freshwater and seawater under abiotic conditions. *Aquaculture* 262:23–28.

Pouliquen H, Thorin C, Verdier M, Morvan ML, Le Bris H (2009). Adsorption and desorption of four aquacultural antibiotics (oxolinic acid, flumequine, oxytetracycline and florfenicol) in natural freshwater sediments. *J Vet Pharmacol Ther* 32:189–189.

Pursell L, Samuelsen OB, Smith P (1995). Reduction in the *in vitro* activity of flumequine against *Aeromonas salmonicida* in the presence of the concentrations of divalent cations found in sea water. *Aquaculture* 135:245–255.

Rangdale RE, Richards RH, Alderman DJ (1997). Minimum inhibitory concentrations of selected antimicrobial compounds against *Flavobacterium psychrophilum* the causal agent of rainbow trout fry syndrome (RTFS). *Aquaculture* 158:193–201.

Rhodes G, Huys G, Swings J, McGann P, Hiney M, Smith P, Pickup RW (2000). Distribution of oxytetracycline resistance plasmids between aeromonads in hospital and aquaculture

environments: Implication of Tn*1721* in dissemination of the tetracycline resistance determinant Tet A. *Appl Environ Microbiol* 66:3883–3890.

Rhodes, LD, Nguyen OT, Deinhard RK, White TM, Harrell LW, Roberts MC (2008). Characterization of *Renibacterium salmoninarum* with reduced susceptibility to macrolide antibiotics by a standardized antibiotic susceptibility test. *Dis Aquat Org* 80(3):173–180.

Ritz DA, Lewis ME, Ma S (1989). Response to organic enrichment of infaunal macrobenthic communities under salmonid seacages. *Marine Biol* 103:211–214.

Rodgers CJ (2001). Resistance of *Yersinia ruckeri* to antimicrobial agents in vitro. *Aquaculture* 196:325–345.

Romalde JL, Ravelo C, Valdés I, Magariños B, de La Fuente E, San Martín C, Avendaño-Herrera R, Toranzo AE (2008). *Streptococcus phocae*, an emerging pathogen for salmonid culture. *Vet Microbiol* 130:198–207.

Saavedra MJ, Guedes-Novais S, Alves A, Rema P, Tacão M, Correia A, Martínez-Murcia A (2004). Resistance to β-lactam antibiotics in *Aeromonas hydrophila* isolated from rainbow trout (*Oncorhynchus mykiss*). *Int Microbiol* 7:207–211.

Samuelsen OB (1989). Degradation of oxytetracycline in seawater at two different temperatures and light intensities, and the persistence of oxytetracycline in the sediment from a fish farm. *Aquaculture* 83:7–16.

Samuelsen OB, Solheim E, Lunestad BT (1991). Fate and microbiological effects of furazolidone in a marine aquaculture sediment. *Sci Total Environ* 108:275–283.

Samuelsen OB, Lunestad BT, Husevag B, Hϕlleland T, Ervik A (1992a). Residues of oxolinic acid in wild fauna following medication in fish farms. *Dis Aquat Org* 12:111–119.

Samuelsen OB, Torsvik V, Ervik A (1992b). Long-range changes in oxytetracycline concentration and bacterial resistance towards oxytetracycline in a fish farm sediment after medication. *Sci Total Environ* 114:25–36.

Samuelsen OB, Lunestad BT, Ervik A, Fjelde S (1994). Stability of antibacterial agents in an artificial marine aquaculture sediment studied under laboratory conditions. *Aquaculture* 126:283–290.

Samuelson OB, Hjeltnes B, Glette J (1998). Efficacy of orally administered florfenicol in the treatment of furunculosis in Atlantic salmon. *J Aquat Anim Health* 10:56–61.

Sandaa R-A (1993). Transfer and maintenance of the plasmid RP4 in marine sediments. *Microb Rel* 2:115–119.

Sandaa R-A, Enger Ø (1994). Transfer in marine sediments of the naturally occurring plasmid pRAS1 encoding multiple antibiotic resistance. *Appl Environ Microbiol* 60:4234–4238.

Sandaa R-A, Enger Ø (1996). High frequency transfer of a broad host range plasmid present in an atypical strain of the fish pathogen *Aeromonas salmonicida*. *Dis Aquat Org* 24:71–75.

Sandaa RA, Torsvik VL, Goksϕyr J (1992). Transferable drug resistance in bacteria from fish-farm sediments. *Can J Microbiol* 38:1061–1065.

Schmidt AS, Bruun MS, Dalsgaard I, Pedersen K, Larsen JL (2000). Occurrence of antimicrobial resistance in fish-pathogenic and environmental bacteria associated with four Danish rainbow trout farms. *Appl Environ Microbiol* 66:4908–4915.

Schmidt AS, Bruun MS, Dalsgaard I, Larsen JL (2001a). Incidence, distribution, and spread of tetracycline resistance determinants and integron-associated antibiotic resistance genes among motile aeromonads from a fish farming environment. *Appl Environ Microbiol* 67:5675–5682.

Schmidt AS, Bruun MS, Larsen JL, Dalsgaard I (2001b). Characterization of class 1 integrons associated with R-plasmids in clinical *Aeromonas salmonicida* isolates from various geographical areas. *J Antimicrob Chemother* 47:735–743.

Schwarz S, Kehrenberg C, Doublet B, Cloeckaert A (2004). Molecular basis of bacterial resistance to chloramphenicol and florfenicol. *FEMS Microbiol Rev* 28:519–542.

Smith JT (1989). Interaction between 4-quinolone antibacterials and multivalent metal ions. *J Chemother* 4:134–135.

Smith P (1995). The meaning of changes in the frequency of resistance to oxytetracycline in the sediments under marine fish farms. *Bull Aquaculture Assoc Can* 95:17–21.

Smith P (1996). Is sediment deposition the dominant fate of oxytetracycline used in marine salmonid farms: A review of available evidence. *Aquaculture* 146:157–169.

Smith P, Samuelsen OB (1996). Estimates of the significance of out-washing of oxytetracycline from sediments under Atlantic salmon sea cages. *Aquaculture* 144:17–26.

Smith P, Donlon J, Coyne R, Cazabon D (1994a). Fate of oxytetracycline in a fresh water fish farm: Influence of effluent treatment systems. *Aquaculture* 120:319–325.

Smith P, Hiney MP, Samuelsen OB (1994b). Bacterial resistance to antimicrobial agents used in fish farming: A critical evaluation of method and meaning. *Annu Rev Fish Dis* 4:273–313.

Smith P, Nyland N, O'Domhnaill F, OTuathaig G, Hiney M (1996). Influence of marine sediment and divalent cations on the activity of oxytetracycline against *Listonella anguillarum*. *Bull Eur Assoc Fish Pathol* 16:54–57.

Sørum H (2006). Antimicrobial drug resistance in fish pathogens. In FM Aarestrup (Ed.), *Antimicrobial Resistance in Bacteria of Animal Origin*. American Society for Microbiology Press, Washington, DC, pp. 213–238.

Sørum H, Roberts MC, Crosa JH (1990). Isolation and characterization of a tetracycline resistance gene from the fish pathogen *Vibrio salmonicida*. In O Olsvik and G Bukholm (Eds.), *Application of Molecular Biology in Diagnosis of Infectious Diseases*. Norwegian College of Veterinary Medicine, Oslo, Norway, pp. 45–48.

Sϕrum H, Roberts MC, Crosa JH (1992). Identification and cloning of a tetracycline resistance gene from the fish pathogen *Vibrio salmonicida*. *Antimicrob Agents Chemother* 36(3): 611–615.

Sϕrum H, L'Abee-Lund TM, Solberg A, Wold A (2003). Integron-containing IncU R plasmids pRAS1 and pAr-32 from the fish pathogen *Aeromonas salmonicida*. *Antimicrob Agents Chemother* 47:1285–1290.

Spanggaard B, Jørgensen F, Gram L, Huss HH (1993). Antibiotic resistance in bacteria isolated from three freshwater fish farms and an unpolluted stream in Denmark. *Aquaculture* 115:195–207.

Starliper CE, Cooper RK (1998). Biochemical and conjugation studies of Romet-resistant strains of *Aeromonas salmonicida* from salmonid rearing facilities in the Eastern United States. *J Aquat Anim Health* 10:221–229.

Stepanauskas R, Glenn TC, Jagoe CH, Tuckfield RC, Lindell AH, King CJ, McArthur JV (2006). Coselection for microbial resistance to metals and antibiotics in freshwater microcosms. *Environ Microbiol* 8:1510–1514.

Teuber M (2001). Veterinary use and antibiotic resistance. *Curr Opin in Microbiol* 4:493–499.

Toranzo AE, Barja JL, Colwell RR, Hetrick FM (1983). Characterization of plasmids in bacterial fish pathogens. *Infect Immun* 48:184–192.

Torsvik VL, Sorheim R, Goksøyr J (1988). Antibiotic resistance of bacteria from fish farm sediments. ICES Report, C. M. 1 988/F:10.

Tsoumas A, Alderman DJ, Rodgers CJ (1989). *Aeromonas salmonicida*: Development of resistance to 4-quinolone antimicrobials. *J Fish Dis* 12:493–507.

Vaughan S, Smith P (1996). Estimations of the influence of river sediment on the biological activity of oxytetracycline HCl. *Aquaculture* 141:67–76.

Vaughan S, Coyne R, Smith P (1996). The critical importance of sample site in the determination of the frequency of oxytetracycline resistance in the effluent microflora of a freshwater fish farm. *Aquaculture* 139:47–54.

Vezzulli L, Chelossi E, Riccardi G, Fabiano M (2002). Bacterial community structure and activity in fish farm sediments of the Ligurian sea (Western Mediterranean). *Aquaculture Int* 10:123–141.

Wiens GD, Rockey DD, Wu Z, Chang J, Levy R, Crane S, Chen DS, Capri GR, Burnett JR, Sudheesh PS, Schipma MJ, Burd H, Bhattacharyya A, Rhodes LD, Kaul R, Strom MS (2008). Genome sequence of the fish pathogen *Renibacterium salmoninarum* suggests reductive evolution away from an environmental *Arthrobacter* ancestor. *J Bacteriol* 190(21):6970–6982.

Wood SC, McCashion RN, Lynch WH (1986). Multiple low-level antibiotic resistance in *Aeromonas salmonicida*. *Antimicrob Agents Chemother* 29:992–996.

Wright GD (2007). The antibiotic resistome: The nexus of chemical and genetic diversity. *Nat Rev Microbiol* 5:175–186.

Wu RSS (1995). The environmental impact of marine fish culture: Towards a sustainable future. *Marine Pollut Bull* 31:159–166.

Zhao J, Aoki T (1992). Nucleotide sequence analysis of the class G tetracycline resistance determinant from *Vibrio anguillarum*. *Microbiol Immunol* 36:1051–1060.

23

EFFECT OF VETERINARY MEDICINES INTRODUCED VIA MANURE INTO SOIL ON THE ABUNDANCE AND DIVERSITY OF ANTIBIOTIC RESISTANCE GENES AND THEIR TRANSFERABILITY

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23.1 INTRODUCTION

In many regions of the world manure fertilization is a routinely used measure in agriculture to maintain soil fertility. The emission of greenhouse gasses (N_2O, CH_4), leaching of NO_3^- to ground and surface water, or heavy-metal contamination (Petersen et al., 2007) are most frequently considered as the main environmental impacts of manure. However, intensification of livestock is typically also accompanied by increasing amounts of antibiotics used. Even in countries that do not permit the application of antibiotics as growth promoter [antibiotic growth promoters were banned in the European Union (EU) in 2006], considerable amounts of antibiotics are used for prophylactic and therapeutic treatments in animal livestock. The chemical characteristics of the antibiotic, as well as numerous biotic and abiotic factors, influence their fate during manure management and after spreading manure onto soil (Halling-Sørensen et al., 1998; Heuer et al., 2008). A recent large-scale monitoring

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study performed by Zhao et al. (2010) showed that residues of antibiotics belonging to the fluoroquinolone, sulfonamide, and tetracycline groups were simultaneously detectable in dung samples from pigs, poultry, and cows originating from 8 provinces in China. In more than 80% of the samples, two or multiple fluoroquinolones were detectable in a single manure sample. A survey of field-scale manure from 16 different farms sampled in Germany in 2006 revealed that manure slurries contained a high load of bacteria carrying transferable antibiotic resistances and mobile genetic elements (Binh et al., 2008, 2009, 2010). The use of veterinary antibiotics in animal husbandry is suspected to affect soil microbial community structure in manure (Schauss et al., 2009a). As spread manure contains antibiotic residues, bacterial populations carrying an enormous diversity of transferable antibiotic resistance genes and nutrients, land application of manure might result in an increased abundance of antibiotic resistance genes through the proliferation of existing subpopulations containing already antibiotic resistance genes or through local adaptation via horizontal transfer. Thus the risks posed by land application of manure need to be evaluated as multiple antibiotic-resistant pathogens pose a major threat for the successful therapeutic use of antibiotics in human and veterinary medicine. The fate of the antibiotics in manure and soil is assumed to be strikingly different and will be determined by the chemical structure of the antibiotic and its metabolites, the amounts excreted, the manure treatment processes, and last but not least by the soil type and various factors influencing microbial activity in soil. Presently, there are no systematic studies available that provide insights in how different manure processing technologies might influence microbial degradation of antibiotic residues, the abundance of antibiotic resistance genes, and microbial community composition and function.

In this chapter, we discuss briefly the methods available for studying the effect of veterinary antibiotics entering via manure into soil on the abundance and transferability of antibiotic resistance genes in soil microbial communities. We also present data from various soil microcosm experiments aimed at investigating the effects of sulfadiazine (SDZ) in piggery manure on the abundance and resistance gene transferability in two different soils. SDZ was chosen as a model substance because sulfonamide antibiotics are among the most frequently used antibiotics for animal husbandry (Mathew et al., 2007), and a very high proportion of the antibiotic compound or its metabolites are excreted. By means of ^{14}C-labeled SDZ administered to pigs, it could be shown that more than 96% of the radioactivity was excreted from the pigs as SDZ, acetyl-SDZ, and 4-OH-SDZ (Heuer et al., 2008). During storage, the acetyl-SDZ was rapidly deacetylated, and thus the amount of SDZ present in manure increased. Therefore, it can be assumed that SDZ used in animal husbandries is spread via manure onto agricultural soils. The research is part of an ongoing project of the German research group FOR566, studying the fate and effects of veterinary antibiotics entering via manure into soil.

23.2 METHODS TO STUDY THE EFFECTS OF VETERINARY MEDICINES ON SOIL MICROBIAL COMMUNITIES

Traditionally, effects of manure treatments on soil microbial communities were determined either by measuring overall soil parameters such as microbial biomass, total N, C_{org}, enzyme activities, or substrate-induced respiration. These methods are

easily applicable to large numbers of samples, but they provide only limited insight into the effects of veterinary medicines entering soil via manure on the abundance and transferability of antibiotic resistance genes.

23.2.1 Cultivation-Based Methods

Cultivation-based methods have been used for decades to explore the effects of veterinary antibiotics on the abundance of antibiotic-resistant bacteria. Serial dilutions of sample material are plated on media supplemented with antibiotics, and colony-forming units (CFU) are determined after incubation. Comparisons of the number of colonies formed on media supplemented with antibiotics can easily be done. However, the cultivation-based approach is impaired as only a rather small proportion of bacteria are able to form colonies on plates and that many bacteria can be intrinsically resistant to various antibiotics. The CFU counts of bacteria resistant to particular antibiotics are influenced by the composition of cultivable bacterial populations present in a sample. This makes comparisons between different soil types less meaningful as the microbial community composition differs between different soils. The clear advantage of cultivation-based methods is that the bacterial hosts carrying respective resistance genes and their genetic localization can be determined (Agersø and Sandvang, 2005; Antunes et al., 2005; Byrne-Bailey et al., 2009; Enne et al., 2008; Moura et al., 2007). However, the number of isolates that need to be screened is critical and resistance genes present in less abundant populations will be missed unless growth conditions are used to select for less abundant taxonomic groups.

23.2.2 Cultivation-Independent Methods

23.2.2.1 Analysis of Total Community DNA (TC-DNA) The analysis of DNA (deoxyribonucleic acid) directly extracted from soil or manure (TC-DNA) became very popular as it can provide insight not only into the microbial composition but also into the abundance and diversity of functional genes (e.g., genes encoding enzymes of the nitrogen cycle or antibiotic resistance genes) or mobile genetic elements independent from the cultivability of their original hosts (Schauss et al., 2009b). Sampling strategies and efficient DNA extraction are crucial for the quality of the data obtained. The DNA extraction protocols used should permit an efficient cell lysis, a high DNA yield, and efficient removal of co-extracted humic acids. Furthermore, the specificity and sensitivity of the detection of antibiotic resistance genes based on polymerase chain reaction (PCR) will be largely determined by the primers that are designed on the basis of known sequences. Here mismatches of the last six bases of the 3′ end of the primer with the target sequence are critical and should be avoided. The primers can be used in an endpoint or quantitative real-time PCR format. In particular, when combined with TaqMan probes, a sensitive and specific quantification in total community DNA becomes feasible. The copy number determined for antibiotic resistance genes in total community DNA can be correlated with different abiotic and biotic factors. In view of comparing treatment effects (e.g., the effects of antibiotic administration), the determination of antibiotic resistance gene copies by means of real-time quantitative PCR is recommended, although the absolute copy number values should not be overinterpreted as these

are influenced by numerous experimental conditions (e.g., DNA extraction and amplification efficiencies). In particular, PCR or real-time quantitative PCR (qPCR) has been used for the detection and quantification of antibiotic resistance genes in DNA directly extracted from different manure or dung samples, soils taken from the vicinity of swine lagoons or from manure-treated soils (Binh et al., 2008; Heuer and Smalla, 2007; Knapp et al., 2008, 2010; Luo et al., 2010; McKinney et al., 2010; Storteboom et al., 2010; Wu et al., 2010). In a recent study, Wu et al. (2010) quantified the abundance of five tetracycline resistance genes in soils sampled in the vicinity of nine swine farms in China and showed that the abundance of tetracycline resistance genes (*tet*) was strongly correlated to the concentrations of tetracycline residues in soil, which were determined by liquid chromatography–electrospray tandem mass spectrometry. Furthermore, the absolute *tet* copy number was strongly related to 16S ribosomal ribonucleic acid (rRNA) gene copy numbers, and organic matter content.

Primers targeting regions of class 1 integron that flank gene cassettes were also used to study the presence of integron gene cassettes in different manure samples and to study the effect of treating soil with manure (Binh et al., 2009; Heuer and Smalla, 2007). Although this approach could have captured unknown gene cassettes, the abundant gene cassettes detected in both studies all belonged to known antibiotic resistance gene cassettes. An interesting observation in the study by Binh et al. (2009) was the high abundance of empty class 1 integrons in manure bacteria.

23.2.2.2 Methods to Capture Antibiotic Resistance Plasmids An elegant method to capture transferable antibiotic resistance genes independent from the cultivability of the original host is the so-called exogenous plasmid isolation into suitable recipients. The exogenous plasmid isolation method can be used as biparental mating by mixing soil or manure containing potential donors of mobile genetic elements (MGE) with a selectable recipient or as triparental mating with an additional donor strain carrying a mobilizable plasmid with selectable markers (see Fig. 23.1). Both techniques were originally established for capturing MGE from river epilithon bacteria (Bale et al., 1988; Hill et al., 1992). While stable replication and the expression of a selectable marker gene are prerequisites for successfully capturing MGE in a biparental mating, in triparental mating the acquisition of the mobilizable plasmid is selected. The ability to mobilize plasmids can be exploited to capture transferable plasmids independent from the presence of selectable markers. However, usually only a small proportion of the transconjugants carry a mobilizing plasmid in addition to the mobilized plasmid (Smalla and Heuer, 2006). In several studies manure bacteria were shown to carry mobilizing plasmids (Götz and Smalla, 1997; Heuer et al., 2002; Smalla et al., 2000). In particular the biparental mating has been employed in different studies on transferable antibiotic resistance plasmids in manure and manure-treated soils (Bahl et al., 2007; Binh et al., 2008; Heuer et al., 2002; Heuer and Smalla, 2007; Smalla et al., 2000; Top et al., 1994). Recipients were mainly *Proteobacteria*. The types of plasmids captured were very much determined by the recipients used (Dronen et al., 1999; Smalla et al., 2000). Thus, ideally, a range of different hosts should be used. However, some recipients display intrinsic antibiotic resistances, and thus the range of suitable

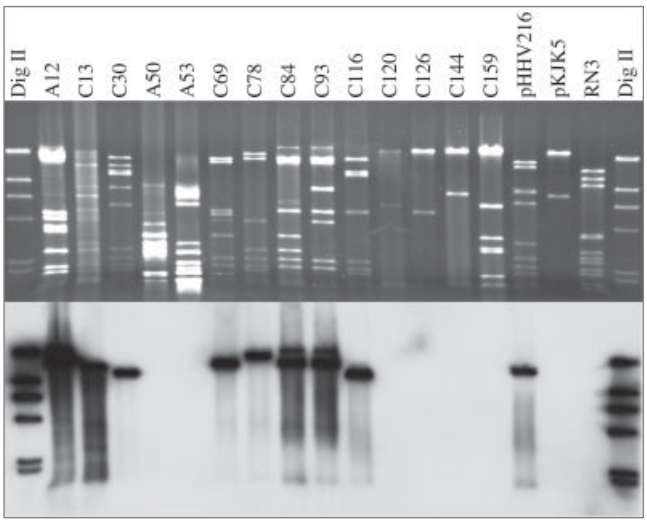

FIGURE 23.1 Upper panel: *Pst1*/*Bst1* restriction digestion patterns of plasmids exogenously captured from manure-treated soils into gfp-tagged *E. coli* CV601 (C) and *Acinetobacter bayleyi* BD413 (A) and of reference plasmids for Low G/C plasmids (pHHV216), IncP-1ε (pKJK5) and IncN (RN3). Dig II stands for digoxygenin-labeled DNA size marker.
Lower panel: Southern blot hybridization with a digoxygenin labeled replicon probe for LowG/C plasmids

recipients depends on the selection marker used. Plasmid-encoded traits can be relatively easy determined, for example, by comparing the resistance patterns of recipient and transconjugant.

23.3 EFFECTS OF SULFADIAZINE SPREAD WITH MANURE ON THE ABUNDANCE OF RESISTANCE GENES AND RESISTANCE TRANSFERABILITY

Various microcosm experiments were recently performed and thoroughly analyzed for effects of SDZ-containing pig manure on the abundance and transferability of resistance genes in soil (Heuer et al., 2008; Heuer and Smalla, 2007). In parallel, the fate of SDZ and its metabolites in manure and soil was investigated. Resistance to the bacteriostatic effect of SDZ is mediated by the genes *sul1*, *sul2,* and *sul3*. They encode dihydropteroate synthases, which are not inhibited by sulfonamides (Perreten and Boerlin, 2003; Sköld, 2000). Mainly, the genes *sul1* and *sul2* were frequently detected in manure (Binh et al., 2008; Heuer et al., 2008).The genes were horizontally transferred to a wide range of bacterial species due to their typical localization on transposable elements and transferable broad-host-range plasmids (Heuer et al., 2004; Schlüter et al., 2003, 2005). The accumulation of *sul* genes in manured soil depends on the input of resistant bacteria from manure, the horizontal transfer of *sul* genes on mobile genetic elements to soil-adapted bacteria, and the input and selective pressure of SDZ in soil.

23.3.1 Design of Microcosm Experiments

We investigated the effects of SDZ and manure in several microcosm experiments using two German agricultural soils that substantially differed physically and chemically. Soil M was a silt loam [Orthic Luvisol, 15.4% clay, 78.2% silt, 6.4% sand, pH ($CaCl_2$) 7.2, organic C 1.2%, maximal water holding capacity (WHC_{max}) 46%] from a field near Merzenhausen, which had no history of previous manure applications. Soil K was a loamy sand from a field near Kaldenkirchen [Gleyic Cambisol, 3.6% clay, 23.1% silt, 73.3% sand, pH ($CaCl_2$) 5.5, organic C 1.0%, WHC_{max} 27%] that was fertilized with manure in previous years. The soils were amended with an amount of manure that corresponded to that of a field application in the top 10 cm (40 g/kg). The manure contained either 0, 250, or 2500 μg/g spiked SDZ. In one microcosm experiment, stored manure from pigs treated with SDZ according to veterinary practice was applied, which contained 120 μg/g SDZ. Unamended soil with the same moisture content served as control treatment. The microcosms were incubated at 10–15°C in the dark. Evaporated water was replaced twice a week. Manure application was done either once or repeated after 63 and 133 days to test for accumulative effects. TC-DNA was extracted from the soil samples and used after purification for molecular analysis. In addition soil bacteria were used as plasmid donors in biparental matings with *gfp*-tagged *Escherichia coli* strains.

23.3.2 Factors Influencing the Relative Abundance of *sul1* and *sul2 G*enes in Soil Bacteria

A single amendment with manure significantly increased the absolute and relative numbers of *sul* genes in both soils analyzed (Heuer et al., 2009; Heuer and Smalla, 2007). The amount of *sul* genes detected in soil one day after manuring partially exceeded the amount that was added with manure, indicating substantial growth of *sul*-carrying populations. The effect of manure on the abundance of *sul1* and *sul2* in soil lasted for at least 2 months after application if the applied manure contained SDZ. This suggested a long-term bioavailability of SDZ, even though it became rapidly bound to the soil matrix. A mathematical model was developed that quantitatively relates the fate of SDZ and its metabolites in soil and the abundance of SDZ resistance genes (Heuer et al., 2008). The model considered the putative cost of *sul* genes on mobile genetic elements, horizontal gene transfer, and selection of the resistant populations in the presence of SDZ. Modeling of data from soil microcosms amended with manure from ^{14}C-SDZ-treated pigs revealed a selective effect of the antibiotic compound in soil on *sul2* even at low concentrations down to 150 μg/kg of extractable SDZ. In our studies, the resistance gene *sul2* was on average one to two orders of magnitude more abundant in manure and soil than *sul1*, as revealed by qPCR, and *sul3* was rarely detected in our studies (Binh et al., 2008; Heuer and Smalla, 2007), but *sul1* was more persistent in soil over 165 days (Heuer et al., 2008).

Repeated application of manure containing SDZ in 2-month intervals resulted in a significant accumulation of *sul1* and *sul2* genes in both soils (Heuer et al., 2011). The accumulation ranged between 40 and 107% of log units per manure application. After the third application the gene *sul1* became more than three orders of magnitude higher in abundance than in the untreated soil M. Estimated from gene copies of *sul1* and *sul2* per ribosomal gene copy, roughly 10% of the soil bacteria became resistant

to sulfonamides. The accumulation was less strong in soil K, which might be due to adaptation of the soil microbial community by manure applications in previous years.

23.3.3 Effects on Transferability

The effect of manure and of SDZ-spiked manure on the transferability of MGE conferring SDZ from soil bacteria was studied by exogenous isolation (Heuer and Smalla 2007). SDZ resistance plasmids were captured in filter matings in *gfp*-tagged *E. coli* recipient directly from different field-scale manures (Binh et al., 2008) and from soil microcosms described above. Plasmids conferring SDZ resistance were captured from all field-scale manures except one at transfer frequencies ranging from 10^{-4} to 10^{-8} (Binh et al., 2008). The frequencies of capturing SDZ resistance from soils nontreated with manure were very low or not detectable for both soil types. Four days after manure application, up to three orders of magnitude increased transfer frequencies compared to the untreated soils were observed for both soil types. At this time point spiking SDZ did not influence the transferability of MGE conferring SDZ resistance. However, the effects of spiking SDZ became clearly visible after 32 days. The transferability was not only affected in a dose but also in a soil-type-dependent manner. In the previously non-manure-treated soil, the S0 and S10 treatments were not significantly different while the transfer frequencies observed for S100 were significantly increased. In the K soil (which was manure treated in the past) an SDZ concentration-dependent increase of transfer frequencies was found. Significant differences among transfer frequencies were observed for the different treatments.

Data from mesocosm and field-scale experiments in which the soils were treated with manure from pigs treated with SDZ or not, also indicated that both the presence of SDZ and the rhizosphere increased the transfer frequencies of capturing MGE conferring SDZ resistance (unpublished data).

The effects of manure and SDZ on the abundance of MGE were studied in the same total community DNA by PCR in combination with Southern blot hybridization and with quantitative real-time PCR for replicon-specific sequences of the two most frequently captured plasmid types IncP-1ε and low guanine–cytosine (GC) plasmids (Heuer et al., 2009). While a significant effect of SDZ spiking on the abundance of low GC plasmids was observed in soil M, no significant effects of manure and SDZ were observed on the IncP-1ε plasmid abundance (unpublished data).

23.3.4 Characterization of Plasmids from Manure and from Manure-Treated Soils

Although the MGE captured were selected based on the SDZ resistance conferred to the *E. coli* recipient, several other resistance genes were located on the same MGE. Plasmid DNA was screened by Southern blot hybridization or by PCR with replicon-specific probes (Binh et al., 2008) for the presence of known plasmid replicons. The majority of plasmids captured from manure-treated soils did not hybridize with probes for broad-host-range plasmids IncP-1α, IncP-1β, IncN, IncW, and IncQ or enterobacterial plasmids but belonged to a novel group of plasmids with low GC content. Three plasmids belonging to this group were completely sequenced (Heuer et al., 2009). All three plasmids had a common backbone of about 30 kb with a guanine–cytosine (G+C) content of 36% and a segment of about 27–28 kb accessory DNA with an average G+C content of 47%. The backbone of all three plasmids was

found to be composed of transfer and maintenance genes that displayed a moderate homology to pIPO2 and a replication module that was clearly of different origin with 5% less G+C content (closest hit *Acinetobacter baumannii*). The accessory genes displayed a remarkable mosaic structure but they shared several genetic elements. All three plasmids carried the genes *rumA* and *rumB,* encoding a DNA polymerase involved in mutagenic repair and genes conferring sulfonamide (*sul2*) or streptomycin resistance (*strA*/*strB*). In addition, plasmid pHH1107 conferred resistance to tetracycline *(tetX*), plasmid pHHV216 to tetracycline (*tetH*), and chloramphenicol (*floR*) and plasmid pHHV35 to gentamicin (*aacC2*). In all three plasmids similar insertion sequence (IS) elements (IS*1422*, IS*1006*, IS*Aba1*, and IS*Aba2*), IS*CR2*, and complete or remnant versions of Tn*5393* were found. These plasmids were captured from various microcosm experiments (Heuer et al., 2009), but also in the collection of exogenously isolated plasmids from the mesocosm and field experiments this plasmid group seemed to dominate. The second most frequently captured group of plasmids belonged to the recently proposed group of IncP-1ε plasmids (Bahl et al., 2009). So far only one representative of this plasmid group was described (pKJK5), which was also isolated from a manure-treated soil in Denmark. Again sequencing of some of these plasmids showed that their diversity lies in the accessory genes while backbone genes were rather stable (unpublished data). On all IncP-1ε plasmids a class 1 integron with a *sul1* gene was detected. The class 1 integrons were either empty or carried gene cassettes *aadA* or *aadB* conferring streptomycin or gentamicin resistance, respectively. Although it cannot be excluded that the plasmids captured originate from manure bacteria, these plasmid groups were detectable by PCR–Southern blot hybridization in total community DNA from nontreated soils.

23.4 CONCLUSION AND OUTLOOK

Based on the data obtained from microcosm, mesocosm, and field experiments, it is concluded that the presence of antibiotics in manure might result in an increased abundance of antibiotic resistance genes, depending on the fate of the antibiotic compound in the soil. Although many antibiotics are rapidly sequestered in soil, our data indicate that they still seem to affect soil bacteria. This increased abundance of antibiotic resistance genes can result from the proliferation of existing populations that carry respective resistance genes and horizontal gene transfer processes. At least transfer frequencies of capturing antibiotic resistance plasmids were always higher for soils treated with pig manure containing SDZ, thus indicating that transferability is enhanced in the presence of a subinhibitory concentration of antibiotics.

Considering the high load of manure with antibiotic resistance genes and mobile genetic elements and residues of antibiotics and metabolites, we strongly propose that the environmental risks of spread manure need to be reconsidered. Measures need to be taken to reduce the unintended release of millions of bacteria carrying multiple antibiotic resistance plasmids together with antibiotics, into agricultural soil. For a long time it was assumed that soils will rapidly sequester antibiotics, and thus no adverse effects from spread manure were expected. Recent studies on the effects of subinhibitory concentrations of antibiotics showed that low concentrations foster horizontal gene transfer processes and gene expression. The reduction of the amount of antibiotics used should be clearly the first option. A second option could

be to evaluate and optimize the potential of different manure processing technologies with respect to degradation of antibiotics and the reduction of the amount of antibiotic resistance genes in manure.

REFERENCES

Agersø Y, Sandvang D (2005). Class 1 integrons and tetracycline resistance genes in *Alcaligenes*, *Arthrobacter*, and *Pseudomonas* spp. isolated from pigsties and manured soil. *Appl Environ Microbiol* 71:7941–7947.

Antunes P, Machado J, Sousa JC, Peixe L (2005). Dissemination of sulfonamide resistance genes (*sul1*, *sul2*, and *sul3*) in Portuguese *Salmonella enterica* strains and relation with integrons. *Antimicrob Agents Chemother* 49:836–839.

Bahl MI, Hansen LH, Goesmann A, Sørensen SJ (2007). The multiple antibiotic resistance IncP-1 plasmid pKJK5 isolated from a soil environment is phylogenetically divergent from members of the previously established alpha, beta and delta sub-groups. *Plasmid* 58:31–43.

Bahl MI, Burmølle M, Meisner A, Hansen LH, Sørensen SJ (2009). All IncP-1 plasmid subgroups, including the novel epsilon subgroup, are prevalent in the influent of a Danish wastewater treatment plant. *Plasmid* 62:134–139.

Bale MJ, Day MJ, Fry JC (1988). Novel method for studying plasmid transfer in undisturbed river epilithon. *Appl Environ Microbiol* 54:2756–2758.

Binh CTT, Heuer H, Kaupenjohann M, Smalla K (2008). Piggery manure used for soil fertilization is a reservoir for transferable antibiotic resistance plasmids. *FEMS Microbiol Ecol* 66:25–37.

Binh CTT, Heuer H, Kaupenjohann M, Smalla K (2009). Diverse *aadA* gene cassettes on class 1 integrons introduced into soil via spread manure. *Res Microbiol* 160:427–433.

Binh CTT, Heuer H, Gomes NCM, Kaupenjohann M, Smalla K (2010). Similar bacterial community structure and high abundance of sulfonamide resistance genes in field-scale manures. In CS Dellaguardia (Eds.), *Manure: Management, Uses and Environmental Impacts*. Nova Science Publishers, Hauppauge, NY, pp. 141–166.

Byrne-Bailey KG, Gaze WH, Kay P, Boxall AB, Hawkey PM, Wellington EMH (2009). Prevalence of sulfonamide resistance genes in bacterial isolates from manured agricultural soils and pig slurry in the United Kingdom. *Antimicrob Agents Chemother* 53:696–702.

Dronen AK, Torsvik V, Top EM (1999). Comparison of the plasmid types obtained by two distantly related recipients in biparental exogenous plasmid isolations from soil. *FEMS Microbiol Lett* 176:105–110.

Enne VI, Cassar C, Sprigings K, Woodward MJ, Bennett PM (2008). A high prevalence of antimicrobial resistant *Escherichia coli* isolated from pigs and a low prevalence of antimicrobial resistant *E. coli* from cattle and sheep in Great Britain at slaughter. *FEMS Microbiol Lett* 278:193–199.

Götz A, Smalla K (1997). Manure enhances plasmid mobilization and survival of *Pseudomonas putida* introduced into field soil. *Appl Environ Microbiol* 63:1980–1986.

Halling-Sørensen B, Nors NS, Lanzky PF, Ingerslev F, Holten Lützhøft HC, Jorgensen SE (1998). Occurrence, fate and effects of pharmaceutical substances in the environment—A review. *Chemosphere* 36:357–393.

Heuer H, Smalla K (2007). Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. *Environ Microbiol* 9:657–666.

Heuer H, Solehati Q, Zimmerling U, Kleineidam K, Schloter M, Müller T, Focks A, Thiele-Bruhn S, Smalla K (2011). Accumulation of sulfonamide resistance genes in arable soils due

to repeated application of manure containing sulfadiazine. *Appl Environ Microbiol* 77:2527–2530.

Heuer H, Krögerrecklenfort E, Wellington EMH, Egan S, van Elsas JD, van Overbeek LS, Collard JM, Guillaume G, Karagouni AD, Nikolakopoulou TL, Smalla K (2002). Gentamicin resistance genes in environmental bacteria: Prevalence and transfer. *FEMS Microbiol Ecol* 42:289–302.

Heuer H, Szczepanowski R, Schneiker S, Pühler A, Top EM, Schlüter A (2004). The complete sequences of plasmids pB2 and pB3 provide evidence for a recent ancestor of the IncP-1β group without any accessory genes. *Microbiology* 150:3591–3599.

Heuer H, Focks A, Lamshöft M, Smalla K, Matthies M, Spiteller M (2008). Fate of sulfadiazine administered to pigs and its quantitative effect on the dynamics of bacterial resistance genes in manure and manured soil. *Soil Biol Biochem* 40:1892–1900.

Heuer H, Kopmann C, Binh CTT, Top EM, Smalla K (2009). Spreading antibiotic resistance through spread manure: Characteristics of a novel plasmid type with low %G+C content. *Environ Microbiol* 11:937–949.

Hill KE, Weightman AJ, Fry JC (1992). Isolation and screening of plasmids from the epilithon which mobilize recombinant plasmid Pd10. *Appl Environ Microbiol* 58:1292–1300.

Knapp CW, Engemann CA, Hanson ML, Keen PL, Hall KJ, Graham DW (2008). Indirect evidence of transposon-mediated selection of antibiotic resistance genes in aquatic systems at low-level oxytetracycline exposures. *Environ Sci Technol* 42:5348–5353.

Knapp CW, Zhang W, Sturm BS, Graham DW (2010). Differential fate of erythromycin and beta-lactam resistance genes from swine lagoon waste under different aquatic conditions. *Environ Pollut* 158:1506–1512.

Luo Y, Mao D, Rysz M, Zhou Q, Zhang H, Xu L, Alvarez JJ (2010). Trends in antibiotic resistance genes occurrence in the Haihe River, China. *Environ Sci Technol* 44:7220–7225.

Mathew AG, Cissel R, Liamthong S (2007). Antibiotic resistance in bacteria associated with food animals: A United States perspective of livestock production. *Foodborne Pathogens Dis* 4:115–133.

McKinney CW, Loftin KA, Meyer MT, Davis JG, Pruden A (2010). *tet* and *sul* antibiotic resistance genes in livestock lagoons of various operation type, configuration, and antibiotic occurrence. *Environ Sci Technol* 44:6102–6109.

Moura A, Henriques I, Ribeiro R, Correia A (2007). Prevalence and characterization of integrons from bacteria isolated from a slaughterhouse wastewater treatment plant. *J Antimicrob Chemother* 60:1243–1250.

Perreten V, Boerlin P (2003). A new sulfonamide resistance gene (*sul3*) in *Escherichia coli* is widespread in the pig population of Switzerland. *Antimicrob Agents and Chemother* 47:1169–1172.

Petersen SO, Sommer SG, Béline F, Burton C, Dach J, Dourmad JY, Leip A, Misselbrook T, Nicholson F, Poulsen HD, Provolo G, Sørensen P, Vinnerås B, Weiske A, Bernal MP, Böhm R, Juhász C, Mihelic R (2007). Recycling of livestock manure in a whole-farm perspective. *Livestock Science* 112:180–191.

Schauss K, Focks A, Heuer H, Kotzerke A, Schmitt H, Thiele-Bruhn S, Smalla K, Wilke BM, Matthies M, Amelung W, Klasmeier J, Schloter M (2009a). Analysis, fate and effects of the antibiotic sulfadiazine in soil ecosystems. *TrAC Trends Anal Chem* 28:612–618.

Schauss K, Focks A, Leininger S, Kotzerke A, Heuer H, Thiele-Bruhn S, Sharma S, Wilke BM, Matthies M, Smalla K, Munch JC, Amelung W, Kaupenjohann M, Schloter M, Schleper C (2009b). Dynamics and functional relevance of ammonia-oxidizing archaea in two agricultural soils. *Environ Microbiol* 11:446–456.

Schlüter A, Heuer H, Szczepanowski R, Forney LJ, Thomas CM, Pühler A, Top EM (2003). The 64508 bp IncP-1β antibiotic multiresistance plasmid pB10 isolated from a waste-water treatment plant provides evidence for recombination between members of different branches of the IncP-1β group. *Microbiology* 149:3139–3153.

Schlüter A, Heuer H, Szczepanowski R, Poler SM, Schneiker S, Pühler A, Top EM (2005). Plasmid pB8 is closely related to the prototype IncP-1 beta plasmid R751 but transfers poorly to *Escherichia coli* and carries a new transposon encoding a small multidrug resistance efflux protein. *Plasmid* 54:135–148.

Sköld O (2000). Sulfonamide resistance: mechanisms and trends. *Drug Resist Updat* 3:155–160.

Smalla K, Heuer H (2006). How to assess the abundance and diversity of mobile genetic elements (MGE) in soil bacterial communities? In P Nannipieri and K Smalla (Eds.), *Nucleic Acids and Proteins in Soil*. Edited Springer-Verlag, Berlin, pp. 313–330.

Smalla K, Heuer H, Götz A, Niemeyer D, Krögerrecklenfort E, Tietze E (2000). Exogenous isolation of antibiotic resistance plasmids from piggery manure slurries reveals a high prevalence and diversity of IncQ-like plasmids. *Appl Environ Microbiol* 66:4854–4862.

Storteboom H, Arabi M, Davis JG, Crimi B, Pruden A (2010). Tracking antibiotic resistance genes in the South Platte River basin using molecular signatures of urban, agricultural, and pristine sources. *Environ Sci Technol* 44:7397–7404.

