# Antimicrobial Resistance in Wastewater Treatment Processes

# Antimicrobial Resistance in Wastewater Treatment Processes

*Edited by Patricia L. Keen and Raphaël Fugère*

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*The editors dedicate this book to the memory of Fred Koch.*

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# Preface

Antimicrobial resistance is arguably the greatest threat to worldwide human health. This book evaluates the roles of human water use, treatment, and conservation in the development and spread of antimicrobial resistance.

The collection of wastewater dates back to the Roman Empire when sewage, surface runoff, and drainage water were received in the Cloaca Maxima and flushed into the Tiber River by water transported from a vast network of aqueducts. By the Middle Ages, several major urban centers developed throughout Europe that included systems of open ditches and wooden, lead, or clay pipes designed for the disposal of sewage. Rapid increases in population densities in major European cities since that time demanded considerable improvements in water distribution systems and wastewater management in order to protect the public health of citizens. However, it was not until the mid-nineteenth century that many of these improvements were, in fact, realized. Canals dedicated to the transport of wastewater for direct discharge into rivers were constructed, although frequently, drinking water pumps were installed in close proximity to the wastewater removal systems. In essentially all situations, wastewater was ultimately returned to the environment in an untreated form.

Since 1850, the increased frequency of disease outbreaks, such as cholera and typhus, has required dedicated engineering efforts for the treatment of wastewater. Early examples of sewage treatment were simply the application of lime to cesspools, intended to reduce foul odors. During the same period, infectious disease epidemics were still believed to be transmitted through the human population by exposure to filth and foul smells and via person-to-person contact. While major advancements in the disciplines of sanitary engineering and health sciences were being made, it became increasingly clear that water played a critical role in the spread of infectious disease among the human population and that the safety of drinking water was compromised by any possibility of exposure to sewage. Water flushed waste through vast networks of ditches and underground sewers, then discharged in major rivers such as the Thames, the Seine, and the Danube. This rapid growth of urban centers was associated with persistent objectionable smells emanating from water courses that bisected large cities. This, in turn, reinforced political will to improve environmental conditions and led to pioneering technologies in sanitary engineering.

The engineering of technologies specific to the treatment of wastewater experienced a period of unprecedented growth beginning in the mid-nineteenth century. Coincidentally, regulations intended to protect the environment from the impacts of discharge of sewage into receiving waters began to appear more frequently in many

major urban centers throughout Europe (Cooper, 2001). The goal of wastewater treatment was to ensure that effluent was sufficiently free of disease-causing entities that it could be released without impacting the safety of drinking water that was being employed for the human population. The secondary objective for improvement in wastewater treatment was driven by the economic incentive linked to the production of artificial guano (Cooper, 2001). Fertility of agricultural lands was declining to such an extent that crop yields were diminishing while the human population in urban centers was constantly growing. To counter this threat to food security, bird droppings were being imported from South America to the United Kingdom for use as agricultural fertilizer. Although land application of domestic waste had been practiced since Roman times, large areas of land adjacent to major urban centers were purchased and designated as "sewage farms." These farms for the land treatment of sewage needed increasing allotments of valuable land. They were subject to a number of weather-related complications and failed to achieve adequate hygiene standards that would ensure the protection of the health of farm workers and citizens at large. In this way, the intimate link between engineered systems for wastewater treatment, agricultural food production, and public health was firmly rooted throughout history.

Antimicrobial resistance in pathogenic organisms is a health risk that has been increasing for the last half century. Domestic sewage contains microbes originating from microbiomes of the human population resident in any community. Wastewater treatment plants receive influent composed largely by domestic sewage and therefore concentrate a vast and diverse collection of microbes in one location. Discharge of effluent from wastewater treatment plants represents the most important source of environmental contaminants, including those that are associated with development of antimicrobial resistance in bacteria.

For some time, antibiotic compounds have been identified as emerging contaminants and included in the category of pharmaceuticals and personal care products. Antibiotics can retain their activity after excretion from human patients such that bacterial communities in biological wastewater treatment systems are impacted by exposure to such contaminants and this antibiotic activity could potentially persist if their removal is incomplete following wastewater treatment. Improvements in instrumentation and tools for the analyses of genes in complex environmental samples have enhanced the ability to track mobile genetic elements of antimicrobial resistance through the wastewater treatment process. Increasing evidence is being gathered to suggest that the dynamic chemical, biological, and ecological conditions of operations in wastewater treatment processes influence the abundance of antimicrobial resistance genes in the effluents discharged to the environment after treatment. Because wastewater treatment plants receive sewage composed of contributions from gut flora of healthy and sick individuals, bacteria that are highly resistant to antibiotic therapy are increasingly detected in wastewater treatment systems.

More than 50% of the world's populations live in cities. Developing nations are witnessing a trend of accelerated urbanization that, in some cases, is accompanied by increased health risks. Clean water, in terms of availability and safe quality, remains a key concern in urban centers. Urban water cycles are now recognized as subsystems where patterns of water use, wastewater treatment, and water reuse play major roles in protection of public health.

Designed as a companion volume to *Antimicrobial Resistance in the Environment* (Wiley-Blackwell, 2012), this book is a multidisciplinary synthesis of topics related to antimicrobial resistance and wastewater treatment processes. Building on the increasing understanding of the central role of the environment in the development and spread of antimicrobial resistance, the book begins with five chapters that describe key issues from a more general perspective before focusing specifically on issues related to wastewater treatment processes. Detailed discussions concerning chemical analyses of antibiotics are included as well as comprehensive examinations of the features of experimental design that are particularly important in studies related to antimicrobial resistance. Advanced treatment strategies for mitigating the effects of factors that influence development and dissemination of antimicrobial resistance in the receiving environment are examined in detail. Several chapters discuss the ever-growing improvements in metagenomics, molecular methods, culture-based analyses, and gene sequencing capabilities, which are becoming popular for the examination of antimicrobial resistance in environmental samples, including those derived from wastewater.

We thank our fantastic team of contributing authors whom we are extremely pleased to regard as both our professional colleagues and our friends. Each chapter of this book has been crafted by some of the world's leading authorities on the topic, in many cases together with early career researchers who continue to explore unanswered questions about risks linked to the development and spread of antimicrobial resistance. We thank the team at Wiley-Blackwell led by Mindy Okura-Marszycki and Kshitija Iyer for guiding us through the entire process of assembling this book from concept to completion. We extend our sincere gratitude to Philippe Raphanel for the use of the image from his wonderful painting on the cover of this book.

Patricia offers her thanks to colleagues from the Department of Civil Engineering at the University of British Columbia and also to her colleagues at the New York Institute of Technology, especially those individuals in the Energy Management Program at the Vancouver Campus, for their roles in making the development of this book a fun and rewarding experience. As well, she would like to express her sincere appreciation to Steve Clark for kindness, patience, and technical support throughout the book preparation process. Raphaël offers his thanks to René Fugère for his encouragement and support throughout all the phases that have led to the completion of this book. This book owes its existence to the inspiration of Julian Davies' letter in *Nature* published in 2012. Patricia and Raphaël are deeply grateful to Julian for his willingness to share his inspiration, knowledge, friendship, and scholarly guidance, which have enabled us to complete this work.

## Reference

Cooper PF (2001). Historical aspects of wastewater treatment. In: *Decentralised Sanitation and Reuse: Concepts, Systems and Implementation*, P Lens, G Zeeman, and G Lettinga (eds). IWA Publishing.

# Préface

La résistance aux antibiotiques est probablement la plus grande menace à la santé humaine. Le présent livre traitera essentiellement des rôles que jouent le traitement, l'utilisation, et la conservation de l'eau dans le développement et la propagation de la résistance aux antibiotiques.

Le premier exemples documenté de collecte des eaux usées daterait de l'empire Romain, où les eaux usées et les eaux pluviales étaient dirigées vers le Cloaca Maxima ("Grand égout collecteur") et rejetées dans le Tibre par un vaste réseau de conduites d'égout. Dès le Moyen Âge, l'Europe vit plusieurs centres urbains se développer, et ces derniers incluaient des systèmes de fossés, ainsi que des conduites de bois, plomb, ou argile, afin d'évacuer les eaux usées. La densification rapide des centres urbains européens à cette époque demanda des avancées majeures afin de protéger la santé publique. Malgré cela, ce n'est pas avant la mi-19ème siècle que la plupart de ces avancées significatives furent réalisées. Des conduites dédiées à la canalisation des eaux usées vers les rivières environnantes furent mises en place, quoique des pompes d'eau potable furent installées à proximité immédiate des points de rejet. La stratégie universelle de gestion des eaux usées et pluviales demeurait le rejet direct au milieu naturel.

Depuis 1850, la fréquence accrue d'épidémies telles que le choléra et le typhus ont requis une ingénierie de systèmes de traitement des eaux usées. Ces mesures techniques étaient à l'origine assez sommaires, tel que le chaulage des fosses septiques afin d'atténuer les odeurs infectes qui s'en dégageaient. Au cours de la même période, la croyance populaire voulait que les épidémies étaient transmises via l'exposition à la "saleté", les mauvaises odeurs et les contacts physiques entre individus. Avec les avancées majeures dans les domaines du génie sanitaire et médical, il devint clair que l'eau jouait un rôle critique dans la propagation des maladies infectieuses au sein des populations humaines, et que l'eau potable était corrompue par tout contact avec des eaux usées. À cette époque, les eaux usées étaient quand même acheminées vers des rivières ou fleuves d'importance tels que la Tamise, la Seine, ou le Danube. Les cours d'eau traversant les grandes villes devinrent synonymes d'odeurs nauséabondes. Ceci eut pour effet de mener à une amélioration des conditions environnementales et le développement de technologies de pointes en génie sanitaire.

Le domaine du génie sanitaire a connu une période de croissance sans précédent vers la mi-19ème siècle. Conséquemment, la réglementation visant à protéger l'environnement de l'impact des rejets d'eaux usées dans les cours d'eau fit son apparition et se répandit à de nombreuses capitales européennes (Cooper, 2001). Le but visé par le traitement des eaux usées était de s'assurer que l'effluent était suffisamment exempt d'entités

causant des maladies pour être rejeté sans impacter directement l'eau potable utilisée par la population. Le but secondaire du perfectionnement du traitement des eaux usées était essentiellement économique et relié à la production de guano artificiel (Cooper, 2001). À cette période, la fertilité des sols était en telle décroissance que les rendements agricoles étaient en forte baisse, alors que la population urbaine croissait sans cesse. Afin de combattre cette menace à la sécurité alimentaire, des excréments d'oiseau étaient importés d'Amérique du Sud vers la Grande-Bretagne et étaient utilisés comme fertilisants agricoles. Malgré le fait que l'épandage d'eaux usées domestiques ait été pratiquée depuis l'époque romaine, de grandes superficies de terrain adjacentes aux centres urbains majeurs furent acquis et désignés comme "fermes de traitement des eaux usées". Ces fermes visant à traiter les eaux usées vinrent à requérir de plus en plus de surface de terrain, ce qui mit leur opération en péril. De plus, elles étaient sujet à un grand nombre de complications reliées à la météo et n'atteignirent jamais des standards d'hygiène suffisants pour permettre la protection des travailleurs agricoles ou des citoyens. C'est ainsi que le lien intime entre les systèmes de traitement des eaux usées, la production agricole et la santé publiques se souda.

La résistance aux antibiotiques des organismes pathogènes est un risque sanitaire qui ne fait que croitre depuis un demi-siècle. Les eaux usées domestiques contiennent des bactéries provenant des microbiomes de la population composant toute communauté urbaine. Les usines d'épuration des eaux usées reçoivent un affluent composé essentiellement d'eaux usées domestiques et se trouvent donc à concentrer une population microbienne vaste et diversifiée en un seul endroit. Les rejets des usines de traitement des eaux usées représentent donc une des plus importantes sources de contaminants environnementaux, incluant ceux qui sont associés avec le développement de la résistance aux antibiotiques chez les bactéries.

Il y a quelques années, les composés antibiotiques ont été identifiés comme contaminants émergents et incorporés à la catégorie des produits de soins personnels et pharmaceutiques. Même après excrétion par des patients, les antibiotiques peuvent préserver leur potentiel biochimique à un point tel qu'ils peuvent avoir un impact significatif sur la diversité microbienne des systèmes de traitement des eaux usées. Cette activité pourrait même persister dans l'environnement advenant une dégradation incomplète dans le système de traitement des eaux usées. Des avancées récentes au niveau de l'instrumentation et des outils d'analyse génétique dans des échantillons environnementaux complexes ont permis de retracer les marqueurs génétiques de la résistance aux antibiotiques tout au long de la chaîne de traitement des eaux usées. Il devient de plus en plus clair que les conditions chimiques, biologiques et écologiques d'opération des procédés de traitement des eaux usées affectent directement la quantité de gènes de résistance aux antibiotiques rejetés à l'environnement après traitement. Étant donné que les usines de traitement des eaux usées recueillent des eaux usées dont une partie provient autant de la flore intestinale de personnes malades que de la flore intestinale de personnes en santé, les bactéries résistantes aux antibiotiques sont de plus en plus détectées à l'affluent de systèmes de traitement des eaux usées.

Plus de cinquante pourcent de la population mondiale vit maintenant en zone urbaine. Les pays en voie de développement voient se développer une tendance d'urbanisation galopante, assortie de risques accrus à la santé publique. L'eau potable - tant en quantité qu'en qualité - demeure une préoccupation de premier plan au sein des centres urbains. Le cycle urbain de l'eau est maintenant reconnu comme tel, et il devient clair que

l'utilisation de l'eau, le traitement des eaux usées et la réutilisation de l'eau jouent des rôles cruciaux dans la protection de la santé publique.

Conçu comme un complément au volume *Antimicrobial Resistance in the Environment* (Wiley-Blackwell, 2012), ce livre est une synthèse multidisciplinaire de sujets touchant à la résistance aux antibiotiques et aux procédés de traitement des eaux usées. Se basant sur la compréhension grandissante du rôle central de l'environnement dans le développement et la propagation de la résistance aux antibiotiques, le livre débute avec cinq chapitres qui décrivent les points saillants d'un point de vue généraliste avant de se concentrer sur les enjeux ayant trait aux procédés de traitement des eaux usées. Des discussions détaillées des analyses chimiques sont présentées, tout comme l'examen en profondeur des particularités des protocoles expérimentaux qui sont particulièrement cruciaux dans le domaine de la résistance aux antibiotiques. Plusieurs procédés de traitement avancé visant à réduire le développement et la dispersion de la résistance aux antibiotiques dans l'environnement sont examinés en détail. Plusieurs chapitres discutent des améliorations dans les domaines de la métagénomique, des méthodes moléculaires, des analyses basées sur les cultures bactériennes et des méthodes de séquençage génétique qui deviennent de plus en plus populaires pour la détermination de la résistance aux antibiotiques dans des échantillons environnementaux, incluant des échantillons d'eaux usées.

Nous remercions notre incroyable équipe d'auteurs-contributeurs que nous considérons comme des collègues professionnels et des amis. Chaque chapitre de ce livre a été rédigé par plusieurs références internationales sur le sujet, en collaboration avec des chercheurs en début de carrière, qui continuent à explorer les questions encore sans réponse des risques reliés au développement et à la propagation de la résistance aux antibiotiques. Nous remercions l'équipe à Wiley-Blackwell, dirigée par Mindy Okura-Marszycki and Kshitija Iyer, qui nous ont guidés tout au long du processus de rédaction, de l'idée conceptuelle jusqu'à la publication. Nous exprimons également notre profonde gratitude envers Philippe Raphanel qui nous a gracieusement permis d'utiliser une photographie d'une de ses peintures pour égayer la couverture du livre.