Top E, De Smet I, Verstraete W, Dijkmans R, Mergeay M (1994). Exogenous isolation of mobilizing plasmids from polluted soils and sludges. *Appl Environ Microbiol* 60:831–839.

Wu N, Qiao M, Zhang B, Cheng WD, Zhu YG (2010). Abundance and diversity of tetracycline resistance genes in soils adjacent to representative swine feedlots in China. *Environ Sci Technol* 44:6933–6939.

Zhao L, Dong YH, Wang H (2010). Residues of veterinary antibiotics in manures from feedlot livestock in eight provinces of China. *Sci Total Environ* 408:1069–1075.

24

TRACKING ANTIBIOTICS AND ANTIBIOTIC RESISTANCE GENES THROUGH THE COMPOSTING PROCESS AND FIELD DISTRIBUTION OF POULTRY WASTE: LESSONS LEARNED

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24.1 INTRODUCTION

Environmental exposure to animal waste may contribute to ecosystem-mediated transport of antibiotic residues, resistant pathogens, and/or resistance genes that, in turn, add to the reservoir of mobile genetic elements that confer resistance. The concomitant risk is that unintended exposure of bacteria to these contaminants may encourage the de novo development of resistance in nontarget species of microorganisms. There is historic evidence to suggest that prevalence of antibiotic resistance elements have increased in ecosystems (Knapp et al., 2010) and specific bacteria species (Grayson et al., 1991; Nemati et al., 2008) since the widespread therapeutic use of antibiotics began. The public health implications of the spread of antibiotic-resistant bacteria and resistance genes between animals and humans are known (Møbak, 2004), and thus transport of animal waste compost as environmental contaminants merits thorough investigation.

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There is growing interest in exploring the persistence and potential transport pathways of manure-borne antibiotic residues or antibiotic resistance genes following land application of animal waste (Baguer et al., 2000; Loke et al., 2003; Boxall et al., 2004; Burkholder et al., 2007). In addition to the directed exposure to foods from animal sources that have been treated with antibiotics, an important pathway for the transfer of antibiotic residues or antibiotic resistance genes is via food crops grown on land fertilized with composted animal manure. Several investigators have demonstrated that plant species, including food crops common in human and animal diets, can take up antibiotics from manure-amended soil (Jjemba, 2002; Migliore et al., 2003; Kumar et al., 2005; Boxall et al., 2006; Kong et al., 2007). Uptake of antibiotics via food crops may influence the potential for human exposure to low concentrations of antibiotic residues or antibiotic resistance genes, although the health consequences of such remain largely unexplored.

Analytical methods have been described for determining concentrations of the tetracycline family of antibiotics in water (Zhu et al., 2001), wastewater (Yang et al., 2005), food (Coyne et al., 1997; Cinquina et al., 2003; Wan et al., 2005), soil (Rabølle and Spliid, 2000; Jacobsen et al., 2004), and animal manure (Loke et al., 2002). O'Connor and Aga (2007) reviewed the analyses of tetracyclines in soil matrices and discussed several important factors that compromise quantification. Both oxytetracycline and chlortetracycline can be measured in poultry tissue and eggs (Meredith et al., 1965), although neither drug was found to persist after normal cooking processes.

In Canada, oxytetracycline and chlortetracycline can be used in poultry without a prescription while tetracycline is generally prescribed by a veterinarian for therapeutic use. Table 24.1 summarizes the approved use of tetracycline antibiotics for use in chickens in Canada. Although antibiotic use in poultry has changed over the past decade, the proven effectiveness of tetracyclines as growth promoters and therapeutic treatments leads to their frequent use in chicken production (Singer and Hofacre, 2006). Antibiotics are prescribed in doses that are frequently only partially

TABLE 24.1 Tetracycline Antibiotics Approved for use in Chickens in Canada

Uses	Antibiotic	CMIB[a] Code	Route
	Nutritional		
Growth promotion and improved feed efficiency	Chlortetracycline hydrochloride	10.1	Feed
Stimulating appetite and maintaining weight gains in times of stress, chronic respiratory disease, or nonspecific enteritis	Chlortetracycline hydrochloride	34	Feed
Stimulating appetite and maintaining weight gains in times of stress, chronic respiratory disease, or nonspecific enteritis	Oxytetracycline hydrochloride	35	Feed
	Therapeutic		
Chronic respiratory disease, bluecomb, cholera, enteritis, and hexamitiasis	Oxytetracycline hydrochloride		Water
	Tetracycline hydrochloride		Water

[a]Compendium of Medicating Feed Ingredients Brochure (Canadian Food Inspection Agency, 2008).

metabolized, leaving a considerable fraction of the drug excreted while retaining its antimicrobial activity (Elmund et al., 1971). It is probable that antibiotics of the tetracycline family of antibiotics can be introduced as environmental contaminants in Canadian agricultural watersheds.

The trend in agricultural production of food animals to increase herd sizes while reducing the number of individual operations is accompanied with the need for disposal of larger amounts of manure. Animal waste compost serves as a valuable resource for improving drainage, structure, and nutrient capacity in agricultural soil. Studies of composted swine manure have reported significant reduction of tetracycline resistance genes, especially ribosomal protection protein genes, over the course of the composting process (Yu et al., 2005), although tetracycline resistance genes have been isolated from various agricultural environments (Kobashi et al., 2005).

Bacteria form one of the most important groups in soil communities by performing crucial ecosystem services, including closing the nutrient and geochemical cycles. Bacteria present in soil can act as reservoirs for antibiotic resistance genes with vast genetic diversity among species (Riesenfeld et al., 2004; Wright, 2007; Dantas et al., 2008). Metal composition of soils can affect bioavailability of introduced antibiotic contaminants (Wang et al., 2008) and can exert selective pressures on indigenous bacteria for long periods of time (Silver and Phung, 1996). Bacteria isolated from soils with higher metal concentrations have been shown to contain more antibiotic-resistant plasmids (Rasmussen and Sorensen, 1998), and it has been suggested that interacting processes in soils between metal-resistant and antibiotic-resistant plasmids influence the mobility of antibiotic resistance genes between populations of indigenous bacteria present in the environment (Alonso et al., 2001; Baker-Austin et al., 2006). The cumulative effect of distribution of agricultural pollutants including metals, stressors that confer resistance, and excess nutrients throughout the environment is likely to impact microbial communities.

In a pivotal study conducted in 1976, Levy et al. demonstrated that tetracycline resistance genes present in *Escherichia coli* strains could be transferred between chickens and from chickens to humans. Members of the tetracycline family of antibiotics are among the most widely prescribed antibiotics in veterinary medicine (Levy, 1998), and tetracycline residues have been previously determined in poultry litter (Webb and Fontenot, 1975). Poultry house litter and indoor air have been demonstrated to be contaminated with antibiotic-resistant bacteria in some cases (Brooks et al., 2010). Recent studies of a major agricultural center in the state of Maryland frequently detected chlortetracycline (and sulfamethoxazole) above the detection limit of 0.001 μg/L in the surface waters adjacent to fields fertilized only with poultry litter (Arikan et al., 2008). Antimicrobial resistance genes and virulence genes in *Enterococcus* spp. have been measured in poultry, specifically broiler chickens, from British Columbia production farms (Diarra et al., 2010).

Mensink and Montforts (2007) reported on a project to search for natural and synthetic hormones in rural watersheds. Since such contaminants would originate from animal manure spread on land, they extended their target analytes to include major classes of antibiotics used in the Netherlands, including tetracyclines, sulfonamides, and some others including flumequine and tylosin. With the knowledge that sulfonamides are likely to be mobile in soil, but not persistent, and that tetracyclines appear to have different mobility characteristics, they also measured tetracycline and sulfonamide resistance genes as a kind of ecoshadow (Midedt, 2004) of the presence

of such contaminants and their carrier matrix (manure). Based on the resistance profiles, the areas where intensive animal husbandry were located (pigs and chickens) were clustered distinctly from the areas with lower intensity (dairy cows). There was also a positive correlation between the profiles of concentrations of antibiotics measured in water and sediment with the intensity of animal husbandry. Higher intensity of animal husbandry was associated with higher antibiotic residues and higher diversity in resistance genes in the nearby watershed. However, these conclusions were based on a single measurement in the spring. Follow-up research including several measurements over the course of the year showed that there was a high seasonal variation at all locations leaving no correlation with agricultural or recreational use or supposedly pristine conditions (M. Montforts and H. Schmitt, personal communication). The divergence between the sampling dates could not be related to weather conditions, indicating runoff drainage of bacteria from soil to surface water was not greatly enhanced during precipitation events.

The main objective of the experiment presented in this chapter was to assess the likelihood that transport of contaminants originating from composted poultry waste could increase the possible opportunities for de novo development of resistance in nontarget environmental bacteria. Samples were taken from a broiler production farm located in the key agricultural watershed in south western British Columbia, Canada, over the course of one year. Complementary methods of microbiological and chemical analyses were used to detect the possible effects of both tetracycline residues and tetracycline resistance genes on environmental bacteria. The investigation measured the abundance of four tetracycline resistance genes chosen from the ribosomal protection protein (RPP) supergroup (*tet*(M), *tet*(O), *tet*(Q), and *tet*(W)) using real-time quantitative polymerase chain reactions (qPCR). Antimicrobial susceptibility to oxytetracycline was measured by standard disk diffusion methods. The investigation attempted to track the presence of tetracycline residues through the composting process and field distribution of composted poultry waste by analyses using electrospray ionization–liquid chromatography–tandem mass spectrometry (ESI–LC–MS/MS).

24.2 EXPERIMENTAL APPROACH AND METHODOLOGY

The study monitored samples of poultry waste compost from one broiler production cycle that began in April 2004. Chickens were housed in an open-floor barn for 38 days and compost was stored in a dedicated concrete floor containment area until it was spread on a nearby field beginning in mid-May 2004. Litter, compost, and soil samples were collected from the medium-sized broiler production facility located in the Sumas watershed between January 24, 2004, and January 25, 2005. Following dispatch of the birds to slaughter, the compost pile was sampled weekly at the surface and inside the pile at a depth of approximately one meter. The compost was distributed on a field that was historically cultivated and fertilized (usually each year) with poultry compost, and both remaining compost and field soil were sampled once per month for one year.

The chemical analyses of poultry litter, compost, and soil samples for five selected tetracycline antibiotics (tetracycline, oxytetracycline, chlortetracycline, doxycycline, and demeclocycline) were conducted using ESI–LC–MS/MS. Detailed analytical procedures are provided in Keen (2009). Quantitative real-time PCR (qPCR) was

used to measure four tetracycline resistance genes [(*tet*(M), *tet*(O), *tet*(Q), and *tet*(W)] and 16S ribosomal ribonucleic acid (rRNA) following deoxyribonucleic acid (DNA) extraction of poultry litter, compost, and soil samples [detailed analytical procedures are provided in Keen (2009)].

Three species of pathogenic bacteria (*E. coli*, *Salmonella* spp., and *Enterococci* spp.) were isolated from the compost samples for susceptibility or resistance to oxytetracycline using the Kirby–Bauer or disk diffusion test following Avian and National Antimicrobial Resistance Monitoring System (NARMS) protocol (U.S. Department of Agriculture, 2000).

24.3 RESULTS AND DISCUSSION

Method development using ESI–LC–MS/MS over the period of 18 months failed to yield a sensitive and reliable procedure for simultaneously determining five tetracycline analytes in the poultry waste compost and soil samples. The preparation of matrix-matched standards was a particular challenge given the heterogeneity of the samples. Initially, a sample of organic poultry compost was selected for use in preparation of compost standards. This material was found to contain concentrations of oxytetracycline above 1000 μg/L before spiking with analyte compounds and thus could not be used for matching of standard matrices. Standards were prepared using material (soil collected from a forest control site) that was most similar to the compost and the soil samples in texture and particle composition. No tetracycline residues were detected in the chosen standard matrix in concentrations above detection limits before spiking.

Over the course of the investigation, refining a suitable extraction method that offered detection limits that were comparable to literature values and instrumental response that was consistent for all compost and soil samples proved to be problematic. A variety of solvent mixtures were tested for the liquid/liquid extraction in combination with several buffers, including tetraflouroacetic acid, formic acid, citrate buffer (at multiple pH values below 4), ammonium acetate buffer, phosphate buffer, and triethylamine. Although samples were consistently "dirty", solid-phase extraction (SPE) did not offer any improvement in separation of the tetracycline analytes in these samples, and thus the adjusted extraction method did not include SPE in sample preparation.

In this investigation, highly variable concentrations of five target compounds from the tetracycline family of antibiotics could be detected in compost samples. However, analyte recoveries were inconsistent, concentration measurements in compost or fertilized soil samples could not be reasonably reproduced, and, thus, no quantitative data for antibiotic residues are presented here.

For the samples evaluated in this study, moisture contents varied considerable, ranging between 4 and 52% moisture among representative samples measured. The lowest moisture contents were observed in the subsurface compost samples, and the highest moisture occurred in soil samples collected in fall or winter months. The quality of compost samples was also highly variable. High lipid (fat) fractions were observed, mostly in surface samples, and thus separation of an extract that contained only the tetracycline analytes of interest was difficult. Following the extraction procedure, several compost samples (mostly compost surface samples) appeared as

brown viscous liquids that were unsuitable for analyses by ESI–LC–MS/MS. A modified procedure of hexane-extractable lipid separation (extraction of 5 g dry weight compost samples with 10 mL hexane, filtration, evaporation, and weighing of residue) determined that lipid fractions of some compost surface samples were within the range of 1.6–3.9% w/w.

Results of the qPCR measurement of tetracycline resistance genes *tet*(M), *tet*(O), *tet* (Q), and *tet*(W) in poultry compost revealed that there was no statistically significant difference in the relative proportion of the tetracycline resistance genes, and there was no trend in the predominant gene class in the compost and soil samples over the duration of the monitoring period. The relative abundance profile of the same four tetracycline resistance genes measured in soil fertilized by poultry compost over 6 months is presented in Figure 24.1. Results of the qPCR for the same four tetracycline resistance genes in the soil control samples were different from those measured in the compost and soil collected from the farm field. The soil from samples collected from a forested mountain slope located near several large trees appeared to be more enriched than the field soil with the abundance of 16S rRNA genes measured to be 8.0×10^{10} copies/mg (average of 3 replicates). The relative abundance of the tetracycline resistance genes in the control soil was *tet*(O) (5.5×10^6 copies/mg), *tet*(Q) (7.0×10^6 copies/mg), *tet*(W) (6.2×10^6 copies/mg), and *tet*(M) (5.2×10^7 copies/mg).

The relative abundance of tetracycline resistance genes was higher (although not statistically significant) in the forested control soil than in the agricultural field soil. For the purposes of this investigation, the experiment was considered as a time-course evaluation using the tetracycline resistance gene abundance from the presprayed soil from September 2004 as the control. Samples of compost and soil after distribution of compost on the field under study were specifically selected to follow persistence of these genes under normal weathering conditions. There was no statistically significant difference between the mean gene abundance of the total of four tetracycline resistance genes in poultry compost or in soil fertilized with the same poultry compost over a period of 6 months (September 2004–January 2005).

Samples of litter and compost (prior to distribution on field soil in mid-May) were collected every 2 weeks for antimicrobial susceptibility (AMS) tests beginning in January 2004. Breakpoints used for the assessment of antimicrobial susceptibility of the three selected bacterial species to tetracyclines were defined by NARMS criteria (susceptible if < 4 μg/mL; resistant if ≥ 16 μg/mL) (U.S. Department of Agriculture, 2000).

Antimicrobial susceptibility testing of isolates of the three species of indicator bacteria revealed that all of the samples that were successfully cultured from the compost were resistant to oxytetracycline and tetracycline (as defined by NARMS criteria for susceptibility/resistance). Other researchers have used the same AMS method to establish positive correlation between the incidence of tetracycline resistance in *Salmonella* spp., *Campylobacter* spp., and *E. coli* isolated from dairy calves and feeding of oxytetracycline-medicated milk replacer (Kaneene et al., 2008). Bunner et al. (2007) conducted a study to compare the antimicrobial susceptibility of *E. coli* isolated from fecal samples from pigs raised in conventional farms and those reared in antibiotic-free conditions. They reported that tetracycline was among the antibiotics found in the three most frequent patterns of multiple resistances in the *E. coli* from pig fecal samples (streptomycin–tetracycline, sulfamethoxazole–tetracycline, and kanamycin–streptomycin–sulfamethoxazole–tetracycline). Results of the

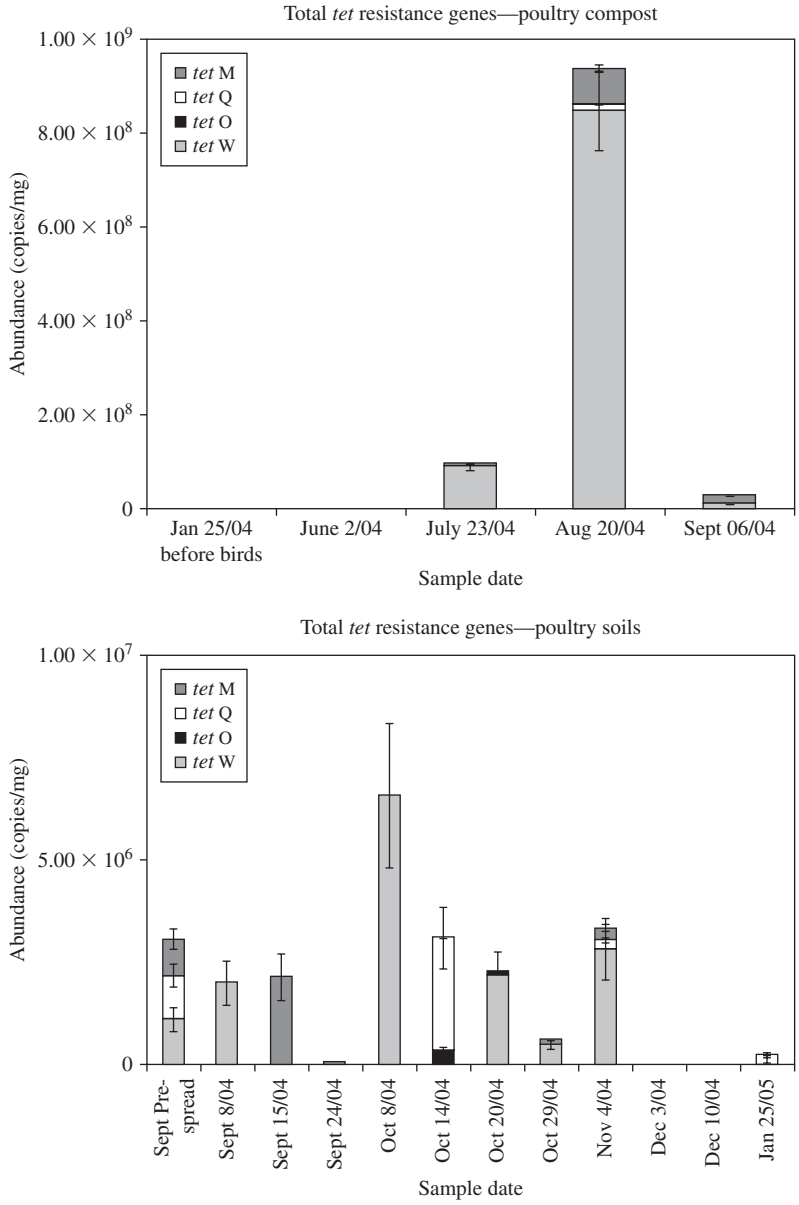

FIGURE 24.1 Relative abundance of tetracycline resistance genes in poultry litter and compost (top) and soil (below)–sampled between January and September 2004 (average values; $n = 3$); error bars represent standard deviation.

present study confirm that tetracycline resistance is often found in some species of pathogenic bacteria found in poultry, cattle, and swine waste. Although the selected bacteria species appeared to die off in the composting process, the abundance of four tetracycline resistance genes that were measured in compost and the abundance measured in field soil prior to fertilization were similar.

24.4 LESSONS LEARNED

Within the Sumas watershed in British Columbia over the past decade, the growth of the poultry (as well as cattle and swine) population within the region is a striking example of intensified agricultural production. Since 1996, data from the Canadian agricultural census reveals that the total population of chickens on census day (broilers, breeding stock, and laying hens) has grown from 872,075 to 8,431,946 (Statistics Canada, 1996, 2001, 2006). The 2006 poultry population was based on a total of 244 farms reporting. Figure 24.2 illustrates the relative increase in poultry (chickens only, excluding turkeys and specialty bird species), swine, and cattle within the Sumas watershed between 1996 and 2001.

The increase in poultry population within the boundaries of an agricultural watershed is accompanied by considerable increase in the volume of manure requiring disposal. The average weekly production of chicken manure using an open-floor housing system ranges between 0.27 kg/week (for layer pullets) and 1.0 kg/week (for broiler breeder) per chicken (BC Ministry of Agriculture Food and Fisheries, 1992). While land application of poultry manure is a valuable soil conditioner, the amount of composted manure that can be distributed on cropland as fertilizer is limited. Agronomic and environmental conditions dictate the acceptable times of the year during which manure may be spread on cropland (usually until mid-October in the south coast of British Columbia), and, thus, compost may be required to remain in storage until appropriate seasonal conditions allow land application of manure. As manure can be a major pollution source and contributor to surface and groundwater contamination, land application of composted manure must be managed such that crop production can be optimized without any accompanying hazard to the receiving environment.

Analyses of compost samples confirmed high variation in compost quality between surface and subsurface samples, most notably in the presence of lipids. In the province of British Columbia, composting of dead birds and broken eggs is an acceptable practice provided that certain guidelines are observed (BC Ministry of

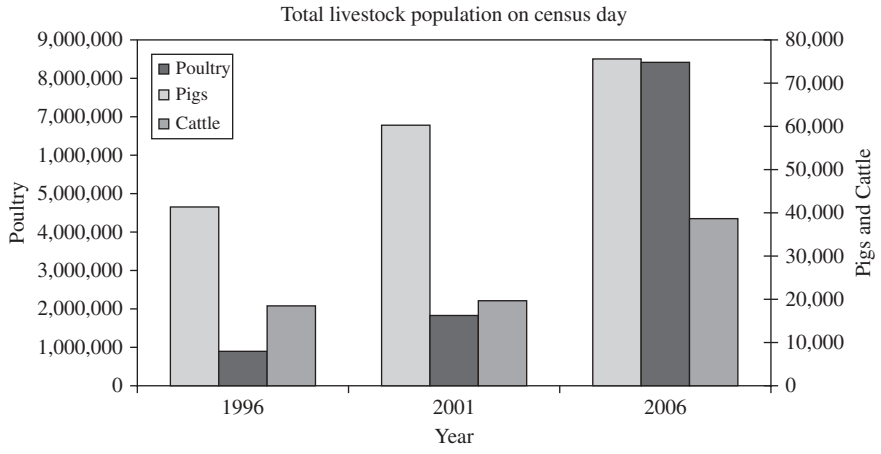

FIGURE 24.2 Livestock in the Sumas Watershed (Statistics Canada, 1996, 2001, 2006).

Agriculture Food and Fisheries, 1992). For this particular broiler cycle, normal mortality rates at the poultry farm were reported. Usually, antibiotics are prescribed by a veterinarian when birds display symptoms of illness and the treatment dose is administered to the flock via their water system. During the period of evaluation, birds did not require medical treatment with antibiotics. In this study, the presence of variable lipid fractions in compost samples compromised the extraction process and affected the chemical determination by ESI–LC–MS/MS. It is possible that the variability of lipid content, especially in samples from the surface of the compost pile, reflected the presence of undigested fats, spilled feed, or some poultry carcasses for which the decomposition process was incomplete.

Conditions of the poultry compost pile under investigation in the present study were favorable for the degradation of tetracycline residues by heat and light. Subsurface temperatures of the compost pile were consistently about 40°C (40.6°C $\pm$ 3.2) and its location allowed exposure to normal daylight cycles. Results of chemical analyses of these compost and soil samples, alone, could not determine whether tetracycline residues contribute to contamination of runoff from fertilized fields. These findings reinforce the need for further research in chemical analyses of tetracycline residues, especially in complex organic matrices, in order to better understand the degradation and transport mechanisms of these compounds in the terrestrial and aquatic environments.

The review by O'Connor and Aga (2007) summarized several of the complications that make chemical analyses of tetracyclines in manure and soil samples particularly challenging. These authors highlighted the wide variability of conditions that affect tetracycline measurement, ranging from field conditions (soil type, drainage, and the amount of mixing that occurs after manure application to soil) to physical chemical processes of extraction (interference of co-extracted organic matter, effects of surface tension, and viscosity). The analytical recoveries for tetracycline residues from soil or manure determined by several mass spectrometry techniques appear to be highly variable and sometimes as low as 30% (O'Connor and Aga, 2007). An earlier review (Anderson et al., 2005) of analytical techniques for measuring tetracycline in various (mostly food-related) sample matrices described many of the aforementioned analytical complications. The results of the present study have demonstrated that some of the challenges previously reported by other research teams have yet to be resolved.

Duration of composting and compost conditions have important consequences on the degradation of antibiotic residues and the potential spread of antibiotic resistance genes through agricultural environments. The tetracycline family of antibiotics is well known to be sensitive to both heat (Loftin et al., 2008) and light, thus temperature within a compost pile and exposure to daylight will affect the persistence of these compounds. When executed properly, the composting process undergoes three phases: mesophilic phase (temperatures between 20 and 40°C), thermophilic phase of intense microbial activity (usually above 40°C but below 60–80°C), and the cooler-temperature-curing phase during which slower processes of decomposition take place (Tiquia and Tam, 2000; Cooke et al., 2001; Wang et al., 2007; Petric and Selimbasic, 2008). Arikan et al. (2007) determined the half-life of oxytetracycline in composted cattle manure/straw/woodchips to be approximately 3.2 days. Other researchers have demonstrated that concentrations of oxytetracycline in manure-amended surface soil declined to below 50% of the initial concentration (270 μg/kg) applied to

experimental land plots after 3 weeks (Aga et al., 2005). Dolliver and Gupta (2008a) demonstrated the effect of seasonal precipitation and land tillage on concentrations of antibiotic residues in runoff from fertilized fields. These investigators concluded that antimicrobial concentrations (chlortetracycline, monensin, and tylosin) were higher in runoff from land that was not tilled during nongrowing periods of higher precipitation. Stoob et al. (2007) reported that sorption of antibiotics (sulfonamides in this example) and surface flow, as a result of weather conditions, influenced the dissipation and transport of antibiotic residues. Studies of Dolliver and Gupta (2008b) also found that antimicrobials (specifically chlortetracycline, monensin, and tylosin) measured in runoff leachate from compost piles were positively correlated to the initial concentration of antibiotics in the manure. Competing physical and chemical processes influence the concentrations of antibiotic residues found in soil and runoff samples.

Analyses of four specific tetracycline resistance genes in compost by qPCR determined relative abundances ranging between 1.35×10^4 copies/mg (June 2, 2004) and 9.38×10^8 copies/mg (August 2000/2004). The relative abundance of *tet* (M), *tet*(O), *tet*(Q), and *tet*(W) in soil varied among samples but appeared to remain constant over time after application of compost onto the field. The abundance of the tetracycline resistance genes neither declined nor increased over the monitoring period. The total abundance of the tetracycline resistance genes for the barn litter prior to the introduction of the birds was 4 orders of magnitude lower than that observed for the compost or soil samples after the broiler production cycle (Fig. 24.1). Of the total tetracycline resistance gene abundance for the litter sample, comparatively higher abundance of *tet*(M) was observed than for any of the other samples measured. In contrast with results reported by Patterson et al. (2007) in which no ribosomal protection protein genes in agricultural soils from several European countries could be detected by microarrays using PCR amplicons, RPP tetracycline resistance genes were observed in soil fertilized by poultry compost in this investigation.

The abundance of the total of four tetracycline resistance genes and 16S rRNA genes as an indication of the total bacterial biomass is shown in Figure 24.3. During the period of July–September, when temperature conditions would favor periods of intense microbial activity, the highest copy numbers of *tet* resistance genes were observed in the compost samples but 16S rRNA gene measurements indicate that higher numbers of total bacterial species were also present in the compost. These results and the results of the antimicrobial susceptibility tests suggest that the higher relative proportion of tetracycline-resistant organisms occur in the bacteria of poultry manure.

Oxytetracycline is a naturally occurring antibiotic produced by *Streptomyces rimosus* (Chopra and Roberts, 2001), and thus it is difficult to determine the background profile of tetracycline resistance genes in soil bacteria from diverse locations. The relative abundance of tetracycline resistance genes in compost or soil is influenced by the resident bacteria communities and the composition of gut microflora of the animals from which the compost manure is derived. As comprehensively described in previous chapters (Chapters 3, 4, and 9), soil bacteria act as a reservoir for a diverse range of genetic elements associated with the development of antibiotic resistance. Every soil ecosystem is unique. Soil microbial communities are constantly exposed to antibiotics as a result of natural production and the result of

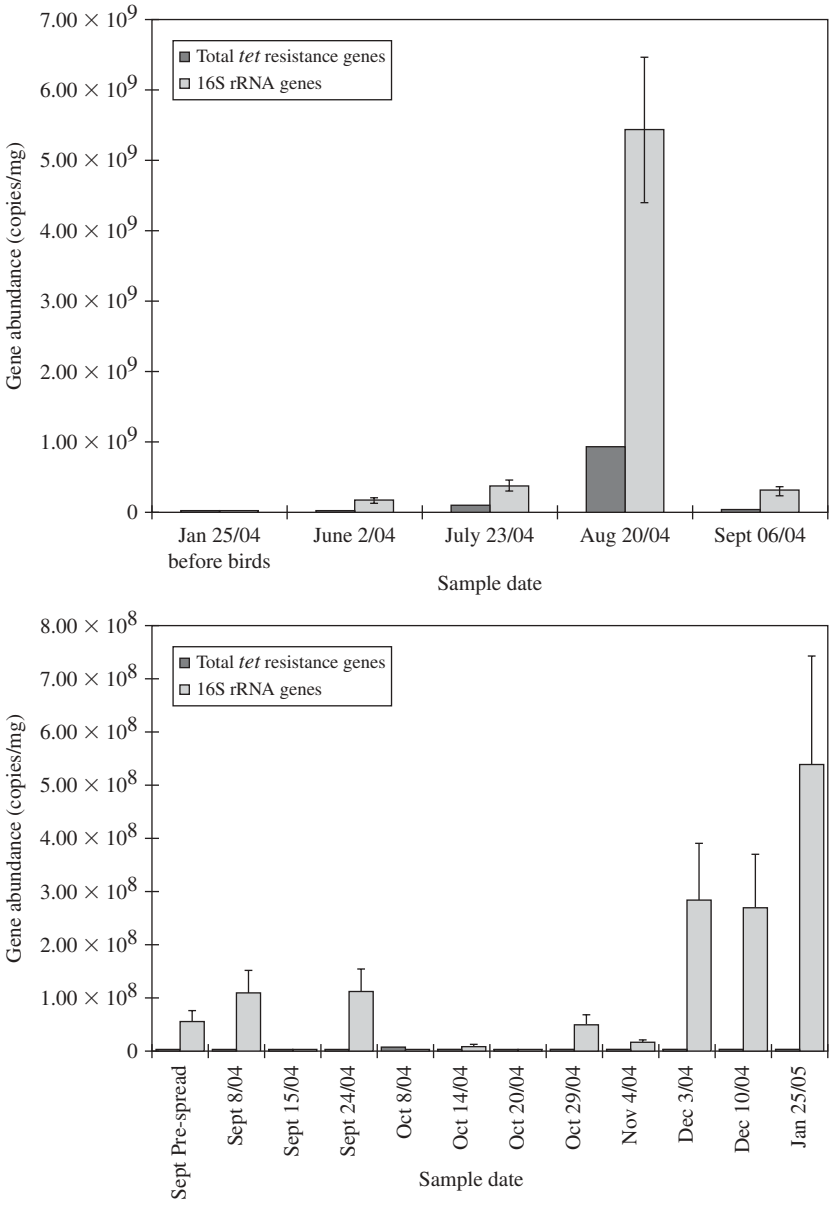

FIGURE 24.3 Total tetracycline resistance and 16S rRNA genes measured in poultry litter and compost (top) and soil (below). Values presented are the average of 3 measurements per sample extract; error bars represent standard deviation.

human activities. However, direct interaction between large quantities of animal manure compost and soil is likely to affect ever-changing ecological processes that govern antibiotic resistance in soil bacteria in unpredictable ways.

Selecting an appropriate control site to compare relative abundance of tetracycline resistance genes in soil bacteria is difficult given the vast diversity in community

structures among various locations. Kobashi et al. (2007) reported that a forest soil reference site was reasonable for use in their evaluation of tetracycline resistance genes in agricultural soil and various animal manures since no tetracycline resistance genes were found in 6 isolates from the forest control soil. Bacteria communities are likely to be different in forested soils near tree roots and in soils collected from flat agricultural fields. Analyses of the control soil used in the present investigation confirmed this and revealed a rich bacterial population with a relative abundance of the four measured tetracycline resistance genes dominated by *tet*(M). In contrast to the findings of Kobashi et al. (2007), soil from the forested site of this investigation could not be used as an appropriate control from which to compare the background tetracycline resistance gene abundance.

Chee-Sanford et al. (2001) confirmed the presence of eight classes of RPP tetracycline resistance genes in lagoons and groundwater under swine production facilities. Smith et al. (2004) observed significantly higher concentrations of *tet*(W) and *tet*(O) genes downstream of feedlot lagoons. Recent studies of sediment from the Mekong River have detected *tet*(W), *tet*(S), and *tet*(O) in most of the samples analyzed (Kobayashi et al., 2007). Exposure to light has been implicated as accelerating the decay rate of tetracycline resistance genes (Engemann et al., 2006). In this experiment, the measured relative abundance of the four common tetracycline resistance genes in soil after application of the poultry compost may reflect conditions achieved after input, transport in surface runoff, and degradation processes reach equilibrium.

Soil column experiments have demonstrated that amplification and attenuation of tetracycline resistance in soil bacteria is influenced by exposure to low concentrations of tetracycline (Rysz and Alvarez, 2004). Experiments by Poté et al. (2003) suggest that there is potential for resistance genes to be transported over long distances in water-saturated soil or groundwater, and this is an important consideration given that the potential for effects on soil bacteria communities and the potential for interference in essential soil functions has already been demonstrated (Schmitt et al., 2006).

Antimicrobial susceptibility testing of isolates of the three species of indicator bacteria revealed that all of the samples that were successfully cultured from the compost were resistant to tetracyclines (as defined by NARMS criteria for susceptibility/resistance). Other researchers have used the same AMS method to establish positive correlation between the incidence of tetracycline resistance in some bacteria and the presence of oxytetracycline in feed (Kaneene et al., 2008; Bunner et al., 2007). Results of the present study confirm that tetracycline resistance genes, often found in common species of indicator bacteria found in poultry, cattle, and swine waste, are likely present in the agricultural watershed that was studied.

Although the selected bacteria species appeared to die off in the composting process, the abundance of four tetracycline resistance genes that were measured in compost and the abundance measured in field soil prior to fertilization were similar. Previous research has suggested that composting of poultry waste at optimal temperatures can limit the opportunity for transport of plasmids and other mobile genetic elements of antimicrobial resistance (Guan et al., 2007). It is possible that the poultry compost system of the current study effectively degraded antibiotic residues and the target tetracycline resistance genes such that they could not be distinguished from natural soil background composition.

This investigation suggests that the bacteria represented by abundance of 16S rRNA genes and four selected tetracycline resistance genes measured on soil fertilized

by poultry compost remains relatively constant throughout the fall–winter season. Measurements of turbidity in the Sumas River increased with more frequent rainfall events in the fall–winter season indicating that soil erosion is likely to be occurring as soil becomes saturated and overland run-off contributes to stream flow (Keen, 2009). For this reason, contribution of tetracycline resistance genes associated with soil bacteria should be considered among the possible contaminants in stream waters and groundwater, although specific source tracking is extremely difficult.

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REFERENCES

Aga DS, O'Connor S, Ensley S, Payero JO, Snow D, Tarkalson D (2005). Determination of the persistence of tetracycline antibiotics and their degradates in manure-amended soil using enzyme-linked immunosorbent assay and liquid chromatography-mass spectrometry. *J Agric Food Chem* 53:7165–7171.

Alonso A, Sanchez P, Martinez JL (2001). Environmental selection of resistance genes. *Environ Microbiol* 3:1–9.

Anderson CR, Rupp HS, Wu W-H (2005). Complexities in tetracycline analysis—Chemistry, matrix extraction, clean-up, and liquid chromatography. *J Chromatog A* 1075:23--32.

Arikan OA, Sikora LJ, Mulbry W, Khan SU, Foster GD (2007). Composting rapidly reduces levels of extractable oxytetracycline in manure from therapeutically treated beef calves. *Biores Tech* 98:169–176.

Arikan OA, Rice C, Cotling E (2008). Occurrence of antibiotics and hormones in a major agricultural watershed. *Desalination* 226:121–133.

Baguer AJ, Jensen J, Krogh PH (2000). Effects of the antibiotics oxytetracycline and tylosin on soil fauna. *Chemosphere* 40:751–757.

Baker-Austin C, Wright MS, Stepanauskas R, McArthur JV (2006). Co-selection of antibiotic and metal resistance. *Trends Microbiol* 14(4):176–182.

Boxall AB, Fogg LA, Blackwell PA, Kay P, Pemberton EJ, Croxford A (2004). Veterinary medicines in the environment. *Rev Environ Contam Toxicol* 180:1–91.

Boxall ABA, Johnson P, Smith EJ, Sinclair CJ, Stutt E, Levy LS (2006). Uptake of veterinary medicines from soils into plants. *J Agric Food Chem* 54:2288–2297.

British Columbia Ministry of Agriculture Fisheries and Food (1992). *Environmental Guidelines for Poultry Producers*. BC MAFF Soils and Engineering Branch Publishers, Abbotsford, British Columbia.

Brooks JP, McLaughlin MR, Scheffler B, Miles DM (2010). Microbial and antibiotic resistant constituents associated with biological aerosols and poultry litter within a commercial poultry house. *Sci Total Environ* 408:4770–4777.

Bunner CA, Norby B, Bartlett PC, Erskine RJ, Downes FP, Kaneene JB (2007). Prevalence and pattern of antimicrobial susceptibility in *Escherichia coli* isolated from pigs reared under antimicrobial-free and conventional production methods. *J Am Vet Assoc* 231:275–283.

Burkholder J, Libra B, Weyer P, Heathcote S, Kolpin D, Thorne PS, Wichman M (2007). Impacts of waste from concentrated animal feeding operations on water quality. *Environ Health Perspec* 115:308–312.

Canadian Food Inspection Agency (2008). Compendium of medicating feed ingredients brochure. Available: http://www.inspection.gc.ca/english/anima/feebet/mib/cmibe.shtml. Accessed February 16, 2011.

Chee-Sanford JC, Aminov RI, Krapac IJ, Garrigues-Jeanjean N, Mackie RI (2001). Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Appl Environ Microbiol* 67:1494–1502.

Chopra I, Roberts M (2001). Tetracycline antibiotics: Mode of action, applications, molecular biology and epidemiology of bacterial resistance. *Microbiol Mol Bio Rev* 65:232–260.

Cinquina AL, Longo F, Anastasi G, Gianetti L, Cozzani R (2003). Validation of a high-performance liquid chromatography method for the determination of oxytetracycline, tetracycline, chlortetracycline and doxycyline in bovine milk and muscle. *J ChromatogNA* 987:227–233.

Cooke CM, Grove L, Nicholson FA, Cook HF, Beck AJ (2001). Effect of drying and composting biosolids on the movement of nitrate and phosphate through repacked soil columns under steady-state hydrological conditions. *Chemosphere* 44:797–804.

Coyne R, Hiney M, Smith P (1997). Transient presence of oxytetracycline in blue mussels *(Mytilus edulis)* following its therapeutic use at a marine Atlantic fish farm. *Aquaculture* 149:175–181.

Dantas G, Sommer MO, Oluwasegun RD, Church GM (2008). Bacteria subsisting on antibiotics. *Science* 320:100–103.

Diarra MS, Rempel H, Champagne J, Masson L, Pritchard J, Topp E (2010). Distribution of antimicrobial resistance and virulence genes in *Enterococcus* spp. and characterization of isolates from broiler chickens. *Appl Environ Microbiol* 76(24):8033–8043.

Dolliver H, Gupta S (2008a). Antibiotic losses in leaching and surface run-off from manureamended agricultural land. *J Environ Qual* 37:1227–1237.

Dolliver HAS, Gupta SC (2008b). Antibiotic losses from unprotected manure stockpiles. *J Environ Qual* 37:1238–1244.

Elmund GK, Morrison SM, Grant DW, Nevens MP (1971). Role of excreted chlortetracycline in modifying the decomposition process of feedlot waste. *Bull Environ Contam Toxicol* 6:129–132.

Engemann CA, Adams L, Knapp CW, Graham DW (2006). Disappearance of oxytetracycline resistance genes in aquatic systems. *FEMS Microbiol Lett* 263:176–182.

Grayson ML, Eliopoulos GM, Wennersten CB, Ruoff KL, DeGirolami PC Ferraro M-J, Moellering RC (1991). Increasing resistance to β-lactam antibiotics among clinical isolates of *Enterococcus faecium*: A 22-year review at one institution. *Antimicrob Agents Chemother* 35(11):2180–2184.

Guan J, Wasty A, Grenier C, Chan M (2007). Influence of temperature on survival and conjugative transfer of multiple antibiotic-resistant plasmids in chicken manure and compost microcosms. *Poultry Sci* 86:610–613.

Jacobsen AM, Halling-Sørensen B, Ingerslev F, Hansen SH (2004). Simultaneous extraction of tetracycline, macrolide and sulfonamide antibiotics from agricultural soils using pressurised

liquid extraction, followed by solid-phase extraction and liquid chromatography–tandem mass spectrometry. *J ChromatogNA* 1038:157–170.

Jjemba PK (2002). The potential impact of veterinary and human therapeutic agents in manure and biosolids on plants grown on arable land: A review. *Agric Ecosyst Environ* 93:267–278.

Kaneene JB, Warnick LD, Bolin CA, Erskine RJ, May K, Miller R (2008). Changes in tetracycline susceptibility of enteric bacteria following switching to nonmedicated milk replacer for dairy calves. *J Clin Microbiol* 46:1968–1977.

Keen PL (2009). Seasonal dynamics of tetracycline resistance genes and antibiotics in a British Columbia agricultural watershed. PhD thesis. University of British Columbia, Vancouver, Canada.

Knapp CW, Dolfing J, Ehlert PA, Graham DW (2010). Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. *Environ Sci Technol* 44:580–587.

Kobashi Y, Hasebe A, Nishio M (2005). Antibiotic-resistant bacteria from feces of livestock, farmyard manure and farmland in Japan—Case report. *Microbes Environ* 20:53--60.

Kobashi Y, Hasebe A, Nishio M, Uchiyama H (2007). Diversity of tetracycline resistance genes in bacteria isolated from various agricultural environments. *Microbes Environ* 22:44–51.

Kobayashi T, Suehiro F, Cach Tuyen B, Suzuki S (2007). Distribution and diversity of tetracycline resistance genes encoding for ribosomal protection proteins in Mekong River sediments in Vietnam. *FEMS Microbiol Ecol* 59:729–737.

Kong WD, Zhu YG, Liang YC, Zhang J, Smith FA, Yang M (2007). Uptake of oxytetracycline and its phytotoxicity to alfalfa (*Medicago sativa* L.). *Environ Poll* 147:187–193.

Kumar K, Gupta SC, Baidoo SK, Chander Y, Rosen CJ (2005). Antibiotic uptake by plants from soil fertilized with animal manure. *J Environ Qual* 34:2082–2085.

Levy SB (1998). The challenge of antibiotic resistance. *Sci Am* 278:46.

Levy SB, Fitzgerald GB, Macone AB (1976). Spread of antibiotic resistance plasmids from chicken to chicken and from chicken to man. *Nature* 260:40–42.

Loftin KA, Adams CD, Meyer MT, Surampalli R (2008). Effects of ionic strength, temperature, and pH on degradation of selected antibiotics. *J Environ Qual* 37:378–386.

Loke M-L, Tjørnelund JJ, Halling-Sørensen B (2002). Determination of the distribution coefficient (log Kd) of oxytetracycline, tylosin A, olaquindox and metronidazole in manure. *Chemosphere* 48:351–361.

Loke M-L, Jespersen S, Vreeken R, Halling-Sørensen B, Tjørnelund J (2003). Determination of oxytetracycline and its degradation products by high-performance liquid chromatography –tandem mass spectrometry in manure-containing test systems. *J ChromatogNB* 783:11–23.

Mensink BJWG, Montforts MHMM (2007). The ecological risks of antibiotic resistance in aquatic environments: A literature review. RIVM report 601500005/2007. RIVM Bilthoven, the Netherlands.

Meredith WE, Weiser HH, Winter AR (1965). Chlortetracycline and oxytetracycline residues in poultry tissues and eggs. *Appl Microbiol* 13:86–88.

Midtvedt T (2004). The ECO-SHADOW concept—A new way of following environmental impacts of antimicrobials. In K Kümmerer (Ed.), *Pharmaceuticals in the Environment: Sources, Fate, Effects, and Risks*, 2nd ed. Springer, Berlin, pp. 311--316.

Migliore L, Cozzolino S, Fiori M (2003). Phytotoxicity and uptake of enrofloxacin in crop plants. *Chemosphere* 52:1233–1244.

Mølbak K (2004). Spread of resistant bacteria and resistance genes from animals to humans—The public health consequences. *J Vet Med* 51:364--369.

Nemati M, Hermans K, Lipinska U, Denis O, Deplano A, Struelens M, Devriese LA, Pasmans F, Haesebrouck F (2008). Antimicrobial resistance of old and recent *Staphylococcus aureus*

isolates from poultry: First detection of livestock-associated methicillin-resistant strain ST398. *Antimicrob Agents Chemother* 52(10):3817–3819.

O'Connor S, Aga DS (2007). Analysis of tetracycline antibiotics is soil: Advances in extraction, clean-up & quantification. *Trends Anal Chem* 26:456–465.

Patterson AJ, Colangeli R, Spigaglia P, Scott KP (2007). Distribution of specific tetracycline and erythromycin resistance genes in environmental samples assessed by macroarray detection. *Environ Microbiol* 9:703–715.

Petric I, Selimbasic V (2008). Composting of poultry manure and wheat straw in a closed reactor: Optimum ratio and evolution of parameters. *Biodegradation* 19:53–63.

Poté J, Ceccherini MT, Van VT, Rosselli W, Wildi W, Simonet P, Vogel TM (2003). Fate and transport of antibiotic resistance genes in soil columns. *Eur J Soil Biol* 39:65–71.

Rabølle M, Spliid N (2000). Sorption and mobility of metronidazole, olaquindox, oxytetracycline and tylosin in soils. *Chemosphere* 40:715–722.

Rasmussen LD, Sorensen SJ (1998). The effect of longterm exposure to mercury on the bacterial community in marine sediment. *Curr Microbiol* 36:291–297.

Riesenfeld CS, Goodman RM, Handlesman J (2004). Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. *Environ Microbiol* 6:981–989.

Rysz, M, Alvarez PJJ (2004). Amplification and attenuation of tetracycline resistance in soil bacteria: Aquifer column experiments. *Wat Res* 38:3705–3712.

Schmitt H, Stoob K, Hamscher G, Smit E, Seinen W (2006). Tetracyclines and tetracycline resistance in agricultural soils: Microcosms and field studies. *Microbol Ecol* 51:267–276.

Silver S, Phung LT (1996). Bacterial heavy metal resistance: New surprises. *Ann Rev Microbiol* 50:753–789.

Singer RS, Hofacre CL (2006). Potential impacts of antibiotic use in poultry production. *Avian Dis* 50:161–172.

Smith MS, Yang RK, Knapp CW, Niu Y, Peak NM, Hanfelt MM, Galland, Graham DW (2004). Quantification of tetracycline resistance genes in feedlot lagoon using real-time PCR. *Appl Environ Microbiol* 70:7372–7377.

Statistics Canada (1996). Agricultural census data for 1996. Summary of 1996 national census. Ottawa, ON. Accessed May 26, 2005. Available: http://www40.statcan.ca/z01/cs0003_e.htm.

Statistics Canada (2001). Agricultural census data for 2001. Summary of 2001 national census. Ottawa, ON. Accessed May 26, 2005. Available: http://www40.statcan.ca/z01/cs0003_e.htm.

Statistics Canada (2006). Agricultural census data for 2006. Summary of 2006 national census. Ottawa, ON. Accessed September 22, 2007. Available: http://www40.statcan.ca/z01/cs0003_e.htm.

Stoob K, Singer HP, Mueller SR, Schwarzenbach RP, Stamm CH (2007). Dissipation and transport of veterinary sulfonamide antibiotics after manure application to grassland in a small catchment. *Environ Sci Technol* 41:7349–7355.

Tiquia SM, Tam NFY (2000). Fate of nitrogen during composting of chicken litter. *Environ Poll* 110:535–541.

U.S. Department of Agriculture (2000). National Antimicrobial Resistance Monitoring System Veterinary Isolates (NARMS). Available: http://www.ars.usda.gov/Main/docs.htm?docid=6750&page=3. Accessed March 18, 2008.

Wan G-H, Cui H, Zheng H-S, Zhou J, Liu L-J, Yu Y-F (2005). Determination of tetracycline residues in honey using high-performance liquid chromatography with potassium permanganate–sodium sulfite–β-cyclodextrin chemiluminescence detection. *J Chromatog, B* 824:57–64.

Wang C-M, Shyu C-L, Ho S-P, Chiou S-H (2007). Species diversity and substrate utilization patterns of thermophilic bacterial communities in hot aerobic poultry and cattle manure composts. *Microbiol Ecol* 54:1–9.

Wang Y-J, Jia D-A, Zhu H-W, Zhou D-M (2008). Adsorption and cosorption of tetracycline and copper (II) on montmorillonite as affected by soil pH. *Environ Sci Technol* 42:3254–3259.

Webb KE, Fontenot JP (1975). Medicinal drug residues in broiler litter and tissue from cattle fed litter. *J Anim Sci* 41(4):1212–1217.

Wright GD (2007). The antibiotic resistome: The nexus of chemical and genetic diversity. *Nat Rev Microbiol* 5:175–186.

Yang S, Cha J, Carlson K (2005). Simultaneous extraction and analysis of 11 tetracycline and sulfonamide antibiotics in influent and effluent domestic wastewater by solid-phase extraction and liquid chromatography-electrospray ionization tandem mass spectrometry. *J Chromatogr A* 1097:40–53.

Yu Z, Michel FC, Hansen G, Wittum T, Morrison M (2005). Development and application of real-time PCR assays for quantification of genes encoding tetracycline resistance. *J Appl Environ Microbiol* 71:6926–6933.

25

ENVIRONMENTAL MICROBIAL COMMUNITIES LIVING UNDER VERY HIGH ANTIBIOTIC SELECTION PRESSURE

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25.1 INTRODUCTION

There is a growing concern within the health care sector, academia, policymakers, and the general public about the risk of antibiotic resistance arising from environmental contamination of anthropogenic antibiotics (Lubick, 2009; Mason, 2009; Swedish Medical Product Agency, 2009). A number of studies have documented environmental pollution approaching or even exceeding therapeutic levels (Babic et al., 2007; Fick et al., 2009; Holm et al., 1995; Larsson et al., 2007; Li et al., 2008a), underlining the importance of studying the consequences of this strong selection force in microbial communities in highly contaminated milieus. It is important to investigate whether such pollution poses risks to humans, animals, and the environment itself, the nature of these risks, and how these risks may be predicted and prevented. The most urgent risk is the development of new resistance mechanisms, either in environmentally occurring pathogens or opportunists such as *Vibrio cholerae* or *Pseudomonas aeruginosa* or in a context that allows resistance genes to spread horizontally to human or animal pathogens. Furthermore, risks for diminished ecosystem services or decreased biodiversity are also evident.

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The difficulty in predicting the risks are compounded by our limited knowledge about the natural role of antibiotics in microbial communities. The vast majority of the antibiotics for human use originate from natural microorganisms, for example, fungi or streptomycetes, even though the molecular structure of most antibiotics in clinical use today are modified to improve their pharmacodynamic and pharmacokinetic properties. It is usually surmised that antibiotics are produced in these natural communities to give their host strain a competitive advantage by selectively killing or preventing growth of competitors and that resistance factors evolved as a defense mechanism in sensitive species. Recent research, however, indicates that some compounds have complex subinhibitory, or hormetic, effects as well (Brazas and Hancock, 2005; Davies et al., 2006; Goh et al., 2002; Yim et al., 2006), showing that their role in natural communities may be more complex than is usually surmised. Therefore, it seems possible that also low levels of anthropogenic antibiotic substances in the environment may have complex effects. It is currently not clear whether low environmental levels of antibiotics adversely affect human or animal health or ecosystem services. The consequences of higher environmental levels of antibiotics have been less explored and the risks may be substantially higher.

In this chapter we will focus on the possible effects and consequences of very high levels of antibiotics in the environment, meaning levels close to or exceeding the therapeutic level. We will summarize some of the studies that have reported antibiotic contamination of the environment and discuss the various sources followed by a presentation of two highly pertinent case studies of extreme levels of antibiotic pollution.

25.2 SOURCES AND FATE OF ANTIBIOTICS IN THE ENVIRONMENT

As indicated above, many of the antibiotics in current use have been developed from naturally produced molecules of bacterial or fungal origin. It is likely that endogenous antibiotics are ubiquitous in natural ecosystems at very low levels (Kümmerer, 2009a). However, a number of studies over the past decades have reported that antibiotics from anthropogenic sources also occur in very diverse environments, both aqueous and terrestrial, including waste, surface, drinking, and groundwater and diverse soil types and sediments.

The sources of these contaminating antibiotics can be divided into two broad categories, either urban or agricultural waste (Kümmerer, 2009a; Segura et al., 2009). Agricultural waste comes mainly from three different activities, growth promotion, veterinary medicine (including aquaculture), and agriculture. Commonly, this waste ultimately ends up either in the aquatic or the terrestrial environment. Antibiotics have been used to treat animal infections nearly as long as for human medical purposes, and they have been used extensively for growth promotion since the 1960s. The rationale for their use in growth promotion is that subtherapeutic concentrations of antibiotics may change the gastrointestinal community structure, resulting in an altered nutrient uptake and metabolism and, in turn, an increased growth rate. Even though the amounts used for growth promotion are always kept at subinhibitory levels in vivo, the contribution to the total antibiotic consumption and consequently expected emissions into the environment remain significant. As in human therapy, much of the antibiotics consumed by animals are excreted. Animal waste is handled differently from human, at least in intensive, industrial agriculture operations, where the majority of agricultural antibiotics are thought to be used. In concentrated

animal feeding operations (CAFO) or other large-scale farms, several hundreds to thousands of animals are housed together, and their waste is typically collected in cesspits before discharge into the environment. These cesspits have been reported to harbor very high concentrations of antibiotics (Campagnolo et al., 2002). Other agricultural uses of antibiotics include spraying fruit orchards with streptomycin or oxytetracyclin to prevent fire blight (McManus et al., 2002). In aquaculture, several antibiotics are used either incorporated into the feed or added directly to the water to prevent and/or treat bacterial infections. In the case of fruit orchards, the resulting concentrations in the soil are not known, but environmental contamination from aquacultural use of antibotics has resulted in very high concentrations, especially in sediments underneath cages with intensively reared fish (Björklund et al., 1991; Coyne et al., 1994; Jacobsen and Berglind, 1988).

Urban waste can be divided into municipal sewage, hospital sewage, and pharmaceutical production waste. In the Western world, these sources are, in most cases, ultimately handled by wastewater treatment plants (WWTPs) before release into the environment, although direct release of raw sewage into the aquatic environment is still common in developing countries. A significant fraction of the anthropogenic antibiotics that are used for human medicine, therefore, ultimately ends up in the aquatic environment. Human sewage may contain substantial amounts of antibiotics (Kümmerer, 2009a; Segura et al., 2009); for several antibiotics more than 70% is not metabolized (Jjemba, 2006) and is thus excreted in its active form. Several studies have documented the levels of antibiotics at several points along the sewage transport system, including hospital effluents (Diwan et al., 2010; Duong et al., 2008; Jarnheimer et al., 2004; Karthikeyan and Meyer, 2006; Martins et al., 2008) and WWTP influents and effluents (Batt et al., 2007; Christian et al., 2003; Gulkowska et al., 2008; Karthikeyan and Meyer, 2006). These studies have shown that before treatment the WWTP levels are typically highest in hospital effluents, whereas household and other everyday sewage contain lower levels. Their larger volume also means that they carry a higher total mass of antibiotics, which is in line with findings that the majority of human antibiotics consumption occurs outside hospitals (de With et al., 2004; Wise, 2002). Levels in treated sewage effluents are typically in the nanograms per liter range, although untreated hospital effluents have reached the micrograms per liter range. In the sludge from WWTPs, high concentrations are frequently encountered, but the bioavailability of these antibiotics is less clear.

By far the highest concentrations reported have been associated with pharmaceutical production, both directly in the effluent from the WWTPs handling production wastewater as well as downstream surface waters, with levels reaching into the milligrams per liter range for several compounds. A recent study from Croatia (Babic et al., 2007) reported concentrations ranging from 1.5 μg/L to 1.5 mg/L of enrofloxacine, oxytetracycline, trimethoprim, penicillin G, and three different sulfonamides in treated effluent from an unnamed pharmaceutical production plant. This study did not apply conclusive analytical methodology; however, given that the effluent investigated came from production of antibiotics, the high levels reported are not unlikely. In treated wastewater from a production plant in China, Li and co-workers measured up to 19.5 mg/L oxytetracycline (Li et al., 2008a). In two recent studies from India, Larsson et al. (2007) and Fick et al. (2009) found up to 31 and 14 mg/L ciprofloxacin, respectively, in the effluent from a WWTP that treats wastewater from a large number of production plants. In the receiving river, levels of up to 6.5 mg/L ciprofloxacin were measured (Fick et al., 2009). The study by Holm

et al. (1995) demonstrated groundwater contamination of sulfonamides as a result from historical releases from a manufacturing site in Denmark. In addition, the very recent study by Phillips et al. (2010) on milligrams per liter levels of nonantibiotic pharmaceuticals in treated effluents in the United States suggest that releases from the manufacturing of drugs, rather than from usage, is indeed the cause of the highest levels found in the environment.

25.3 EFFECTS OF ANTIBIOTICS IN THE EXTERNAL ENVIRONMENT

The widespread occurrence of antibiotic compounds in a great variety of microbial ecosystems has attracted significant research interest lately. However, the number of studies on the effects of very high antibiotic concentration (milligrams per liter) in the external environment is still very limited, undoubtedly partly because most sources of contamination do not give rise to such high environmental levels. There are some metagenomic studies of the effects of therapeutic levels of antibiotics in the human gut (Dethlefsen et al., 2008; Dethlefsen and Relman, 2010; Jakobsson et al., 2010) that may, in part, be extrapolated to highly contaminated environmental communities with one major difference being the duration of the exposures.

Considering that many bacterial species have intrinsic resistance and that many antibiotics are designed to specifically target certain groups of bacterial species (such as Gram-positive or Gram-negative), changes in the taxonomic composition of a community are expected in response to antibiotic selection pressure. In recent articles, Dethlefsen et al. have characterized the changes in community structure of the human distal gut microbiota following administration of ciprofloxacin (Dethlefsen et al., 2008; Dethlefsen and Relman, 2010). These studies show that phylotype abundances after repeated treatment with ciprofloxacin are surprisingly robust, although marked changes are observed in some individuals. Jakobsson et al. (2010) have reported similar findings from a long-term study of patients after *Helicobacter pylori* eradication with metronidazole and clarithromycin. Other studies have documented changes in bacterial density and community structure following administration of antibiotics in a model system of activated sludge (Al-Ahmad et al., 1999, 2009). Thus, it seems likely that community changes in response to antibiotics do occur in very different ecosystems but that the overall taxonomic composition may be quite robust even under high concentrations, at least if enough time is given for recovery. However, the human gut studies show that superficially similar milieus (different individuals) may respond rather differently to antibiotic exposure. We do not yet know what factors determine resilience in microbial ecosystems exposed to long-term, high-level antibiotic selection pressure.

Recent studies have shown that many bacterial communities contain a diverse collection of resistance genes (D'Costa et al., 2006, 2007; Kümmerer, 2009b; Sommer et al., 2009; Zhang et al., 2009). This pool of genetic material has been called the *resistome,* and it is known to contain mechanisms for defending bacteria against many classes of antibiotics used in human therapy. The environmental resistome has consequently been hypothesized to constitute a reservoir of resistance genes that may spread to human pathogenic bacteria (Allen et al., 2010; Canton, 2009). A rapid selection for resistant strains is likely to occur more or less whenever an antibiotic selection pressure is applied. Interestingly, some studies have suggested

that acquired resistance genes may remain in the community even after the antibiotic is removed, perhaps most notably in the case of the *sul2* gene (Bean et al., 2009), which confers resistance to sulfonamides.

Resistance genes are typically found in mobile elements such as plasmids and integrons, and studies have shown that horizontal transfer of resistance may occur in, for instance, sewage (Ohlsen et al., 2003). The indirect evidence for acquisition of antibiotic resistance genes through horizontal gene transfer clearly warrants further characterization of the associated genetic mobility factors (the *mobilome*) and the dynamics thereof under antibiotic selection pressure. The type of mobility factors that are associated with promoted resistance genes are likely of great importance for their potential to be transferred to other bacteria. The direct effects of high antibiotic concentrations on mobile elements in complex environmental communities have, however, not been extensively explored. The recently characterized activity of gene transfer agents (GTA) in the marine environment (McDaniel et al., 2010) should also serve as a reminder that there may yet be mechanisms, including those propagating resistance transfer, that we do not know of today.

In addition to antibiotic resistance, there may be other effects of antibiotic selection pressure that are particularly important to consider in future studies. These include decreased ecosystem services rendered by microbial communities in diverse environmental processes. In particular, nitrification and other degradation processes, including those utilized in wastewater treatment, but also anabolic processes such as nitrogen capture, may be negatively affected (Dokianakis et al., 2004; Gomez et al., 1996; Kümmerer et al., 2004). Similarly, community-level changes, among them enrichment for sulfate reducers, have been documented in response to ciprofloxacin addition to salt marsh sediments (Cordova-Kreylos and Scow, 2007). The recently described association of the microbiota with human metabolic syndromes (Ley et al., 2005; Turnbaugh et al., 2006, 2009) also suggests that possible causal relationships with antibiotic therapy may be of clinical interest and serves as a reminder that symbiotic relationships between bacteria and multicellular organisms may be highly complex and sensitive to perturbations also in the external environment.

When assessing risks for ecosystem and human health both density and diversity of the local bacterial community are important, as these parameters will affect ecosystem dynamics under a high selection pressure. A high bacterial density increases the frequency of physical contacts between bacteria and thus possibilities for horizontal gene transfer to occur. On the other hand, a high density may also result in a more rapid metabolism of antibiotics, potentially decreasing the selection pressure. A greater diversity may imply a greater reservoir of resistance determinants that may be promoted within the community (D'Costa et al., 2006). Environmental bacterial communities contaminated by human or animal sewage may provide a situation where environmental bacteria may have the opportunity to share resistance factors from their gene pool with human or animal commensals or even pathogens. Similarly, such exchanges may conceivably also occur if environmental bacteria, particularly from contaminated environments where resistance factors are strongly promoted, are ingested and meet the intestinal microflora of humans or animals. If these humans or animals then are under antibiotic therapy, the risks for recruitment of resistance factors from the environmental resistome are likely increased.

Time is also important to consider; different systems and locations are exposed to antibiotic contamination for different time periods. Antibiotics have significantly

different acute and chronic toxicity profiles (Froehner et al., 2000; Kümmerer et al., 2004; Thomulka et al., 1993). In human therapy, the exposure times are most often limited to days or weeks, although there are a few situations where much longer therapy is required. Seasonal concentration peaks, such as those often found in urban wastewater (Giger et al., 2003; Göbel et al., 2005), or constant high levels for prolonged time periods, where environments contaminated by pharmaceutical production wastewater may constitute an exceptional example, may thus affect the microbial communities quite differently, and a stronger selection pressure would be expected with chronic exposure. Another important parameter to consider is the mixture of the contaminating antibiotics. In human therapy, one or at the most a few antibiotics are used at a time. This is radically different from the external environment, where multiple antibiotics are often present and also in combination with other chemical stressors. Generally, the number of antibiotics found in urban waste is higher than in agricultural waste, simply because many compounds are licensed exclusively for human use. Hospital effluents may not only quantitatively but also qualitatively differ from urban wastewater because some antibiotics are used only in hospitals and clinics.

In summary, it is important to study sites combining high bacterial diversity and density with long-term exposure to high environmental levels of antibiotics. Pharmaceutical production wastewater and surrounding ecosystems are, therefore, of urgent concern to investigate.

25.4 CASE STUDY: EFFECTS OF PENICILLIN G AND OXYTETRACYCLINE PRODUCTION WASTE ON BACTERIAL ISOLATES FROM THE RECEIVING RIVER

To our knowledge, the first comprehensive analyses of the effects of pharmaceutical production effluents on exposed aquatic microbial communities are those from the rivers Wangyang and Xiao in Hebei, China (Li et al., 2008a, 2008b, 2009, 2010). In these studies by Li et al. bacteria were isolated directly from WWTP effluent and from the river, downstream and upstream from the discharge site. The samples were analyzed using microbiological and molecular methods, combined with chemical analyses of selected antibiotics.

25.4.1 Penicillin G Production Site at the Wangyang River

The first set of studies were conducted on the Wangyang River where the WWTP mainly handles waste from a production plant manufacturing penicillin G (Li et al., 2008b). In this first study, the efficacy of the waste treatment process and resultant levels of penicillin G and degradation products in the WWTP effluent and the river system are also described. The main pollutant found in raw wastewater was the degradation product penilloic acid (up to 389 mg/L), whereas penicillin G was found at much more modest concentrations (153 μg/L). Penicillin G levels decreased substantially to 1.68 μg/L in the treated WWTP effluent and were below detection limit (0.03 μg/L) at the downstream river sampling site. The findings are not surprising since β-lactams are well known to be quickly and efficiently hydrolyzed in both WWTPs and in aquatic environments. However, what makes the subsequent

microbiological study interesting despite modest levels of penicillin G found in the recipient is the fact that the WWTP includes a biological treatment step, meaning that microbes inside of the treatment plant are exposed to continuous selection pressure from high levels of penicillin. Some of these bacteria are subsequently released into the river.

In the follow-up microbiological study from 2009 (Li et al., 2009), a total of 417 strains were isolated by standard culture methods, with 179 from the effluent, 163 from the downstream site, and 75 from the upstream site. The community structures in the different sites were analyzed using Polymerase chain reaction (PCR) amplification of 16S sequences followed by restriction fragment length polymorphism (RFLP) analysis and sequencing of selected amplicons to determine the taxonomic identity of the isolates. The minimum inhibitory concentration (MIC) of 18 antibiotics was then determined for the 406 strains belonging to a species or genus for which protocols existed at the time of the study. The 18 compounds covered 7 classes of antibiotics, including β-lactams, aminoglycosides, macrolides, phenicols, tetracyclines, quinolones, and ansamycins. Using results from the MIC analysis, the ratio between the resistant and sensitive strains were determined for each site and each compound. For several antibiotics, including β-lactams, the exposed downstream site contained significantly higher levels of resistant isolates compared to the upstream site. For quinolones, however, higher levels of resistant isolates were found upstream. Another notable difference between the sites was that a significantly higher relative frequency of isolates from effluent and downstream was resistant to β-lactams compared to the other classes of antibiotics. At the upstream sites, β-lactam resistance was not more frequent than resistance to most other classes of antibiotics investigated.

The study also describes the occurrence of resistance to multiple antibiotics within the same isolate. About 80% of strains isolated from the effluent and 50% of the downstream strains were resistant to at least 10 of the 18 tested compounds, in stark contrast to the upstream strains where less than 5% were resistant to more than 7 compounds. As mentioned, the culture-based MIC analyses were complemented with PCR analyses of selected resistance genes. For this purpose, the isolates were screened for the presence of 5 β-lactamase genes (bla_{TEM}, bla_{SHV}, bla_{OXA}, $bla_{\mathrm{CTX\text{-}M}}$, and bla_{IMP}). The bla_{TEM} gene was identified in 31 effluent isolates and 18 downstream isolates, but in none of the upstream strains. The other *bla* genes were not identified in any isolates. In addition to *bla* genes, the isolates were also screened for the presence of class 1 integrons, which was identified in 25 effluent and 15 downstream strains. Eighteen and 8 of these also carried bla_{TEM}, respectively. No integrons could be detected in the upstream isolates. Interestingly, sequencing revealed that the identified integrons primarily contained different variants of aminoglycoside resistance genes *aadA*, *aadB,* and *aacA* together with the multidrug exporter encoding gene *qacH*, which confers resistance to quaternary ammonium compounds, a commonly used class of disinfectants.

25.4.2 Oxytetracycline Production Site on the Xiao River

In the second set of studies, Li et al. investigated the pollution levels and correlated bacterial community changes in river water up- and downstream from the discharge of a WWTP handling wastewater from a factory producing oxytetracycline,

ivermectin, and avermectin. The authors note that ivermectin and avermectin are strict antiparasitic drugs and therefore omit these from further analyses. In the wastewater, the authors reported 19.5 mg/L oxytetracycline, which is diluted to 641 μg/L in the river water at the discharge site and further to 377 μg/L approximately 20 km downstream from the WWTP (Li et al., 2008a). Upstream from the WWTP, oxytetracycline levels were below detection limit (1 μg/L). The levels found at the discharge site and downstream are thus close to the human therapeutic plasma concentration of approximately 1 μg/mL. In the follow-up microbiological study (Li et al., 2010), 189, 87, and 65 bacterial isolates from waste, downstream river, and upstream river water, respectively, were analyzed for antibiotic resistance patterns in a similar fashion to isolates from the Wangyang River, although 10 rather than 18 compounds covering 7 classes of antibiotics were used in the resistance screen. All isolates were further analyzed for the presence of class 1 integrons using PCR and sequencing of selected amplicons.

Downstream and wastewater isolates were further analyzed for the presence of 14 relevant resistance genes, including tetracycline resistance-associated efflux pumps, 8 genes conferring ribosomal tetracycline protection, and 1 gene conferring resistance through enzymatic modification of tetracycline. Isolates from the downstream site and wastewater were resistant to the tested compounds in significantly higher numbers, and no differences were found between the downstream and wastewater isolates in regard to resistance patterns. Interestingly, the isolates were not significantly more frequently resistant to tetracycline compared to the other compounds throughout the three sites. The analyses also showed that a majority of isolates (>80%) were resistant to higher levels of tetracycline than the corresponding concentration measured for the respective site. With regard to multidrug resistance, the wastewater and downstream isolates were on average co-resistant to 5 classes of drugs (range 0–7), whereas upstream isolates were, on average, resistant to 1 class (range 0–3). Approximately 95% of wastewater and downstream isolates carried the tetracycline resistance genes with no significant differences in gene abundances between isolates from the two sites. Additionally, nearly 90% of isolates from these sites carried multiple tetracycline resistance genes. Finally, class 1 integrons were identified in 97% of wastewater and 86% of downstream isolates, but only in 3% of upstream isolates. Following sequencing, the integrons were found to contain mainly aminoglycoside resistance genes (*aadA1*, *aadA2,* and *aadA2a*), trimethoprim resistance genes (*dfrA1* and *dfrA12*), and, in one isolate only, a quaternary ammonium resistance gene (*qacG*).

25.5 CASE STUDY: EFFECTS ON BACTERIAL COMMUNITIES IN RIVER SEDIMENTS EXPOSED TO VERY HIGH LEVELS OF FLUOROQUINOLONES

A remarkable example of environmental exposure to very high levels of antibiotics was recently found at a WWTP in Patancheru, near Hyderabad in India (Larsson et al., 2007). The treatment plant receives and treats wastewater from a nearby cluster of chemical industries mainly manufacturing active pharmaceutical ingredients for a wide range of drugs, including antibiotics. In the treated effluent leaving the WWTP, high levels of fluoroquinolones were detected with ciprofloxacin as the most

abundant at concentrations up to 31 mg/L (Larsson et al., 2007). Similarly high levels were confirmed in samples taken 1.5 years later (Fick et al., 2009). The surface water in the downstream river also contained high levels of ciprofloxacin and the sediments were shown to be contaminated at least 17 km downstream from the discharge site (Kristiansson et al., 2011). The groundwater and the drinking water in nearby villages were also found to be contaminated with a range of drugs (Fick et al., 2009). Together this suggests that the pharmaceutical pollution in Patancheru has been going on for extended periods of time, possibly dating back to the establishment of the site as a major production area for the bulk drug industry sector in the late 1970s. Interestingly, there were also moderate levels of ciprofloxacin detected in the water (Fick et al., 2009) and sediment (Kristiansson et al., 2011) upstream from the treatment plant at concentrations still expected to pose a selection pressure on many bacterial species. The source(s) of antibiotics found upstream from the treatment plant have not been identified. However, local authorities have documented illegal dumping of untreated industrial waste to be very frequent in this region, particularly in the past, and this may be part of the explanation.

The concentrations of ciprofloxacin found both in the effluent and in the receiving river are higher than known inhibitory levels for many bacterial strains. Additional classes of antibiotics were also identified in the effluent, albeit at substantially lower levels. The high levels of antibiotics together with the long-term exposure provided the rationale for an in-depth characterization of the effects on exposed environmental microbial communities. The approach taken was through culture-independent metagenomics using next-generation sequencing technology (Metzker, 2010). River sediments were collected at two sites upstream and three sites downstream from the treatment plant, and the deoxy ribonucleic acid (DNA) of the complex microbial communities were sequenced (study fully described in Kristiansson et al., 2011). In addition, river sediment collected up and downstream from a Swedish sewage treatment plant with no input of pharmaceutical industry waste was used as a reference site. Sequencing of all samples was performed using massively parallel pyrosequencing generating 441,000 DNA fragments (reads).

25.5.1 Species and Functional Diversity

In all of the study sites, about half of DNA reads could be annotated, and most of these were found to be of bacterial origin (84%) while only a smaller part matched viruses, eukaryotes, and archea. The proportion of bacteria downstream from the treatment plant was slightly lower than upstream, but the difference was small (86% downstream and 93% upstream). The biodiversity in the sediments was further investigated by rarefaction curve analysis, a nonparametric technique based on random resampling of observations (reads), enabling direct comparison of the different samples (Hurlbert, 1971). Both the species diversity, using taxonomic information from the reads annotated to known proteins, and the functional diversity, using the identified COG (clusters of orthologous groups of proteins) gene families in the metagenomes, was compared between the sites (Tringe et al., 2005). The difference in species diversity between the up and downstream sediments was surprisingly small. In addition, the diversity in the highly contaminated sediment in Patancheru was also similar to the diversity of the Swedish sediments (Kristiansson

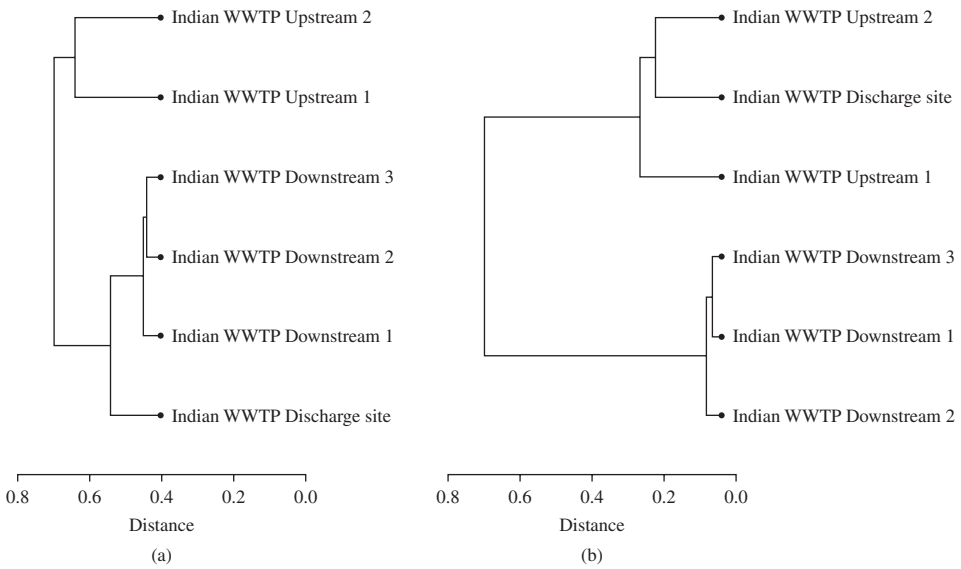

FIGURE 25.1 Hierarchical cluster analyses based on DNA shotgun sequencing of bacterial communities in sediment samples from an Indian river receiving antibiotic-contaminated wastewater. The two dendrograms correspond to the taxonomic (a) and functional (b) diversities in the metagenomes. The difference in functional diversity between the up- and downstream sediments appeared to be more marked than the corresponding difference in taxonomic composition.

et al., 2011). The difference in functional diversity appeared to be more marked, and this discrepancy could also be seen when the abundance profiles of the bacterial genera and gene families were compared using hierarchical clustering (Fig. 25.1). Interestingly, the discharge site where the effluent from the wastewater treatment is mixed with the upstream water seems to be taxonomically more similar to the downstream sites but functionally more similar to the upstream sites. At present we do not have a simple explanation for these observations. The functional discrepancies between the up and downstream sites were further investigated by comparative metagenomics (Kristiansson et al., 2009). The analysis revealed that genes representing several functional categories were found in significantly lower levels downstream compared to upstream, for example, amino acid transport and metabolism, energy production and conversion, and secondary metabolite biosynthesis, transport, and catabolism Table 25.1. There were, however, also functional categories such as coenzyme transport and metabolism, cytoskeleton, and defense mechanisms with a higher relative abundance downstream than upstream. The differences in functional composition between the exposed downstream sites and the upstream sites suggest that exposure to high levels of antibiotics affect the functional diversity of microbial communities and, by extension, possibly ecosystem services.

25.5.2 The Resistome

The river sediment metagenomes were compared against a large database of known resistance genes comprising more than 24,000 peptides representing 380 known mobile

TABLE 25.1 Analyses of Changes in Functional Gene Categories of Bacterial Communities in River Sediment up- and Downstream from an Indian Treatment Plant Discharging Highly Antibiotic-Contaminated Wastewater

COG Functional Category	Fold-Change[a]	Adjusted p value[b]
Coenzyme transport and metabolism (H)	1.97	$<10^{-16}$
Function unknown (S)	0.83	2.1×10^{-8}
Amino acid transport and metabolism (E)	0.85	2.5×10^{-8}
Energy production and conversion (C)	0.86	9.1×10^{-6}
Secondary metabolites biosynthesis, transport, and catabolism (Q)	0.76	3.5×10^{-5}
Inorganic ion transport and metabolism (P)	0.88	2.2×10^{-3}
Cytoskeleton (Z)	3.42	4.0×10^{-3}
Signal transduction mechanisms (T)	0.89	7.3×10^{-3}

[a]Difference in abundances (measured as fold-change) between the samples down and upstream. A number above 1 thus indicates overrepresentation downstream.
[b]Adjusted p value according to the Bonferroni method.

resistance genes [taken from the Antibiotic Resistance Database and other sources (Liu and Pop, 2009)]. Differences in relative abundance of resistance genes found at the different sites were then statistically assessed (Kristiansson et al., 2009). The analysis revealed that as much as 1.71% of the DNA from the downstream sites could be matched to known resistance genes while the corresponding number in the upstream sites was 0.22%. In the sediments from the Swedish reference sites, the proportion of resistance genes was substantially lower with 0.05 and 0.02% in the up and downstream sediments, respectively.

The most abundant resistance gene was *sul2*, which encodes for a sulfonamide-resistant variant of dihydropteroate synthase, an enzyme essential in the folate synthesis (Skold, 2000). The abundance of *sul2* in the downstream sediments was very high and constituted 3.4% of the annotated bacterial genes, 66 times higher than the upstream sites. The downstream sediments also contained considerable levels of *strA* and *strB*, two genes providing resistance to aminoglycosides through phosphorylation. These genes, overrepresented by 22 and 54 times in the downstream sites, constituted 0.37 and 0.53%, respectively, of the total bacterial genes present here. The upstream sediments also contained high levels of antibiotic resistance genes. *Qnr* genes constitute a class of resistance genes that provides resistance to moderate levels of fluoroquinolones. The levels of *qnr* genes were more than 26 times higher compared to the downstream sites and three different forms were identified, that is, *qnrD*, *qnrS,* and *qnrVC*. The considerably lower levels of *qnr* genes in the metagenomes from the downstream sites are likely a consequence of their inability to provide resistance to the high levels of fluoroquinolones present there.

Resistance genes are mobilized by gene transfer mechanisms, such as integrons, transposons, and plasmids. Interestingly, the metagenomes from the downstream sediments contained a high abundance of integrases associated with class 1 integrons, which are known to transfer a wide range of resistance genes (Boerlin and Reid-Smith, 2008; Partridge et al., 2009). There were also significantly higher levels of a transposase associated with the insertion common sequence region class 2, a transposon known to mobilize several forms of resistance genes, including *sul2*

(Toleman et al., 2006). Moreover, the amount of DNA associated with plasmids was surprisingly high in the downstream sediments where as much as 7.25% of the reads could be matched to plasmids. The corresponding number was substantially lower in the upstream sites (0.60%) and very low in the samples from the Swedish treatment plant, 0.097 and 0.033% from the up and downstream samples, respectively. The majority of the plasmid-associated DNA in downstream sites originated from a novel plasmid, pHIRE-D1, containing the *sul2* resistance gene. Two highly abundant previously characterized *sul2*-carrying resistance plasmids were also found (RSF1010, pMTSm3). Moreover, a novel plasmid was also found abundant in the upstream sites containing the *qnrD* resistance gene (pHIRE-U1).

25.6 SUMMARY OF EFFECTS OBSERVED IN HIGHLY ANTIBIOTIC-CONTAMINATED ENVIRONMENTS

The results summarized within this book chapter illustrate fundamental effects on environmental bacterial communities exposed to very high levels of antibiotics. Experimental short-term studies exposing bacterial communities to high levels of antibiotics often report gross and rapid changes in taxonomic composition. Interestingly, the Indian case study, representing a real case of a very long-term exposure to high levels of antibiotics, showed that although there were some differences in taxonomic composition between upstream and downstream sites, the difference in biodiversity was limited (Kristiansson et al., 2011). In addition, differences in functional diversity were demonstrated between up and downstream sites, suggesting that antibiotics may have direct consequences on the ecosystem services provided by environmental bacterial communities. However, further functional studies with endpoints specifically reflecting such services will be needed to fully elucidate these observations.

Both Li et al. (2010) and Kristiansson et al. (2011) found a significant increase of antibiotic resistance genes in exposed communities. Surprisingly, these genes represented several classes of resistance mechanisms even though only one single antibiotic class was detected at high levels at each site. In the absence of more complete chemical analyses of other substances, we can only hypothesize that the high diversity of resistance genes are due to co-resistance that may be genetically linked, possibly in combination with an increased frequency of horizontal gene transfer events. In fact, the study from the Indian treatment plant (Kristiansson et al., 2011) found high levels of a plasmid carrying only one known resistance gene (*sul2*) in the bacterial communities exposed to high levels of fluoroquinolones, even though no sulfonamides could be detected.

The genetic mobility of resistance genes depends on integrons, transposons, and plasmids. Information about the mechanisms used to horizontally transfer resistance genes in exposed bacterial communities may, therefore, provide information about the risk of spread to human pathogens. For example, both the studies from China and India identify high levels of class I integrons, a general and common form of integron present in a wide range of bacterial genomes.