Patricia offre ses remerciements à ses collègues du département de génie civil à University of British Columbia, ainsi qu'à ses collègues du New York Institute of Technology, particulièrement aux membres du programme de gestion de l'énergie au campus de Vancouver pour avoir fait de la rédaction de ce livre une expérience agréable et enrichissante. Elle voudrait également exprimer sa profonde appréciation à Steve Clark pour sa gentillesse, sa patience et son support technique tout au long du projet. Raphaël remercie chaleureusement René Fugère pour son support indéfectible et ses encouragements tout au long de cette expérience. Le projet de livre a été inspiré par une lettre de Julian Davies dans le journal *Nature*, publiée en 2012. Patricia et Raphaël tenaient à exprimer leur sincère reconnaissance envers Julian pour son enthousiasme à partager son inspiration, ses connaissances, son amitié et rigueur académique.

## Référence

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## About the Cover Artist

Paris-born Canadian painter Philippe Raphanel has a deeply held passion for the natural environment, which is reflected in nearly all of his work. As a member of the Young Romantics, a Vancouver-based group of artists whose work in the mid-1980s signaled a distinct shift in contemporary painting, Philippe refined a unique visual language that consistently references beauty in nature. Having spent much of his life on the west coast of Canada, his paintings are imbued with a similar reverence for sensuality in nature as that also captured in works by Emily Carr, Gordon Smith, and members of the Group of Seven. His work constantly explores the eternal bond between humans and the environment.

As an artist, Philippe is one of very few individuals whose formative years included a direct link to the realm of microbiology. As a young man in Paris, Philippe was close to long-time family friends and well-known microbiologists Germaine Stanier Cohen-Bazire and Roger Stanier. Known for their pioneering research in ultrastructure and physiology of cyanobacteria, Germaine and Roger Stanier introduced Philippe to the natural beauty of the microbiological world during their time at the Pasteur Institute, and later they invited him to their summer home in British Columbia. That appreciation for nature at the cellular level and, of course, the friendship has lasted a lifetime.

Philippe has been recognized with multiple awards celebrating achievement in contemporary art throughout his career and he is currently a lecturer at the Emily Carr University of Arts and Design in Vancouver, BC. His paintings can be found in museums, public institutions, corporate collections, and private collections worldwide. We are very pleased that a segment of Philippe's painting "Quick Sands" is presented on the cover of this book.

## List of Abbreviations

| | |
|---|---|
| ACs | antibiotic compounds |
| AOP | advanced oxidation process |
| AR | antibiotic/antimicrobial resistance |
| ARB | antibiotic/antimicrobial resistant bacteria |
| ARGs | antibiotic/antimicrobial resistance genes |
| BACI | before-after-control-impact |
| BHR | broad host range |
| CAS | conventional activated sludge |
| CEC | contaminant of emerging concern |
| CFU | colony forming unit |
| CPO | carbapenamase-producing organisms |
| DAGs | directed acyclic graphs |
| DGGE | denaturing gradient gel electrophoresis |
| DOC | dissolved organic carbon |
| DS | dissolved solids |
| dsDNA | double strand DNA |
| ESBL | extended-spectrum beta-lactamase |
| FTICR | Fourier transform ion cyclotron resonance |
| GC | gene cassette |
| GTA | gene transfer agent |
| HBP | human bacterial pathogen |
| HGT | horizontal gene transfer |
| HRMS | high-resolution mass spectrometry |
| HRT | hydraulic retention time |
| HWW | hospital wastewater |
| Inc | plasmid incompatibility |
| IR | inverted repeat |
| IS | insertion sequence |
| LC | liquid chromatography |
| LCMS | liquid chromatography tandem mass spectrometry |
| LOD | limit of detection |
| LOQ | limit of quantification |
| MBR | membrane bioreactor |
| MDR | multidrug resistant |

| | |
|---|---|
| MECs | measured environmental concentrations |
| MGEs | mobile genetic elements |
| MIC | minimal inhibitory concentration |
| MLOQ | method limits of quantification |
| MLS | macrolides-lincosamides and streptogramin |
| MPN | most probable number |
| MRM | multiple reaction monitoring |
| MRSA | methicillin resistant *Staphylococcus aureus* |
| MS | mass spectrometry |
| MST | microbial source tracking |
| NOM | natural organic matter |
| NPS | nonpoint source |
| ORF | open reading frame |
| PCR | polymerase chain reaction |
| PECO | population; exposure; comparator; outcome |
| PECs | predicted environmental concentrations |
| PS | point source |
| QACs | quaternary ammonium compounds |
| QMRA | quantitative microbial risk assessment |
| qPCR | quantitative polymerase chain reaction |
| QqQ | triple quadrupole mass spectrometry |
| RIs | resistance integrons |
| ROS | reactive oxygen species |
| RCTs | randomized control trials |
| RWI | reclaimed water irrigated |
| SAT | soil aquifer treatment |
| SMX | sulfamethoxazole |
| SPE | solid phase extraction |
| SRT | solid retention time |
| SS | suspended solids |
| TIAC | total investigated antibiotic concentration |
| TMP | trimethoprim |
| ToF | MS time of flight mass spectrometry |
| TOC | total organic carbon |
| TPs | transformation products |
| TRACA | transposon-aided capture |
| TWW | treated wastewater |
| UV | ultraviolet |
| UWWTP | urban wastewater treatment plant |
| VRE | vancomycin resistant enterococci |
| WW | wastewater |
| WWTP | wastewater treatment plant |

# 1

# Antimicrobial Resistance Genes and Wastewater Treatment

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Since ancient times, humans have randomly disposed of waste into the environment, such as in rivers and cesspits. The industrial revolution of the late eighteenth and early ninteenth centuries was a period that saw increased disposal of toxic organic chemicals by direct release into the environment. Many of these toxic molecules had antimicrobial activity, and it can be assumed that microbes resistant to these toxins multiplied in such environments. As a modern example, one can cite the concentrations of heavy oils that were dumped near detection stations in the distant early warning line at the end of the Second World War. These sites are now excellent sources of bacteria with enhanced biodegradation capacities and have been extensively studied in recent years.

Following the discovery of the chemically synthesized sulphonamides and trimethoprim and the identification of dual resistance in 1969, the subsequent and most disastrous environmental pollution has come from the disposal of antibiotic production wastes in various forms. These discarded products were developed as food supplements for farm animals to promote weight gain in all aspects of animal and fish husbandry worldwide. The amounts of antibiotics and antibiotic wastes disposed in this way cannot be accurately identified. However, according to recent estimates by the Union of Concerned Scientists in the United States, antibiotic use for nontherapeutic purposes in three major livestock sectors (chickens, cattle, and swine) was about eight times more than the consumption for human medicine (Mellon et al., 2001).

In the past 50 years, we have seen the rapid evolution of a new plague—that of worldwide antibiotic resistance. Though not a disease in itself, antimicrobial resistance (AR) results in the failure to effectively prevent and treat many diseases, leading to widespread untreatable microbial infections and greatly increased morbidity and mortality: a plague of resistance genes (Davies and Davies, 2010). The global use of antibiotics at low cost, auto medication, and short duration of treatment has accelerated, extended, and expanded the spectra of resistance worldwide. The earth has been continuously bathed in a dilute solution of antibiotics for more than half a century.