These studies also reveal several methodological issues to consider for future studies. The great diversity of resistance genes found in different environments as well as the sheer complexity of environmental bacterial communities emphasize the

need for exploratory methods, such as metagenomic sequencing. High-throughput sequencing of 16S ribosomal ribonucleic acid (rRNA) genes have proved useful to determine changes in the phylogenetic composition of a microbial community under strong antibiotic selection (Dethlefsen et al., 2008; Dethlefsen and Relman, 2010; Jakobsson et al., 2010), but open shotgun sequencing as performed by Kristiansson et al. (2011) is necessary to identify also the resistance genes present. A high diversity of resistance genes stresses the need for vast amount of sequence data if the aim is to cover less common resistance factors. Kristiansson et al. (2011) also show that shotgun sequencing allows characterization of possible changes in the functional repertoire of the metagenomes, changes that may be greater than those in the phylogenetic structure (Fig. 25.1). Furthermore, functional screens of cloned metagenomic libraries combined with sequencing (functional metagenomics) has proved highly useful to identify novel resistance factors in soil (Allen et al., 2009; Donato et al., 2010; Lang et al., 2010; Riesenfeld et al., 2004) and human gut microflora (Kazimierczak et al., 2008; Sommer et al., 2009) and would also be valuable to apply in highly contaminated environmental bacterial communities.

More traditional molecular and culture-based approaches also have considerable advantages, as highlighted in the studies by Li et al. Conventional PCR, especially when combined with amplicon sequencing, has the potential to identify the presence of low-abundant genes that may remain elusive in metagenomic shotgun sequencing efforts. Quantitative PCR can be used to compare the relative frequencies of specific resistance genes between different sites or over time (Knapp et al., 2010). However, current PCR technologies have considerable limitations in the number of genes that are possible to investigate in parallel. Finally, despite the fact that the vast majority of environmental bacteria are unculturable using standard media (Hugenholtz et al., 1998), culture-based methods remain important for detailed characterization of individual bacterial strains and mobile genetic elements such as plasmids.

25.7 SOME CONCLUDING ASPECTS ON RISK ASSESSMENT AND MANAGEMENT

Without doubt, the increased global health problems with antibiotic-resistant pathogens are strongly connected to our use, overuse, and misuse of antibiotics. We also have good reasons to believe that the external environment serves as a recruitment pool for resistance genes to human pathogens (Canton, 2009). The role of antibiotic pollution in this context is, however, not clear. Several factors probably determine the risk for antibiotic pollution to contribute, that is, the type, level, and duration of antibiotic selection pressure, the diversity and number of microbes exposed, the possibilities for resistant environmental bacteria to physically interact with the human microflora (both commensals and pathogens), as well as the type of genetic elements facilitating the mobility of resistance genes in these bacteria. In most environments, even those under strong influence of human activities, the detected levels of antibiotics are relatively low. However, as described in this chapter, there are some sites, particularly in the vicinity of pharmaceutical production plants in India and China, where there is evidence for widespread contamination of antibiotics leading to resistance in environmental bacteria (Fig. 25.2). Knowing that there are external environments where there is a high likelihood that resistance is promoted,

FIGURE 25.2 Sampling of Indian river sediment contaminated with broad-spectrum antibiotics (up to ~1 g ciprofloxacin per kg organic material). Pyrosequencing of the microbial communities within these sediments revealed high levels of resistance and gene transfer elements (Kristiansson et al., 2011). *(See color insert.)*

a more important bottleneck may rather be the possibilities that resistant environmental bacteria come into contact with human microflora in sufficient numbers and under sufficiently favorable conditions to transfer their resistance or cause disease, if they are pathogens themselves. Water treatment facilities and hygiene standards are often much less advanced in developing regions, and this most likely contributes to the risks. A concurrent use of antibiotics among the people living in highly antibiotic-contaminated milieus can be expected to facilitate the establishment of resistant bacteria in the human microflora and provide the selection pressure required to promote horizontal gene transfer there.

If antibiotic pollution does play a role in the accelerating, global resistance development, the price tag, at the end of the day, could be immensely high. The precautionary principle would therefore advice immediate measures to prevent such releases. The responsibility for taking action is, at least from a moral standpoint, shared between different stakeholders, including regulatory agencies, manufacturers of antibiotics, importers, and the health care sector (Larsson and Fick, 2009; Larsson, 2010). In this context, one can argue that increased ability to improve the situation is linked to responsibility. Clearly, abilities are very different for different players in the "antibiotic chain," and, unfortunately, increased ability does not always go hand in hand with increased (economical) incentives to act. New management strategies for reducing the pollution are therefore needed, and it is probably wise to work on parallel tracks (Larsson, 2010).

Given that human bacteria, pathogens as well as commensals, are often transported rapidly across the world, it seems relatively unimportant if resistance in environmental bacteria is promoted in China, India, Europe, or North America. If

resistance genes from the external environment become resident in the human microflora at one site, it is likely to be only a matter of time until they pose a challenge for other countries and regions. One can therefore argue that efforts to minimize risks should be focused on those sites where the risks are perceived the largest, regardless of national borders. This is a major challenge, particularly as the means to enforce measures to limit antibiotic pollution in, for example, China and India, are restricted through European and American law. The Swedish government recently commissioned the Swedish Medical Product Agency (MPA) to investigate possibilities of finding ways to reduce environmental pollution from drug manufacturing sites on a global level. One of several suggestions from the MPA was to expand the framework of good manufacturing practices (GMP) for drugs to also encompass environmental aspects. Currently, the role of GMP is limited to ensuring the quality of the products. As anyone who wants to sell their drugs on the European market is required to comply with GMP standards, this would have a global impact (Swedish Medical Product Agency, 2009). In addition to this, we have proposed an increased transparency in the production chain of drugs to provide incentives for manufacturers to reduce environmental discharges (Larsson and Fick, 2009). Some of the major international companies manufacturing antibiotics have also announced that they are now investing more efforts into incorporating the environmental standards in their production chains. Very recently, the two largest county councils in Sweden have also begun to include aspects on environmental emissions from production sites (including production of active pharmaceutical ingredients) within their procurement of drugs.

It is thus positive to see that some actions are taken, but there is probably a long way to go until we no longer have "hot spots" in the environment with very high levels of antibiotics. Studying such hot spots will improve our ability to assess associated risks, and the result from such research may provide the necessary leverage for scientifically founded mitigations. Another important reason for studying hot spots is that they contain diverse collections of bacteria living under unique conditions of long-term antibiotic selection pressure. As such, they may provide a possibility to discover resistance genes that we may face among pathogens in the clinic tomorrow.

REFERENCES

Al-Ahmad A, Daschner FD, Kümmerer K (1999). Biodegradability of cefotiam, ciprofloxacin, meropenem, penicillin g, and sulfamethoxazole and inhibition of wastewater bacteria. *Arch Environ Contam Toxicol* 37(2):158–163.

Al-Ahmad A, Haiss A, Unger J, Brunswick-Tietze A, Wiethan J, Kümmerer K (2009). Effects of a realistic mixture of antibiotics on resistant and nonresistant sewage sludge bacteria in laboratory-scale treatment plants. *Arch Environ Contam Toxicol* 57(2):264–273.

Allen HK, Moe LA, Rodbumrer J, Gaarder A, Handelsman J (2009). Functional metagenomics reveals diverse beta-lactamases in a remote alaskan soil. *ISME J* 3(2):243–251.

Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J (2010). Call of the wild: Antibiotic resistance genes in natural environments. *Nat Rev Microbiol* 8(4):251–259.

Babic S, Mutavdzic D, Asperger D, Horvat AJM, and Kastelan-Macan M (2007). Determination of veterinary pharmaceuticals in production wastewater by hptlc-videodensitometry. *Chromatographia* 65(1–2):105–110.

Batt AL, Kim S, Aga DS (2007). Comparison of the occurrence of antibiotics in four full-scale wastewater treatment plants with varying designs and operations. *Chemosphere* 68(3):428–435.

Bean DC, Livermore DM, Hall LMC (2009). Plasmids imparting sulfonamide resistance in *Escherichia coli*: Implications for persistence. *Antimicrob Agents Chemother* 53(3): 1088–1093.

Björklund HV, Rabergh CMI, and Bylund G (1991). Residues of oxolinic acid and oxytetracycline in fish and sediments from fish farms. *Aquaculture* 97(1):85–96.

Boerlin P, Reid-Smith RJ (2008). Antimicrobial resistance: Its emergence and transmission. *Anim Health Res Rev* 9(2):115–126.

Brazas MD, Hancock REW (2005). Ciprofloxacin induction of a susceptibility determinant in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 49(8):3222–3227.

Campagnolo ER, Johnson KR, Karpati A, Rubin CS, Kolpin DW, Meyer MT, Esteban JE, Currier RW, Smith K, Thu KM, McGeehin M (2002). Antimicrobial residues in animal waste and water resources proximal to large-scale swine and poultry feeding operations. *Sci Total Environ* 299(1–3):89–95.

Canton R (2009). Antibiotic resistance genes from the environment: A perspective through newly identified antibiotic resistance mechanisms in the clinical setting. *Clin Microbiol Infect* 15 Suppl 1 :20–25.

Christian T, Schneider RJ, Farber HA, Skutlarek D, Meyer MT, Goldbach HE (2003). Determination of antibiotic residues in manure, soil, and surface waters. *Acta Hydrochim Hydrobiol* 31(1):36–44.

Cordova-Kreylos AL, Scow KM (2007). Effects of ciprofloxacin on salt marsh sediment microbial communities. *ISME J* 1(7):585–595.

Coyne R, Hiney M, Oconnor B, Kerry J, Cazabon D, Smith P (1994). Concentration and persistence of oxytetracycline in sediments under a marine salmon farm. *Aquaculture* 123(1–2):31–42.

D'Costa VM, McGrann KM, Hughes DW, Wright GD (2006). Sampling the antibiotic resistome. *Science* 311(5759):374–377.

D'Costa VM, Griffiths E, Wright GD (2007). Expanding the soil antibiotic resistome: Exploring environmental diversity. *Curr Opin Microbiol* 10(5):481–489.

Davies J, Spiegelman GB, Yim G (2006). The world of subinhibitory antibiotic concentrations. *Curr Opin Microbiol* 9(5):445–453.

Dethlefsen L, Relman DA (2011). Microbes and health sackler colloquium: Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *Proc Natl Acad Sci USA* 108(supplement 1):4554–4561.

Dethlefsen L, Huse S, Sogin ML, Relman DA (2008). The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16s rrna sequencing. *PLoS Biol* 6(11): 2383–2400.

de With K, Schroder H, Meyer E, Nink K, Hoffmann S, Steib-Bauert M, Kammerer R, Ruess S, Daschner FD, Kern WV (2004). Antibiotic use in Germany and Europe. *Dtsch Med Wochenschr* 129(38):1987–1992.

Diwan V, Tamhankar AJ, Khandal RK, Sen S, Aggarwal M, Marothi Y, Iyer RV, Sundblad-Tonderski K, Stalsby-Lundborg C (2010). Antibiotics and antibiotic-resistant bacteria in waters associated with a hospital in Ujjain, India. *BMC Public Health* 10:414.

Dokianakis SN, Kornaros ME, Lyberatos G (2004). On the effect of pharmaceuticals on bacterial nitrite oxidation. *Water Sci Technol* 50(5):341–346.

Donato JJ, Moe LA, Converse BJ, Smart KD, Berklein FC, McManus PS, Handelsman J (2010). Metagenomic analysis of apple orchard soil reveals antibiotic resistance genes encoding predicted bifunctional proteins. *Appl Environ Microbiol* 76(13):4396–4401.

Duong HA, Pham NH, Nguyen HT, Hoang TT, Pham HV, Pham VC, Berg M, Giger W, Alder AC (2008). Occurrence, fate and antibiotic resistance of fluoroquinolone antibacterials in hospital wastewaters in Hanoi, Vietnam. *Chemosphere* 72(6):968–973.

Fick J, Söderström H, Lindberg RH, Phan C, Tysklind M, Larsson DGJ (2009). Contamination of surface, ground, and drinking water from pharmaceutical production. *Environ Toxicol Chem* 28(12):2522–2527.

Froehner K, Backhaus T, Grimme LH (2000). Bioassays with *Vibrio fischeri* for the assessment of delayed toxicity. *Chemosphere* 40(8):821–828.

Giger W, Alder AC, Golet EM, Kohler HPE, McArdell CS, Molnar E, Siegrist H, Suter MJF (2003). Occurrence and fate of antibiotics as trace contaminants in wastewaters, sewage sludges, and surface waters. *Chimia* 57(9):485–491.

Göbel A, Thomsen A, Mcardell CS, Joss A, Giger W (2005). Occurrence and sorption behavior of sulfonamides, macrolides, and trimethoprim in activated sludge treatment. *Environ Sci Technol* 39(11):3981–3989.

Goh EB, Yim G, Tsui W, McClure J, Surette MG, Davies J (2002). Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics. *Proc Natl Acad Sci USA* 99(26):17025–17030.

Gomez J, Mendez R, Lema JM (1996). The effect of antibiotics on nitrification processes—Batch assays. *Appl Biochem Biotechnol* 57–58:869–876.

Gulkowska A, Leung HW, So MK, Taniyasu S, Yamashita N, Yeunq LWY, Richardson BJ, Lei AP, Giesy JP, Lam PKS (2008). Removal of antibiotics from wastewater by sewage treatment facilities in Hong Kong and Shenzhen, China. *WaterRes* 42(1–2):395–403.

Holm JV, Bjerg PL, Rugge K, Christensen TH (1995). Occurrence and distribution of pharmaceutical organic-compounds in the groundwater downgradient of a landfill (Grindsted, Denmark)—response. *Environ Sci Technol* 29(12):3074–3074.

Hugenholtz P, Goebel BM, Pace NR (1998). Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J Bacteriol* 180(24):6793–6793.

Hurlbert SH (1971). Nonconcept of species diversity—critique and alternative parameters. *Ecology* 52(4):577–587.

Jacobsen P, Berglind L (1988). Persistence of oxytetracycline in sediments from fish farms. *Aquaculture* 70(4):365–370.

Jakobsson HE, Jernberg C, Andersson AF, Sjölund-Karlsson M, Jansson JK, Engstrand L (2010). Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. *PLoS One* 5(3).

Jarnheimer PA, Ottoson J, Lindberg R, Stenström TA, Johansson M, Tysklind M, Winner MM, Olsen B (2004). Fluoroquinolone antibiotics in a hospital sewage line; occurrence, distribution and impact on bacterial resistance. *Scand J Infect Dis* 36(10): 752–755.

Jjemba PK (2006). Excretion and ecotoxicity of pharmaceutical and personal care products in the environment. *Ecotoxicol Environ Saf* 63(1):113–130.

Karthikeyan KG, Meyer MT (2006). Occurrence of antibiotics in wastewater treatment facilities in wisconsin, USA. *Sci Total Environ* 361(1–3):196–207.

Kazimierczak KA, Rincon MT, Patterson AJ, Martin JC, Young P, Flint HJ, Scott KP (2008). New tetracycline efflux gene, tet(40), is located in tandem with tet(o/32/o) in a human gut firmicute bacterium and in metagenomic library clones. *Antimicrob Agents Chemother* 52 (11):4001–4009.

Knapp CW, Dolfing J, Ehlert PAI, Graham DW (2010). Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. *Environ Sci Technol* 44(2): 580–587.

Kristiansson E, Hugenholtz P, and Dalevi D (2009). Shotgunfunctionalizer: An r-package for functional comparison of metagenomes. *Bioinformatics* 25(20):2737–2738.

Kristiansson E, Fick J, Janzon A, Grabic R, Rutgersson C, Weijdegård B, Söderström H, Larsson DGJ (2011). Pyrosequencing of antibiotic-contaminated river sediments reveals high levels of resistance and gene transfer elements. *PLoS ONE* 6(2):e17038.

Kümmerer K (2009a). Antibiotics in the aquatic environment—A review—Part I. *Chemosphere* 75(4):417–434.

Kümmerer K (2009b). Antibiotics in the aquatic environment—A review—Part II. *Chemosphere* 75(4):435–441.

Kümmerer K, Alexy R, Huttig J, Scholl A (2004). Standardized tests fail to assess the effects of antibiotics on environmental bacteria. *Water Res* 38(8):2111–2116.

Lang KS, Anderson JM, Schwarz S, Williamson L, Handelsman J, Singer RS (2010). Novel florfenicol and chloramphenicol resistance gene discovered in Alaskan soil by using functional metagenomics. *Appl Environ Microbiol* 76(15):5321–5326.

Larsson DJ (2010). Release of active pharmaceutical ingredients from manufacturing sites—need for new management strategies. *Integr Environ Assess Manag* 6(1):184–186.

Larsson DGJ, Fick J (2009). Transpararency throughout the production chain—a way to reduce pollution from the manufacturing of pharmaceuticals. *Regul Toxicol Pharmacol* 53:161–163.

Larsson DGJ, de Pedro C, Paxeus N (2007). Effluent from drug manufactures contains extremely high levels of pharmaceuticals. *J Hazard Mater* 148(3):751–755.

Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI (2005). Obesity alters gut microbial ecology. *Proc Natl Acad Sci USA* 102(31):11070–11075.

Li D, Yang M, Hu J, Ren L, Zhang Y, Li K (2008a). Determination and fate of oxytetracycline and related compounds in oxytetracycline production wastewater and the receiving river. *Environ Toxicol Chem* 27(1):80–86.

Li D, Yang M, Hu JY, Zhang Y, Chang H, Jin F (2008b). Determination of penicillin g and its degradation products in a penicillin production wastewater treatment plant and the receiving river. *Water Res* 42(1–2):307–317.

Li D, Yang M, Hu JY, Zhang J, Liu RY, Gu X, Zhang Y, Wang ZY (2009). Antibiotic-resistance profile in environmental bacteria isolated from penicillin production wastewater treatment plant and the receiving river. *Environ Microbiol* 11(6):1506–1517.

Li D, Yu T, Zhang Y, Yang M, Li Z, Liu MM, Qi R (2010). Antibiotic resistance characteristics of environmental bacteria from an oxytetracycline production wastewater treatment plant and the receiving river. *Appl Environ Microbiol* 76(11):3444–3451.

Liu B, Pop M (2009). Ardb-antibiotic resistance genes database. *Nucleic Acids* Res 37: D443–D447.

Lubick N (2009). India's drug problem. *Nature* 457(7230):640–641.

Martins AF, Vasconcelos TG, Henriques DM, Frank CD, Konig A, Kümmerer K (2008). Concentration of ciprofloxacin in Brazilian hospital effluent and preliminary risk aassessment: A case study. *Clean-Soil Air Water* 36(3):264–269.

Mason M (2009). World's highest drug levels entering india stream. Associated Press. Retrieved from http://www.washingtontimes.com/news/2009/jan/26/worlds-highest-drug-levels-entering-india-stream/

McDaniel LD, Young E, Delaney J, Ruhnau F, Ritchie KB, Paul JH (2010). High frequency of horizontal gene transfer in the oceans. *Science* 330(6000):50.

McManus PS, Stockwell VO, Sundin GW, Jones AL (2002). Antibiotic use in plant agriculture. *Annu Rev Phytopathol* 40:443–465.

Metzker ML (2010). Sequencing technologies—The next generation. *Nat Rev Genet* 11(1): 31–46.

Ohlsen K, Ternes T, Werner G, Wallner U, Loffler D, Ziebuhr W, Witte W, Hacker J (2003). Impact of antibiotics on conjugational resistance gene transfer in *Staphylococcus aureus* in sewage. *Environ Microbiol* 5(8):711–716.

Partridge SR, Tsafnat G, Coiera E, Iredell JR (2009). Gene cassettes and cassette arrays in mobile resistance integrons. *FEMS Microbiol Rev* 33(4):757–784.

Phillips PJ, Smith SG, Kolpin DW, Zaugg SD, Buxton HT, Furlong ET, Esposito K, Stinson B (2010). Pharmaceutical formulation facilities as sources of opioids and other pharmaceuticals to wastewater treatment plant effluents. *Environ Sci Technol* 44(13):4910–4916.

Riesenfeld CS, Goodman RM, Handelsman J (2004). Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. *Environ Microbiol* 6(9):981–989.

Segura PA, Francois M, Gagnon C, Sauve S (2009). Review of the occurrence of anti-infectives in contaminated wastewaters and natural and drinking waters. *Environ Health Perspect* 117 (5):675–684.

Skold O (2000). Sulfonamide resistance: Mechanisms and trends. *Drug Resis Updat* 3(3): 155–160.

Sommer MOA, Dantas G, Church GM (2009). Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science* 325(5944):1128–1131.

Swedish Medical Product Agency (2009). Report on government commission regarding possibilities to require stricter environmental standards during the manufacturing of pharmaceuticals including active substances. In *Rapport från läkemedelsverket*, December 16, 2009 [in Swedish with english summary]. Available at: http://www.Lakemedelsverket.Se/upload/nyheter/2009/2009–12–16_rapport_miljökrav-läkemedel.pdf.

Thomulka KW, Mcgee DJ, Lange JH (1993). Detection of biohazardous materials in water by measuring bioluminescence reduction with the marine organism *Vibrioharveyi*. *J Environ Sci Health A Environ Sci Eng Toxic Hazard Subst Control* 28(9):2153–2166.

Toleman MA, Bennett PM, Walsh TR (2006). Iscr elements: Novel gene-capturing systems of the 21st century? *Microbiol Mol Biol Rev* 70(2):296–316.

Tringe SG, von Mering C, Kobayashi A, Salamov AA, Chen K, Chang HW, Podar M, Short JM, Mathur EJ, Detter JC, Bork P, Hugenholtz P, Rubin EM (2005). Comparative metagenomics of microbial communities. *Science* 308(5721):554–557.

Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444 (7122):1027–1031.

Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, Jones WJ, Roe BA, Affourtit JP, Egholm M, Henrissat B, Heath AC, Knight R, Gordon JI (2009). A core gut microbiome in obese and lean twins. *Nature* 457(7228):480–U487.

Wise R (2002). Antimicrobial resistance: Priorities for action. *J Antimicrob Chemother* 49 (4):585–586.

Yim G, de La Cruz F, Spiegelman GB, Davies J (2006). Transcription modulation of *Salmonella enterica* serovar *typhimurium* promoters by sub-mic levels of rifampin. *J Bacteriol* 188(22):7988–7991.

Zhang XX, Zhang T, Fang H (2009). Antibiotic resistance genes in water environment. *Appl Microbiol Biotechnol* 82(3):397–414.

26

ANTIBIOTIC USE DURING AN INFLUENZA PANDEMIC: DOWNSTREAM ECOLOGICAL EFFECTS AND ANTIBIOTIC RESISTANCE

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The years 2009–2010 saw the first pandemic virus in several decades. Only in retrospect has the low pathogenicity of the virus been able to be confirmed. The pandemic saw as many deaths per capita as a seasonal influenza virus, but with the significant difference that the young (<18 years) were atypically impacted over those >18 years old (Kamigaki and Oshitani, 2009). Pharmaceuticals played an important part of health care during the influenza pandemic. Many nations implemented huge stockpiles of antivirals in response to the pandemic, but owing to the low pathogenicity of the virus, there was a negligible increase in existing antibiotic use over interpandemic usage. However, current estimates for antiviral and antibiotic use during a moderate and severe influenza pandemic are without historical precedent (Singer et al., 2011). Here we discuss the environmental and human health implications of a moderate or severe influenza pandemic with regard to Tamiflu itself and the use of antibiotics to treat secondary bacterial infections. Antibiotic use will be framed in the context of existing paradigms of antibiotic treatment and how these practices already contribute to human and environmental hazards and how these hazards might be minimized in the event of a moderate or severe influenza pandemic.

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26.1 PANDEMIC INFLUENZA

26.1.1 Introduction

The World Health Organization (WHO) has recently documented that infectious diseases are not only spreading faster but emerging at an unprecedented rate of one or more per year, resulting in the addition of ~40 infectious diseases that were unknown only a generation ago (WHO, 2007). As evident from the H1N1 pandemic of 2009, we cannot be certain whether highly pathogenic avian influenza A H5N1, H9N2, or another subtype will spark a human pandemic (Wan et al., 2008), but what does appear certain is that another influenza pandemic will occur at some point in the future in the absence of a universal influenza vaccine (Cabinet Office, 2008; U.S. Homeland Security Council, 2007). In response to this pressure, many countries worldwide have published pandemic preparedness plans (European Influenza Surveillance Scheme, 2008; Mounier-Jack and Coker, 2006). The aims of these plans are to maintain essential services, reduce disease transmission, minimize the socio-economic consequences of a pandemic, and reduce clinical cases, hospitalizations, and deaths (WHO, 2005). Key to the plans is slowing the spread of pandemic influenza through: (i) vaccine development, stockpiling, and distribution (Department of Health and Human Services and Department of Homeland Security, 2007), (ii) nonpharmaceutical measures (U.S. Centers for Disease Control and Prevention, 2007), and (iii) antiviral and antibiotic stockpiling and distribution (Hampson, 2008; NAS, 2008; U.S. Department of Health and Human Services, 2007). While the existence of preparedness plans in many countries highlights the attention that is given to the effects of a pandemic on society, possible implications of pharmaceutical therapies on the environment have been addressed much less. This chapter addresses how concentrations of antivirals and antibiotics occurring in wastewater treatment plants (WWTP) and surface water can be simulated, and whether these concentrations could have detrimental environmental effects or favor resistance development.

26.1.2 Influenza—Symptoms and Treatment

Influenza in adults is characterized by the abrupt onset of fever, myalgia, headache, malaise, nonproductive cough, sore throat, and rhinitis. Otitis media, nausea, and vomiting are the more commonly reported symptoms of influenza in children. Pronounced elevation of proinflammatory cytokines during H5N1 influenza virus infection, known as the "cytokine storm," is hypothesized to be the main cause of pathology and ultimately of death from uncomplicated influenza (Salomon et al., 2007). Although uncomplicated influenza typically resolves within 7 days, a cough and malaise can persist for >2 weeks. Influenza can cause viral pneumonia, exacerbate underlying medical conditions (e.g., pulmonary or cardiac disease), and lead to secondary bacterial pneumonia.

A vaccine is the first line of defense against influenza virus infections; however, its production is hampered by the inability to predict the antigentic details of the pandemic strain before it arrives. Moreover, a vaccine will only become available roughly 4–6 months after the outset of a pandemic as a result of an outdated vaccine production system (e.g., egg culture) and insufficient production capacity (WHO, 2006a). Owing to this delay, it is estimated that only 14% of the world's population

are predicted to have available to them a vaccine for the pandemic influenza strain within the first year (Osterholm, 2005; Uscher-Pines et al., 2006). Given these uncertainties, the WHO has recommended a number of mitigating strategies to help slow the spread of the pandemic, thereby providing additional time for a vaccine to be developed, distributed, and administered. Antivirals are to play a key role in this mitigation strategy.

Treatment for influenza using antivirals must be achieved within 48 hours of the onset of symptoms. Empirical antiviral therapy will be used in the absence of rapid diagnostic tests for influenza infection (Lim, 2007; U.S. Department of Health and Human Services, 2008). Currently, licensed antivirals for therapy and/or prophylaxis of influenza fall into two classes—the adamantanes (amantadine and rimantadine), M2 ion channel inhibitors effective against influenza A viruses only, and the neuraminidase inhibitors [NAI; oseltamivir ethylester phosphate (OE-P; Tamiflu) and zanamivir (Relenza)]—that are effective against both influenza A and B viruses. Although amantadine (Symmetrel) and rimantadine (Flumadine) are approved by the U.S. Food and Drug Administration (FDA) for the treatment and/or prevention of influenza, adamantane resistance is high and growing and it is therefore not recommended for use during an influenza pandemic (Barr et al., 2008; Hurt et al., 2007; Jefferson, 2007). Neuraminidase inhibitors (NAI), such as Tamiflu, Relenza, and Peramivir, are sialic acid analogs that inhibit the influenza neuraminidase enzyme, which is required for the release of progeny virions from infected cells. An inhibited neuraminidase ensures that the virions remain tethered to the infected cell surface, thereby limiting viral shedding (Ong and Hayden, 2007). Neuraminidase enzyme IC_{50} values for oseltamivir for clinically isolated influenza A ranged from 0.1 to 1.3 nM, and for influenza B was 2.6 nM (Roche, 2007).

The WHO has strongly recommended the use of Tamiflu (oseltamivir carboxylate; produced and distributed by Hoffmannn–La Roche) as the primary choice for combating an influenza pandemic as: (i) it is easy to administer (capsule), (ii) it is systemically active, and (iii) it is effective against characterized influenza A and B viruses (Roche Group, 2006; WHO, 2006b).

26.1.3 Antiviral Stockpiling

Many countries have stockpiled antivirals as part of their preparedness plans. Even before the 2009 pandemic, Roche reported orders for Tamiflu from more than 80 countries, equating to approximately 215 million treatments (Tierney and Reddy, 2007). Many countries, including the United States and the United Kingdom, are supplementing their antiviral stockpiles (Reuters, 2007) to include Relenza, (Zanamivir, marketed and distributed by GlaxoSmithKline), with the expectation of further supplementation with drugs soon to be available, such as Peramivir (marketed and distributed by BioCryst Pharmaceuticals) (Morse, 2007; Pharmaceuticals, 2007) and A-315675 (produced by Abbott Laboratories) (Kati et al., 2002), among many others in development.

26.1.4 Secondary Bacterial Infections

The main complication from influenza is secondary bacterial infection, particularly pneumonia (Brundage, 2006; Brundage and Shanks, 2008; Gupta et al., 2008;

Morens et al., 2008; New England Journal of Medicine Editors, 2009; Peltola et al., 2005; Rainsford, 2006; Schwarzmann and Sullivan, 1971; UK Department of Health, 2007b). The most common bacterial etiologies for influenza-associated community-acquired pneumonia (IA-CAP) are *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Staphylococcus aureus* (Lim, 2007; Schwarzmann and Sullivan, 1971; Siquier et al., 2006; UK Department of Health, 2007b). IA-CAP is characterized by fever (97% of cases), malaise (80%), respiratory symptoms, for example, cough, chest pain, shortness of breath (80–85%), headache (65%), and others (Bannister et al., 2006; Lim, 2007; Louria et al., 1959). Mortality of viral or combined viral-bacterial pneumonia is $\sim$10–12% (Public Health Agency of Canada, 2004).

Chemotherapy guidelines for treating IA-CAP have been recently published with the backing of the British Infection Society, British Thorax Society, and the Health Protection Agency (Lim, 2007). This guidance is tailored toward pandemic IA-CAP, which distinguishes itself from previously published CAP guidelines, for example, American Thoracic Society (ATS)(ATS, 2005). Antibiotic therapy for IA-CAP will be empirical as a result of the anticipated overwhelming surge of patients during a pandemic (Gupta et al., 2008; Nap et al., 2007; UK Department of Health, 2007a).

Patients with IA-CAP infections receive antibiotic therapy and a range of over-the-counter (OTC) medications, for example antipyretics, antiphlogistics, cough medicine, and decongestants, used to relieve some of the discomfort of the illness (U.S. Department of Health and Human Services, 2005). In addition to treating the influenza virus infection, there will be considerable use of OTC drugs including analgesics and antiphlogistics. Personal stockpiles of these drugs have been recommended for all families within the U. S. Pandemic Flu Preparedness Checklist for Individuals & Families (Meltzer et al., 1999; U.S. Department of Health and Human Services, 2006).

The availability of antibiotics within a country is considerably less defined than it is for antivirals. Recently, the UK Department of Health announced the intention to purchase 14.7 million courses of antibiotics, to cover at-risk groups totaling 25% of the population (Department of Health, 2007). However, to date, there was no indication of the nature of the stockpile. Owing to the threat of bioterrorism, many countries maintain a stockpile of antibiotics, primarily ciprofloxacin, for postexposure anthrax treatment (Gupta et al., 2008). The United States stockpiles sufficient antibiotics (ciprofloxacin and doxycycline) to treat 60 million people. Both of these antibiotics could be mobilized to treat some, but not all, of the causes of IA-CAP (Fowler et al., 2005; Gupta et al., 2008).

26.2 EXPOSURE ASSESSMENT OF ANTIBIOTICS AND ANTIVIRALS DURING A PANDEMIC

In order to determine risks of pandemic antiviral and antibiotic loads in the aquatic environment, knowledge on the concentrations in WWTPs and surface water as well as on possible effects on WWTP bacterial communities and occurrence of antibiotic resistance is needed. In this section, we summarize how concentrations can be predicted from coupling a spatially structured global epidemic model with a GIS-

based watershed model. To our knowledge, the modeling approaches described in (Singer et al., 2011) and in this chapter are the first investigations of possible antibiotic concentrations during an influenza pandemic.

The spatiotemporal pattern of the pandemic and its concurrent secondary infections were simulated by use of the global epidemic and mobility (GLEaM) model (Balcan et al., 2009a), which maps 6 billion individuals and integrates their mobility data. Parameters of the infection dynamics consisted of transmission of influenza under seasonal variation and included secondary infections according to the UK Pandemic Assumptions for complication, hospitalization, and intensive care unit admission rates (Balcan et al., 2009b) (UK Department of Health, 2009). In order to incorporate a wide range of possible influenza transmission potentials, we explored a mild pandemic ($R_0 = 1.65$), a moderate ($R_0 = 1.9$), as well as a severe ($R_0 = 2.3$) scenario. R_0, the basic reproductive number, is the average number of secondary infections produced by a single infected individual while he or she is infectious in an entirely susceptible population. This is a measure of the degree of transmissibility of an infection (Anderson and May, 1991). No large-scale antiviral treatment was included in the mild scenario. For the other two scenarios, Tamiflu antiviral treatment was foreseen, with the assumption that 30% of the cases were detected and antiviral treated. It was assumed that antiviral treatment reduced the infectious period and the rate of development of secondary infections (Kaiser et al., 2003; Nicholson et al., 2000; Treanor et al., 2000; Whitley et al., 2001).

Within each scenario, we explored the effect of antiviral prophylaxis for 2 or 4 weeks from the start of the outbreak per country, as well as the effect of no prophylaxis. Antibiotics were assumed to be used according to guidelines for treating influenza-associated pneumonia sanctioned by the British Infection Society, British Thoracic Society, and the UK Health Protection Agency (Lim, 2007) (Table 26.1). It should be noted that this is only a small subset of the drugs that have been proposed to be used during a pandemic. Table 26.2 presents data on the environmental fate of a wide range of antibiotics that may be used during a pandemic, reflecting the considerable variation in antibiotic prescribing that exists regionally, nationally, and globally (Coenen et al., 2006; de Neeling et al., 2001; Ferech et al., 2006; García-Rey et al., 2002; Muller et al., 2007; Priest et al., 2001); and the possibility that, dependent on the extent of any stockpile, shortages are likely to occur and a range of antibiotics might be used (Gupta et al., 2008). Table 26.3 summarizes data on concentrations in WWTPs and surface water for pharmaceuticals that have been proposed for use during an influenza pandemic.

Simulations of an influenza pandemic (R_0) resulted in estimates of the cases of influenza and pneumonia at each stage of disease progression, together with the quantities of drugs used. Simulations were able to project cases and drugs down to the spatial resolution scale of ¼° and a time resolution of one day. A water quality model, Low Flows 2000—Water Quality Extension (LF2000-WQX) (Rowney et al., 2009), then predicted the environmental release of antivirals and antibiotics. Values for excretion of active substances within the feces and/or urine were taken from the literature (see Table 26.4). Dilution processes in the river were modeled using the annual mean flow from each river stretch, while a worst-case scenario of no pharmaceutical degradation or loss to sorption in WWTPs and rivers was applied (see Fig. 26.1). The Thames catchment in the southern United Kingdom was chosen as a model test case.

TABLE 26.1 Preferred and Alternative Empirical Antibiotic Treatment Regimens for Pneumonic Influenza-Associated Complications

CURB-65 Score	Compartment	Preferred Treatment Regimen	Alternative Treatment Regimen	Duration
0–2	P^{I}, P^{II}	Co-amoxiclav 625 mg tds PO **or** doxycycline 200 mg stat and 100 mg od PO	Macrolide (erythromycin 500 mg qds PO **or** clarithromycin 500 mg bd PO) **or** fluoroquinolone (e.g., levofloxacin 500 mg od PO or moxifloxacin 400 mg od PO)	7 days
3–5	P^{III}	Co-amoxiclav 1.2 g tds IV **or** cefuroxime 1.5 g tds IV **or** cefotaxime 1 g tds IV **plus** macrolide (erythromycin 500 mg qds IV **or** clarithromycin 500 mg bd IV)	Fluoroquinolone with some enhanced pneumococcal activity (e.g., levofloxacin 500 mg od IV or moxifloxacin 400 mg od PO) **plus, either** macrolide (erythromycin 500 mg qds IV ***or*** clarithromycin 500 mg bd IV) **or** β-lactamase stable antibiotic (i.e., co-amoxiclav 1.2 g tds IV **or** cefuroxime 1.5 g tds IV **or** cefotaxime 1 g tds IV)	10 days

Source: From Lim (2007).

A survey of the literature as well as an examination of the STPWIN model (U.S. Environmental Protection Agency, 2007) within the Estimation Program Interface (EPI) SuiteTM 4.0 indicates low (<20%) removal for most antibiotics in WWTPs, inclusive of loss due to sorption and biodegradation (Fig. 26.1). A literature search revealed that most antibiotics, particularly those not containing a β-lactam moiety, are resistant to metabolism in vivo as well as in the environment (Al-Ahmad et al., 1999; Alexy et al., 2004; Benotti and Brownawell, 2009; Brain et al., 2004a; 2004b; Gartiser et al., 2007; Junker et al., 2006; Kümmerer et al., 2000), with half-lives of days to weeks (Benotti and Brownawell, 2009; Christensen, 1998). For these reasons, we feel there was justification in assuming a conservative pharmaceutical biodegradation model of zero degradation/sorption.

26.2.1 Interpandemic Antibiotic and Antiviral Use

A very wide range of pharmaceuticals are in constant use in a population and thus will be present in the wastewater during a pandemic (Kümmerer, 2009a, 2009b). While the relative contribution of single antibiotic classes to overall use does not greatly vary between countries (Fig. 26.2), the absolute amount of defined daily

TABLE 26.2 List of Pharmaceuticals Proposed or Likely to Be Used During an Influenza Pandemic[a]

ATC Code	Common Name	CAS	Daily Dose (g)	Total Excretion (%)[b]	Total STW Removal (%)	STW Biodegradation Removal (%)	STW Adsorption Removal (%)
			β-Lactam Antibacterials				
J01AA07	Tetracycline	60-54-8	1	91	8.78	7.13	1.65
J01AA02	Doxycycline	564-25-0	0.1	80	8.78	7.13	1.65
J01AA06	Oxytetracycline	2058-46-0	1	35	8.78	7.13	1.65
J01AA03	Chlortetracycline	57-62-5	1	20	1.85	0.09	1.76
J01AA01	Demeclocycline	127-33-3	0.6	85	8.78	7.13	1.65
J01BA01	Chloramphenicol	56-75-7	3	11	8.86	7.16	1.70
J01CE01	Benzylpenicillin	61-33-6	3.6	89	22.60	20.90	1.68
J01CE02	Phenoxymethyl-penicillin	87-08-1	2	35	23.10	21.22	1.88
J01CF05	Floxacillin (Flucloxacillin)	5250-39-5	2	80	3.34	0.10	3.24
J01CA04	Amoxicillin	26787-78-0	1	75	22.04	20.57	1.47
[c]	Clavulanic acid	58001-44-8	0.375	38	92.06	91.72	0.33
J01DB01	Cefalexin	15686-71-2	2	100	22.02	20.56	1.46
J01DC02	Cefuroxime	55268-75-2	0.5	95	21.98	20.53	1.45
J01DC04	Cefaclor	53994-73-3	1	85	21.99	20.54	1.45
J01DD01	Cefotaxime	63527-52-6	4	61	22.01	20.55	1.46
J01DD04	Ceftriaxone	73384-59-5	2	100	8.78	7.13	1.65
J01DH51	Imipenem	74431-23-5	2	70	75.06	74.44	0.62
			Sulfonamides and Trimethoprim Antibacterials				
J01EA01	Trimethoprim	738-70-5	0.4	100	8.83	7.15	1.68
J01EC01	Sulfamethoxazole	723-46-6	2	100	22.05	20.57	1.47
			Imidazole Antibacterial				
J01XD01	Metronidazole	443-48-1	1.5	100	21.98	20.53	1.45

(*Continued*)

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TABLE 26.2 (Continued)

ATC Code	Common Name	CAS	Daily Dose (g)	Total Excretion (%)[b]	Total STW Removal (%)	STW Biodegradation Removal (%)	STW Adsorption Removal (%)
			Macrolide Antibacterials				
J01FA01	Erythromycin	114-07-8	1	100	6.23	0.13	6.11
J01FA06	Roxithromycin	80214-83-1	0.3	60	4.05	0.11	3.94
J01FA10	Azithromycin	83905-01-5	0.3	85	30.99	0.33	30.66
J01FA09	Clarithromycin	81103-11-9	0.5	55	7.30	0.14	7.17
J01FF01	Clindamycin	18323-44-9	1.2	14	9.66	7.46	2.20
			Aminoglycoside Antibacterial				
J01GB03	Gentamicin	1403-66-3	0.24	80	21.97	20.53	1.44
			Fluoroquinolone Antibacterials				
J01MA02	Ciprofloxacin	85721-33-1	1	100	8.79	7.13	1.66
J01MA12	Levofloxacin	100986-85-4	0.5	96	1.88	0.09	1.79
J01MA06	Norfloxacin	70458-96-7	0.8	40	8.78	7.13	1.65
J01MA02	Ofloxacin	82419-36-1	0.4	85	1.85	0.09	1.76
J01MA14	Moxifloxacin	354812-41-2	0.4	100	1.88	0.09	1.79
			Glycopeptide Antibacterial				
J01XA01	Vancomycin	1404-90-6	2	75	1.85	0.09	1.76
			Analgesic/Antiphlogistics				
M01AB01	Indometacin	53-86-1	0.1	95	78.45	46.71	31.74
M01AB05	Diclofenac	15307-86-5	0.1	95	86.57	46.94	39.63
M01AE01	Ibuprofen	15687-27-1	1.2	100	94.93	80.36	14.57
M01AE02	Naproxen	22204-53-1	0.5	90	83.68	79.66	4.02
M01AE03	Ketoprofen	22071-15-4	0.15	100	82.89	79.26	3.63
N02BE01	Acetaminophen	103-90-2	3	94	75.09	74.46	0.63
N02BA01	Acetylsalicylic acid	50-78-2	3	100	92.11	91.74	0.37
R05DA04	Codeine	76-57-3	0.1	100	8.87	7.16	1.71

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			Antivirals				
J05AH02	Oseltamivir ethylester-P	204255-11-8	0.15	20	22.06	20.58	1.48
[d]	Oseltamivir carboxylate	187227-45-8	0.15	80	75.07	74.45	0.62
J05AH01	Zanamivir	139110-80-8	0	100	92.06	91.72	0.33
N04BB01	Amantadine	768-94-5	0.2	100	48.58	46.41	1.91
J05AC02	Rimantadine	13392-28-4	0.2	100	38.81	30.11	8.17
			Statins (HMG CoA Reductase Inhibitors)				
C10AA01	Simvastatin	79902-63-9	0.015	73	96.49	58.76	37.73
C10AA02	Lovastatin	75330-75-5	0.03	70	90.7	64.4	26.3
C10AA05	Atorvastatin	134523-00-5	0.01	88	97.29	25.72	71.57
C10AA03	Pravastatin	81093-37-0	0.02	78	94.93	92.26	2.67
			Uricosurics (Transporting Inhibitors)				
M04AB01	Probenecid	57-66-9	1	88	34.8	28.2	6.6
			Decongestants				
R01BA02	Pseudoephedrine	90-82-4	0.24	90	75.2	74.5	0.65
R01AA04	Phenylephrine	59-42-7	0.004	3	92.1	91.7	0.4
R01BA01	Phenylpropanolamine	14838-15-4	0.1	90	75.1	74.5	0.64

[a] including their defined daily dose (DDD); % excretion as parent or active metabolite; % loss in sewage treatment plant (STW) owing to biodegradation and adsorption.
[b] Total excretion is the sum of the % parent chemical released in the urine and feces. Metabolites were only included in the sum if the metabolite has a known bioactivity.
[c] There is no official DDD for clavulanic acid. Clavulanic acid is administered in conjunction with amoxicillin in the form of Co-amoxiclav. In this case we used a typical adult dose of 0.125 g and assumed 3 doses per day, equating to a DDD equivalent of 0.375 g.
[d] Oseltamivir carboxylate (OC) is the active metabolite of the prodrug oseltamivir ethylester phosphate (OE-P); 20% of OE-P is excreted in feces, 80% of OE-P is excreted in urine as OC (He et al. 1999).