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Aquatic ecosystems have been identified as hotspots of resistance mechanisms (Rizzo et al., 2013). This is due to the large diversity of pathogenic and commensal microorganisms and the continuous discharge of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) into these environments. As part of aquatic ecosystems, urban wastewater treatment systems (collecting sanitary sewage, hospital effluents, and storm water runoff) possess all the components required to ensure the acquisition of all varieties of resistance genes. The antimicrobials present in wastewater due to incomplete degradation by humans and animals, disposal of unused drugs, and runoff losses from land application, together with environmental and pathogenic bacteria in nutrient-rich engineered systems, provide all the necessary requirements to support a breeding ground for horizontal gene transfer and the propagation of resistance genes (Davies and Davies, 2010; Ferreira da Silva et al., 2006; Kim and Aga, 2007; Lefkowitz and Duran, 2009).

Since 1890 with the building of the first biological wastewater treatment plant (WWTP) in Worcester, Massachusetts, advances in wastewater treatment technology have been improving the efficient removal of biodegradable organic pollutants. Currently, enhanced biological phosphorus removal processes have not only enabled the removal of traditional carbonaceous contaminants but also reduced phosphorus concentrations to very low levels (<0.1 mg/L) in the effluent discharge (Zuthi et al., 2013).

Over the past 15 years, increasing attention has shifted toward the identification and removal mechanisms of micropollutants from wastewater and sludge. Micropollutants are persistent organic or mineral substances such as pharmaceuticals and personal care products, detergents, and pesticides whose discharge, even at very low concentrations, is a constant growing environmental contamination (Luo et al., 2014).

Despite the evolution of wastewater treatment technologies from conventional to advanced treatment configurations, existing urban biological wastewater treatment systems are not designed to remove micropollutants and ARGs. Studies on antibiotics as emerging classes of micropollutants have confirmed the high frequency of antimicrobial resistant genotypes as well as ARB in wastewater treatment systems, including constructed wetlands and WWTPs (Martins da Costa et al., 2006; Kim et al., 2010; Volkmann et al., 2004; Luczkiewicz et al., 2010; Reinthaler et al., 2003).

In a landmark series of papers published between 2003 and 2009, Szczepanowski and colleagues presented the first extensive DNA sequence–based screening of a large set of known ARGs in samples of activated sludge and the final effluent of a WWTP in Bielefeld-Heepen, Germany. This comprehensive survey identified 140 different clinically relevant antimicrobial resistant genotypes and contaminants. From these investigations, it is evident that such treatment systems may play important roles in the development and assortment of multidrug-resistant (MDR) bacteria among complex populations.

The occurrence of ARB and ARGs in the two main by-products of wastewater treatment systems (biosolids and effluent discharge) has been reported frequently. Currently, effluent water quality standards, prior to discharge, are limited to controlling the concentrations of carbonaceous biochemical oxygen-demanding matter, suspended solids, total residual chlorine and un-ionized ammonia. There exist no regulatory guidelines to monitor and control the levels of ARGs in bacteria and extracellular DNA from lysed microbial cells in the effluent discharge. Accordingly, studies have reported that

antibiotic resistance determinants and MDR pathogens are transported from the effluent to the receiving water (Iwane et al., 2001; Galvin et al., 2010; Goñi-Urriza et al., 2000). For example, LaPara et al. (2011) showed that the quantities of three tetracycline resistance genes were significantly higher in a tertiary treated effluent discharge than in receiving water samples in the St. Louis River, Duluth-Superior Harbor, and Lake Superior, USA.

Despite the evidence for the occurrence of resistance genes in effluent discharge points, the overall impact of treated wastewater applications on irrigation processes is unclear. Some studies have observed an increase in soil microbial activity and biomass after irrigation by treated wastewater as shown by a shift in the composition of soil bacterial communities (Oved et al., 2001; Broszat et al., 2014). However, recent studies have observed no significant impact on AR in the wastewater-irrigated soil microbiome (Gatica and Cytryn, 2013; Negreanu et al., 2012).

The presence of ARB and ARGs in biosolids-amended soils is well documented (Brooks et al., 2006; Rahube et al., 2014). Biosolids are the treated and stabilized nutrient-rich organic residuals produced as a by-product of wastewater treatment and widely used as fertilizer to stimulate plant growth (Lu and Stoffella, 2012). Recent studies have demonstrated that complementary technologies such as aerobic digestion and lime stabilization can be used as approaches to reduce the quantities of ARGs in biosolids (Munir et al., 2011). However, ARG concentrations and corresponding decay rates can be variable depending on the application methods, biosolids treatment reactor design, storage conditions, the specific ARGs involved, and the frequency of biosolids application (Burch et al., 2013; Miller et al., 2014).

Although ARB and genes encoding antibiotic resistance have been commonly detected in wastewater and the by-products of treatment systems, the role of wastewater treatment processes in the dissemination of AR is not clear. In recent years, a number of studies have investigated the variables affecting the patterns of ARB and ARGs during treatment processes (Xia et al., 2012; Yuan et al., 2014). However, in spite of many studies indicating a contribution from treatment processes to the evolution, spread, and positive selection of antimicrobial resistant isolates, it has been shown that wastewater treatment process can act as efficient barriers to decrease the number of ARB and concentrations of ARGs (Gao et al., 2012; Duong et al., 2008; Nagulapally et al., 2009). The reasons for such discrepancies are the large number of variables in conditions such as influent source, input quality, treatment process configurations, and operating conditions.

Hospital wastewater is probably a major contributor to the spread of pathogenic MDR bacteria in WWTPs (Brown et al., 2006). Due to the presence of constant subinhibitory levels of broad-spectrum antimicrobials, hospital sewage creates a perfect situation for the exchange of ARGs and their combinations between clinical pathogens and environmental bacteria (Amador et al., 2015; Santoro et al., 2015). In this respect, the ratios of influent wastewater from institutions (including hospitals), blackwater (excreta, urine, and fecal sludge), graywater (kitchen and bathing wastewater), storm water, and other urban runoff sources are important determinants of the input quality, the frequency of detection of ARGs and pathogenic ARB, and the dissemination of antibiotics and AR from treatment plants (Harris et al., 2013).

Over the past few years, some European countries have constructed specialized WWTPs to provide separate treatment of hospital wastewater (HWW). With membrane

bioreactors as a pretreatment, ozonation, and powdered and granulated activated carbon have been proposed as the most attractive options to remove micropollutants from HWW (Beier et al., 2010; Beier et al., 2012; Kovalova et al., 2013). Very recently, Chonova and coworkers (2016) published a comparative study on the efficiency of the removal of antibiotics from parallel wastewater systems providing separate treatment of hospital and urban wastewater. Despite the higher concentration of antibiotics in the hospital influent as well as treated effluent, the results indicated increased removal efficiency of antibiotics during the separate treatment of HWW. It was also demonstrated that biofilm communities receiving hospital treated effluent had lower bacterial diversity and less developed biomass. Observations from this study confirm the adaptations of wastewater bacterial communities receiving HWW. With respect to the dedicated treatment of hospital waste, more studies are needed to reveal the mechanisms by which adapted biofilm microbial communities can be transferred to aquatic environments.

Advanced wastewater disinfection technologies such as ultraviolet radiation and ozonation are effective approaches to decrease the extent of ARB and levels of ARGs (Zhang et al., 2015). However, other research has observed higher survival rates of resistant strains compared to sensitive bacteria, selection of ARGs, and shifts in bacterial population in the effluent after advanced treatments (Lüddeke et al., 2015; McKinney and Pruden, 2012; Alexander et al., 2016; Hu et al., 2016). Variations in reports on the efficiency of advanced approaches to wastewater treatment in controlling AR may be due to underestimates of the roles of variable operating conditions.

Solids retention time (SRT) is a design and operational parameter that has a crucial impact on the performance of activated sludge processes. SRT or the mean cell residence time is defined as total solids mass present in the system divided by solids mass disposed of per day (Clara et al., 2005). As SRT controls the net growth rate of the entire system, it is the main factor influencing dominant composition of a wastewater microbial community (Benefield and Randall, 1980; Xia et al., 2012). As an example, Liu and Wang (2014) showed that the nitrite-oxidizing bacteria/ammonia-oxidizing bacteria ratio is significantly influenced by variations in SRT.

A recent approach to wastewater management minimizes sludge production through microbial predation and metabolic changes (Amanatidou et al., 2015). One of the key factors that influences bacterial ecosystem manipulation and reduces the excess production of sludge is operation of the system at high SRTs (Yoon et al., 2004; Li and Wu, 2014). However, the role of prolonged SRT on the composition of bacterial processes contributing to AR is not yet clear. Although antibiotic degradation is maximized by prolonged cell residence time, extended exposure of bacteria to antibiotics from the source may increase the potential for development of AR (Walston, 2013; Xia et al., 2012). Meanwhile, environmental concerns associated with transformation of antibiotics into other biologically active compounds during the extended SRT operations have not been considered in many cases. More detailed research is required to detect antimicrobial degradation products in the treatment process and to investigate the optimal SRT required to achieve the best ARG removal.

Another serious operating challenge in wastewater management is the control of filamentous bulking and foaming. Although filamentous microorganisms support the activated sludge floc formation, their overabundance in WWTPs causes considerable operational difficulties such as poor sludge settling and thickening (Cydzik-Kwiatkowska and Zielińska, 2016; Pal et al., 2014). Different strategies have been employed to control

foaming, including polymer addition, the application of disinfectants such as chlorine, and the use of foam-classifying selectors to skim and remove foaming bacteria (Parker et al., 2003). The use of bacteriophages to reduce the concentration of filamentous bacteria is one of the most promising environmentally friendly approaches to control foaming (Liu et al., 2015). Despite the role of foaming bacteria on the efficiency of the treatment process and the environmental risks associated with foam disposal or formation of undesirable chlorinated by-products, no studies of antibiotic resistance patterns in foam-causing bacteria have been reported. More detailed studies of the impact of chemical disinfectants on the susceptibility profiles of foam-causing bacteria and the survival and gene transfer after disposal of resistant foaming bacteria and their survival are required.

For more than a decade, culture-dependent approaches have been the most common methods to study antimicrobial resistance in WWTPs (Al-Bahry et al., 2009; Okoh and Igbinosa, 2010). In these studies, resistance profiles of pathogenic population subsets of bacterial communities downstream of the effluent discharge were studied. (Lefkowitz and Duran, 2009; Akiyama and Savin, 2010; Zhang et al., 2009).

Culture-dependent methods have also been used to investigate the role of mobile genetic elements (MGEs) in the dissemination of antibiotic resistance genes in WWTPs. It has been shown that MGEs influence bacterial evolution, adaptation, and the roles that genetic elements play in the emergence, recombination, and propagation of antibiotic resistance (Jackson et al., 2011). Studies to date have documented the incidence of integron-associated ARG cassettes on MGEs such as plasmids in WWTP samples (Tennstedt et al., 2003; Koczura et al., 2012; Kotlarska et al., 2015; Ma et al., 2011). In this respect, understanding the correlation between the distribution of pathogenic bacteria and associated integron patterns will aid in clarifying resistance mechanisms in WWTPs.

In addition to resistance genes and associated elements, it is probable that virulence and biodegradation gene clusters are propagated in WWTPs. In a recent publication, Olaniran and colleagues (2015) detected four virulence-associated genes in *Listeria* and *Aeromonas* spp. isolated from treated effluents of two WWTPs and receiving waters in Durban, South Africa. This study emphasizes the need for more investigation of virulent bacteria found in WWTPs and the co-occurrence of virulence genes and ARGs.

Despite the advantages of culture-dependent techniques, including low cost and the potential to combine with other methods, the availability of culture-based methods for studies of environmental microbes gives a highly restricted view of microbial community structure in environmental ecosystems (Mahmod, 2014; Heidrich et al., 2016). Hence, culture-based approaches are not appropriate for comprehensive studies of the diversity and abundance of ARGs as well as the incidence of MGEs in WWTPs (Wang et al., 2013). To date, a variety of molecular approaches have been applied to study the relationships between wastewater microbial communities and treatment process performance (Shah, 2014; Jabari et al., 2016; Gómez-Villalba, 2006; Kim et al., 2013; McIlroy et al., 2015; Ju et al., 2014). A brief summary of the most commonly used molecular techniques is provided in Table 1.1.

The application of targeted (PCR- and/or microarray-based) and sequenced-based metagenomics provides more extensive and accurate assessments of the abundance of ARGs and the phylogenetic and functional diversity of wastewater resistome (Schmieder and Edwards, 2012; Parsley et al., 2010; Ma et al., 2016). As an example, Wang and

**Table 1.1** Summary of culture-independent approaches used to identify the diversity and activity of microorganisms in wastewater treatment processes (adapted from Ahmad et al., 2011, page 32).

| Partial Community Analysis Approaches | Whole Community Analysis Approaches |
|---|---|
| Denaturing gradient gel electrophoresis (DGGE) | Whole genome sequencing |
| Temperature gradient gel electrophoresis (TGGE) | Sequenced-based metagenomics |
| Single strand conformation polymorphism (SSCP) | G + C fractionation |
| Deoxyribonucleic acid (DNA) microarrays | Functional metagenomics |
| Real time-polymerase chain reaction (RT-PCR) | Metatranscriptomics |
| Fluorescence in situ hybridization (FISH) | Metaproteomics |

coworkers (2013) performed a metagenomic study of MGEs and ARGs in both anaerobic and aerobic sludge of a tannery WWTP in China. Metagenomic analyses showed that the taxonomic classification, as well as the abundance of functional genes in aerobic and anaerobic sludge microbial communities, was different. They also observed a high prevalence of insertion sequences (ISs) and integron-integrase genes highlighting the important role of MGEs in gene transfer in the tannery WWTP.

Recently, Li and coworkers (2015) compared the metagenomic libraries of total and plasmid DNA from influent, activated sludge and digested sludge of two WWTPs in Hong Kong. They observed that, compared to DNA metagenomes, plasmid metagenomes encoded significantly higher numbers of ARGs. This emphasizes the significant role of horizontal gene transfer in WWTPs. They also observed a prominent decrease in the number of ARGs in activated and digested sludge metagenomes compared to the influent metagenome.

Despite the many advantages of high-throughput shotgun sequencing methodologies to identify the structure of biological wastewater communities as well as WWTP resistomes, these approaches do not provide definitive relationships of ARGs to their host microorganisms. This may lead to varied correlations between WWTP resistome content and corresponding microbiome (Noyes et al., 2016). In addition, the scope of metagenomic read mapping approaches is limited to prior knowledge of resistance genes (i.e., through comparison of the sequence reads to known ARGs). In this respect, function-based metagenomics are more valuable approaches as they have the potential to identify novel ARGs and MGEs and to correlate resistance genes with the community structure. A functional metagenomics approach involves construction of metagenomic libraries through extraction of DNA, cloning DNA fragments, heterologous expression in surrogate hosts, and screening for specific activities (Lam et al., 2015).

In recent years, functional metagenomic studies of antibiotic resistance in environmental microbiomes such as soil and marine water have added considerably to our knowledge of the diversity of the natural gene pool of ARGs and revealed many unknown functions (Torres-Cortés et al., 2011; Donato et al., 2010; Schmieder and Edwards, 2012; Hatosy and Martiny, 2015). However, only limited studies have constructed functional metagenomic libraries from compartments of wastewater treatment processes and studied the diversity of ARGs and their host organisms (Amos et al., 2014; Uyaguari et al., 2011; Li et al., 2015). In a recent publication, Munck and coworkers (2015) combined

metagenomic functional selections and deep metagenomic sequencing data to identify the diversity of ARGs in a core WWTP resistome in Denmark. They found that the core resistome consists of stably maintained and (mostly) novel ARGs that confer resistance to the 15 antimicrobials tested. They also showed that the WWTP microbial community is remarkably stable with a strong correlation between the resistome and the microbial composition and limited gene transfer with the human gut microbiota.