TABLE 26.3 Pharmaceuticals Proposed for Use During an Influenza Pandemic[a,b]

Drug	Influent (mean–high) (ug/L)	Ref.	Effluent (mean–high) (μg/L)	Ref.	River Water (mean–high) (μg/L)	Ref.
Tetracycline	0.738–1.41	25, 26, 48, 56	0.337–0.977	25, 34, 37, 48, 51, 56	0.11[c]	4, 8
Doxycycline	0.157–0.22	25, 26	0.073–0.046	22, 26, 34	[c]	4, 8, 22
Oxytetracycline	7.76–23.62	25, 26	0.66[a]	[26]	0.34[c]	4, 8
Chlortetracycline	52.63–171	25, 26	0.28[a]	26	0.69[a]	4, 8
Demeclocycline	1.45–3.15	25, 26	0.65–1.12	26	—	
Chloramphenicol	0.19	14	0.396–0.56	8, 14, 30	0.124–0.266 $\times 10^{-3}$	8, 55
Benzylpenicillin	29[a]	48	[c]	48	[c]	8
Phenoxymethylpenicillin					[a]	8
Amoxicillin					*0.104–0.187	55
Cefalexin	1.73–2900	35, 48	16.3–78.2	35, 48	0.021–0.026	35
Ceftriaxone					[c]	10
Cefotaxime	0.006 – 0.024	48	0.009 – 0.034	48		
Trimethoprim	1.44–7.9[c]	14, 24, 39, 48, 56, 59, 60	0.025.5–2.5[c]	8, 14, 22, 24, 28, 30, 37, 39, 48, 51, 53, 56, 57, 59, 60, 61	0.073–0.71[c]	4, 8, 22, 28, 52, 57, 59, 60
Sulfamethoxazole	0.304–1	14, 24, 26, 25, 39, 56	0.702–2.0	8, 14, 22, 24, 28, 30, 37, 39, 51, 53, 56, 57	0.077–1.9[c]	4, 8, 17, 27, 28, 33, 37, 39, 52, 57
Metronidazole			0.050–0.080[a]	8, 14, 22, 24, 28, 30, 37, 39, 51, 53, 56, 57	0.0143–0.043[c]	22
Erythromycin	0.215–0.810	14, 24, 59, 48	0.645 – 0.85[c]	8, 14, 22, 24, 28, 30, 34, 45, 48, 49, 57, 59	1.955–67.0[a]	2, 4, 8, 10, 22, 27, 28, 33, 52, 55, 57, 59
Roxithromycin	0.056	14	2.743–1.0	14, 30, 34, 45	0.080–0.18[c]	4, 8, 27, 55
Azithromycin			0.059–0.077	63	0.022–0.047[c]	63
Clarithromycin	[c]	14	0.180–0.536	8, 14, 30, 34, 45	0.059–0.26[a]	8, 27
Clindamycin	[c]	14	0.050–0.11[c]	14, 37	0.034–0.14	10, 37
Ciprofloxacin	0.558–1.4	1, 3, 35, 56	0.340–0.97[c]	1, 3, 9, 9, 22, 34, 35, 37, 51, 56, 61	0.107–0.36[c]	1, 4, 35, 37, 52
Levofloxacin					[a]	50
Norfloxacin	0.32–0.6	1, 3, 48	0.121–0.367[a]	1, 3, 9, 9, 22, 34, 35, 48, 61	0.06–0.251[c]	1, 4, 22, 35, 55

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Ofloxacin	0.421–1	1, 14, 39	0.205–0.58[a]	1, 14, 22, 34, 39, 61	0.047–0.109[c]	1, 22, 52, 55
Indometacin			0.27–0.6	7	0.04–0.2	7
Diclofenac	0.757–1.92	20, 23, 24, 32, 41, 59, 60	0.480–5.45	19, 21, 23, 24, 28, 30, 32, 40, 41, 42, 44, 53, 57, 59, 60, 61	0.061–0.360[a]	21, 27, 28, 29, 32, 53, 57, 60
Ibuprofen	25.56–68,700	5, 20, 23, 24, 36, 38, 41, 59, 60	3.42–28[c]	5, 6, 7, 19, 20, 21, 23, 24, 28, 30, 36, 38, 40, 41, 42, 44, 53, 57, 59, 60, 61	1.01–3.1[a]	4, 5, 6, 10, 21, 27, 28, 33, 38, 43, 53, 57, 59, 60
Naproxen	33.49–280	5, 7, 20, 24, 36, 37, 41, 60	33–3.3[c]	5, 6, 8, 19, 20, 21, 24, 30, 36, 40, 41, 44, 53, 57, 60, 61	0.75–0.85[c]	5, 6, 21, 27, 29, 53, 57, 60
Ketoprofen	0.959–2.9	5, 7, 20, 41, 60	1.39–36[c]	5, 7, 19, 20, 21, 30, 40, 41, 44, 53, 60, 61	0.050–0.290[a]	5, 21, 53, 60
Acetaminophen	117.6–281	23, 24, 38	1.5–11.5[c]	6, 19, 23, 24, 38, 40, 57	0.025–10[c]	4, 6, 27, 28, 33, 37, 38, 57
Acetylsalicylic acid			0.037–0.057	19	0.025–0.037[c]	43
Codeine	5.2–11	23	3.7–8.1	23	0.004 – 1.0[c]	4, 33, 43
Simvastatin	0.004	47	0.001	47	ND	47
Lovastatin	0.049	47	0.014	47	0.0183[c]	47, 62
Atorvastatin	0.076	47	0.037	47	ND	47
Pravastatin	0.117	47	0.059	47	ND	47

[a] The absence of a pharmaceutical from this table should not be interpreted as its absence from the environment. The mean is from all the reported concentrations from the noted references and the max is the highest reported concentration from the references.

[b] Including their occurrence in STW influent and effluent and river water.

[c] Denotes a study that looked for the drug but did not detect it.; [1] (Vieno et al. 2006); [2] (Sacher et al. 2001); [3] (Giger et al. 2003); [4] (Kolpin et al. 2002); [5] (Lindqvist et al. 2005); [6] (Ternes 1998); [7] (Heberer 2002); [8] (Hirsch et al. 1999); [9] (Golet et al. 2001); [10] (Zuccato et al. 2000); [11] (Alexy et al. 2004); [12] (Gartiser et al. 2007); [13] (Junker et al. 2006); [14] (Alexy et al. 2006); [15] (Ternes et al. 2002); [16] (Lindberg et al. 2007); [17] (Liebig et al. 2006); [18] (Kümmerer et al. 2000); [19] (Rabiet et al. 2006); [20] (Vieno et al. 2005); [21] (Tixier et al. 2003); [22] (Wennmalm 2005); [23] (Gomez et al. 2007); [24] (Trenholm et al. 2006); [25] (Choi et al. 2007); [26] (Yang and Carlson 2003); [27] (Wiegel et al. 2004); [28] (Ashton et al. 2004); [29] (Ahrer et al. 2001); [30] (Ternes 2001); [31](Carlsson et al. 2006); [32] (Buser et al. 1998); [33] (Stackelberg et al. 2004); [34] (Miao et al. 2004); [35] (Costanzo et al. 2005); [36] (Carballa et al. 2004); [37] (Batt et al. 2006); [38] (Bound and Voulvoulis 2006); [39] (Brown et al. 2006); [40] (Brun et al. 2006); [41] (Brun et al. 2006); [42] (Reemtsma et al. 2006); [43] (Moldovan 2006); [44] (Drewes et al. 2003); [45] (McArdell et al. 2003); [46] (Joss et al. 2006); [47] (Miao and Metcalfe 2003); [48] (Gulkowska et al. 2008); [49] (Cordy et al. 2004); [50] (Conleya et al. 2008); [51] (Sacher et al. 2001); [52] (Batt and Aga 2005); [53] (Haggard et al. 2006); [54] (Metcalfe et al. 2003); [55] (Al-Ahmad et al. 1999); [56] (Batt et al. 2007); [57] (Kim et al. 2007); [58] (EA 2003); [59] (Roberts and Thomas 2006); [60] (Bendz et al. 2005); [61] (Andreozzi et al. 2003); [62] (Conlea et al., 2008); [63] (Jones-Lepp 2006).

TABLE 26.4 Percentage of Parent Pharmaceuticals Investigated in This Study Excreted in the Feces and Urine unchanged and/or as a Bioactive Metabolite

Pharmaceutical	% Excreted as Parent or Bioactive Metabolite
Tamiflu	100
Amoxicillin	75
Clavulanate	38
Doxycycline	80
Cefuroxime	95
Cefotaxime	61
Erythromycin	100
Clarithromycin	55
Levofloxacin	96
Moxifloxacin	100

Source: From Dollery (1999) and Wishart et al. (2006).

dosages per 1000 inhabitants per day ranges between 10 and >20 in Europe (Coenen et al., 2006; Ferech et al., 2006; Muller et al., 2007). If one was to use the average annual antibiotic usage within England during an interpandemic period (NHS BSA, 2008) as a first approximation, 62 μg antibiotics/L would be present in the UK WWTPs, which was assumed to be diluted by the median volume of wastewater in WWTP within the Thames catchment of 230 L/head/day (Table 26.5).

Table 26.3 and Figure 26.3 give an extensive overview on the concentrations of antibiotics that have been found in WWTP and surface water globally as well as in the United Kingdom. When comparing predicted and actual concentrations of antibiotics in WWTPs (Table 26.5 vs Table 26.3), it should be noted that veterinary use significantly contributes to concentrations in surface water (Hurd and Raef, 2010; Smith et al., 2002) such that WWTP effluent is not the only source of antibiotic residues in the aquatic environment. Over-and underestimations of predicted concentrations of antibiotics in the environment can also be caused by unforeseen losses from sorption and/or degradation, the impact of climate on degradation, difference in antibiotic use between different regions within a country, as well as country-specific differences, seasonal fluctuations in drug use, as well as seasonal fluctuations on WWTPs and river dilution (ter Laak et al., 2010). Hence, it is very difficult to project the concentration of antibiotics in use during interpandemic periods for any one location at any particular time. For this study we employed annual usage statistics for England (2007) with no further modification or adjustment.

Unlike antibiotics, interpandemic usage of Tamiflu in the United Kingdom has been reported to be negligible (Kramarz et al., 2009), implying that any substantial increase will be a result of the influenza pandemic.

26.2.2 Projected Concentrations of Antibiotics and Tamiflu in WWTP and Rivers During a Pandemic

In WWTPs, a mild pandemic scenario was projected to increase interpandemic antibiotic use of the same antibiotics (see Section) by only approximately 1% (95%

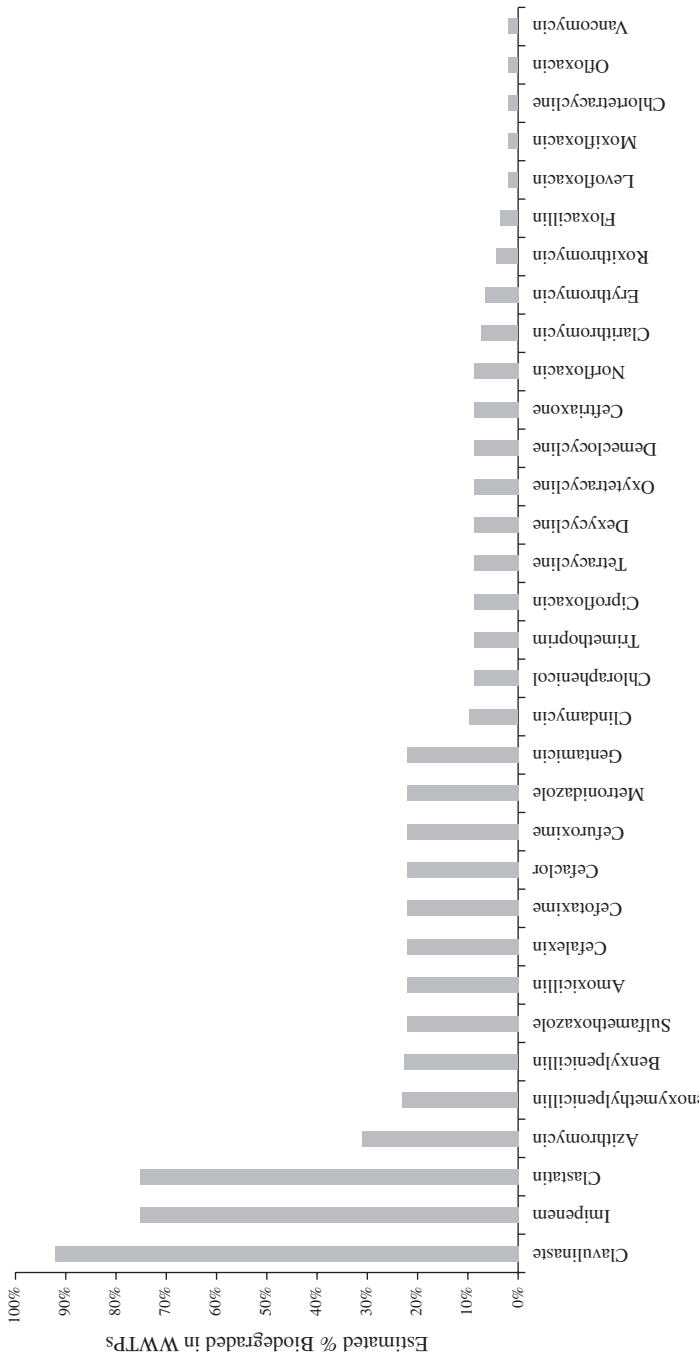

FIGURE 26.1 Percent removal of a range of antibiotics in WWTPs, from biodegradation.

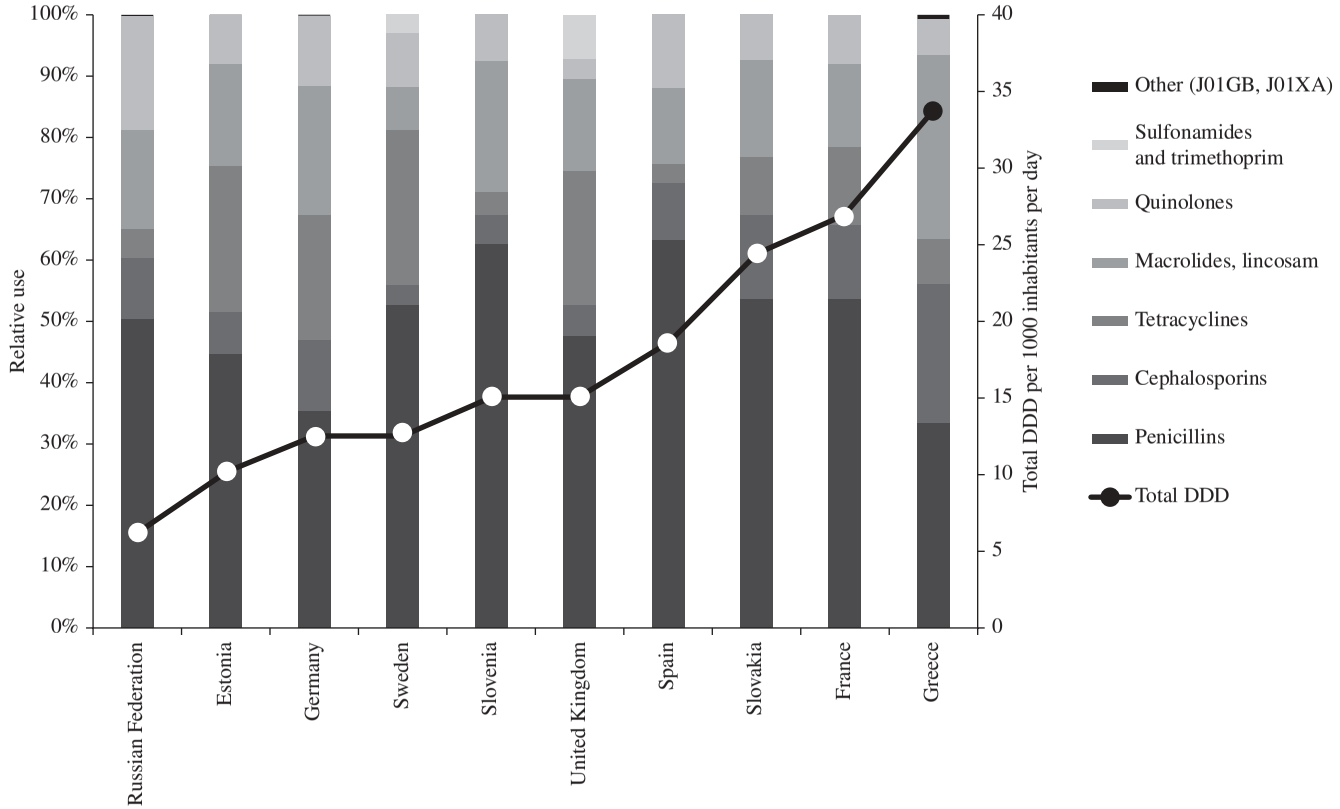

FIGURE 26.2 Outpatient antibiotic use in 10 European countries in 2005 for each of seven major classes of antibacterials and the total DDD per 1000 inhabitants per day.

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TABLE 26.5 Estimated Concentration of Antibiotics in English WWTP During an Interpandemic Period[a]

	Combined μg/head/d	% of Total Antibiotics in Use (mass basis)	Estimated Concentration in WWTP (μg/L)
Floxacillin + co-fluampicil	4068	28.0	17.5
Amoxicillin + co-amoxiclav	3698	25.4	15.9
Cefalexin	2023	13.9	8.68
Erythromycin	1391	9.6	5.97
Ampicillin	840	5.8	3.61
Ciprofloxacin	582	4.0	2.50
Penicillin V	531	3.6	2.28
Trimethoprim	387	2.7	1.66
Cefradine	253	1.7	1.09
Clarithromycin	156	1.1	0.671
Cefaclor	129	0.9	0.553
Cefadroxil	126	0.9	0.543
Clavulanate	108	0.7	0.464
Oxytetracycline	66	0.5	0.285
Lymecycline	47	0.3	0.202
Sulfamethoxazole	36	0.3	0.156
Cefuroxime	24	0.2	0.103
Minocycline	30	0.2	0.128
Doxycycline	12	0.1	0.052
Azithromycin	16	0.1	0.069
Ofloxacin	9	0.1	0.041
Norfloxacin	8	0.1	0.035
Levofloxacin	7	0.1	0.031
Moxifloxacin	5	<0.1	0.022

[a]Drug use was as reported by the National Health Service Business Services Authority (NHS BSA, 2008). Where the ADQ (average daily quantity) was unknown, the DDD (defined daily dose) (World Health Organization, 2004) was used to calculate the mass of drug used per head per day (population of England served by the NHS: 54,180,000).

reference range, RR, of the stochastic epidemic model: 0.4–23%). An increase by 13% (95% RR, 1–83%) and 252% (95% RR, 158–279%) was determined for the moderate and severe scenarios. In absolute concentrations, a moderate pandemic would increase interpandemic concentrations by 1.2– 3 μg/L (total of all antibiotics; mean concentration of all 135 WWTP in the Thames catchment area for the median epidemic scenario, Table 26.6). A severe pandemic would cause total antibiotic concentrations to reach 55–60 μg/L on average, with a maximum of 800 μg/L for the WWTP with the lowest dilution. Amoxicillin and erythromycin have the greatest share of the total antibiotic load (59 and 18%, respectively). Tamiflu concentrations greatly vary with the epidemic scenario for mild and moderate R_0 and are highest if antiviral prophylaxis is assumed for 10% of the population (around 45 μg/L in the mild and moderate scenario). A severe pandemic would increase Tamiflu concentrations to >80 μg/L on average (Table 26.6).

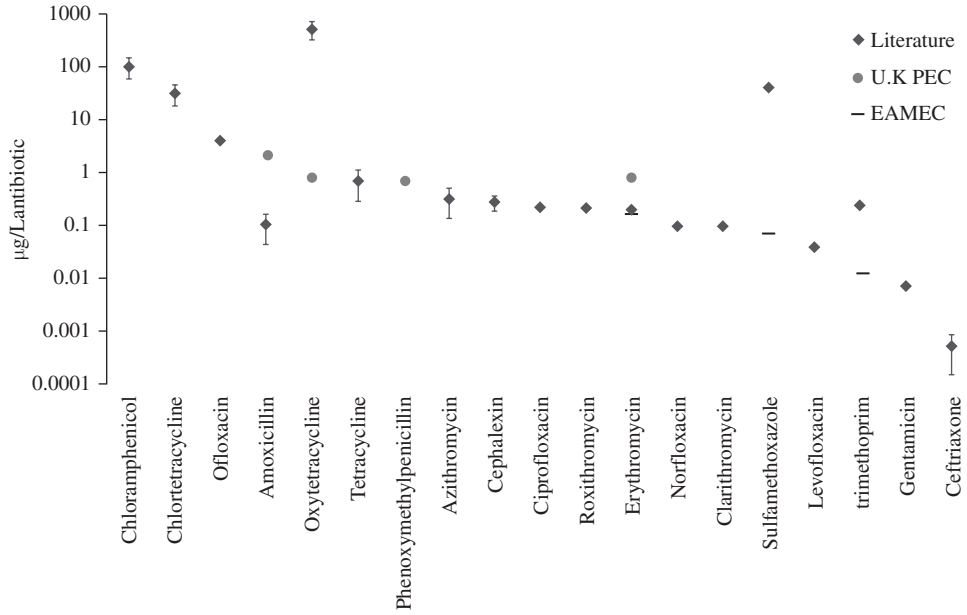

FIGURE 26.3 Prepandemic river water concentration of pharmaceuticals that have been examined in STW effluent and/or river water.

Mean concentrations of total antibiotics in the Thames catchment were projected to be <0.09 and <0.8 μg/L for a mild and moderate pandemic, respectively (Table 26.7). On the other hand, a severe pandemic was projected to achieve <15 μg/L total antibiotics, with a maximum of 80 μg/L. A mild and moderate pandemic with AVP>0 was projected to generate mean concentrations of Tamiflu in the Thames catchment between 1.1 and 11.5 μg/L (Table 26.7). A more severe pandemic, regardless of AVP, was projected to result in mean concentrations of Tamiflu in the Thames catchment in excess of 100 μg/L, consistent with previous projections of a severe pandemic in southern England (Singer et al., 2007).

26.3 EFFECTS ASSESSMENT OF ANTIBIOTICS DURING A PANDEMIC

Elevated concentrations of antibiotics during a pandemic will, in the first instance, affect microbial consortia in WWTPs and surface waters. In order to evaluate such potential effects of antibiotics, we determined the fraction of bacteria that were potentially affected by a given antibiotic exposure [(potentially affected fraction, PAF). This approach projects effects on whole communities from toxicities of a substance to single members of the community compiled in species-sensitivity distributions (SSD) (Newman et al., 2002)]. As experimental data on the toxicity of the chosen antibiotics is almost entirely lacking, we based our analyses on minimum inhibitory concentrations (MIC) of human pathogens from the EUCAST database (EUCAST, 2009). Through the application of models for mixture toxicity (De Zwart and Posthuma, 2005), we accounted for the presence of all eight antibiotics simultaneously (clavulanate was omitted because no MICs were present for this

TABLE 26.6 Projected Mean Concentrations of Antibiotics and Tamiflu in WWTP in the Thames Basin[a]

	Antibiotics (μg/L)		Tamiflu (μg/L)	
Scenario[b]	Mean ± Stdev	Maximum	Mean ± Stdev	Maximum
$R_0$1.65				
s1	0.36 ± 0.4	4.76	0.78 ± 0.87	10.41
s2	0.34 ± 0.37	4.49	4.63 ± 5.14	61.55
s3	0.34 ± 0.37	4.46	4.86 ± 5.4	64.65
s4	0.3 ± 0.33	3.97	45.35 ± 50.37	603.42
s5	0.29 ± 0.32	3.83	45.43 ± 50.46	604.4
s6	0.05 ± 0.06	0.73	0.11 ± 0.12	1.49
$R_0$1.9				
s1	2.99 ± 3.32	39.8	4.02 ± 4.46	53.44
s2	2.85 ± 3.17	38	4.79 ± 5.32	63.67
s3	2.97 ± 3.3	39.5	6.05 ± 6.72	80.53
s4	2.27 ± 2.52	30.2	45.3 ± 50.4	603.3
s5	1.71 ± 1.9	22.8	47 ± 52.3	625.9
s6	1.2 ± 1.33	15.9	1.52 ± 1.69	20.22
$R_0$2.1				
s1	60.2 ± 66.8	800.6	87.9 ± 97.6	1169.5
s2	59.7 ± 66.3	793.6	87.8 ± 97.5	1167.6
s3	59.3 ± 65.9	789.6	87.6 ± 97.3	1166.1
s4	57 ± 63.3	757.8	85.5 ± 94.9	1137.2
s5	53.7 ± 59.6	714	80.1 ± 89	1065.6
s6	54.2 ± 60.2	721.3	80.7 ± 89.6	1073.6

[a]Mean values are inclusive of all excreted antibiotics. Values reflect the median epidemic scenario for each condition and reflect the mean concentration for all 461 river stretches utilized within LF2000-WQX of parent pharmaceuticals investigated in this study excreted in the feces and urine unchanged and/or as a bioactive metabolite.

[b]s1 = where AVP = 0, rate of AVT = 30%, limited supply of Tamiflu.
s2 = where 2 wk AVP, AVP = 1%, rate of AVT = 30%, limited supply of Tamiflu.
s3 = where 4 wk AVP, AVP = 1%, rate of AVT = 30%, limited supply of Tamiflu.
s4 = where 2 wk AVP, AVP = 10%, rate of AVT = 30%, limited supply of Tamiflu.
s5 = where 4 wk AVP, AVP = 10%, rate of AVT = 30%, limited supply of Tamiflu.
s6 = where AVP = 0, rate of AVT = 30%, unlimited supply of Tamiflu.

substance on its own). Toxicities were determined for WWTP and river stretches in the Thames catchment area from antibiotic concentrations predicted through exposure modeling. The results of this determination of the potentially affected fraction of bacteria in the community based on simulated antibiotic concentrations are shown in Figures 26.4 and 26.5. For a mild pandemic, projected toxicity in WWTP was well below 1% PAF (less than 1% of the community might be growth inhibited, Fig. 26.4a). During a moderate pandemic, toxicities >5% were predicted in 74% of the WWTP for concentrations at the upper bound of the 95% RR of the stochastic model, while no toxicity was predicted for the lower bound (Fig. 26.4a). A proportion of growth-inhibited species >5% was chosen as a pragmatic threshold for possible effects on community functioning (European Chemicals Agency, 2008). The severe pandemic was projected to affect between 8 and 32% of the microbial species in WWTP (Figs. 26.4b and 26.5c).

TABLE 26.7 Projected Mean Concentrations of Antibiotics and Tamiflu in Rivers in the Thames Basin[a]

Scenario[b]	Antibiotics (μg/L)		Tamiflu (μg/L)	
	Mean ± Stdev	Maximum	Mean ± Stdev	Maximum
$R_0$1.65				
s1	0.085 ± 0.088	0.476	0.186 ± 0.192	1.04
s2	0.082 ± 0.084	0.445	1.12 ± 1.15	6.09
s3	0.083 ± 0.084	0.447	1.20 ± 1.21	6.47
s4	0.073 ± 0.074	0.400	11.1 ± 11.2	60.8
s5	0.070 ± 0.072	0.384	11.1 ± 11.3	60.6
s6	0.013 ± 0.014	0.073	0.027 ± 0.027	0.149
$R_0$1.9				
s1	0.741 ± 0.744	3.95	1.00 ± 1.00	5.31
s2	0.690 ± 0.706	3.77	1.16 ± 1.19	6.33
s3	0.719 ± 0.731	3.90	1.47 ± 1.49	7.96
s4	0.552 ± 0.563	3.01	11.0 ± 11.2	60.0
s5	0.418 ± 0.427	2.27	11.5 ± 11.7	62.4
s6	0.294 ± 0.298	1.59	0.37 ± 0.38	2.02
$R_0$2.1				
s1	14.8 ± 15.0	80.5	21.3 ± 21.3	102
s2	14.5 ± 14.8	80.6	21.0 ± 21.3	103
s3	14.5 ± 14.8	79.9	21.1 ± 21.2	102
s4	14.0 ± 14.2	75.9	20.7 ± 20.8	99.1
s5	13.1 ± 13.3	69.3	19.6 ± 19.9	103
s6	13.2 ± 13.4	72.3	19.6 ± 20.0	108

[a]Mean values are inclusive of all excreted antibiotics. Values reflect the median epidemic scenario for each condition and reflect the mean concentration for all 461 river stretches utilized within LF2000-WQX of parent pharmaceuticals investigated in this study excreted in the feces and urine unchanged and/or as a bioactive metabolite.

[b]s1 = where AVP = 0, rate of AVT = 30%, limited supply of Tamiflu.
s2 = where 2 wk AVP, AVP=1%, rate of AVT = 30%, limited supply of Tamiflu.
s3 = where 4 wk AVP, AVP=1%, rate of AVT = 30%, limited supply of Tamiflu.
s4 = where 2 wk AVP, AVP=10%, rate of AVT = 30%, limited supply of Tamiflu.
s5 = where 4 wk AVP, AVP=10%, rate of AVT = 30%, limited supply of Tamiflu.
s6 = where AVP=0, rate of AVT = 30%, unlimited supply of Tamiflu.

Absolute toxicity in rivers was projected to be slightly lower than in WWTPs, with the maximum PAF for any river stretch in a moderate pandemic being ~15% (Fig. 26.4e). During a severe pandemic, the 5% threshold of toxicity would be exceeded in about half of the river stretches at the upper bound of the 95% R (Fig. 26.4c), corresponding to about one third of total river length (Figs. 26.4d and 26.5f). Maximum toxicity during a severe pandemic was simulated to reach 30% (Fig. 26.4e).

The same effect models, when applied to the background concentrations of the eight investigated antibiotics, yield toxicities (PAF) in Thames catchment WWTP of between 4 and 17%. At these concentrations, however, no major functional breakdowns have been recorded. Reasons for the tolerance of WWTP communities to chronic exposure to antibiotics might be: (1) reduction of bioavailable concentrations through degradation or sorption, (2) acquired community tolerance through

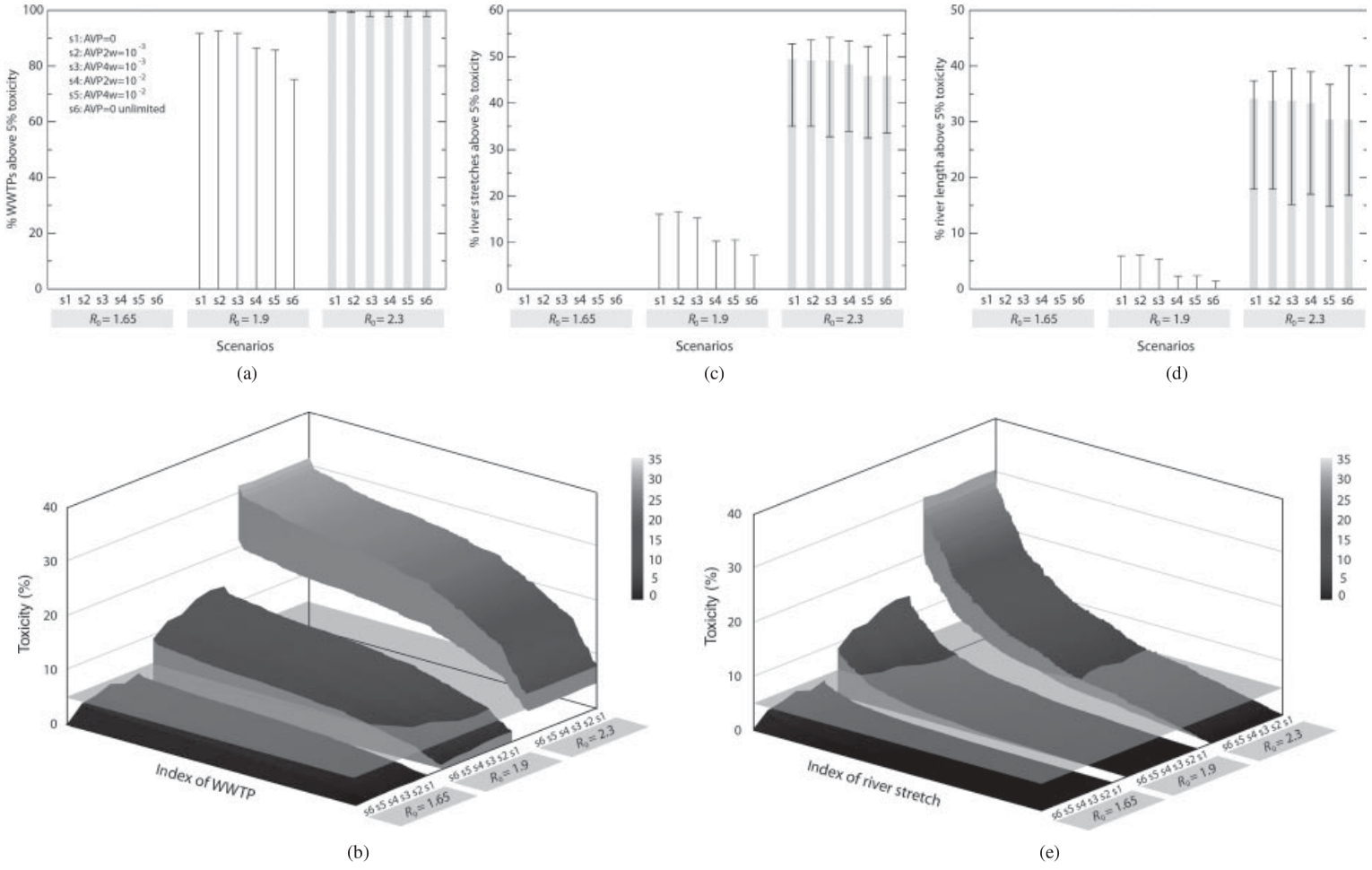

FIGURE 26.4 Predicted toxicity in WWTPs and river stretches. *(See color insert.)*

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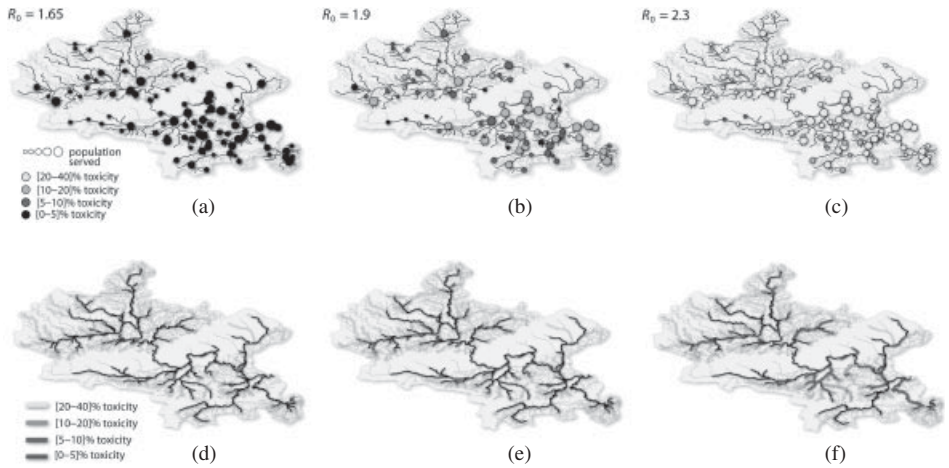

FIGURE 26.5 Predicted toxicity maps. *(See color insert.)*

selection for more tolerant or resistant bacteria, (3) differences between the antibiotic sensitivity of human pathogens and WWTP bacteria, and (4) overcapacity of WWTPs, thereby enabling some decline in function to occur with no significant loss in overall sewage treatment. As only the severe scenario increases the PAF substantially, we conclude that a mild or moderate pandemic would not be likely to affect WWTP consortia.

Notably, effects modeling in this study were based on MICs of human pathogens instead of effects observed in WWTP toxicity testing. Arguably, the toxicity of antibiotics to functionally active WWTP bacteria might differ from pathogen MICs. Also, the predominance of biofilms in WWTPs as flocs will influence antibiotic toxicity. There is little experimental data to verify whether modeled toxicity matches experimental data. Also, often, studies are based on test systems that differ from a full-scale WWTP, such as through the use of batch studies or through the use of synthetic sewage as medium.

Erythromycin is the only antibiotic investigated in this study for which substantial data on toxicity in WWTPs exists. For example, in batch studies with activated sludge inoculum from French WWTPs (Louvet et al., 2010a) and raw wastewater as medium, a range of effects of erythromycin were observed. After exposure to the antibiotic for less than one hour, direct toxicity was seen in cell staining experiments. In 24-hour batch experiments with inocula collected at nine different time points, 10 mg/L erythromycin (equivalent to a PAF of 80% according to our models) inhibited COD (chemical oxygen demand) removal by 79% on average. Inhibition strongly varied between different inocula, highlighting the dependence of batch toxicity studies on the initial inoculum. Inhibition of nitrification was 40% on average in similar experiments.

Inhibition was also found at lower concentrations of erythromycin (see Table 26.8). Further, erythromycin interfered with floc structure: flocs divided and biomass was transferred to the foam layer on top of the batch reactors (Louvet et al., 2010a). At longer exposure times of batch reactors (5 days), effects on soluble COD were observed at erythromycin concentrations as low as 4 μg/L (Louvet et al., 2010b),

TABLE 26.8 Effects of Antibiotics on Wastewater Treatment Plant Bacterial Consortia and Processes

Class	Compound	Concen-tration (mg/L)	PAF (%)	PAF (%) of Group Member	Size of Effect Measured (%)	Toxicity Parameter	N	Refs.
	Erythromycin	0.1	22		10–35	Reduction in live bacteria in mixed liquor samples after 20–45 min	3	1
	Erythromycin	1	56		50–62	Reduction in live bacteria in mixed liquor samples after 25–45 min	3	1
	Erythromycin	0.004	0.3		13/31/0	Batch reactors with activated-sludge-fed raw wastewater: decreased NH_4 reduction after 40/65/90 h	2	1
	Erythromycin	0.1	22		55/90 (activated sludge from two different STP)	Batch reactors with activated-sludge fed raw wastewater: inhibition of specific N-NH_4 evolution rate after 4 h	2	2
	Erythromycin	0.1	22		6/89 (activated sludge from two different STP)	Batch reactors with activated-sludge-fed raw wastewater: inhibition of the specific COD evolution rate after 4 h	2	2
	Erythromycin	0.5	46		62/44/29	Batch reactors with activated-sludge-fed raw wastewater: decreased nitrification (nitrate evolution) after 40/65/90 h	2	1

(*Continued*)

TABLE 26.8 (Continued)

Class	Compound	Concen-tration (mg/L)	PAF (%)	PAF (%) of Group Member	Size of Effect Measured (%)	Toxicity Parameter	N	Refs.
	Erythromycin	1	56		36/92 (activated sludge from two different STP)	Batch reactors with activated-sludge-fed raw waste-water: inhibition of specific N-NH_4 evolution rate after 4 h	3	2
	Erythromycin	1	56		51/70 (activated sludge from two different STP)	Batch reactors with activated-sludge-fed raw wastewater: inhibition of the specific COD evolution rate after 4 h	3	2
	Erythromycin	5	75		32	Batch reactors with activated-sludge-fed raw wastewater: inhibition of the initial ammonia uptake rate over 24 h	Not stated	3
	Erythromycin	10	80		46	Batch reactors with activated-sludge-fed raw wastewater: inhibition of nitrification over 48 h	Not stated	3
	Erythromycin	10	80		79 (standard deviation: 34)	Batch reactors with activated-sludge-fed raw waste water: inhibition of the specific COD evolution rate after 4 h	13	2
	Erythromycin	10	80		40 (standard deviation: 25)	Batch reactors with activated-sludge-fed raw wastewater: inhibition of	13	2

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					specific N-NH_4 evolution rate after 4 h		
	Erythromycin	10	80	38/−12 (activated sludge from two different STP)	Batch reactors with activated-sludge-fed raw wastewater: inhibition of the specific nitrification rate	13	2
	Erythromycin	20	85	66	Batch reactors with activated-sludge-fed raw wastewater: inhibition of the initial ammonia uptake rate over 24 h	Not stated	3
	Erythromycin	20	85	72	Batch reactors with activated-sludge-fed raw wastewater: inhibition of nitrification over 48 h	Not stated	3
	Erythromycin	0.003	0.1	Not stated	Reduction in CFU, development during CBT	11	4
	Erythromycin, roxithromycin, clarithromycin, trimethoprim, and sulfamethoxazole	mix of 0.04 mg/L of each of the antibiotics			Food chain effects: food preference behavior of *Gammarus fossarum*		5
	Clarithromycin	3	78	Not stated	Reduction in CFU, development during CBT	11	4
Fluoro-quinolones	Ciprofloxacin	3.5	86 (levofloxacin) / 91 (moxifloxacin)	Not stated	CFU development in CBT	Not stated	6
Fluoro-quinolones	Ciprofloxacin	0.08	31 (levofloxacin) / 26 (moxifloxacin)	50%	growth inhibition of Pseudomonas putida (GIT)	Not stated	6

(*Continued*)

TABLE 26.8 (Continued)

Class	Compound	Concen-tration (mg/L)	PAF (%)	PAF (%) of Group Member	Size of Effect Measured (%)	Toxicity Parameter	N	Refs.
Third-generation cephalosporins	Ceftriaxone disodium	0.005		11 (cefotaxime)	Not stated	Reduction in CFU, development during CBT	Not stated	6
Tetracyclines	Chlortetracycline HCl	0.003		0.7	Not stated	Reduction in CFU, development during CBT	11	4
	Clarithromycin	3		78	Not stated	Reduction in CFU, development during CBT	11	4
Tetracyclines	Tetracycline	16.8		88 (doxycycline)	100%	Inhibition of degradation of control substance in closed bottle test (OECD 301 B) over 31 days	1	7
Tetracyclines	Tetracycline	37		93 (doxycycline)	Weak	COD removal in semiindustrial bioreactor	1	7
Tetracyclines	Tetracycline	11		84 (doxycycline)	41	Inhibition of degradation of control substance in closed bottle test (OECD 301 B) over 31 days	2	8
Fluoro-quinolones	Ofloxacin	12		93 (levofloxacin) / 96 (moxifloxacin)	Non-competitive inhibition constant	Inhibition of nitrification in batch reactors with nitrifying communities	1	9

Sources: [1](Louvet et al. 2010b); [2](Louvet et al. 2010a); [3](Alighardashi et al. 2009); [4](Alexy et al. 2004); [5](Bundschuh et al. 2009); [6](Al-Ahmad et al. 1999); [7](Prado et al. 2009); [8](Prado et al. 2010); [9](Dokianakis et al. 2004).

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which is equivalent to a PAF of 0.2%. Concentrations higher than 500 μg/L also inhibited nitrification. At 4 μg/L, erythromycin-treated reactors showed higher nitrification as compared to control reactors. This was explained through the extra source of nitrogen provided by dead bacteria.

In contrast, Fan et al. (2009) found that erythromycin (at concentrations of 500 μg/L or a single-substance PAF of 46% according to our calculations) did not have pronounced effects on nitrogen and phosphorus removal in sequencing batch reactors fed synthetic wastewater during long incubations (Fan et al., 2009). Still, phylogenetic investigations showed that the diversity of ammonium-oxidizing bacteria and of nitrifying bacteria had declined by up to 80%, highlighting effects of the antibiotic on the composition of the functional community. Further, short-term toxicity measurements with higher concentrations of erythromycin and H_2O–erythromycin showed that while the adapted nitrifier and ammonium oxidizer communities were more tolerant to high doses of antibiotics, functional parameters were still affected at concentrations equal or higher than the long-term concentration (Fan et al., 2009). Thus, shock concentrations of antibiotics during the onset of a pandemic might affect WWTP functioning, although almost all treatment plants will be acclimated to lower antibiotic concentrations during the predominant interpandemic period. Further, while relatively low concentrations of single antibiotics are able to select for equally functional, but less diverse, antibiotic-tolerant communities, the question remains as to whether joint effects of several antibiotics might further reduce diversity and therefore functioning.

As seen above, investigations with full-scale treatment plants sometimes show a high functional stability also for other antibiotics. Most relevant to this study, Al-Ahmad et al. (1999) found that a mixture of 12 antibiotics tested in laboratory-scale treatment plants did not have effects on DOC elimination during 84 days of operation, even at concentrations that were 100 times higher than average influent concentrations. In contrast, effects of a sudden change in wastewater composition were apparent in two WWTPs purifying waste water from a pharmaceutical production site. Failure in ammonium oxidation and changes in the composition of ammonium-oxidizing bacterial communities were found when the plant started producing imidazoles, and problems continued for a few weeks (Wittebolle et al., 2005).

To conclude, congruence of modeled toxicity with experimental data on toxicity of shock loads of antibiotics suggests that a severe pandemic might indeed provide antibiotic concentrations capable of affecting microbial WWTP consortia, at least for a short time. This could compromise vital and obligate microbial functions such as nitrification, phosphorus, and COD removal. With longer exposure duration, changes in community composition, together with an increase in genetically encoded antibiotic resistance (see Section 1.3.2), might preserve WWTP functioning.

26.3.1 Effects on Antibiotic Resistance

The projected widespread use of antibacterials during an influenza pandemic introduces an opportunity for the rapid spread of antibiotic resistance. Bacteria can become resistant to antibiotics by mutations or the acquisition of appropriate genes from other microorganisms (Verhoef and Fluit, 2006). Many publications have shown that WWTPs are a hot spot for the occurrence of resistance genes (Schluter

et al., 2003; Szczepanowski et al., 2009), showing that the human community discharges bacteria and mobile genetic elements carrying resistance genes to the WWTP in interpandemic times. Further, WWTPs have been discussed as environments for gene transfer between bacteria (Geisenberger et al., 1999), and the presence of residual antibiotics could speed such genetic exchange. In any case, massive use of antibiotics during the pandemic will further resistance development in human microbiota (Barlow, 2009), and a significant proportion of these will reach WWTPs and be discharged with WWTP effluent after incomplete purification.

An additional question is whether elevated concentrations of antibiotics during a pandemic might be high enough for selection and recombination of resistant bacteria in the WWTP. There is little experimental evidence to prove or disprove this idea, as most studies so far have investigated treatment plants that also received human waste, presumably containing resistant bacteria. However, recent investigations in WWTPs treating waste from antibiotic production sites showed that high concentrations of antibiotics can indeed select for resistant populations that can subsequently be discharged to the receiving rivers (Li et al., 2009; 2010). In the first production plant, concentrations of penicillin G were around 150 μg/L (PAF of amoxicillin: 34%). Extremely high MICs for β-lactam antibiotics were observed in isolates from the WWTP, while resistance to unrelated antibiotics was also elevated. In the second plant, which produced oxytetracycline (30.5 mg/L in the sludge, equivalent to a doxycycline PAF of 92%), extremely high MICs were again observed in isolates tested for resistance to different tetracyclines. Also, many isolates showed multiresistance to unrelated antibiotics. Isolates often contained class I integrons, genetic elements that can integrate a multitude of resistance genes into bacterial genomes. From these two studies, it indeed appears that antibiotics can select for resistance in WWTPs.

26.4 CONCLUSIONS

Through epidemiological simulations and environmental and toxicity modeling, the concentrations and effects of antibiotics during an influenza pandemic were determined. It was shown that a severe pandemic might affect WWTP consortia and lead to increased nitrogen and COD release, at least for short periods. Pandemic concentrations are also likely to increase antibiotic resistance beyond levels already observed in WWTP. In line with modeling results obtained for a mild pandemic, no widespread WWTP failures were observed during the 2009/2010 influenza pandemic. Still, current experimental data is insufficient to disprove possible effects during an influenza pandemic characterized by a higher infectivity.

REFERENCES

Ahrer W, Scherwenk E, Buchberger W (2001). Determination of drug residues in water by the combination of liquid chromatography or capillary electrophoresis with electrospray mass spectrometry. *J Chromatogr* A 910(1):69–78.

Al-Ahmad A, Daschner FD, Kümmerer K (1999). Biodegradability of cefotiam, ciprofloxacin, meropenem, penicillin G, and sulfamethoxazole and inhibition of waste water bacteria. *Arch Environ Contam Toxicol* 37(2):158–163.

Alexy R, Kumpel T, Kümmerer K (2004). Assessment of degradation of 18 antibiotics in the closed bottle test. *Chemosphere* 57(6):505–512.

Alexy R, Sommer A, Lange FT, Kümmerer K (2006). Local use of antibiotics and their input and fate in a small sewage treatment plant—Significance of balancing and analysis on a local scale vs. nationwide scale. *Acta Hydrochim Hydrobiol* 34(6):587–592.

Alighardashi A, Pandolfi D, Potier O, Pons MN (2009). Acute sensitivity of activated sludge bacteria to erythromycin. *J Hazard Mater* 172(2–3):685–692.

Anderson RM, May RM. 1991. *Infectious Diseases of Humans: Dynamics and Control*. Oxford University Press, Oxford.

Andreozzi R, Raffaele M, Nicklas P (2003). Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment. *Chemosphere* 50(10):1319–1330.

Ashton D, Hilton M, Thomas KV (2004). Investigating the environmental transport of human pharmaceuticals to streams in the United Kingdom. *Sci Total Environ* 333(1–3):167–184.

American Thoracic Society (ATS) (2005). Guidelines for the management of adults with hospital-acquired, ventilator-associated, and healthcare-associated pneumonia. *Am J Respir Crit Care Med* 171:388–416.

Balcan D, Colizza V, Gonçalves B, Hu H, Ramasco JJ, Vespignani A (2009a). Multiscale mobility networks and the large scale spreading of infectious diseases. *Proc Nat Acad Sci* 106:21484–21489.

Balcan D, et al. (20090b). Modeling the critical care demand and antibiotics resources needed during the Fall 2009 wave of influenza A(H1N1) pandemic. *PLoS Currents Influenza*. Available: http://knol.google.com/k/duygubalcan/modeling-the-critical-care-demand-and/3cbpxfuorniyr/9. Accessed June 30, 2010.

Bannister B, Gillespie S, Jones J. (2006). *Infection: Microbiology and Management*, 3rd ed. Blackwell, Oxford.

Barlow GD (2009). Swine flu and antibiotics. *J Antimicrob Chemother* 64(5):889–894.

Barr IG, Deng YM, Iannello P, Hurt AC, Komadina N (2008). Adamantane resistance in influenza A(H1) viruses increased in 2007 in South East Asia but decreased in Australia and some other countries. *Antiviral Research* 80(2):200–205 doi:10.1016/j.antiviral.2008.06.008

Batt AL, Aga DS (2005). Simultaneous analysis of multiple classes of antibiotics by ion trap LC/MS/MS for assessing surface water and groundwater contamination. *Anal Chem* 77:2940–2947.

Batt AL, Bruce IB, Aga DS (2006). Evaluating the vulnerability of surface waters to antibiotic contamination from varying wastewater treatment plant discharges. *Environ Pollut* 142(2):295–302.

Batt AL, Kim S, Aga DS (2007). Comparison of the occurrence of antibiotics in four full-scale wastewater treatment plants with varying designs and operations. *Chemosphere* 68(3):428–435.

Bendz D, Paxeus NA, Ginn TR, Loge FJ (2005). Occurrence and fate of pharmaceutically active compounds in the environment, a case study: Hoje River in Sweden. *J Hazard Mater* 122(3):195–204.

Benotti MJ, Brownawell BJ (2009). Microbial degradation of pharmaceuticals in estuarine and coastal seawater. *Environ Pollut* 157(3):994–1002.

Bound JP, Voulvoulis N (2006). Predicted and measured concentrations for selected pharmaceuticals in UK rivers: Implications for risk assessment. *Water Res* 40(15):2885–2892.

Brain RA, et al. (2004a.) Microcosm evaluation of the effects of an eight pharmaceutical mixture to the aquatic macrophytes *Lemna gibba* and *Myriophyllum sibiricum*. *Aquatic Toxicolo* 70(1):23–40.

Brain RA, Johnson DJ, Richards SM, Sanderson H, Sibley PK, Solomon KR (2004b). Effects of 25 pharmaceutical compounds to *Lemna gibba* using a seven-day static-renewal test. *Environ Toxicol Chem* 23(2):371–382.

Brown KD, Kulis J, Thomson B, Chapman TH, Mawhinney DB (2006). Occurrence of antibiotics in hospital, residential, and dairy effluent, municipal wastewater, and the Rio Grande in New Mexico. *Sci Total Environ* 366(2–3):772–783.

Brun GL, Bernier M, Losier R, Doe K, Jackman P, Lee H-B (2006). Pharmaceutically active compounds in Atlantic Canadian sewage treatment plant effluents and receiving waters, and potential for environmental effects as measured by acute and chronic aquatic toxicity. *Environ Toxicol Chemi* 25(8):2163–2176.

Brundage JF (2006). Interactions between influenza and bacterial respiratory pathogens: Implications for pandemic preparedness. *Lancet Infecti Dis* 6(5):303–312.

Brundage JF, Shanks GD (2008). Deaths from bacterial pneumonia during 1918–19 influenza pandemic. *Emerg Infect Dis* 14(8):1193–1198.

Bundschuh M, Hahn T, Gessner MO, Schulz R (2009). Antibiotics as a chemical stressor affecting an aquatic decomposer-detritivore system. *Environ Toxicol Chem* 28(1):197–203.

Buser HR, Poiger T, Muller MD (1998). Occurrence and fate of the pharmaceutical drug diclofenac in surface waters: Rapid photodegradation in a lake. *Environ Sci Technol* 32(22):3449–3456.

Cabinet Office (2008). National Risk Register. Available: http://www.cabinetofficegovuk/~/media/assets/www.cabinetofficegovuk/publications/reports/national_risk_register/national_risk_register%20pdfashx.

Carballa M, et al. (2004). Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. *Water Res* 38(12):2918–2926.

Carlsson C, Johansson A-K, Alvan G, Bergman K, Kuhler T (2006). Are pharmaceuticals potent environmental pollutants? Part I: Environmental risk assessments of selected active pharmaceutical ingredients. *Sci Total Environ* 364(1–3):67–87.

Choi K-J, Kim S-G, Kim C-w, Kim S-H (2007). Determination of antibiotic compounds in water by on-line SPE-LC/MSD. *Chemosphere* 66(6):977–984.

Christensen FM (1998). Pharmaceuticals in the environment: A human risk. *Regul Toxicol Pharmacol* 28:212–221.

Coenen S, et al. (2006). European surveillance of antimicrobial consumption (ESAC): Outpatient cephalosporin use in Europe. *J Antimicrob Chemother* 58(2):413–417.

Conleya JM, Symes SJ, Kindelberger SA, Richards SM (2008). Rapid liquid chromatography–tandem mass spectrometry method for the determination of a broad mixture of pharmaceuticals in surface water. *J Chromatogr A* 1185(2):206–215.

Cordy GE, et al. (2004). Do pharmaceuticals, pathogens, and other organic waste water compounds persist when waste water is used for recharge? Ground *Water Monitor Remediat* 24(2):58–69.

Costanzo SD, Murby J, Bates J (2005). Ecosystem response to antibiotics entering the aquatic environment. *Marine Pollut Bull* 51(1–4):218–223.

de Neeling AJ, Overbeek BP, Horrevorts AM, Ligtvoet EEJ, Goettsch WG (2001). Antibiotic use and resistance of *Streptococcus pneumoniae* in The Netherlands during the period 1994–1999. *J Antimicrob Chemother* 48(3):441–444.

Department of Health (2007). Government launches new flu pandemic plan. Available:http://ndscoigovuk/environment/fullDetailasp?ReleaseID=332808&NewsAreaID=2&NavigatedFromDepartment=False. Accessed July 16, 2008.

Department of Health and Human Services, Department of Homeland Security (2007). Draft guidance on allocating and targeting pandemic influenza vaccine. Available: http://www.pandemicflugov/vaccine/prioritizationpdf.

De Zwart D, Posthuma L (2005). Complex mixture toxicity for single and multiple species: Proposed methodologies. *Environ Toxicol Chem* 24(10):2665–2676.

Dokianakis SN, Kornaros ME, Lyberatos G (2004). On the effect of pharmaceuticals on bacterial nitrite oxidation. *Water Sci Technol* 50(5):341–346.

Dollery C. 1999. *Therapeutic Drugs*, 2nd ed. Harcourt Brace, London.

Drewes JE, Heberer T, Rauch T, Reddersen K (2003). Fate of pharmaceuticals during ground water recharge. *Ground Water Monitor Remediat* 23(3):64–72.

Environment Agency (EA) (2003). Targeted monitoring programme for pharmaceuticals in the aquatic environment. R&D Technical Report P6-012/06/TR. EA, Bristol.

EUCAST (2009). Antimicrobial wild type distributions of microorganisms. Available: http://217703399/Eucast2/. Accessed January 12, 2010.

European Chemicals Agency (2008). Guidance on information requirements and chemical safety assessment, Chapter R.10: Characterisation of dose [concentration]-response for environment.

European Influenza Surveillance Scheme (2008). Pandemic plans on the Internet. Available: http://www.eissorg/html/pandemic_planshtml. Accessed July 16, 2008.

European Surveillance of Antimicrobial Consumption project (ESAC) (2006). ESAC interactive database. Available: http://www.esacuaacbe/esac_service/applet/eidbhtml.

Fan C, et al. (2009). Influence of trace erythromycin and eryhthromycin-H_2O on carbon and nutrients removal and on resistance selection in sequencing batch reactors (SBRs). *Appl Microbiol Biotechnol* 85(1):185–195.

Ferech M, et al. (2006). European surveillance of antimicrobial consumption (ESAC): Outpatient penicillin use in Europe. *J Antimicrob Chemother* 58(2):408–412.

Fowler RA, et al. (2005). Cost-effectiveness of defending against bioterrorism: A comparison of vaccination and antibiotic prophylaxis against anthrax. *Ann Intern Med* 142(8):601–610.

García-Rey C, Aguilar L, Baquero F, Casal J, Dal-Re R (2002). Importance of local variations in antibiotic consumption and geographical differences of erythromycin and penicillin resistance in *Streptococcus pneumoniae*. *J Clin Microbiol* 40(1):159–164.

Gartiser S, Urich E, Alexy R, Kümmerer K (2007). Ultimate biodegradation and elimination of antibiotics in inherent tests. *Chemosphere* 67(3):604–613.

Geisenberger O, Ammendola A, Christensen BB, Molin Sr, Schleifer K-H, Eberl L (1999). Monitoring the conjugal transfer of plasmid RP4 in activated sludge and in situ identification of the transconjugants. *FEMS Microbiol Lett* 174(1):9–17.

Giger W, et al. (2003). Occurrence and fate of antibiotics as trace contaminants in wastewaters, sewages ludges, and surface waters. *CHIMIA Int J Chem* 57:485–491.

Golet EM, Alder AC, Hartmann A, Ternes TA, Giger W (2001). Trace determination of fluoroquinolone antibacterial agents in urban wastewater by solid-phase extraction and liquid chromatography with fluorescence detection. *Anal Chem* 73(15):3632–3638.

Gomez MJ, Martinez Bueno MJ, Lacorte S, Fernandez-Alba AR, Aguera A (2007). Pilot survey monitoring pharmaceuticals and related compounds in a sewage treatment plant located on the Mediterranean coast. *Chemosphere* 66(6):993–1002.

Gulkowska A, et al. (2008). Removal of antibiotics from wastewater by sewage treatment facilities in Hong Kong and Shenzhen, China. *Water Res* 42(1–2):395–403.

Gupta RK, George R, Nguyen-Van-Tam JS (2008). Bacterial pneumonia and pandemic influenza planning. *Emerg Infect Dis* 14(8):1187–1192.

Haggard BE, Galloway JM, Green WR, Meyer MT (2006). Pharmaceuticals and other organic chemicals in selected North-Central and Northwestern Arkansas streams. *J Environ Qual* 35(4):1078–1087.

Hampson AW (2008). Vaccines for pandemic influenza. The history of our current vaccines, their limitations and the requirements to deal with a pandemic threat. *Ann Acad Med Singapore* 37(6):510–518.

He G, Massarella J, Ward P (1999). Clinical pharmacokinetics of the prodrug oseltamivir and its active metabolite Ro 64–0802. *Clin Pharmacokinet* 37(6):471–484.

Heberer T (2002). Tracking persistent pharmaceutical residues from municipal sewage to drinking water. *J Hydrol* 266(3–4):175–189.

Hirsch R, Ternes T, Haberer K, Kratz K-L (1999). Occurrence of antibiotics in the aquatic environment. *Sci Total Environ* 225(1–2):109–118.

Hurd HS, Raef TA (2010). ISU Associate Professor and Former USDA Deputy Undersecretary Food Safety Responds to CBS News Segments on Antibiotics—Feb. 9 and 10. Available: http://vetmediastateedu/news/isu-associate-professor-and-former-usda-deputy-undersecretary-food-safety-responds-cbs-news-seg.

Hurt AC, Selleck P, Komadina N, Shaw R, Brown L, Barr IG (2007). Susceptibility of highly pathogenic A(H5N1) avian influenza viruses to the neuraminidase inhibitors and adamantanes. *Antiviral Res* 73(3):228–231.

Jefferson T (2007). Look at all the evidence before stockpiling amantadine. *BMJ* 334 (7591):439-b–.

Jones OAH, Voulvoulis N, Lester JN (2002). Aquatic environmental assessment of the top 25 English prescription pharmaceuticals. *Water Res* 36(20):5013–5013.

Jones-Lepp TL (2006). Chemical markers of human waste contamination: Analysis of urobilin and pharmaceuticals in source waters. *J Environ Monitor* 8:472–478.

Joss A, et al. (2006). Biological degradation of pharmaceuticals in municipal wastewater treatment: Proposing a classification scheme. *Water Res* 40(8):1686–1686.

Junker T, Alexy R, Knacker T, Kümmerer K (2006). Biodegradability of ^{14}C-labeled antibiotics in a modified laboratory scale sewage treatment plant at environmentally relevant concentrations. *Environ Sci Technol* 40(1):318–324.

Kaiser L, Wat C, Mills T, Mahoney P, Ward P, Hayden F (2003). Impact of oseltamivir treatment on influenza-related lower respiratory tract complications and hospitalizations. *Arch Intern Med* 163(14): 1667–1672.

Kamigaki T, Oshitani H (2009). Epidemiological characteristics and low case fatality rate of pandemic (H1N1) 2009 in Japan. *PLoS Curr* 1:RRN1139.

Kati WM, et al. (2002). In vitro characterization of A-315675, a highly potent inhibitor of A and B strain influenza virus neuraminidases and influenza virus replication. *Antimicrob Agents Chemother* 46(4):1014–1021.

Kim SD, Cho J, Kim IS, Vanderford BJ, Snyder SA (2007). Occurrence and removal of pharmaceuticals and endocrine disruptors in South Korean surface, drinking, and waste waters. *Water Res* 41(5):1013–1021.

Kolpin DW, et al. (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999–2000: A national reconnaissance. *Environ Sci Technol* 36(6):1202–1211.

Kramarz P, Monnet D, Nicoll A, Yilmaz C, Ciancio B (2009). Use of oseltamivir in 12 European countries between 2002 and 2007—Lack of association with the appearance of oseltamivir-resistant influenza A(H1N1) viruses. *Euro Surveill* 14(5).

Kümmerer K (2009a). Antibiotics in the aquatic environment—A review—Part I. *Chemosphere* 75(4):417–434.

Kümmerer K (2009b). Antibiotics in the aquatic environment—A review—Part II. *Chemosphere* 75(4):435–441.

Kümmerer K, Al-Ahmad A, Mersch-Sundermann V (2000). Biodegradability of some antibiotics, elimination of the genotoxicity and affection of wastewater bacteria in a simple test. *Chemosphere* 40(7):701–710.

Li D, et al. (2009). Antibiotic-resistance profile in environmental bacteria isolated from penicillin production wastewater treatment plant and the receiving river. *Environ Microbiol* 11(6):1506–1517.

Li D, et al. (2010). Antibiotic resistance characteristics of environmental bacteria from an oxytetracycline production wastewater treatment plant and the receiving river. *Appl Environ Microbiol* 76(11):3444–3451.

Liebig M, Moltmann JF, Knacker T (2006). Evaluation of measured and predicted environmental concentrations of selected human pharmaceuticals and personal care products. *Environ Sci Pollut Res* 13(2):110–119.

Lim WS (2007). Pandemic flu: Clinical management of patients with an influenza-like illness during an influenza pandemic. *Thorax* 62(Suppl1):1–46.

Lindberg RH, Bjorklund K, Rendahl P, Johansson MI, Tysklind M, Andersson BAV (2007). Environmental risk assessment of antibiotics in the Swedish environment with emphasis on sewage treatment plants. *Water Res* 41(3):613–619.

Lindqvist N, Tuhkanen T, Kronberg L (2005). Occurrence of acidic pharmaceuticals in raw and treated sewages and in receiving waters. *Water Res* 39(11):2219–2228.

Lishman L, et al. (2006). Occurrence and reductions of pharmaceuticals and personal care products and estrogens by municipal wastewater treatment plants in Ontario, Canada. *Sci Total Environ* 367(2–3):544–558.

Louria DB, Blumenfeld HL, Ellis JT, Kilbourne ED, Rogers DE (1959). Studies on influenza in the pandemic of 1957–1958. II. Pulmonary complications of influenza. *J Clin Invest* 38(1 Pt 1–2):213–265.

Louvet JN, Giammarino C, Potier O, Pons MN (2010a). Adverse effects of erythromycin on the structure and chemistry of activated sludge. *Environ Pollut* 158(3):688–693.

Louvet JN, Heluin Y, Attik G, Dumas D, Potier O, Pons MN (2010b). Assessment of erythromycin toxicity on activated sludge via batch experiments and microscopic techniques (epifluorescence and CLSM). *Process Biochem* 45(11):1787–1794.

McArdell CS, Molnar E, Suter MJF, Giger W (2003). Occurrence and fate of macrolide antibiotics in wastewater treatment plants and in the Glatt Valley Watershed, Switzerland. *Environ Sci Technol* 37(24):5479–5486.

Meltzer MI, Cox NJ, Fukuda K (1999). The economic impact of pandemic influenza in the United States: Priorities for intervention. *Emerg Infect Dis* 5(5):659–671.

Metcalfe CD, Miao XS, Koenig BG, Struger J (2003). Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. *Environ Toxicol Chem* 22(12):2881–2889.

Miao X-S, Metcalfe CD (2003). Determination of cholesterol-lowering statin drugs in aqueous samples using liquid chromatography-electrospray ionization tandem mass spectrometry. *J Chromatogr A* 998(1–2):133–141.

Miao XS, Bishay F, Chen M, Metcalfe CD (2004). Occurrence of antimicrobials in the final effluents of wastewater treatment plants in Canada. *Environ Sci Technol* 38(13):3533–3541.

Moldovan Z (2006). Occurrences of pharmaceutical and personal care products as micropollutants in rivers from Romania. *Chemosphere* 4(11):1808–1817.

Morens DM, Taubenberger JK, Fauci AS (2008). Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: Implications for pandemic influenza preparedness. *J Infect Dis* 198(7):962–970.

Morse SS (2007). The US pandemic influenza implementation plan at six months. *Nat Med* 13 (6):681–684.

Mounier-Jack S, Coker R (2006). Pandemic influenza: Are Europe's institutions prepared? *Eur J Public Health* 16(2):119–120.

Muller A, Coenen S, Monnet DL, Goossens H, group TEp (2007). European surveillance of antimicrobial consumption (ESAC): Outpatient antibiotic use in Europe, 1998–2005. *Eurosurveillance* 12(41):1.

Nap RE, Andriessen MPHM, Meessen NEL, van der Werf TS (2007). Pandemic influenza and hospital resources. *Emerg Infect Dis* 13(11). Available:http://www.cdc.gov/EID/content/13/11/1714.htm.

NAS (2008). Antivirals for pandemic influenza: Guidance on developing a distribution and dispensing program. Available: http://booksnapedu/openbookphp?record_id = 12170&page = R1.

National Centers for Coastal Ocean Science (19xx). Pharmaceuticals in the environment, information for assessing risk. Available:http://www.chbrnoaagov/peiar/defaultaspx.

New England Journal of Medicine Editors (2009). When to consider the use of antibiotics in the treatment of 2009 H1N1 influenza-associated pneumonia. *N Engl J Med*. Available: http://h1n1.nejm.org/?p = 1234&query = TOC#.

Newman M, Ownby D, Mezin L, Powell D, Christensen T, Lerberg S (2002). Species sensitivity distributions in ecological risk assessment: Distributional assumptions, alternate bootstrap techniques and estimation of adequate number of species. In L. Posthuma, G. Suter, and T Traas (Eds), *Species Sensitivity Distributions in Ecotoxicology*, CRC Press, Boca Raton, FL, p. 616.

NHS BSA (2008). Prescribing analysis charts: National antibiotics charts. Available:http://www.nhsbsa.nhs.uk/PrescriptionServices/Documents/NPC_Antibiotics_July_2008.ppt. Accessed June 30, 2010.

Nicholson KG, et al. (2000). Efficacy and safety of oseltamivir in treatment of acute influenza: A randomised controlled trial. Neuraminidase Inhibitor Flu Treatment Investigator Group. *Lancet* 355(9218):1845–1850.

Ong AK, Hayden FG (2007). John F. Enders Lecture 2006: Antivirals for Influenza. *J Infect Dis* 196(2):181–190.

Osterholm M (2005). Preparing for the next pandemic. *Foreign Affairs* 84. Available:http://www.foreignaffairs.org/20050701faessay84402/michael-t-osterholm/preparing-for-the-next-pandemic.html.

Peltola Ville T, Murti KG, McCullers Jonathan A (2005). Influenza virus neuraminidase contributes to secondary bacterial pneumonia. *J Infect Dis* 192(2):249–257.

Pharmaceuticals B (2007). Biocryst awarded $102.6 million from U.S. Department of Health an Human Services to develop Peramivir for seasonal and pandemic influenza.

Prado N, Ochoa J, Amrane A (2009). Biodegradation by activated sludge and toxicity of tetracycline into a semi-industrial membrane bioreactor. *Bioresource Technol* 100(15):3769–3774.

Prado N, Montéléon C, Ochoa J, Amrane A (2010). Evaluation of the toxicity of veterinary antibiotics on activated sludge using modified Sturm tests—Application to tetracycline and tylosine antibiotics. *J Chem Technol Biotechnol* 85(4):471–477.

Priest P, Yudkin P, McNulty C, Mant D, Wise R (2001). Antibacterial prescribing and antibacterial resistance in English general practice: cross sectional study commentary: Antibiotic resistance is a dynamic process. *BMJ* 323(7320):1037–1041.

Public Health Agencyof Canada (2004). Canadian pandemic influenza plan for the health sector. Annex G, health services: Clinical care guidelines and tools, Appendix 5. IV. Antibiotics. Available:http://www.phac-aspcgcca/cpip-pclcpi/pdf-e/16-CPIP-Appendix-G-Clinical_epdf. Accessed July 28, 2008.

Rabiet M, Togola A, Brissaud F, Seidel JL, Budzinski H, Elbaz-Poulichet F (2006). Consequences of treated water recycling as regards pharmaceuticals and drugs in surface and ground waters of a medium-sized Mediterranean catchment. *Environ Sci Technol* 40 (17):5282–5288.

Rainsford K (2006). Influenza ("Bird Flu"), inflammation and anti-inflammatory/analgesic drugs. *Inflammopharmacology* 14(1):2–9.

Reemtsma T, et al. (2006). Polar pollutants entry into the water cycle by municipal wastewater: A European perspective. *Environ Sci Technol* 40(17):5451–5458.

Reuters (2007). Roche gives workers flu drug in case of pandemic. Available:http://www.reuterscom/article/governmentFilingsNews/idUSN0834658920070308?pageNumber = 1. Accessed March 8, 2007.

Roberts PH, Thomas KV (2006). The occurrence of selected pharmaceuticals in wastewater effluent and surface waters of the lower Tyne catchment. *Sci Total Environ* 356(1–3):143–153.

Roche H-L (2007). Tamiflu: Pharmacological properties. Available:http://emcmedicinesorguk/emc/industry/defaultasp?page = displaydocasp&documentid = 10446. Updated September 12, 2007.

Roche Group (2006). Factsheet Tamiflu. March 20.

Sacher F, Lange FT, Brauch H-J, Blankenhorn I (2001). Pharmaceuticals in groundwaters: Analytical methods and results of a monitoring program in Baden-Wurttemberg, Germany. *J Chromatogr A* 938(1–2): 199–210.

Salomon R, Hoffmann E, Webster RG (2007). Inhibition of the cytokine response does not protect against lethal H5N1 influenza infection. *PNAS* 104(30):12479–12481.

Schluter A, et al. (2003). The 64 508 bp IncP-1{beta} antibiotic multiresistance plasmid pB10 isolated from a waste-water treatment plant provides evidence for recombination between members of different branches of the IncP-1{beta} group. *Microbiology* 149 (11):3139–3153.

Schwarzmann SW, Sullivan RJ (1971). Bacterial pneumonia during the Hong Kong influenza epidemic of 1968–1969. *Arch Internal Med* 127:1037–1041.

Singer AC, Nunn MA, Gould EA, Johnson AC (2007). Potential risks associated with the widespread use of tamiflu. *Environ Health Perspect* 115(1):102–106.

Singer AC, Colizza V, Schmitt H, Andrews J, Balcan D, Huang WE, Keller VDJ, Vespignani A, Williams RJ (2011). Assessing the ecotoxicologic hazards of a pandemic influenza medical response. *Environmental Health Perspectives*. 119(8):1084–1090 doi:10.1289/ehp.1002757

Siquier B, et al. (2006). Efficacy and safety of twice-daily pharmacokinetically enhanced amoxicillin/clavulanate (2000/125 mg) in the treatment of adults with community-acquired pneumonia in a country with a high prevalence of penicillin-resistant *Streptococcus pneumoniae*. *J Antimicrob Chemother* 57(3):536–545.

Smith DL, Harris AD, Johnson JA, Silbergeld EK, Morris JG Jr (2002). Animal antibiotic use has an early but important impact on the emergence of antibiotic resistance in human commensal bacteria. *PNAS* 99(9):6434–6439.

Stackelberg PE, Furlong ET, Meyer MT, Zaugg SD, Henderson AK, Reissman DB (2004). Persistence of pharmaceutical compounds and other organic wastewater contaminants in a conventional drinking-water-treatment plant. *Sci Total Environ* 329(1–3):99–113.

Szczepanowski R, et al. (2009). Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. *Microbiology* 155(7):2306–2319.

ter Laak TL, van der Aa M, Houtman CJ, Stoks PG, van Wezel AP (2010). Relating environmental concentrations of pharmaceuticals to consumption: A mass balance approach for the river Rhine. *Environ Int* 36(5):403–409.

Ternes TA (1998). Occurrence of drugs in German sewage treatment plants and rivers. *Water Res* 32(11):3245–3260.

Ternes TA (2001). Analytical methods for the determination of pharmaceuticals in aqueous environmental samples. *TrAC Trends Anal Chem* 20(8):419–434.

Ternes TA, et al. (2002). Removal of pharmaceuticals during drinking water treatment. *Environ Sci Technol* 36(17):3855–3863.

Thomas KV, Hilton M (2003). Targeted monitoring programme for pharmaceuticals in the aquatic environment. UK Environment Agency R&D Technical Report P6–012/06. Environment Agency, United Kingdom.

Tierney E, Reddy D (2007). Roche media briefing: Update on current developments around Tamiflu (Basel, 26 April 2007). Available:http://www.rochecom/media/events/media-briefing-tamiflu-2007htm. Accessed January 12, 2010.

Tixier C, Singer HP, Oellers S, Muller SR (2003). Occurrence and fate of carbamazepine, clofibric acid, diclofenac, ibuprofen, ketoprofen, and naproxen in surface Waters. *Environ Sci Technol* 37(6):1061–1068.

Treanor JJ, et al. (2000). Efficacy and Safety of the oral neuraminidase inhibitor oseltamivir in treating acute influenza. *JAMA* 283(8):1016–1024.

Trenholm RA, Vanderford BJ, Holady JC, Rexing DJ, Snyder SA (2006). Broad range analysis of endocrine disruptors and pharmaceuticals using gas chromatography and liquid chromatography tandem mass spectrometry. *Chemosphere* 65(11):1990–1998.

U.S. Centers for Disease Control and Prevention (2007). Interim pre-pandemic planning guidance: Community strategy for pandemic influenza mitigation in the United States: Early, targeted, layered use of nonpharmaceutical interventions. Available:http://health-vermontgov/panflu/documents/0207interimguidancepdf.

Uscher-Pines L, Omer SB, Barnett DJ, Burke TA, Balicer RD (2006). Priority setting for pandemic influenza: An analysis of national preparedness plans. *PLoS Medi* 3(10):e436.

UK Department of Health (2007a). Pandemic flu: A national framework for responding to an influenza pandemic (22 November). Available:http://www.dhgovuk/en/Publicationsandstatistics/Publications/PublicationsPolicyAndGuidance/dh_080734. Accessed October 2, 2009a.

UK Department of Health (2007b). The use of antibiotics for pandemic influenza: Scientific evidence base. (Available:http://www.dh.gov.uk/prod_consum_dh/groups/dh_digitalassets/@dh/@en/documents/digitalasset/dh_077274.pdf. Accessed June 30, 2010.

UK Department of Health (2009). Swine Flu: UK planning assumptions. Available:http://www.dh.gov.uk/en/Publicationsandstatistics/Publications/PublicationsPolicyAndGuidance/DH_104844. Accessed April 7, 2010.

US Department of Health and Human Services (HHS) (2005). Pandemic influenza plan. Available:http://www.hhs.gov/pandemicflu/plan/pdf/HHSPandemicInfluenzaPlan.pdf. Accessed June 30, 2010.

U.S. Department of Health and Human Services (HHS) (2006). Pandemic flu planning checklist for individuals and families. Available:http://www.pandemicflugov/plan/individual/checklisthtml. Accessed July 7, 2008.

U.S. Department of Health and Human Services (HHS) (2007). Community strategy for pandemic influenza mitigation. Availale: http://www.pandemicflugov/plan/community/commitigationhtml#IV. Accessed July 14, 2008.

U.S. Department of Health and Human Services (HHS) (2008). HHS awards contracts for the development of faster influenza diagnostic tests. Available:http://www.hhsgov/news/press/2008pres/06/20080612ahtml. Accessed July 7, 2008.

U.S. Environmental Protection Agency (2007). Estimation Program Interface (EPI) Suite v3.2. Available:http://www.epagov/oppt/exposure/pubs/episuitehtm. Accessed January 12, 2010.

U.S. Homeland Security Council (2007). National strategy for pandemic influenza: Implementation plan one year summary. Available:http://snipurlcom/2okhb.

Verhoef J, Fluit A (2006). Surveillance uncovers the smoking gun for resistance emergence. *Biochem Pharmacol* 71(7):1036–1036.

Vieno NM, Tuhkanen T, Kronberg L (2005). Seasonal variation in the occurrence of pharmaceuticals in effluents from a sewage treatment plant and in the recipient water. *Environ Sci Technol* 39(21):8220–8226.

Vieno NM, Tuhkanen T, Kronberg L (2006). Analysis of neutral and basic pharmaceuticals in sewage treatment plants and in recipient rivers using solid phase extraction and liquid chromatography-tandem mass spectrometry detection. *J Chromatogr A* 1134(1–2):101–111.

Wan H, et al. (2008). Replication and transmission of H9N2 influenza viruses in ferrets: Evaluation of pandemic potential. *PLoS ONE* 3(8): e2923.

Wennmalm AGB (2005). Public health care management of water pollution with pharmaceuticals: Environmental classification and analysis of pharmaceutical residues in sewage water. *Drug Inform J* 39(3).

Whitley RJ, et al. (2001). Oral oseltamivir treatment of influenza in children. *Pediat Infect Dis J* 20(2):127–133.

Wiegel S, et al. (2004). Pharmaceuticals in the river Elbe and its tributaries. *Chemosphere* 57 (2):107–126.

Wishart DS, et al. (2006). DrugBank: A comprehensive resource for in silico drug discovery and exploration. *Nucl Acids Res* 34(Suppl 1):D668–672.

Wittebolle L, Boon N, Vanparys B, Heylen K, De Vos P, Verstraete W (2005). Failure of the ammonia oxidation process in two pharmaceutical wastewater treatment plants is linked to shifts in the bacterial communities. *J Appl Microbiol* 99(5):997–1006.

World Health Organization (WHO) (2004). The anatomical therapeutic chemical classification system with defined daily doses (ATC/DDD). Available:http://www.whocc.no/atcddd/indexdatabase/index.php. Accessed July 8, 2008.

World Health Organization (WHO) (2005). WHO global influenza preparedness plan. Available:http://www.whoint/csr/resources/publications/influenza/GIP_2005_5Ewebpdf.