Recently, integrated "omics" analyses have provided an enhanced understanding of the species and their functions in wastewater microbial systems (Narayanasamy et al., 2015). As an example, Roume and coworkers (2015) showed that seasonal variations did not significantly affect the expression of enzymes involved in nitrogen metabolism in the anoxic tank of a biological WWTP in Luxembourg. However, in winter, when lipid accumulation was higher, they observed significant expression of enzymes involved in glycerolipid metabolism. As an integrated "omics" analysis identifies the links between genes encoding key biological functionalities and functionally important community members, it can be used to optimize the wastewater treatment processes. This can be done through enrichment of favourable microorganisms such as lipid-accumulating organisms as proposed by Roume and coworkers (2015).

There is much current research focused on gaining a better understanding of the role of wastewater treatment in propagation and selection of antimicrobial resistance. Current information suggests that WWTPs serve as a nexus between contaminants in human waste and the environment. However, there are still many gaps in our knowledge that need to be addressed to help understand whether WWTPs are a minor, major, or variable contributor to the worldwide problem of antibiotic resistance.

A potential approach to the global concern of AR is to generate truly novel antibiotics with narrow spectra of action that can be combined with inhibitors of AR function. There is no shortage of potential therapeutic agents in nature; there are many novel antibiotics to be discovered and current methodology comes nowhere near to exhausting the molecular richness of natural environments. Creative screening approaches that rely on properties such as signaling will likely lead to a constant supply of novel bioactive compounds by using bioinformatic-heterologous expression approaches (Donia and Fischbach, 2015). However, new antibiotics will have short useful lives unless there is strict control of their use. AR is an evolutionary response by microbes that has had drastic consequences for the human race. It is essential that studies of the origins of AR and their "natural" functions be a priority. A key component will be to understand how AR diversity is generated as a result of rapid gene transfer and turnover. Finally, it must be recognized that the worldwide plague of AR was entirely manmade and could/should have been prevented and/or contained by stricter control of the use of antibiotics. Without appropriate regulations and strict compliance, the evolution and dissemination of AR will never be prevented.

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# 2

# When Pathogens and Environmental Organisms Meet

## Consequences for Antibiotic Resistance

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It is generally assumed that antibiotic resistance genes currently present in human bacterial pathogens originated in environmental microorganisms. The origins of only a few resistance genes, such as *qnr* or $bla_{\text{CTX-M}}$, have been investigated; in all cases they have been tracked to environmental bacteria that, in these cases, were not antibiotic producers. Together with functional metagenomic studies, which show that environmental microbiota contain a large number of genes capable of conferring resistance to heterologous hosts, this indicates that the first event in the acquisition of resistance requires the coexistence of the recipient pathogens with the donor environmental microorganisms. In this chapter, we will review the different bottlenecks involved in the transfer of resistance genes from environmental microorganisms to bacterial pathogens. In addition, we will discuss the effect that the constant release of plasmid-encoded resistance genes in natural ecosystems may have for the spread of antibiotic resistance.

## Introduction

Antibiotic resistance is a relevant problem for human health (WHO, 2000; WHO, 2010; WHO, 2014) and is also one of the few evolution processes that does not require geological times to happen (Martinez et al., 2009a). Indeed, the spread of resistance among human pathogens has occurred in a human lifetime, and the emergence and spread of resistance to a new antibiotic usually requires about 20 years or even less to take place (Bush et al., 2011). Bacterial pathogens were susceptible when antibiotics were introduced for therapy in the first half of the last century. Nevertheless, resistant microorganisms were soon selected by two different genetic events: mutation (Martinez and Baquero, 2000) and horizontal gene transfer (Ochman et al., 2000; Baquero et al., 2002; Normark and Normark, 2002; Baquero and Coque, 2011). Mutation is a traceable event; in vitro studies of experimental evolution allow the determination of all potential

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mutations involved in the acquisition of resistance and even the prediction, to some extent, of those that have not yet been found among antibiotic resistant clinical isolates of bacterial pathogens (Munck et al., 2014; Salverda et al., 2010; Novais et al., 2010; Barlow and Hall, 2003a; Barlow and Hall, 2003b; Shcherbakov et al., 2010). Reconstruction (and prediction) of the evolution of resistance is by far more difficult in the case of the acquisition of antibiotic resistant genes. This uncertainty is because the first events of such acquisition usually happen outside clinical settings, and in most cases the original host of the resistance gene is ignored. Despite these problems, there are some rules that may help in predicting the emergence of resistance and, more importantly, the capability of a given gene to spread among the populations of bacterial pathogens (Martinez et al., 2011; Martinez et al., 2007).

To implement such rules, however, we need to precisely define an antibiotic resistance gene (Martinez et al., 2015b). In a situation where the low price of high-throughput sequencing methods allows exploration of all potential resistance determinants in any ecosystem, without culturing the organisms, getting a precise definition of resistance so as to avoid false positives is highly relevant. It should be noted here that, while some studies of the resistome are based in functional metagenomic assays (Sommer et al., 2009; Forsberg et al., 2012), for several other studies the identification of resistance genes is based on homology criteria. Whereas for highly similar (nearly identical) genes this is a valid approach, sequence-based predictions are not sufficiently robust when the identity threshold is relaxed (Martinez et al., 2015a; Martinez et al., 2015b).

In addition, the fact that environmental microorganisms are not included in the breakpoint-based clinical databases that classify bacteria as resistant or susceptible makes it difficult to study resistance in the case of nonpathogenic microorganisms, even when functional approaches are implemented (Berendonk et al., 2015). The clinical definition of antibiotic resistance is based on the likelihood that the treatment of an infection by the given pathogen would not result in clinical success (Turnidge and Paterson, 2007). Obviously, this definition cannot stand for nonpathogenic bacteria. However, the epidemiological (ecological) definition of resistance is based on the screening of a large number of isolates of a given bacterial population and the identification of those (the resistant ones) presenting higher minimal inhibitory concentrations than the bulk of the population (Baquero, 1990; Kahlmeter et al., 2003; Kronvall et al., 2011). This second method can be used for nonpathogenic environmental microorganisms as well as for antimicrobials that are not used for treating infections. We can expect that, in the absence of a relevant exposure to antibiotics in natural environments, populations of environmental bacterial strains should present monomodal distributions, around peaks corresponding to the natural level of susceptibility (modal MIC) to the different species. Analysis of these distributions searching for bi- or multimodality could be of interest to infer the evolution of natural bacterial communities toward antibiotic resistance, and to assess the influence of eventual anthropogenic releases of antimicrobial agents on the acquisition of antibiotic resistance.

A more technical definition can be applied to describe antibiotic resistance in other kinds of organisms, based on the comparison of the susceptibility phenotype of a parental strain with another one harboring a mutation or a putative resistance gene. This approach is more suitable for application in the case of functional metagenomics as well as on experimental studies on the spread of antibiotic resistance (Martinez et al., 2015a). Using this last definition, several works have already shown that antibiotic resistance

genes can be found in any ecosystem and that their numbers exceed those actually found in human pathogens by several orders of magnitude (Bush et al., 2011; Sommer et al., 2009; Forsberg et al., 2012; Forsberg et al., 2014; D'Costa et al., 2011; Wright, 2010; Kaminski et al., 2015; Clemente et al., 2015). This situation indicates that there are serious bottlenecks that modulate the transfer of a resistance gene from the original, mainly environmental, microorganism to bacterial pathogens and that identifying these bottlenecks may prove valuable for implementation of new strategies for fighting resistance from a one-health standpoint (Martinez, 2011).