World Health Organization (WHO) (2006a). Global pandemic influenza action plan to increase vaccine supply. Available:http://www.whoint/csr/resources/publications/influenza/CDS_EPR_GIP_2006_1pdf.

World Health Organization (WHO) (2006b). World Health Organization pandemic influenza draft protocol for rapid response and containment (Updated draft 17 March 2006). Available:http://tinyurlcom/g5col. Accessed May, 15 2008.

World Health Organization (WHO) (2007). The World Health Report: A safer future: Global health security in the 21st century. Available:http://www.whoint/whr/2007/whr07_enpdf.

Yang S, Carlson K (2003). Evolution of antibiotic occurrence in a river through pristine, urban and agricultural landscapes. *Water Res* 37(19):4645–4656.

Zuccato E, Calamari D, Natangelo M, Fanelli R (2000). Presence of therapeutic drugs in the environment. *Lancet* 355(9217):1789–1789.

27

USE OF VETERINARY ANTIBACTERIAL AGENTS IN EUROPE AND THE UNITED STATES

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27.1 INTRODUCTION

Because of concerns about emerging antimicrobial resistance and its apparent relation with usage of antibacterial agents, monitoring of antibiotic usage became a priority for policymakers and pharmacists in the early 1980s (Anonymous, 1984). Standardized collection and analysis of data on human use was initiated (see http://www.whocc.no/atc_ddd_methodology/history/) and subsequently associated with data on occurrence of antimicrobial resistance (McGowan, 1983, 1987; Anonymous, 1984; Franklin et al., 2001; Silley et al., 2011). Currently European antibiotic usage data in humans are reported annually [European Surveillance of Antimicrobial Consumption (ESAC)] and related to data on antimicrobial resistance in European and national reports (EARSS, Nethmap, DANMAP, SVARM, Norm). Veterinary use data on antimicrobial agents are still scarce. Moreover, the collection and analysis of veterinary use data is not yet standardized (Silley et al., 2011). Most European countries report use data based on sales figures of the pharmaceutical industry or prescriptions by veterinarians. In some countries a specific veterinary

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antimicrobial use monitoring program was developed as in the Netherlands and Belgium (Catry et al., 2007; MARAN, 2011).

Essential for comparisons of antimicrobial usage data is to relate the amount of sold or prescribed antibiotics to a relevant denominator. In human medicine antimicrobial usage is expressed per 1000 inhabitants for general pactitioner (GP) prescription and per 100 hospital bed-days for hospitals. In veterinary medicine the denominator is not yet standardized. Grave et al. (2010) related the sales of antimicrobials per country to the biomass (kilograms) produced. The biomass was estimated based on the numbers of slaughtered pigs, cattle and poultry and the number of live dairy cows present in a certain year. However, in the European Union (EU), millions of food-producing animals are exported annually as live animals and slaughtered abroad. Therefore, a more accurate denominator is the average biomass (kilograms) of live animals present in a country, based on the average numbers of animals present in a given time period (Eurostat, 2011).

For optimum comparisons, it is also essential to define animal daily dosages (ADD) for each antimicrobial pharmaceutical compound and each animal species (Jensen et al., 2004). Using ADDs and standardized average weights assigned for each animal species and age groups, the antimicrobial sales data (in kilograms) can be transformed to numbers of ADDs administered to a certain animal species per time period. This system, currently applied by the Netherlands, Denmark, and Belgium, provides optimum information of exposure of animals to antimicrobials and optimum comparison of antimicrobial use data between countries.

It is the purpose of the present chapter to quantify the veterinary antimicrobial use from 2001 to 2009 as numbers of ADDs per animal year for nine European countries and the United States. The calculation is based on reported veterinary antimicrobial sales or prescription data, defined ADDs, and average numbers of live food-producing animals present from Eurostat and the Food and Agriculture Organization (FAO) databases.

27.2 METHODS

Animal daily dosages (ADD) are defined for each antimicrobial pharmaceutical compound characterized by the ATC-vet classification system: For instance, oral gentamicin (oral anti-infective) has a different ADD than intravenous or intramuscular gentamicin.

Subsequently, the total numbers of ADDs per animal per year are calculated for each country, expressed as dd/ay (daily dosages per animal year, i.e., the average number of treatment days per animal per year). Human data are converted to the same definition by multiplying DDD/1000 Person-days with 0.365, and, when necessary hospital use by converting DDD/100 bed-days to DDD/1000 Person-days, and then multiplying with 0.365.

27.2.1 ADD Determination

We calculated the mean Dutch licensed dose in milligrams per kilogram per route of administration for each ATC-vet code for every animal species. Subsequently, the mean weighted dose for each pharmacotherapeutic group (e.g., tetracyclines) was

calculated, based on the relative contribution of every active ingredient belonging to that group, determined by its number of licensed products. In this way we reduced all individual antimicrobial pharmaceutical substances to 11 main therapeutic groups in 4 animal species and 5 routes of administration (see Fig.27.1). Figure 27.1 illustrates the available routes of administration per species and compiles all groups in one bar. The height of this bar is more or less indicative of the interspecies differences in dosing: Poultry have high doses (especially in oral anti-infectives and oral TMPS), while intramammary doses of penicillins are relatively low.

Finally, per country the mean dose per group was calculated taking into account the main route of administration, orally for most groups in pigs and parenterally in cattle, exclusively parenteral for aminoglycosides (because the orally applied aminoglycosides belong to the ATC-vet group of oral anti-infectives), and third- or fourth- generation cephalosporins and the composition of the livestock (Table 27.1).

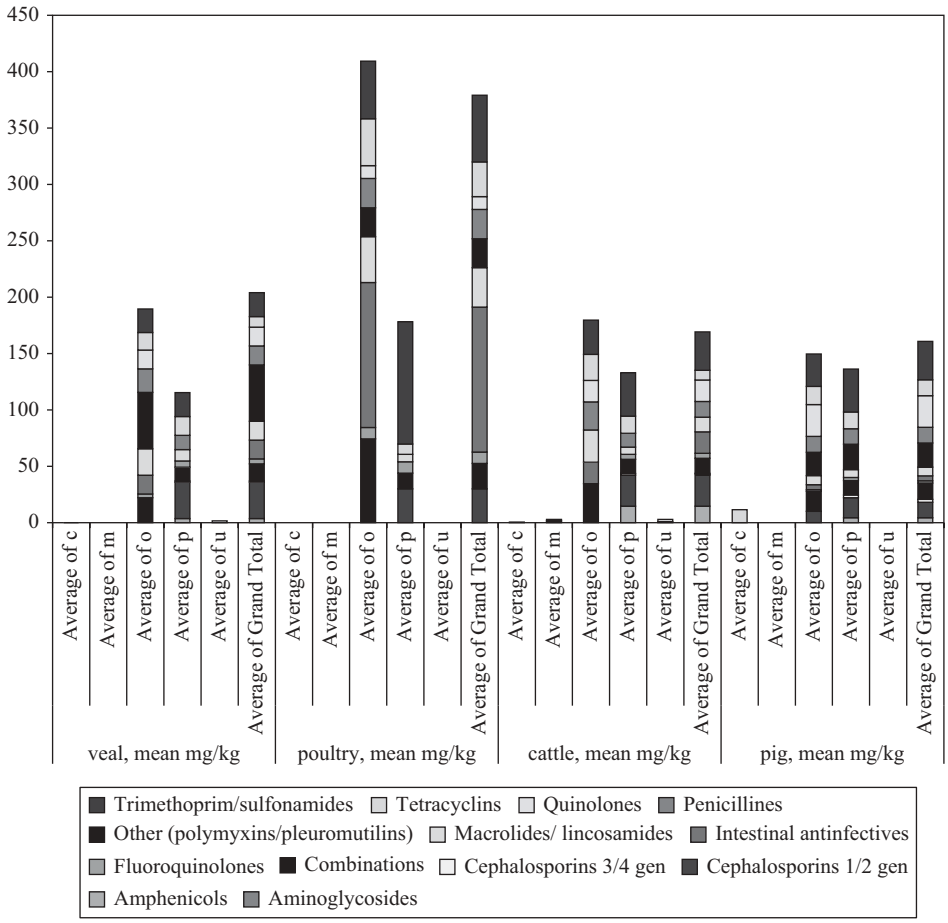

FIGURE 27.1 Calculated doses per pharmacotherapeutic group differentiated per species and by way of administration, based on all for livestock-authorized medicinal products in the Netherlands ultimo 2010 (c cutaneous, m intramammary, o oral, p parenteral, and u intrauterine). *(See color insert.)*

TABLE 27.1 Calculated Mean Dose (mg/kg) per Group per Country, Depending on Livestock Composition[a]

Dose (mg/kg)	min	max	NL	DK	Dui	Fr	UK	Swe	Fi	No	Swi	USA
Tetracyclines	9.09	41.54	16.09	15.89	15.90	16.09	15.83	15.57	15.75	15.88	15.50	16.56
Oral anti-infectives/ionophores	4.41	128.52	17.19	12.52	17.87	20.93	20.59	18.15	17.99	19.73	18.33	22.97
Amphenicols	9.97	32.22	22.88	18.69	23.83	26.07	26.69	25.37	24.49	25.78	25.79	26.12
Trimethoprims/sulfonamides	20.96	108.39	34.95	33.69	36.58	37.85	38.27	37.32	36.95	37.44	37.62	38.26
β-Lactams/penicillins	12.29	26.11	13.68	13.40	12.99	12.94	12.67	12.69	12.83	12.92	12.58	13.1
Cephalosporins, first and second generation	0.54	0.83	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54
Cephalosporins, third and fourth generation	0.25	2.41	0.86	0.66	1.03	1.18	1.26	1.17	1.10	1.16	1.21	1.7
Aminoglycosides	3.67	14.67	10.99	9.41	12.38	13.63	14.15	13.33	12.83	13.37	13.64	13.74
Macrolides/lincosamides	6.25	40.66	8.60	7.49	7.35	7.58	7.06	6.86	7.07	7.45	6.67	8.04
Quinolones	11.29	28.13	21.28	23.54	20.79	19.56	19.29	20.06	20.48	19.77	19.85	19.41
Fluoroquinolones	1.33	10.00	3.60	2.91	3.86	4.30	4.39	4.08	3.96	4.19	4.15	4.42
Combinations	1.12	74.37	16.13	15.82	14.97	15.07	14.26	13.94	14.48	14.71	13.65	16.09
Other (polymyxines, pleuromutilins)	20.58	50.00	25.78	20.69	21.50	23.81	22.09	21.81	21.13	24.70	20.91	22.39

[a]Min and max: calculated minimum and maximum (reflects the dose in one species for one route of administration).

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Thus the resulting mean ADD for the 11 groups is higher in countries with a large poultry production, such as the Netherlands, while for the countries with a lot of cattle (such as Switzerland) penicillin ADD is relatively low.

27.2.2 Population Data

Eurostat data (Eurostat, 2011) on livestock, namely number of heads at the most recently reported time, were applied, assuming this adequately represents the mean number of animals present in a country.

27.2.3 Antimicrobial Sales or Prescription Data

The volume and composition of antibiotic use in the years 2001–2009 in Sweden (SVARM, 2011), Norway (NORM/NORM-VET, 2011), Finland (Finnish Medicines Agency, 2011), Denmark (DANMAP, 2011), Germany (2003 and 2005 data only) (GERMAP, 2008), Switzerland (Swissmedic, 2011), The Netherlands (FIDIN, 2011), United Kingdom (Veterinary Medicines Directorate, 2011), France (ANSES, 2011) and the United States (U.S. FDA, 2011) were obtained. Only for Denmark and Sweden (starting 2003) were prescription data on a national level available, all other countries report total sales data.

Human-use data collected for the ESAC project (ESAC, 2011) were used, except for Denmark (DANMAP, 2011), the Netherlands (NETHMAP, 2011), and Norway (NORM/NORM-VET, 2011).

Usage data stem from different sources, comparable to the veterinary situation. Sweden and the Netherlands are prescription data, Finland and France report Medicines Agency data, the United Kingdom reports only ambulatory data from an unknown source, and the remaining countries report sales figures.

27.2.4 Evaluation of Antibiotic Prescription Guidelines

With antibiotic usage described this way, a more reliable evaluation of national formulary choices of pharmacotherapeutic groups is feasible.

27.2.5 Expression of Antibiotic Use Per Square Kilometre

Most antibiotics are extensively metabolized before excretion, but they do extend their influence beyond the treated animal alone, as the antimicrobial colonization of the animal and probably its environment will change during and after a treatment with antibiotics. We have examined the relationship between daily dosages per animal year and the amount of animals per square kilometer to evaluate the relative environmental antimicrobial pressure. All available usage data in daily dosage per animal-year were plotted against kilograms of animal per square kilometer.

In the visualization of the comparison of veterinary use to human use, the density of animals and human per square kilometer was depicted in the same figure.

27.3 RESULTS

Figure 27.2 shows the calculated daily dosages of antibiotics that is administered in the various countries per average animal year. The error bars indicate the results for

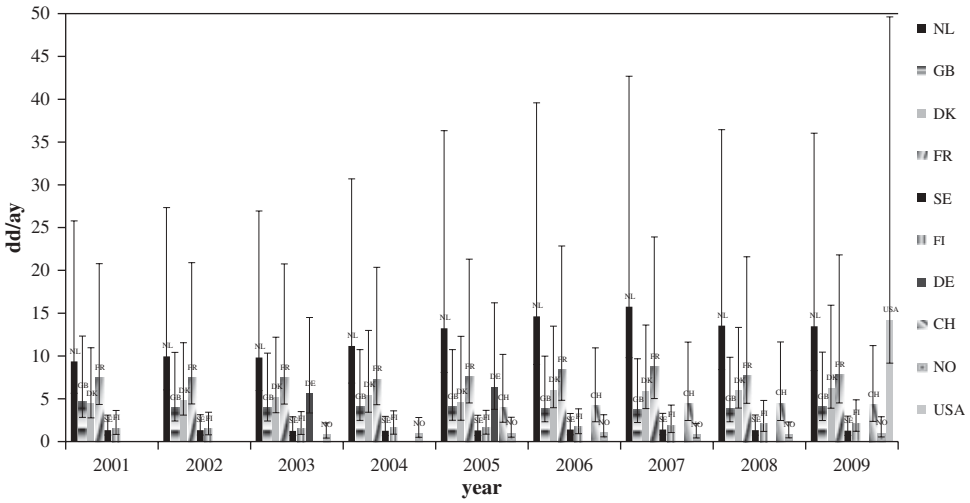

FIGURE 27.2 Daily dosages per animal year in Europe and the United States. Error bars depict the range between min and max ADD for the amount of kilograms.

calculation with the lowest to highest dose in a pharmacotherapeutic group. For instance, for the tetracyclines the lowest dose is the mean parenteral dose in poultry (9.09 mg/kg) and the highest is the oral dose in poultry (41.54 mg/kg) (Table 27.1).

The average animal in the Netherlands is administered a daily dosage of antibiotics on approximately 9 days (2001) to 13 days (2009) a year. The estimated average number of daily dosages is significantly lower in some other countries, although the situation might differ for specific types of animals. The Dutch and French antibiotic uses have increased until 2007; in both countries this trend seems to have stopped in 2008 and 2009. Use was also increasing in Germany (but only 2003 and 2005 data are available) and Denmark (continuing to increase in 2009), whereas uses in the other countries are relatively stable.

An overview of European antibiotic use by group of medicines gives an insight into the veterinary antibiotics policy pursued in the various countries (see Fig. 27.3).

A distinction can be made between three general treatment strategies in Europe. The Scandinavian countries: a strategy primarily based on β-lactam antibiotics, Denmark a strategy based on tetracyclines + macrolides + β-lactams, and other European countries: a strategy primarily based on tetracyclines.

Norway uses relatively large amounts of fluoroquinolones (15%) in the fish-breeding sector, which is more than the amount prescribed to humans in ambulatory setting in any country (3–8%) or in the Netherlands in hospitals (14%).

All countries use roughly the same percentage of trimethoprim/sulfonamides combinations (approx. 8%), except for Switzerland.

The above comparison has been made for all years from 2001 to 2008, and no major differences could be detected between years in applied therapeutic groups. Because not all usage data for 2009 were available, the year 2008 was depicted; these data were not available for the United States.

To explore the usage of antibiotic medicines in livestock in relation to the amount of animals per country surface, the amount livestock in kilograms of animal per

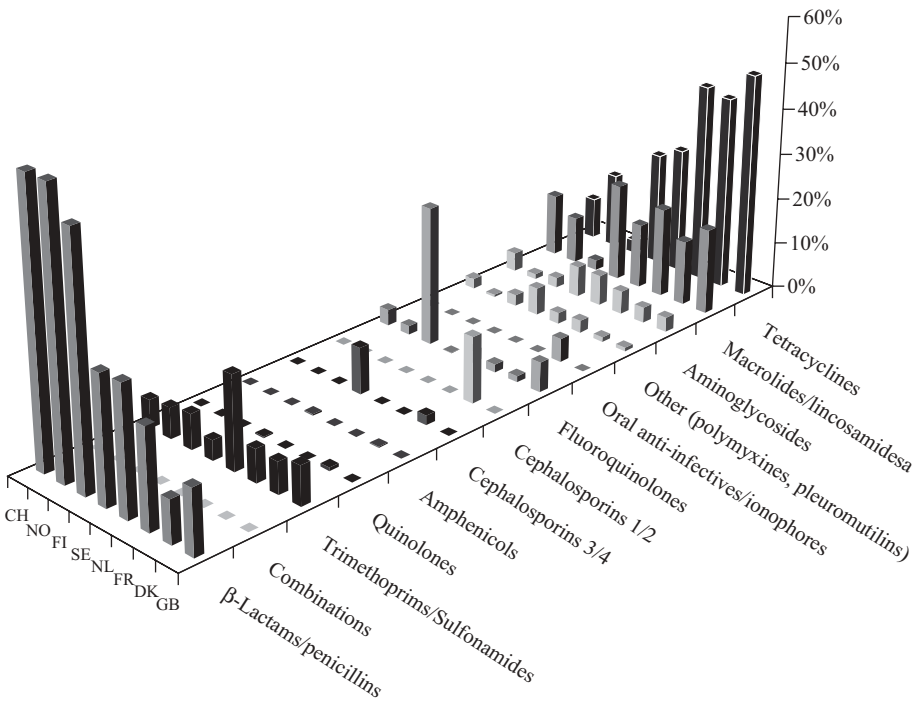

FIGURE 27.3 Percentages (%) of the total antibiotic use (expressed in calculated daily dosages) of the 11 main groups of antimicrobial preparations in 8 European countries in 2008 (Germany and the United States not available) (CH Switzerland, NO Norway, FI Finland, SE Sweden, NL Netherlands, FR France, DK Denmark, and GB United Kingdom).

square kilograms per country is depicted versus the estimated number of daily doses per animal year (Fig. 27.4). In Europe a rather continuous relation between amount of animals per square kilograms and application of antibiotics seems to exist. The position of the United States indicates that most probably other mechanisms determine the antibiotic usage as well. Note that the livestock density was calculated based on total country surface (excluding water surfaces), whereas the area of agricultural or livestock production regions could have been a more accurate denominator for this exploratory analysis. The reason is that in most countries the livestock farms are not evenly distributed. We expect that a further analysis would reveal relations with other important factors, as, for example, farm size and animal health management. In the Netherlands, in a sample of 94 Dutch pig farms, we did find a statistically significant relation between farm size and antibiotic usage (Bondt et al., 2009).

For comparison of human use of antimicrobials to veterinary use, the ambulatory and hospital human use in DDD/Person-year (daily dosage per person-year) is presented in a plot with inhabitant density and veterinary use and animal density (Fig. 27.5). Animal daily dosage per animal year seem to increase with number of animal per square kilometer, while the number of human daily dosage per person-year does not show a relationship with number of inhabitants.

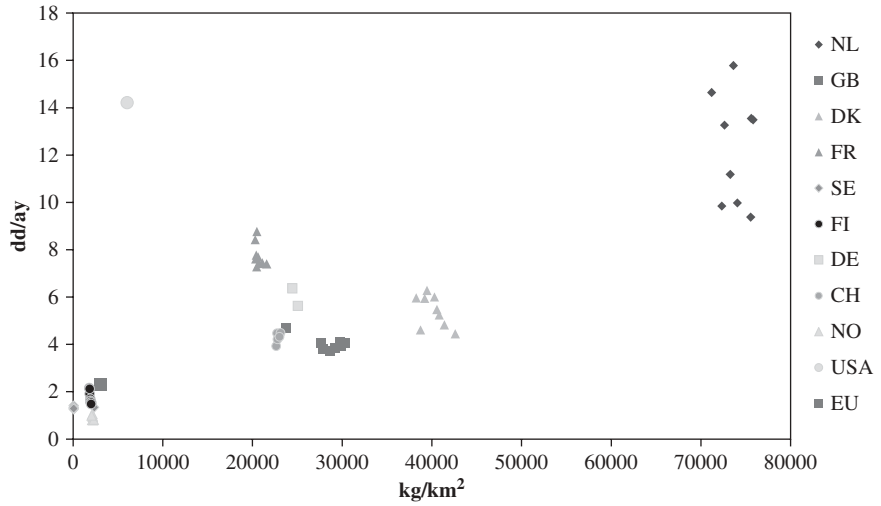

FIGURE 27.4 Daily dosages per animal year versus kilograms of animal per square kilometer. The different dots per country represent different years (2001–2009); for the United States only 2009 data are available, for Germany only 2003 and 2005 data. EU is the mean of all European countries and all years. (NL Netherlands, GB United Kingdom, DK Denmark, FR France, SE Sweden, FI Finland, DE Germany, CH Switzerland, and NO Norway).

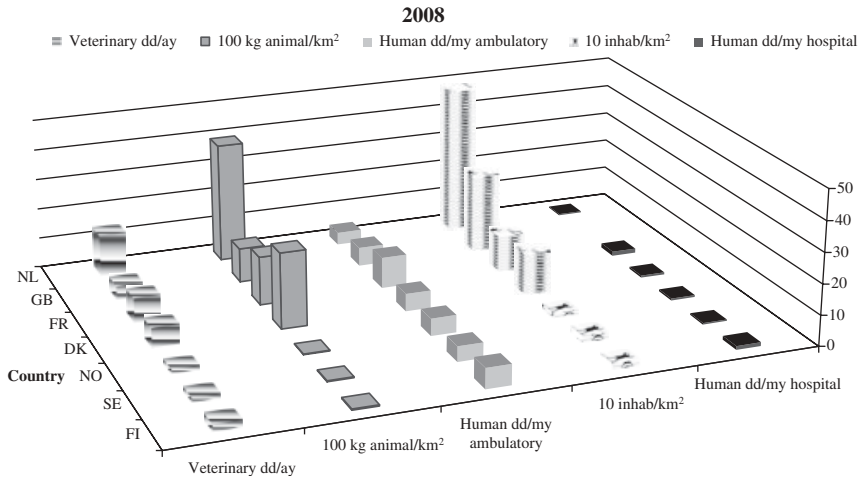

FIGURE 27.5 Veterinary and human antimicrobial drug consumption in 7 European countries in 2008, in relation to number of animals (expressed in 100 kg/km^2) in casu number of inhabitants (expressed in 10 inhabitants/km^2); for United States and Switzerland no human data available, Germany no veterinary data available (NL Netherlands, GB United Kingdom, FR France, DK Denmark, NO Norway, SE Sweden, and FI Finland).

27.4 DISCUSSION

Direct comparisons of antibiotic usage are complicated by the differences in the level at which records are kept: at a national sales level (Netherlands, France, Germany, United Kingdom, Finland, Norway) or at farm level (prescription level) [Denmark and Sweden (as from 2003)].

The big differences in antibiotic use will be due at least partly to the major differences in the national animal demography and in husbandry systems (denominator data). France and the United Kingdom, for example, have large numbers of beef cattle and sheep that are always outdoors and receive very limited amounts of antibiotics. In the Netherlands, the use reflects the larger proportion of intensive animal production. However, reports previously published in countries such as Denmark, where livestock farming exhibits more similarities with the Netherlands, indicate that antibiotic use is actually lower. The United States appears to have an antibiotic usage comparable to the Netherlands. This use is probably limited to a few states with large amounts of livestock.

A tentative analysis shows that the veterinary use of antimicrobials seems to be higher in countries with a higher density of livestock. The higher density could imply an increased risk for transmission of diseases, which could lead to more treatments. In human ambulatory use no relation with density of inhabitants is found. Remarkable is the big difference between human and veterinary use in the Netherlands, whereas France displays relatively high use in both animals and humans, and Norway shows overall low usage.

REFERENCES

Anonymous (1984). Control of antibiotic-resistant bacteria: Memorandum from a WHO meeting. World Health Organization Scientific Working Group on Antimicrobial Resistance. *Am J Hosp Pharm* 41:1329–1337.

ANSES (2011). *Sales survey of veterinary medicinal product containing antimicrobials in France*. Laboratoire de Fougères, Fougères, France. Available: http://www.anmv.anses.fr/en_anmv/ Accessed March 2011.

Bondt N, Puister L, Bergevoet R (2009). *Antibioticagebruik op melkvee-, varkens- en pluimveebedrijven in Nederland*. [Antibiotic usage on dairy, pig and poultry farms in the Netherland]. Wageningen UR, Den Haag, Netherlands.

Catry B, Dewulf J, Opsomer G, Vanrobaeys M, Decostere A, Haesebrouck F, de Kruif A (2007). *Antibioticumgebruik en antimicrobiële resistentie bij rundvee ontwikkeling van een surveillancesysteem op bedrijfsniveau*. FOD, Veiligheid van de Voedselketen en Leefmilieu Belgium.

DANMAP (2011). Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, foods and humans in Denmark; Danish Integrated Antimicrobial Resistance Monitoring and Research Programme, National Food Institute, Technical University of Denmark, Danish Zoonosis Centre Denmark. Available: http://www.danmap org/ Accessed February 2011.

ESAC (2011). The European Surveillance of Antimicrobial Consumption (ESAC) project. European Centre for Disease Prevention and Control (ECDC) Sweden. Available: http://www.esac.ua.ac.be. Accessed February 2011.

Eurostat (2011). European Commission, Luxembourg. Available: http://epp.eurostat.ec.europa.eu. Accessed February 2011.

FIDIN (2011). *Antibioticarapportage werkgroep antibioticabeleid*. FIDIN The Hague, The Netherlands. Available: http://www.fidin.nl. Accessed February 2011.

Finnish Medicines Agency (2011). Lääketukuista myydyt, eläimille tarkoitetut mikrobilääkkeet 2001–2009 (kg vaikuttavaa ainetta). Finnish Medicines Agency Finland. Available: http://www.fimea.fi/elainlaakkeet/kulutus/mikrobilaakkeiden_kulutus_elaimilla/selite. Accessed March 2011.

Franklin A, Acar J, Anthony F, Gupta R, Nicholls T, Tamura Y, Thompson S, Threlfall EJ, Vose D, van Vuuren M, White DG, Wegener HC, Costarrica ML, Office International des Epizooties Ad Hoc Group (2001). Antimicrobial resistance: Harmonisation of national antimicrobial resistance monitoring and surveillance programmes in animals and in animal-derived food. *Rev Sci Tech* 20:859–870.

GERMAP (2008). *Antibiotika-Resistenz und -Verbrauch*.. Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL) Berlin, Germany. Available: http://www.bvl.bund.de. Accessed February 2011.

Grave K, Torren-Edo J, Mackay D (2010). Comparison of the sales of veterinary antibacterial agents between 10 European countries. *J Antimicrob Chemother* 65:2037–2040.

Jensen VF, Jacobsen E, Bager F (2004). Veterinary antimicrobial-usage statistics based on standardized measures of dosage. *Prev Vet Med* 64:201–215.

MARAN (2011). *Monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands*. Central Institute for Animal Disease Control (CIDC) Lelystad, The Netherlands. Available: http://www.cvi.wur.nl/NL/publicaties/rapporten/maranrapportage.

McGowan JE Jr (1987). Is antimicrobial resistance in hospital microorganisms related to antibiotic use? *Bull NY Acad Med* 63:253–268.

McGowan JE Jr (1983). Antimicrobial resistance in hospital organisms and its relation to antibiotic use. *Rev Infect Dis* 5:1033–1048.

NETHMAP (2011). Consumption of antimicrobial agents and antimicrobial resistance among medically important bacteria in the Netherlands. SWAB—RIVM studios Bilthoven, The Netherlands. Available: http://www.swab.nl. Accessed February 2011.

NORM/NORM-VET (2011). Usage of antimicrobial agents and cccurrence of antimicrobial resistance in Norway. National Veterinary Institute Norway. Available: http://www.vetinst.no. Accessed February 2011.

Silley P, de Jong A, Simjee S, Thomas V (2011). Harmonisation of resistance monitoring programmes in veterinary medicine: An urgent need in the EU? *Int J Antimicrob Agents*.

SVARM (2011). Swedish veterinary antimicrobial resistance monitoring. National Veterinary Institute (SVA), Uppsala, Sweden. Available: http://www.sva.se. Accessed February 2011.

Swissmedic (2011). Report on sales of antibiotics in veterinary medicine 2005–2008. Swissmedic, Swiss Agency for Therapeutic Products Switzerland. Available: http://www.swissmedic.ch. Accessed February 2011.

U.S. Food and Drug Administration (FDA) (2011). SUMMARY report on antimicrobials sold or distributed for use in food-producing animal. Department of Health and Human Services, Center for Veterinary Medicine Silver Spring, MD. Available: http://www.fda.gov. Accessed February 2011.

Veterinary Medicines Directorate (2011). Sales of antimicrobial products authorised for use as veterinary medicines, antiprotozoals, antifungals, growth promoters and coccidiostats, in the UK. Veterinary Medicines Directorate Surrey, United Kingdom. Available: http://www.vmd.gov.uk. Accessed February 2011.

28

REGULATORY RESEARCH ON ANTIMICROBIAL RESISTANCE IN THE ENVIRONMENT

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28.1 AN ENVIRONMENTAL PERSPECTIVE

There are a number of settings in which microorganisms are controlled: clinical (humans and animals), food production (animal, plant, and fungi), industry, and households. An antibiotic can be functionally described as a molecule that has a useful therapeutic activity in killing or inhibiting microbial growth (Yim et al., 2007). The antibiotic paradox (Levy, 2002) is that any use of antibiotics also selects for resistance. We are, in turn, tightly connected to the settings wherein resistance may be selected (health care, food production and consumption, drinking water production and consumption, air, outdoor recreational activities, etc.).

Scientific literature provides a variety of names for what surrounds the communities we research, such as environment, ecosystem, and habitat. These concepts are not used consistently across disciplines, nor necessarily across individuals within a discipline. It is, therefore, necessary to define the use of such terms when putting laboratory results in an environmental context or interpreting surveys on presence, abundance, or transport, as one does in risk assessment. Environmental scientists describe the environment in a variety of ways: as compartments (soil, water, air), biogeographical classes (ecoregions), use functions (such as rural areas), and via organization levels (ecosystem, biotope, community).

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For environmental scientists, the human gut, or the clinic, or just any biofilm is not "the environment," although these settings are "environments" to microbiologists. When researching antimicrobial resistance in the environment, it is important to be very specific when using terminology, to avoid the use of terms such as "environmental," "global", or "ecological" as containers for everything that may occur outside the controlled experiment (Janssen et al., 2009). For the purposes of this writing, the term "environment" refers to the external elements of any ecosystem, including air, water, soil, and related media.

The problem of antimicrobial resistance (AMR) is usually described in terms of areas of use and product types. Such socioeconomic categorization identifies stakeholders and responsible parties and has a direct link to regulatory instruments such as regulation of quality (and production) of consumer goods (food and nonfood) and authorization of products (medicines and biocides). Clearly, in such hot spots there is much to be gained by controlling AMR. However, conclusions about where such areas are located, what separates them, where the resistance originated and whether such areas are communicating cannot yet be made. Heuer and Smalla (2007) summarized the problem concisely: They point out that the majority of scientific work (focusing particularly on food-borne zoonotic organisms) has argued that most human pathogens showing antibiotic resistance have no link with animals and that evidence for transfer of resistance genes to the human microflora remains sparse (Phillips et al., 2004; Wassenaar, 2005) [for a more recent example see Leverstein-van Hall et al., 2011)]. However, this argument ignores the fact that humans are continuously exposed to exogenous bacteria and are living in an interconnected microbial world.

Moriarty (1983) defined a pollutant as a substance that occurs in the environment at least in part as a result of human activities that have a negative effect on living organisms. Can we then consider antibiotic resistance (AR) determinants as pollutants (even though they are not a "substance" in the general parlance) (Pruden et al., 2006)? Negative effects need not be the result of direct intoxication but can also become manifest through more indirect ecological connections. AR determinants lead to indirect negative effects (the risk of antimicrobial resistance) and can be considered as pollutants. However, we do not yet know the risk of the environmental routes of exposure: How do the strength and frequency of contact with bacteria or their resistance elements in the environment compare to that of clinical settings, animal husbandry practices (direct contact to antibiotics via air, hand–mouth, and animal contact), and contact via food? Within a risk assessment framework such questions can be answered. When assessing the risks of antimicrobial resistance in the environment one must:

- Discern between background levels and added anthropogenic levels.
- Identify and quantify sources and emissions of antibiotic resistance and/or resistant organisms.
- Identify the in situ environmental exposure to organisms and identify which organisms are exposed and possibly affected.
- Estimate the adverse effects of antibiotic resistance on exposed organisms, preferably in a quantitative way.
- Assess the risks by assessing the likelihood and severity of such adverse effects in relation to the exposure.

This whole process is generally referred to as environmental risk assessment (Vermeire et al., 1997).

With respect to background levels, a number of scientific studies have reported that it is difficult to separate autochthonous resistance from anthropogenic resistance as genetic mobile elements encoding for resistance may be transferred between bacteria (e.g., Henriques et al., 2006). Various studies on sources of resistance, emissions, and in situ environmental exposure indicate that antibiotic resistance is ubiquitous in aquatic and terrestrial environments. Anthropogenic sources of resistance genes are, for instance, enteric bacteria in waste from humans and husbandry animals treated with antibiotics. AMR genes reach aquatic environments after emission from households, hospitals, slaughterhouses, and municipal wastewater or sewage treatment plant (STP) effluents, and via manure amended soils (Mensink and Montforts, 2007).

The behavior and fate of antibiotic resistance genes in aquatic and terrestrial environments is complex. When characterizing AMR in the environment (such as in a soil sample or groundwater aquifer), the specific spatial and organizational context should be carefully considered. In experiments, observations are made within a particular setting, for example, the Petri dish, the patient, the crop, the surface, the herd, the farm, the hospital, or the soil sample. A sample taken is like a snapshot, without information on rates or directions. The challenge is to manipulate or measure the appropriate parameters at the relevant scale, in time, in space, and at the exact microlocation. Of equal importance is the notion that we are not looking at distribution, degradation, and impact of chemicals, but at evolution of traits and possible biological amplification (Antonovics et al., 2007). Unlike chemicals, the abundance of bacteria and their genes may increase under conditions that favor their expression. Where chemicals dilute and degrade within environmental mediums, a gene can be amplified. Indeed, this has been observed in our environment: The volume of antibiotics released in effluents from hospitals, households, and production sites causes a selection pressure on bacteria (Kümmerer and Henninger, 2003; Larsson et al., 2007). Environmental conditions (meaning all stressors and not only antibiotic residues) may select further for unique resistant strains. Antibiotics can also increase the proportion of strong mutators in a bacterial population, leading to an increased capacity of acquiring resistance mutations (Blázques et al., 2002).

Risk assessment for antimicrobial resistance should ideally consider both potential public health and potential ecological effects. From the public health point of view, the environment is a genetic pool directly affecting the exposure of the human population (and its pathogens) to resistance genes. Risk assessment considering public health impacts must also consider other exposure routes such as clinical and occupational settings and transmission via food. To understand how antimicrobial resistance operates and spreads, we need to understand what sets different settings apart: What properties define a setting, and what properties describe the barriers between settings? Because AMR moves within the confines of particular "settings", and individual organisms move in and out of these settings, the boundary between a setting and the rest of the environment is not absolute (see, e.g., Singer et al., 2006).

The spreading of resistance genes may affect the ecological balance. Hughes Martiny et al (2006) chose to describe the biotic communities at the taxon level, not at the species level. Should we look at the taxa in communities, or at biocenoses in total, such as biofilms? Or should we exclusively focus on genes and not on species?

For example, we know now that the tetracycline resistome is extremely large (Schmitt et al., 2006; Thaker et al., 2010). The term resistome, first coined by D'Costa et al., (2006), refers to the aggregate of all antibiotic resistance mechanisms (Thaker et al., 2010). Aminov (2009) uses the term environmental antibiome. Thus, the main question is: Where and what to look for? There are no studies available that focus on the effects of AMR on autochthonous microbial communities and higher organisms. That the contamination with antimicrobial resistance genes may change the genotype of autochthonous bacteria has been shown in various studies (Pruden et al., 2006; Schwartz et al., 2003). These genetic changes, however, will presumably only be expressed in the presence of substances that act as antibiotics or other stressors that illicit their expression. In addition, resistance to antimicrobial substances is part of the natural evolution of microbial species. This was recently demonstrated by Van de Velde et al., who showed that antibiotic plant peptides induce irreversible terminal differentiation in symbiotic rhizobia associated with *Medicago truncatula* (Van de Velde et al., 2010). The rhizobia then continue as a population (resist destruction via the antimicrobial peptides) but are irreversibly linked to the *M. truncatula*. Thus, it may be extremely difficult to discern the harm or benefit of the presence of resistance genes or the induction of resistance mechanisms in environmental microbial communities.

It remains to be demonstrated if the term environment, for the purpose of understanding the phenomena of antimicrobial resistance, is currently defined (and applied) in a useful way. A pragmatic approach may be taken where efforts to understand AMR are combined with efforts to describe environment and ecology in a way that adds meaning to the results. Although landscape ecology holds useful tools and models to unravel the mechanisms and implications of AMR in the environment (Singer et al., 2006), it may be that we need different tools and models to understand the environment from the microbiological perspective (Hughes Martiny et al., 2006), as well as different tools to assess the risk of AMR in the environment (Midtvedt, 2004).

28.2 REGULATION OF RESISTANCE IN THE ENVIRONMENT

The need for assessment and regulation of the potential risk of AMR in the environment has been recognized by regulatory bodies worldwide. Here the efforts made by the European Union (EU) and the U.S. government are described. A transatlantic task force on antimicrobial resistance issues was created by the 2009 EU-US Summit (EU-US, 2009). This task force worked until March 2011 on urgent antimicrobial resistance issues, such as appropriate therapeutic use of antimicrobial drugs in the medical and veterinary communities, prevention of both health-care- and community-associated drug-resistant infections, and strategies for improving development of new antimicrobial drugs. The task force builds on the research and recommendations reported in the previous decade by the World Heal Orgenization (WHO), the European Union, and the United States.

28.2.1 The European Union

In 1998, the Danish government in Copenhagen, Denmark, hosted the Invitational European Union Conference on "The Microbial Threat" (September 9–10, 1998),

and its report was endorsed by the European Council (Rosdahl and Pedersen, 1998). The report concluded that there is an established relationship between the consumption of antimicrobial agents and the prevalence of drug resistance in microorganisms. It also concluded that dissemination of resistant microorganisms occurs both inside and outside hospitals and that the major route of transmission of resistant microorganisms from animals to humans is through the food chain. Following that, in the 1999 Opinion of the Scientific Steering Committee (SSC) to the European Commission, the prevalence of resistance in the environment and its health implications was also considered, emphasizing the global ecology of resistance and the lack of knowledge on fate and behavior of antibiotics (Section 3.4 in SSC, 1999). The European Community strategy against antimicrobial resistance that the Commission then developed called upon the member states and the Commission to take actions on all the relevant aspects to tackle this threat. This was based on the provisions of Article 152 of the treaty establishing the European Community, which provides that a high level of health protection shall be ensured in the definition and implementation of all Community policies and activities. On this basis, the Commission proposed to put in place a Community strategy on four key areas of action (EC, 2001):

1. Surveillance
2. Prevention of communicable diseases, and infection control, including the prudent use of antimicrobial agents for which the European Council issued a recommendation (2002/77/EC)
3. Research and product development
4. International cooperation

The Commission is also funding several projects related to antimicrobial resistance through its Health Programme and monitoring the antimicrobial resistance risk, with the support of the European Centre for Disease Prevention and Control and the European Food Safety Authority. The research includes the European Antimicrobial Resistance Surveillance System (EARSS) of the National Institute of Public Health and the Environment (RIVM) in the Netherlands and the European Surveillance of Antimicrobial Consumption (ESAC) of Antwerp University in Belgium.

In 2008, the European Commission requested that the European Food Safety Authority (EFSA), the European Medicines Agency (EMA), the European Centre for Disease Prevention and Control (ECDC), and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) provide a scientific report on AMR. The ECDC is the EU agency that prevents and controls infectious disease in the EU. In the report, it was noted that the main reservoir of antimicrobial-resistant strains of these zoonotic bacteria is the gastrointestinal tract of healthy food animals, particularly poultry, cattle, and pigs (EFSA, 2009b). The fate of these bacteria in the environment after spreading of manure was not considered in the report. The assessment of a possible contribution of other agents in the selection of antimicrobial-resistant microorganisms was recommended, including biocides, detergents, and bacteria as such, as a source of AMR mechanisms and as a vector of dissemination of AMR genes. The role of bacterial biofilms in the colonization process, in surviving antimicrobial treatments, and in the sources and dissemination of AMR genes, should be established. The somewhat unspecific use of the words such

as "environment" and related concepts (environmental isolates in all areas of biocide usage; environments that facilitate bacterial gene transfer; role of bacterial biofilms; the diffusion of antimicrobial-resistant bacteria) indicates that the manner in which different areas of use are connected is a subject that needs more attention.