## Origin of Antibiotic Resistance Genes

Antibiotics have been developed against particular bacterial pathogens, which mean that such pathogens must necessarily be susceptible to such antibiotics; otherwise the drug would not be introduced for therapy. This does not mean that bacterial pathogens do not harbor resistance genes. Indeed, the analysis of the intrinsic resistome of bacterial pathogens has shown that they present a large number of genes capable of conferring resistance upon expression in a heterologous host or increase the susceptibility of the organism naturally hosting them if they are inactivated (Olivares et al., 2013; Cox and Wright, 2013; Blake and O'Neill, 2013; Martinez, 2012b; Alvarez-Ortega et al., 2011; Alvarez-Ortega et al., 2010; Tamae et al., 2008; Fajardo et al., 2008; Fernandez et al., 2013). Nevertheless, until now, the contribution of these intrinsic resistance genes to the spread of resistance through horizontal gene transfer is negligible. Further, the analysis of plasmids present in human pathogens before and after the generalized use of antibiotics for therapy has shown that there has not been an enrichment of plasmids previously carrying resistance genes that evolved toward resistance before the antibiotic era. Rather, the backbone of the plasmids before and after the antibiotic era is very similar, but the latter now contain resistance genes (Datta and Hughes, 1983). This indicates that the major force driving the horizontal gene transfer and the fixation of antibiotic resistance genes is the use of antibiotics, mainly in farms and for therapy of human infectious diseases. As the consequence of this extensive use, natural ecosystems are now polluted with antibiotics, which constitute a second cause for the emergence, evolution, and spread of antibiotic resistance (Berendonk et al., 2015; Martinez, 2012b; Martinez, 2009a; Martinez, 2009b; Martinez, 2008). The effects of antibiotics on the selection and the evolution of antibiotic resistance in soils are probably enhanced by common fertilization strategies (Nolvak et al., 2016). Altogether, this indicates that, at least in the case of the selection of antibiotic resistant organisms, humans constitute the greatest evolutionary force (Palumbi, 2001).

Since most antibiotics currently used for therapy are produced by environmental bacteria (Waksman and Woodruff, 1940), it was earlier thought that resistance genes should originate from the organisms producing them (Davies, 1994; Davies, 1997). Further, it was discussed that DNA from producers, present in the antibiotic preparations as a result of fermentation-based production, might be a source of antibiotic resistance genes (Webb and Davies, 1993). It is true that the genomes of producers must harbor genes encoding decontamination elements that are capable of impeding the action of the antibiotic that they synthesize in the producing cells. It is also true that resistance genes present in antibiotic producers frequently belong to the same families

as those that bacterial pathogens have acquired by horizontal gene transfer (Benveniste and Davies, 1973). However, until now, among clinically significant resistance genes that human pathogens have acquired, none have been shown to originate in an antibiotic producer. Indeed, in the few cases in which the origin of antibiotic resistance genes has been unequivocally tracked, they were documented in nonpathogens, and likely playing housekeeping functions that differ from their proposed role as antibiotic resistance determinants (Martinez et al., 2009a; Baquero et al., 2009).

One of such genes is *qnr*A, which encodes a low-level quinolone resistance determinant (Martinez-Martinez et al., 1998). It is important to note that quinolones are synthetic antibiotics, and hence it is unlikely that quinolone resistance genes have evolved in nature to counteract the action of these antimicrobials (Hernandez et al., 2011). It has been hypothesized that *qnr*A might be involved in the bacterial response to the presence of phages, to DNA damage or to cold shock (Kim et al., 2011; Wang et al., 2009). For achieving this function, *qnr*A binds the bacterial topoisomerases, which are the targets of the quinolones, and this binding modifies the affinity of quinolones for such targets (Tran et al., 2005). As the consequence of this activity, the microorganisms harboring *qnr*A are less susceptible to the action of quinolones, although the original role of such determinant was not antibiotic resistance. The same happens for multidrug efflux pumps (Garcia-León et al., 2014; Alvarez-Ortega et al., 2013; Martinez et al., 2009b), or antibiotic inactivating enzymes such as CTX-M, chromosomally encoded *Amp*C beta-lactamase (Henderson et al., 1997), or the *Providencia stuartii* 2'-N-acetyltransferase (Macinga and Rather, 1999; Canton et al., 2012; Humeniuk et al., 2002). Their natural substrates are structurally similar to antibiotics currently used in therapy, which means that they can extrude or modify these compounds despite the fact that their original function was not antibiotic resistance (Baquero et al., 2009; Fajardo et al., 2009). Under this scope, nearly any microorganism can be a source of antibiotic resistance genes (Martinez, 2008); thus, predicting which one among those potential resistance genes present in a natural ecosystem will transfer into a human pathogen as a response to the introduction of a new antimicrobial drug is likely beyond the possibilities of current knowledge in the field (Martinez et al., 2007).

## Ecological Connectivity

While the prediction of the next antibiotic resistance gene to transfer into a pathogen is a nearly impossible task, at least using the currently available tools, the analysis of the most suitable reservoirs where this event may happen is more suitable. Different reports have shown that so-called "resistance genes" are present everywhere, including locations where the presence of bacterial pathogens is unsuitable, such as the ice core (D'Costa et al., 2011), permafrost (Hultman et al., 2015), or the deep terrestrial subsurface (Brown and Balkwill, 2009), including isolated caves (Bhullar et al., 2012).

Although the identification and analysis of resistance genes from environmental organisms may have evolutionary and ecological value, their influence in public health will remain unlikely. The possibility of being transferred to a human pathogen should be rare, since these two types of microorganisms would never (or rarely) share the same microbial ecosystem. It could be argued that a chain of sequential events may allow such transfer. However, this situation would require selection to occur, and it has been

stated that the concentrations of free antibiotics of microbial origin is likely low in nature (Yim et al., 2007; Yim et al., 2006).

It has been proposed that several growth-inhibitory substances produced by microorganisms may have a defensive function to prevent the invasion by competitors, but this requires a high density of producer cells, already established in a niche (Wiener et al., 1998). Although local high antibiotic concentrations in the vicinity of producers, allowing selection of resistant clones, are possible (Laskaris et al., 2010), the occurrence of the chain of events leading to the acquisition by a pathogen of a resistance gene originated in an organism colonizing a distant habitat (ecologically disconnected) from those usually colonized by bacterial pathogens is unlikely.

When looking at the structure of ecosystems that might favor the spread of antibiotic resistance, two aspects have to be taken into consideration. One is the presence of the pathogen, second is the presence of the environmental donor of resistance. Human-linked ecosystems such as hospitals, day-care centers, or long-term care facilities would favor the spread of those resistance genes already present in the bacterial populations, especially if we take into consideration that these places present crowded human populations and a high antibiotic load, which favor respectively the spread and the selection of antibiotic resistance determinants (Baquero et al., 2013; Baquero, 2004). Nevertheless (and with the exception of the acquisition of resistance in pathogens by recombination with genes originated and transmitted from commensals, such as the mosaic penicillin-binding proteins (PBPs)) (Sibold et al., 1994; Dowson et al., 1993; Spratt et al., 1992), the primary event in acquisition of resistance is not expected to occur in clinical settings but rather in ecosystems where the environmental donor and the pathogenic receptor can meet.

As discussed in other chapters in this volume, this situation puts the focus on wastewater treatment plants (Baquero et al., 2008). These ecosystems obviously harbor environmental bacteria and may receive as well the influx of wastes from homes, hospitals, farms, and industries (including pharma antibiotic-producing companies). These wastes contain human-linked microbiota, both commensals and bacterial pathogens, which may harbor gene-transfer elements containing the resistance genes that are actually problematic for the treatment of infectious diseases in human patients (Brechet et al., 2014). In addition, wastewater treatment plants may receive antibiotics or other antimicrobials, such as heavy metals or biocides, the presence of which can respectively select and coselect antibiotic resistant microorganisms (Baquero et al., 2008; Kümmerer, 2009; Pauwels and Verstraete, 2006). It could be argued that the concentration of these selective agents is likely too low to allow selection of antibiotic resistance. However, the finding that antibiotic resistant mutants (or plasmids harboring resistance genes) can be selected along antibiotic gradients (Baquero and Coque, 2014), including very low concentrations of antibiotics, well below their minimal inhibitory concentrations (Gullberg et al., 2014; Gullberg et al., 2011), indicates that selection of resistance can be at work even in habitats such as wastewater treatment plants where the concentrations of the selective agents are usually low.