Recommendations from other scientific opinions do, however, specifically recognize that AMR has an overall environmental context, as already signaled by the SSC (1999). For example, in the EFSA Opinion "Foodborne Antimicrobial Resistance as a Biological Hazard" it was highlighted that "the role of food, water and the environment in the spread of apparently epidemic plasmids encoding multiple resistance is not clear, but deserves immediate attention" (EFSA, 2008). In the Joint scientific report of ECDC, EFSA and EMA, on methicillin-resistant *Staphylococcus aureus* (MRSA) in livestock, companion animals, and foods, it was recommended that the factors responsible for host specificity, persistence in different environments, transmission routes (including airborne transmission) and vectors, should be investigated (EFSA, 2009a). In the CVMP 2009 reflection paper on MRSA in food-producing and companion animals in the European Union, the risk of disseminating the pathogens, resistance, or antibiotic residues into the environment is, however, not addressed (CVMP, 2009c). In contrast, in the revised reflection paper on the use of third- and fourth- generation cephalosporins in food-producing animals in the European Union, all routes (via food, via direct contact with infected animals, or indirectly via the environment) are mentioned (CVMP, 2009a). The environmental example here was pet treats containing dried beef (Pitout et al., 2003). This example also gives some perspective to the earlier discussion of the best operational description of "environment."

Regarding the Council Recommendation 2002/77/EC, in 2009 the European Commission reported on the progress and concluded that the areas on which future work could be focused include addressing the global dimension of antimicrobial resistance and monitoring the environmental impact of the use of antimicrobials (EC, 2009a).

In 2009, the European Cooperation in the field of Scientific and Technical Research—COST—granted a European Concerted Research Action designated as COST Action TD0803: Detecting evolutionary hot spots of Antibiotic Resistances in Europe (DARE). The main objective of this action is to identify and characterize environmental hot spots for antimicrobial resistance emergence and spreading of antibiotics and antibiotic resistance patterns, aiming at the development of measures to control antibiotic resistance evolution.

In the fields of feed additives, plant protection products and of veterinary medicines, human medicines, and biocides, regulatory action has been taken. The resulting guidelines are briefly introduced below with a special emphasis on the environmental connection.

28.2.1.1 Feed Additives Feed additives are not considered in depth since within the EU the use of antibiotics as growth promoters, following the Swann report (1969), has been under tight control since the European Regulation of 1970 (EC, 1970; Pugh, 2002) and is actually banned since January 1, 2006 [Regulation (EC) No. 1831/2003; EC, 2003].

28.2.1.2 Plant Protection Products Considering plant protection products, the antibiotics polyoxin, kasugamycin, streptomycin, and oxytetracyclin have been used

before 2007 in several European member states as plant protection products. Notably, oxytetracyclin has never been listed for inclusion in the Community regulation on plant protection products. Following recommendations of the Scientific Steering Committee (SSC, 1999), the agricultural application of streptomycin was phased out by Commission Decision 2004/129/EC (EC, 2004) and kasugamycin and polyoxin have been phased out by Commission Decision 2005/303/EC (EC, 2005). Regarding the emergency use of these compounds, the Commission recalled in 2009 that use of antibiotics in general must be minimized in order to avoid antimicrobial resistance (SCFCAH, 2009).

28.2.1.3 Veterinary Medicines The EMA Committee on Veterinary Medicinal Products (CVMP) report on antibiotic resistance in the European Union associated with therapeutic use of veterinary medicines in July 1999 (CVMP, 1999a) mentions that resistance genes may have already existed in environmental bacteria and have been transferred to species that are of more (veterinary) medical interest and that the environment is a reservoir readily accessible to bacteria. Reference is made to Davies (1994) and Linton (1986), where the latter specifically refers to animal husbandry as a source of contamination of the environment, raising the expectation that a strategy to assess this risk would be developed. However, in the risk assessment section, the development and/or emission of resistance via the waste products of this food chain (manure) is not considered. The strategy document released in July 1999 (CVMP, 1999b) focuses on ensuring effective use of antibiotics, including external communication to promote effective and prudent use of antimicrobial products. It also permits coordinated action across traditional boundaries. The recommendations within the remit of the CVMP are followed by a section on political considerations, which should be used to advise the European Commission. It is here that the CVMP highlights that "ecological issues related to disposal of waste products containing antimicrobials, require consideration during the authorization process (CVMP, 1999b: p. 6)".

With respect to the protection of the environment from antimicrobials, the CVMP refers for the next 5 years (2006–2010) (CVMP, 2006) to the VICH Guidelines 6 (2000) and 38 (2004): "As for any other veterinary medicinal product, the assessment of environmental safety needs to be carried out when the dossier for marketing authorization is applied (CVMP, 2006: p. 12)". However, these guidelines deal with traditional ecotoxicological risk assessment of the pharmaceutical substances and not with the risk of spreading antimicrobial resistance in the environment.

The Commission Directive 2009/9/EC of February 10, 2009, amending Directive 2001/82/EC, on the Community code relating to medicinal products for veterinary use provides a legal basis to assess the microbiological risk addressing both the development of AMR in bacteria of the human gut flora and disruption of the colonization barrier (EC, 2009a). Applicants are now required to address the microbiological properties of residues and the development of resistance (including resistance of relevance for clinical use in animals). Potentially, regulatory conditions and availability of information would enable the development of guidelines to incorporate the added risk of the environmental exposure in the risk–benefit analysis.

28.2.1.4 Human Medicines In 2010, the Committee for Human Medicinal Products (CHMP) released the draft Guideline on the Evaluation of Medicinal Products Indicated for Treatment of Bacterial Infections (CHMP, 2010). This guideline

specifies that activity against pathogens that are resistant to other antibacterial agents, including agents of the same class if this is applicable, should be explored. At the time of first approval of a new antibacterial agent, applicants should have plans in place to assess the emergence of resistance to the test antibacterial agent over a period of approximately 3–5 years. Data from surveillance networks should be used for this purpose.

Although the CHMP guideline sets requirements on research into resistance from a broad perspective, the issue of antimicrobial resistance in the environment is not specifically mentioned.

28.2.1.5 Biocides In 2008 the European Commission asked the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) to assess the antibiotic resistance effects of biocides. The SCENIHR opinion delivered in January 2009 (SCENIHR, 2009) confirmed that at least some resistance mechanisms are common to both biocides and antibiotics. Scientific evidence from bacteriological, biochemical, and genetic data does indicate that the use of active molecules in biocidal products may contribute to the increased occurrence of antibiotic-resistant bacteria. In view of the large and increasing use of biocides and the continuous increase of bacterial resistance to antibiotics, the SCENIHR identified a number of data and knowledge gaps to be filled, especially:

1. Quantitative data on exposure to biocides
2. Standards and methods to evaluate the ability of a biocide to induce/select for resistance against biocides and antibiotics
3. Environmental studies focusing on the identification and characterization of resistance and cross resistance to antibiotics following use and misuse of biocides

In particular, the recommendation to develop standard protocols for the evaluation of antimicrobial resistance induced by biocides was considered to be valuable in the review program of the Biocides Directive (98/8/EC) where active substances used in biocidal products are currently being evaluated for their risks to human health and the environment.

To enable the European Commission to propose the most relevant research topics on this issue for future funding, the SCENIHR was given a new term of reference in which the research recommendations should be further developed and if necessary expanded. The pursuant opinion (SCENIHR, 2010) was aimed at addressing knowledge gaps about bacterial resistance and cross resistance to biocides. The knowledge gaps identified in the opinion are:

1. Environmental studies focusing on the identification and characterization of resistance and cross resistance to antibiotics following use and misuse of biocides.
2. In vitro studies demonstrate that some biocides used at sublethal concentrations trigger the emergence of antibiotic resistance and/or select bacteria resistant to antibiotics. Despite this mechanistic evidence from in vitro data, epidemiological data indicating public health relevance are lacking.

3. Knowledge of (measured) concentrations of biocides in various environmental matrices is required to assess biocide exposure especially of the environment.

The opinion indeed considers exposure of the environment (air, soil, water, groundwater) and acknowledges that the same lack of information on emerging resistance or selection for resistance may also concern other microorganisms, notably fungi and protozoa (SCENIHR, 2010).

28.2.2 The United States of America

In 2003, the Institute of Medicine (IOM) of the National Academies of Science (NAS) of the United States released a report on major microbial threats to human health (IOM, 2003). The report discussed 13 factors that account for the greatest potential for public health disasters and also noted that "the convergence of any number of factors can create an environment where infectious diseases can emerge—" (p. 4). The first of the 13 factors was "microbial adaptation and change," reflecting the understanding in the U.S. scientific and regulatory community of the importance of AMR as a threat to public health.

A number of U.S. federal government bodies have potential oversight of the issue of antimicrobial resistance (AMR) in the United States. Some of the most important include the U.S. Department of Health and Human Services (DHHS) and under it the Centers for Disease Control and Prevention (CDC) and the Food and Drug Administration (FDA), the U.S. Department of Agriculture (USDA), and the U.S. Environmental Protection Agency (EPA). Each of these regulatory bodies assesses and addresses the subject of antimicrobial resistance differently, as appropriate to their regulatory authority and mission; however, all are participants in a variety of interagency work groups designed to address the issue of antimicrobial resistance.

A point that should be addressed early is that of the structure of public health regulation in the United States, which is such that much of the existing public health law is in the hands of the states, as opposed to the federal government (Fidler, 1998), since the U.S. Constitution did not grant the federal government explicit public health powers (IOM, 1998a). This means that, in practice, antimicrobial resistance is addressed by the federal government agencies in conjunction with state and local public health agencies. For example, many of the surveillance programs that have been instituted in the United States depend on partnerships with states (each of whom has the option to opt in or out of the program). In addition, partnerships between the many agencies that have oversight or impact on AMR are imperative. To that end, the Interagency Task Force on Antimicrobial Resistance is the most notable regulatory mechanism for understanding and addressing AMR in the United States.

In 1999 an Interagency Task Force on Antimicrobial Resistance was formed, following a Congressional hearing on the topic ("Antimicrobial Resistance: Solutions to a Growing Public Health Problem") with the CDC, FDA, and National Institutor of Health (NIH) as co-chairs, and also including the USDA, EPA, Department of Defense, Department of Veterans Affairs, Agency for Healthcare Research and Quality, Health Care Financing Administration, and the Health Resources and Services Administration. The task force was intended as a means of addressing the many facets of AMR that overlap between the various federal

governmental entities in the United States. In 2001, the task force released a "Public Health Action Plan to Combat Antimicrobial Resistance: Part I: Domestic Issues" in which the wide variety of issues surrounding antimicrobial resistance are addressed and how the task force plans to deal with them is outlined. The report is a result of a June 1999 meeting of the task force partner agencies, as well as a wide variety of public and private stakeholders and state and local government officials (ITFAR, 2001). It builds on previous work by a number of federal and private entities and working groups on the issue of AMR, including the U.S. Congress Office of Technology Assessment (1995), National Academy of Sciences, Institute of Medicine (IOM, 1998b), the American Society of Microbiology (1995), and the General Accounting Office (1999a, 1999b). In addition, several WHO reports (2000, 2001) were utilized in the development of the Task Force Action Plan (ITFAR, 2001).

This first version of the Action Plan focuses on domestic issues (within the United States) and outlines four areas specifically addressed: surveillance, prevention and control, research, and product development. Within each of these areas are specific action items. The Priority Goals and Action Items section mentions the environment only once, in the research section, saying, "Understanding the fundamental processes involved in antimicrobial resistance within microbes and the resulting impact on humans, animals, and the environment forms an important basis for influencing and changing these processes and outcomes. Basic and clinical research provides the fundamental knowledge necessary to develop appropriate responses to antimicrobial resistance emerging and spreading in hospitals, communities, farms, and the food supply" (ITFAR, 2001, p. 5). This priority item specifically mentions understanding the emergence and spread of AMR in communities and on farms, which would seem to indicate that this is what is meant by "the environment" in the prior sentence. This focus on farms and food supply is echoed in the other four areas Priority Goals and Action Items: In Surveillance it is noted that "[a]gricultural surveillance data will also help improve understanding of the relationship between antimicrobial drug and pesticide use and the emergence of drug resistance" (p. 3). The Prevention and Control section notes the need for "a regulatory framework to address the need for antimicrobial drug use in agriculture and veterinary medicine while ensuring that such use does not pose a risk to human health" (p. 4). And Product Development mentions the need to encourage development of new veterinary antimicrobials and "improve or reduce the agricultural use of particular antimicrobial drugs, as well as ways to prevent infection, such as the use of veterinary vaccines, changes in animal husbandry, and the use of competitive exclusion products (i.e., treatments that affect the intestinal flora of food animals)" (p. 6).

The Action Plan has had yearly updates on the many projects under the umbrella of the Interagency Task Force, starting in 2001, with the most recent annual report available from 2008. The contents of the 2008 Annual Report (IFTAR, 2009) will be discussed below, when we address actions by the various individual agencies and entities.

On December 12, 2007, the task force met again, along with 50 U.S. and 9 international consultants, to discuss and obtain advice and commentary on updating the Public Health Action Plan. Each of the recommendations and comments of the consultants are being reviewed by the task force members, and the updated Action Plan was slated to be available for public comment last year, although it is not yet available at the time of this writing (ITFAR, 2011). Therefore, we cannot comment

as to whether there was any increase in the mention of the environment or if projects involving the environment have become a higher priority.

Another interagency work group that deals directly with the environment was formed in 2006. The Pharmaceuticals in the Environment Working Group (or PiE Group) was formed by the Toxics and Risk Subcommittee, under the auspices of the Committee on the Environment and Natural Resources, to specifically address and assess the potential human health and ecological risk from pharmaceuticals in the environment. Representatives from the EPA, FDA, USGS, USDA, CDC, National Oceanic and Atmospheric Administration (NOAA), and National Institute of Environmental Health Sciences (NIEHS) were included (EPA OW, 2008; Larsen, 2009). The PiE includes a subworking group specifically focusing upon AMR, with representatives from the CDC, USGS, FDA, USDA, and NOAA. The PiE Final Report has not yet been released to the public.

There are many ongoing AMR projects undertaken by the major regulatory bodies that oversee AMR in the United States. An excerpt is discussed below, focusing on those that deal with AMR in the environment. This overview is not meant to be exhaustive.

28.2.2.1 Food and Drug Administration The U.S. FDA specifically addressed the subject of antimicrobial use in food animals as a potential mechanism for resistance spread in the Center for Veterinary Medicine (CVM) Guidance 152 for Industry (CVM, 2003). This guidance "is premised on the concept that increasing the exposure of bacterial populations to antimicrobial drugs increases the risk of generating resistance to those antimicrobial drugs." It takes into account other factors such as the extent of use of the drug in question, the mechanism of action and mechanism(s) of resistance of the drug, the prevalence of zoonotic food-borne bacteria in the species for which the drug is intended, and the importance of the drug for human therapies. The CVM Guidance considers risk mitigation methods, as well, such as limitations on the use of the drug or use by or on the order of a veterinarian only (CVM, 2003).

The FDA CVM recently replaced Guidance 152 with draft Guidance 209 (CVM, 2010), which states that the "overall weight of evidence available to date supports the conclusion that using medically important antimicrobial drugs for production purposes is not in the interest of protecting and promoting the public health". It also mentions that the "FDA considers the issue of antimicrobial resistance as part of its human food safety review related to new animal drugs used in food-producing animals" and is also found on page 13. FDA CVM considers an antimicrobial new animal drug to be "safe" if the agency concludes that there is "reasonable certainty of no harm to human health from the proposed use of the drug in food-producing animals" (CVM, 2010). Ergo, the FDA CVM considers the potential for development and spread of AMR when deliberating upon the safety and efficacy of a potential new animal drug. Neither Guidance 152 nor Guidance 209 request specific testing or data be submitted as relates to the development or spread of AMR beyond that which is already required. Neither is the environment at large specifically mentioned as being part of the analysis (nor is it discounted).

The CVM has a specific research project under the auspices of the task force to isolate and identify antimicrobial-resistant bacteria from aquaculture facilities (ITFAR, 2009) and is also the main coordinator of the National Antimicrobial Resistance Monitoring System (NARMS), which also involves several centers within

the USDA and the CDC (which oversees surveillance in the human population). The goal of NARMS is to monitor the existence and spread of antimicrobial resistance in the animal and human population and provide that information to researchers and stakeholders to better understand and control that spread (NARMS, 2011).

Within the Center for Drug Evaluation and Research (CDER), which regulates human small-molecule drug products, antimicrobial resistance is also considered as part of the drug approval process. The Division of Anti-Infective Drug Products released a draft Guidance for Industry on Microbial Data for Systemic Antibacterial Drug Products—Development, Analysis, and Presentation in September of 2009 (CDER, 2009). This guidance indicates that the division specifically considers the potential for resistance development (or pre-existing/known resistance) in the safety and efficacy assessment for the drug, particularly the mechanism of action and potential mechanisms for development of resistance and potential for cross resistance. This guidance applies purely to systemically administered anti-infectives (i.e., topical anti-infectives are outside the scope of the guidance). The environment is not mentioned (CDER, 2009). In addition, CDER has been working to provide specific guidance on improving clinical trial designs, in order to assure noninferiority, on the development of new antimicrobials and vaccines, and on diagnostics to allow physicians to better judge when antimicrobial use is warranted (Woodcock, 2010). The Guidance for Industry on Environmental Assessment for Drug and Biologics Applications mentions that a drug that will have an effect on a naturally occurring organism (such as microbial organisms) should address the potential effects on that organism (and possibly other organisms) in the environment as part of the environmental assessment (EA) for that drug (CDER/CBER, 1998).

28.2.2.2 Environmental Protection Agency The EPA directly performs or funds a variety of research relating to AMR in the environment, particularly under the umbrella of the Interagency Task Force. EPA has funded or performed a number of projects looking at AMR in the environment, including methods for detecting and the presence of antimicrobials themselves in a variety of environmental media, and the persistence and spread of particular strains of bacteria as well as resistance genes in the environment after they are introduced. These studies looked at the spread of resistance both in the presence and absence of pressure from antimicrobials themselves in the environment. In 2002–2003 an EPA grant supported work out of Oregon State University looking at the survival of fecal bacteria of the *Bacteroidales* spp. in the environment, and the presence of *Tet*Q resistance genes in surface waters and in wild animals (ITFAR, 2009). Several articles have been published on the EPA's work on environmental sinks for illicit drugs and macrolide antibiotics (Jones-Lepp, 2004; Loganathan, 2009). They are currently funding research on bacterial resistance to kasugamycin in the blossom, leaf surface, and soil bacterial communities prior to, and after the application of kasugamycin to control fire blight (i.e., agricultural use as a plant protection product). The research is intended to identify any kasugamycin-resistant (KmR) bacterial isolates and determine the occurrence of KmR genes in these environmental isolates, with data expected to have been submitted by the end of 2010 (ITFAR, 2009). Besides these research projects, the

EPA conducts and funds a number of projects on methods of detection and levels of pharmaceuticals and emerging contaminants in the environment, including a wide variety of antimicrobials (ITFAR, 2009).

When EPA promulgated its new antimicrobial pesticides data requirements in 2008, it specifically addressed the fact that those data requirements did not address AMR, saying that "research being conducted by the collaborating federal agencies [in the Interagency Task Force], which is primarily focused on antibiotics, may eventually form the basis for the Pesticide Programs' approach to potential resistance as a result of the use of pesticide products" (EPA, 2008, p. 47). It also point out that the EPA has have "the authority to require studies on a case-by-case basis and to revise our data requirements in the future, if appropriate" (p. 47). The authority and ability to request data in the future, or should a need be identified, is a common mechanism for regulatory agencies in the United States, and is utilized by FDA in its evaluations of human and veterinary medicines, as well as EPA for pesticides. However, the EPA also points out that it has "neither determined the extent of the problem nor how data requirements could be developed to address the issue" (p. 47). It should be noted that this new data requirements rule is not yet final, though a final rule is expected in April 2011.

28.2.2.3 U.S. Department of Agriculture As a key member of the Interagency Task Force, USDA oversees a number of research projects, particularly relating to food safety and antimicrobial resistance as a result of animal agriculture. These include projects to understand the role that waterways play in the movement of bacteria and antimicrobials originating from animal production facilities, research on the role that protozoa play in the survival and transfer of pathogenic and resistant bacteria on farms, and projects collecting bacteria from dairy farms, including blood, manure, milk tank, and environmental surveys, to measure resistance and encourage environmental stewardship. Early studies looked at mechanisms of streptogramin resistance in enterococci from animals and the environment and found Q/D resistance genes including *vat*D, *vat*B, and *vga*B (ITFAR, 2009). USDA also collaborates with the other agencies to oversee several monitoring programs for AMR, particularly within the veterinary arm of NARMS.

Recently, the House Committee on Energy and Commerce, Subcommittee on Health held three separate hearings on the subject of antimicrobial resistance (U.S. Congress, 2010a, 2010b, 2010c). The first, on April 28, 2010, addressed the growing problem of AMR and its impact on human health. The second, June 9, 2010, specifically addressed judicious use of antibiotics in clinical settings and the development of new antibiotics (though use in food animal production was mentioned by several witnesses). The final hearing, on July 12, 2010, focused specifically on the use of antimicrobials in food animal production and links to public health. None of the witnesses called upon to testify in the hearings mentioned potential environmental components, such as reservoirs for resistance or resistance transfer.

That the hearings were called demonstrates the U.S. government's increasing concern regarding the imminent threat of antimicrobial resistance. However, it should not be assumed that the lack of mention of antimicrobial resistance in the environment per se signifies a lack of thought on the matter.

28.3 PERSPECTIVES

The overwhelming evidence is that environmental organisms harbor a previously underappreciated density of antibiotic resistance genes (Aminov, 2009; Bacquero et al., 2008; D'Costa et al., 2007; Martinez, 2009a; Wright, 2007). Currently, only a fraction of the available resistance traits have been identified in pathogens and other bacteria. This conclusion should have a paradigm shifting impact on our understanding of the judicious use of antibiotics and the drug discovery process. Proliferation of new resistance traits is an ongoing process, as is the continuous introduction of large numbers of AMR organisms into the environment together with both nutrients (waste) and stressors (antibiotics, metals, disinfectants). As put forward by Aminov (2009) and Baquero et al. (2009), we have not sufficiently investigated if the reverse phenomenon is occurring in nature, that is, if the dispersal of these "second-age" antibiotic resistance genes might facilitate the environmental adaptation of pathogens or alter the natural ecological adaptation of environmental organisms. In this context, the environment is not some remote pristine area but the ecosystem we, as a society, interact with in our daily life.

The European Union and United States have taken regulatory action the past decade to reduce the threat of antimicrobial resistance, initially focusing on the areas where policy instruments were already at hand. Regulatory action is gradually but continuously expanded in terms of depth (more focused information enabling more detail, continuous checking and balancing efforts and results) and of scope (considering the need for attention to combined stressors, the necessity of international cooperation, and the exploration of the environmental dimensions). This reflects not only the growing understanding of the potential scope of the problem but also the changing views on the idea of public health. Risk assessment for antimicrobial resistance should ideally consider both potential public health and potential ecological effects. As mentioned above, from the public health point of view, the environment is a genetic pool directly affecting the exposure of the human population (and its pathogens) to resistance genes. Consideration of the environment for many aspects of public health has increased in the past decade, where a growing body of literature focusing on socioecologic links as a basis for health has emerged (McMichael, 1999; Krieger, 2001; Parkes and Horwitz, 2009; Waltner-Toews, 2004). Work in this area may become more and more vital for addressing cross-border and cross-setting risks such as antimicrobial resistance.

REFERENCES

American Society for Microbiology (ASM) (1995). Report of the ASM task force on antibiotic resistance. ASM, Washington, DC.

Aminov RI (2009). The role of antibiotics and antibiotic resistance in nature. *Environ Microbiol* 11(12):2970–2988.

Antonovics J, Abbate JL, Baker CH, Daley D, Hood ME, Jenkins CE, Johnson LJ, Murray JJ, Panjeti V, Rudolf VHW, Sloan D, Vondrasek J (2007). Evolution by any other name: Antibiotic resistance and avoidance of the e-word. *PLos Biol* 5(2):e30.

Baquero F, Martinez JL, Canton R (2008). Antibiotics and antibiotic resistance in water environments. *Curr Opin Biotechnol* 19:260–265.

Baquero F, Alvarez-Ortega C, Martínez JL (2009). Ecology and evolution of antibiotic resistance. *Environ Microbiol Reports* 1(6):469–476.

Blázquez J, Oliver A, Gómez-Gómez J-M (2002). Mutation and evolution of antibiotic resistance: Antibiotics as promoters of antibiotic resistance? *Curr Drug Targets* 3: 345–349.

Center for Drug Evaluation and Research/Center for Biologics Evaluation and Research) CDER/CBER (1998). *Guidance for industry: Environmental assessment for human drug and biologics applications*. CDER/CBER, Rockville, MD.

Center for Drug Evaluation and Research (CDER), Division of Anti-Infective Drug Products (2009). Draft guidance for industry: Microbiological data for systemic antibacterial drug products—Development, analysis, and presentation. CDER, Silver Spring, MD.

Center for Veterinary Medicine (CVM), United States Food and Drug Administration (2003). Guidance #152 for industry: Evaluating the safety of antimicrobial new animal drugs with regard to their microbiological effects on bacteria of human health concern. Docket No.98D-1146. CVM, Rockville, MD.

Center for Veterinary Medicine (CVM), United States Food and Drug Administration (2010). Draft guidance #209: The judicious use of medically important antimicrobial drugs in food-producing animals. CVM, Rockville, MD.

CHMP (2010). Guideline on the evaluation of medicinal products indicated for treatment of bacterial infections—Draft. CHMP/EWP/558/95 rev 2. EMEA, London.

CVMP (1999a). Antibiotic resistance in the EU associated with therapeutic use of veterinary medicines. EMEA/CVMP/342/99-corr-final. EMEA, London.

CVMP (1999b). A risk management strategic plan for controlling antimicrobial resistance through the authorisation of veterinary medicines—Recommendations consequent to the report and qualitative risk assessment of the CVMP. EMEA/CVMP/818/99-Final. EMEA, London.

CVMP (2006). CVMP strategy on antimicrobials 2006–2010 and status report on activities on antimicrobials. EMEA/CVMP/353297/2005. EMEA, London.

CVMP (2009a). Revised reflection paper on the use of 3rd and 4th generation cephalosporins in food producing animals in the European Union: Development of resistance and impact on human and animal health. EMEA/CVMP/SAGAM/81730/2006-Rev.1. EMEA, London.

CVMP (2009b). Concept paper on the use of macrolides, lincosamides and streptogramins in food-producing animals in the European Union: Development of resistance and impact on human and animal health. EMEA/CVMP/SAGAM/113420/2009-CONSULTATION. EMEA, London.

CVMP (2009c). Reflection paper on MRSA in food producing and companion animals in the European Union: Epidemiology and control options for human and animal health. EMEA/CVMP/SAGAM/68290/2009. EMEA, London.

Davies J (1994). Inactivation of antibiotics and the dissemination of resistance genes. *Science* 264:375–382.

D'Costa VM, McGrann KM, Hughes DW, Wright GD (2006). Sampling the antibiotic resistome. *Science* 311:374–377.

D'Costa VM, Griffiths E, Wright GD (2007). Expanding the soil antibiotic resistome: Exploring environmental diversity. *Cur, Opin Microbiol* 10:481–489.

Environmental Protection Agency (EPA) (2008). Proposed rule: Data requirements for antimicrobial pesticides (RIN 2070-AD30). *Fed Reg* 73(196).

European Commission (EC). (1970). Council Directive 70/524/EEC of 23 November, 1970 concerning additives in feedingstuffs.

European Commission (EC). (2001). Communication from the Commission on a Community Strategy against antimicrobial resistance. COM/2001/0333 final Volume I. June 2001. Available: http://ec.europa.eu/health/antimicrobial_resistance/policy/index_en.htm.

European Commission (EC). (2003). Regulation (EC) no 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition.

European Commission (EC). (2004). Commission Decision of 30 January 2004 concerning the non-inclusion of certain active substances in Annex I to Council Directive 91/414/EEC and the withdrawal of authorisations for plant protection products containing these substances.

European Commission (EC). (2005). Commission Decision 2005/303/EC of 31 March 2005 concerning the non-inclusion of cresylic acid, dichlorophen, imazamethabenz, kasugamycin and polyoxin in Annex I to Council Directive 91/414/EEC and the withdrawal of authorisations for plant protection products containing these substances.

European Commission (EC). (2009a). Commission Directive 2009/9/EC of 10 February 2009 amending Directive 2001/82/EC of the European Parliament and of the Council on the Community code relating to medicinal products for veterinary use.

European Commission (EC) (2009b). Second report from the Commission to the Council on the basis of Member States' reports on the implementation of the Council Recommendation (2002/77/EC) on the prudent use of antimicrobial agents in human medicine. SANCO-2009–11004–00–00-EN-REV-00.

European Food Safety Authority (EFSA). (2008). Scientific Opinion of the Panel on Biological Hazards on a request from the European Food Safety Authority on foodborne antimicrobial resistance as a biological hazard. *EFSA J*. 765:1–87.

European Food Safety Authority (EFSA). (2009a). Joint scientific report of ECDC, EFSA and EMEA on methicillin resistant *Staphylococcus aureus* (MRSA) in livestock, companion animals and foods. EFSA-Q-2009–00612. *EFSA* J 301:1–10.

European Food Safety Authority (EFSA). (2009b). Joint report on antimicrobial resistance (AMR) focused on zoonotic infections of the European Centre for Disease Control and Prevention; Scientific Opinion of the Panel on Biological Hazards; Opinion of the Committee for Medicinal Products for Veterinary Use; Scientific Opinion of the Scientific Committee on Emerging and Newly Identified Health Risks. *EFSA* J 7(11):1372.

Environmental Protection Agency (EPA). OW (2008). Response letter to Senator James M. Inhofe from Assistant Administrator Benjamin Grumbles. April 4, 2008. Available: http://epw.senate.gov/public/index.cfm?FuseAction=Files.View&FileStore_id=d8eed081–070c-43ab-b4cd-3a60d9918bce.

EU-US (2009). 2009 EU-U.S. Summit Declaration 3 November 2009. Available: http://www.eeas.europa.eu/us/sum11_09/docs/declaration_en.pdf. Accessed March 3, 2011.

Fidler DP (1998). Legal issues associated with antimicrobial drug resistance. *Emerg Infectious Dis* 174(4): No. 2, p. 169–177.

General Accounting Office (1999a). Antimicrobial resistance: Data to assess public health threat from resistant bacteria are limited (GAO/HEHS/NSIAD/RCED-99–132). U.S. Government Printing Office, Washington, DC.

General Accounting Office (1999b). Food safety: The agricultural use of antibiotics and its implications for human health (GAO/RCED-99–74). U.S. Government Printing Office, Washington, DC.

Henriques I, Moura A, Alves A, Saavedra MJ, Correia A (2006). Analysing diversity among beta-lactamase encoding genes in aquatic environments. *FEMS Microbiol Ecol* 56:418–429.

Heuer H, Smalla K (2007). Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. *Environ Microbiol* 9(3): 657–666.

Hughes Martiny JB, Bohannan BJM, Brown JH, Colwell RK, Fuhrman JA, Green JL, Horner-Devine MC, Kane M, Krumins JA, Kuske CR, Morin PJ, Naeem S, Øvreås L, Reysenbach A-L, Smith VH, Staley JT (2006). Microbial biogeography: Putting microorganisms on the map. *Nat Rev Microbiol* 4:102–112.

Interagency Task Force on Antimicrobial Resistance (ITFAR). (2001). A public health action plan to combat antimicrobial resistance, Part 1: Domestic issues. Centers for Disease Control and Prevention, Atlanta, GA.

Interagency Task Force on Antimicrobial Resistance (ITFAR). (2009). Inventory of progress, progress report: Implementation of a public health action plan to combat antimicrobial resistance. Centers for Disease Control and Prevention, Atlanta, GA.

Interagency Task Force on Antimicrobial Resistance (ITFAR). (2011). Official website of the ITFAR: http://www.cdc.gov/drugresistance/actionplan/actionPlanUpdate.html. Accessed January 2011.

IOM (1998a). The future of public health. National Academies Press, Washington, DC.

IOM (1998b). Forum on emerging infections, antimicrobial resistance: Issues and options. Workshop report. National Academies Press, Washington, DC.

IOM (2003). Microbial threats to health: Emergence, detection, and response. National AcademiesPress, Washington, DC.

Janssen S, Ewert F, Li H, Athanasiadis IN, Wien JJF, Thérond O, Knapen MJR, Bezlepkina I, Alkan-Olsson J, Rizzoli AE, Belhouchette H, Svensson M, van Ittersum MK (2009). Defining assessment projects and scenarios for policy support: Use of ontology in integrated assessment and modelling. *Environ Modelling Software* 24:1491–1500.

Jones-Lepp TL, Alvarez DA, Petty JD, Huckins JN (2004). Polar organic chemical integrative sampling and liquid chromatography-electrospray/ion-trap mass spectrometry for assessing selected prescription and illicit drugs in treated sewage effluents. *Arch Environ Control Toxicol* 47(4):427–439.

Krieger N (2001). Theories for social epidemiology in the 21st century: An ecosocial perspective. *Int J Epidemiol* 30:668–677.

Kümmerer K, Henninger A (2003). Promoting resistance by the emission of antibiotics from hospitals and households into effluent. *Clin Microbiol Infect* 9(12):1203–1214.

Larsen (2009). Overdose: How drugs and chemicals in water supplies and the environment are harming our fish and wildlife. Testimony before the Committee on Natural Resources, Subcommittee on Insular Affairs, Oceans and Wildlife, Washington, DC, June 9, 2009.

Larsson DGJ, de Pedro C, Paxeus N (2007). Effluent from drug manufactures contains extremely high levels of pharmaceuticals. *J Hazard Mat* 148(3): 751–755.

Leverstein-van Hall MA, Dierikx CM, Cohen-Stuart J, Voets GM, Van den Munckhof MP, van Essen-Zandbergen A, Platteel T, Fluit AC, Van de Sande–Bruinsma N, Scharinga J, Bonten MJM, Mevius DJ, on behalf of the national ESBL surveillance group (2011). Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. *Clin Microbiol Infect*. Available:http://dx.doi.org/10.1111/j.1469–0691.2011.03497.

Levy SB (2002). *The Antibiotic Paradox: How the Misuse of Antibiotics Destroys Their Curative Powers*. Perseus Cambridge, MA.

Linton AH (1986). Flow of resistance genes in the environment and from animals to man. *J Antimicrob Chemother* 15:385–386.

Loganathan B, Phillips M, Mowery H, Jones-Lepp TL (2009). Contamination profiles and mass loadings of macrolide antibiotics and illicit drugs from a small urban wastewater treatment plant. *Chemosphere* 75(1):70–77.

Martinez JL (2009a) Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environ Pollut* 157:2893–2902.

McMichael AJ (1999). Prisoners of the proximate: Loosening the constraints on epidemiology in an age of change. *Am J Epidemiol* 148:887–897.

Mensink BJWG, Montforts MHMM (2007). The ecological risks of antibiotic resistance in aquatic environments: A literature review. RIVM report 601500005/2007. RIVM Bilthoven, The Netherlands.

Midtvedt T (2004). The ECO-SHADOW concept—A new way of following environmental impacts of antimicrobials. In K Kümmerer (Ed.), *Pharmaceuticals in the Environment: Sources, Fate, Effects, and Risks*, 2nd ed. Springer; Berlin, pp. 311–316.

Moriarty F. (1983). *Ecotoxicology*. *The Study of Pollutants in Ecosystems*. Academic, New York.

National Antimicrobial Resistance Monitoring System (NARMS) (2011). Official FDA website of NARMS. http://www.fda.gov/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/default.htm. Accessed January 20, 2011.

Parkes MW, Horwitz P (2009). Water, ecology and health: ecosystems as settings for promoting health and sustainability. *Health Promotion Int* 24(1):94–102.

Phillips I, Casewell M, Cox T, de Groot B, Friis C, Jones R, Nightingale C, Preston R, Waddell J (2004). Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *J Antimicrob Chemother* 53:28–52.

Pitout JDD, Reisbig MD, Mulvey M, Chui L, Loue M, Crowe L, Church DL, Elsayed S, Gregson D, Ahmed R, Tilley P, and Hanson ND (2003). Association between handling of pet treats and infection with Salmonella enterica serotype Newport expressing the AmpC beta-lactamase, CMY-2. *Journal of Clincal Microbiology*. 41, 4578–82

Pruden A, Pei R, Storteboom H, Carlson KH (2006). Antibiotic resistance genes as emerging contaminants: Studies in Northern Colorado. *Environ Sci Technol* 40:7445–7450.

Pugh DM (2002). The EU precautionary bans of animal feed additive antibiotics. *Toxicol Lett* 128:35–44.

Rosdahl VT, Pedersen KB (Eds.) (1998). The Copenhagen Recommendations. Report from the Invitational EU Conference on the Microbial Threat, Copenhagen, Denmark, September 9 – 10, 1998

SCENIHR (2009). Assessment of the antibiotic resistance effects of biocides. European Commission, Brussels.

SCENIHR (2010). Research strategy to address the knowledge gaps on the antimicrobial resistance effects of biocides. European Commission, Brussels.

SCFCAH (2009). Standing Committee on the Food Chain and Animal Health of 2–3 July 2009. D(2009) 411097. Availeble: http://ec.europa.eu/food/committees/regulatory/scfcah/phytopharmaceuticals/sum_0203072009_en.pdf.

Schmitt H, Stoob K, Hamscher G, Smit E, Seinen W (2006). Tetracyclines and tetracycline resistance in agricultural soils: Microcosm and field studies. *Microb Ecol* 51:267–276.

Schwartz T, Kohnen W, Jansen B, Obst U (2003). Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS Microbiol* Ecol 43:325–336.

Scientific Steering Committee (SSC) (1999). Opinion of the Scientific Steering Committee on antimicrobial resistance of 28 May 1999. Available: http://ec.europa.eu/food/fs/sc/ssc/out50_en.pdf.

Singer RS, Ward MP, Maldonado G (2006). Can landscape ecology untangle the complexity of antibiotic resistance? *Nat Rev Microbiol* 4:943–952.

Swann MM (1969). Swann Report. Joint Committee on the use of Antibiotics in Animal Husbandry and Veterinary Medicine. Her Magesty's Stationery Office, London.

Thaker M, Spanogiannopoulos P, Wright GD (2010). The tetracycline resistome. *Cell Mol Life Sci* 67:419–431.

U.S. Congress House Committee on Energy and Commerce, Subcommittee on Health (2010a). Hearing on "Antibiotic Resistance and the Threat to Public Health," April 28, 2010, Rm 2123 Rayburn House Office Building, Washington, DC. Available: http://democrats.energycommerce.house.gov/index.php?q = hearing/antibiotic-resistance-and-the-threat-to-public-health. Accessed January 20, 2011.

U.S. Congress House Committee on Energy and Commerce, Subcommittee on Health (2010b). Hearing on "Promoting the Development of Antibiotics and Ensuring Judicious Use in Humans," June 9, 2010, Rm 2123 Rayburn House Office Building, Washington, DC. Available: http://democrats.energycommerce.house.gov/index.php?q = hearing/hearing-on-promoting-the-development-of-antibiotics-and-ensuring-judicious-use-in-humans. Accessed, January 20, 2011.

U.S. Congress House Committee on Energy and Commerce, Subcommittee on Health (2010c). Hearing on "Antibiotic Resistance and the Use of Antibiotics in Animal Agriculture", July 12, 2010, Rm 2123 Rayburn House Office Building, Washington, DC. Available: http://democrats.energycommerce.house.gov/index.php?q = hearing/hearing-on-antibiotic-resistance-and-the-use-of-antibiotics-in-animal-agriculture. Accessed January 20, 2011.

U.S. Congress Office of Technology Assessment (1995). *Impacts of Antibiotic-Resistant Bacteria*. OTA-H-629. U.S. Government Printing Office, Washington, DC.

Van de Velde W, Zehirov G, Szatmari A, Debreczeny M, Ishihara H, Kevei Z, Farkas A, Mikulass K, Nagy A, Tiricz H, Satiat-Jeunemaître B, Alunni B, Bourge M, Kucho K, Abe M, Kereszt A, Maroti G, Uchiumi T, Kondorosi E, Mergaert P (2010). Plant peptides govern terminal differentiation of bacteria in symbiosis. *Science* 327:1122–1126.

Vermeire TG, Jager DT, Bussian B, Devillers J, den Haan K, Hansen B, Lundberg I, Niessen H, Robertson S, Tyle H, Van der Zandt PT (1997). European Union System for the Evaluation of Substances (EUSES). Principles and structure. *Chemosphere* 34(8):1823–1836.

VICH (2000). Environmental Impact Assessment (EIAs) for veterinary medicinal products (VMPs)—Phase 1. Available: http://vich.eudra.org/htm/guidelines.htm. EMEA, London.

VICH (2004). Draft Environmental Impact Assessment (EIAs) for veterinary medicinal products (VMPs)—Phase II. Available: http://vich.eudra.org/htm/guidelines.htm. EMEA, London.

Waltner-Toews D (2004). *Ecosystem Sustainability and Health: A Practical Approach*. Cambridge University Press, Cambridge.

Wassenaar TM (2005). Use of antimicrobial agents in veterinary medicine and implications for human health. *Crit Rev Microbiol* 31:155–169.

Woodcock, J. (2010). Promoting the development of antibiotics and ensuring judicious use in humans. Testimony before the U.S. House of Representatives, House Committee on Energy and Commerce, Subcommittee on Health, Washington, DC, June 9, 2010.

World Health Organization (WHO) (2000). World Health Organization Report on Infectious Diseases 2000: Overcoming Antimicrobial Resistance (WHO/CDS/2000.2). WHO, Geneva.

World Health Organization (WHO) (2001). Global Strategy for the Containment of Antimicrobial Resistance (WHO/CDS/CSR/DRS/2001.2). WHO, Geneva.

Wright GD (2007). The antibiotic resistome: The nexus of chemical and genetic diversity. *Nat Rev Microbiol* 5:175–186.

Yim G, Wang HH, Davies JE (2007). Antibiotics as signalling molecules. *Philos Trans R Soc B* 362:1195–1200.

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