One characteristic of water systems is that they form a continuum of different habitats, which favor selection even at long distances. In this regard it is worth mentioning that, while the concentration of chemical pollutants diminishes with time (because of their degradation) and space (because they are diluted), this does not necessarily apply in the case of autoreplicative pollutants such as resistant bacteria or antibiotic resistance

genes (Martinez et al., 2007; Martinez, 2009a). In this situation, selection may occur in any place of this interconnected set of habitats.

One of the water habitats where the antibiotic selective pressure is higher consists of fish farms (Cabello et al., 2013; Cabello, 2006). Although the use of antibiotics for fish farming has been banned in different countries, a large number of fish farms still use antimicrobials, several of them belonging to the same families as those used for treating human infectious diseases. As a consequence, selection of resistance to drugs with clinical relevance is a frequent event in these locations (Kümmerer, 2004; Giraud et al., 2004; Halling-Sørensen et al., 1998).

Among the types of events (mutation and horizontal gene transfer) leading to resistance of natural microorganisms present in water, the most important one from the point of view of human health is the integration of resistance genes in plasmids, even when those plasmids are present in environmental nonpathogenic organisms. Once these genes are present in gene-mobile elements, the chance of their dissemination, eventually toward pathogens, is high. Indeed, it has been described that the origin of such well-known resistance genes as *qnr*A or oxacillinases as $bla_{\text{OXA48}}$ is the water-dwelling bacteria *Shewanella* (Poirel et al., 2005; Poirel et al., 2004). Further, it has been described that similar *qnrS2*-encoding plasmids are present in environmental *Aeromonas* spp. isolates from water at distant places of the Seine River in France (Cattoir et al., 2008). This indicates that water bodies are relevant elements for the acquisition of resistance, and a first event in such acquisition might be the integration of the resistance determinant in a gene mobile element of an environmental microorganism. The spread of this gene toward human pathogens will obviously be favored if the gene is integrated in a broad host range plasmid and when the environmental donor and the pathogenic organism belong to the same exchange community (Skippington and Ragan, 2011). In this regard, it is worth mentioning that the transfer from an environmental organism to a bacterial human commensal (more abundant in stools than pathogens) can be an intermediate step in the acquisition of resistance by human pathogens. Indeed, studies of the resistome of the human gut in nontreated healthy individuals have shown that commensals may harbor resistance genes acquired through horizontal gene transfer (Sommer et al., 2009). This situation has been considered to be the consequence of the acquisition by the commensal microorganism of the resistance genes from pathogens previously harboring them. However, the opposite situation might also happen. The possibility of a first event of acquisition of resistance genes by the commensal, in non-clinical ecosystems, followed by a secondary transfer toward the pathogen in the hospital setting (or still at natural habitats) should be taken into consideration.

## Microcompartmentalization and Granularity

One of the significant frontiers of our knowledge in the dynamics of genetic interaction between environmental microorganisms, commensals, and pathogens is the incomplete definition of spatial microenvironments (Baquero and Coque, 2014), where bacteria capable of exchanging antibiotic resistance genes can meet. The trading space depends on the habitat compartmentalization. In fact, the interactive network of genetic interactions typically occurs within very short distances, between spatially close populations, and also inside them, where selection for variants might occur (Slater et al., 2008; Slater

et al., 2010). Bacteria sharing the same microenvironment probably have close, if not shared, functional niches; because of this, environmental bacteria, commensals, and pathogens phylogenetically linked have more probability of coexisting and exchanging antibiotic resistance genes as genetic exchange communities (Skippington and Ragan, 2011; Skippington and Ragan, 2012). Note that even if particular resistomes are hosted in quantitatively dominant species, the possibility of transferring genes to pathogens that occupy different niches should be low.

The spatial position as a determinant of genetic exchange is particularly evident in surface-attached bacterial communities that are dense in number and spatially structured (Kim et al., 2014). Future studies on co-colonization of particular environmental or mucosal surfaces are critical in the understanding of the spread of antibiotic resistance.

The problem of "where pathogens and environmental or commensals meet" is also an ecological complication to "where the environments to which pathogens and commensals belong might meet." We have already considered the spatial "reactors" (Baquero et al., 2008) where most of these encounters might occur. Of course, the anthropogenic artificial mixture of human and animal microbiota (with a certain degree of presence of pathogens) and environmental bacteria might create mixed or recombinant environments.

In fact, the physical structure of environments might also influence the pathogen-commensal-environmental bacterial interactions involving transfer of antibiotic resistance. In the human- or animal-polluted soil or in the water sediments and loamy sands, organic and mineral particles (such as clay) might serve as substrates for heterogeneous bacterial populations that originate from different niches; the adsorption of nutrients, humic substances, and antibiotics to these granules released into the environment might create conditions for growth, gene transfer, and selection. This is expected to occur when attached microorganisms are exposed to antibiotics released by desorption from particles (Halling-Sørensen et al., 2002). Physical forces of anthropogenic origin may also influence the antibiotic resistance gene flow. For instance, sepionite (clay mineral) is an additive for animal feed (E-562) that reduces the speed of food passage (allowing a more efficient digestion of proteins); it has also been used for heavy metal removal from wastewater (Alvarez Ayuso and Garcia-Sanchez, 2003). Clay-promoted friction forces generated in the interface of ground material promote bacterial genetic material release and transformation, resulting in a significant increase in gene acquisition (Rodriguez-Beltran et al., 2013). It remains to be known if human-driven actions involving soil mechanical manipulation or natural phenomena such as floods might influence environmental merging, thus creating neutral spaces of coexistence and facilitating environmental-commensal-pathogen interactions.

## Founder Effect

The consequences of horizontal gene transfer of novel genes from environmental commensal populations to pathogens are modulated by the founder effect, impeding in several cases the assimilation of genetic novelties in bacterial populations. In fact, the astonishingly low variability of resistance genes acquired by human pathogens in comparison to those regularly found using functional metagenomic studies in complex

ecosystems (where environmental commensal bacteria prevail) can be explained by this founder effect. Horizontal gene transfer is likely a frequent event in any ecosystem, but we can only track the spread of those genes that are widespread in the population. Acquisition (and extinction) of a given gene may happen rather frequently, but the consequences of this situation will be just transient. Only in the case of positive selection of the phenotype conferred by the transferred gene, will the gene spread and its presence in the population be detected. In the case of antibiotic resistance genes, they are selected when the bacteria harboring them are under selective pressure by antibiotics or another coselector (such as heavy metals or biocides). This selective pressure is strong in the case of antibiotic-susceptible populations, as they will be inhibited unless they acquire a resistant phenotype, but it is very weak (if anything) if the population is already resistant. Under this scope, the spread of a given resistance gene (the founder) among the population may impede the entrance of another gene conferring the same or a very similar phenotype. The success of a resistance gene to spread after the first acquisition event by a bacterial pathogen depends on different factors, each one at a different level of complexity (Baquero et al., 2002; Andam et al., 2011). First, the gene should enter into a mobile element that can be shared between different pathogens, which constitutes a gene exchange community. Second, the spread of the gene will be favored if the gene mobile element enters into a pandemic clone. Finally, as we will see later on, the fitness costs associated with the acquisition of resistance may preclude the spread of a resistance determinant because resistant bacteria will be outcompeted by susceptible ones in the absence of antibiotics.

Even if resistance determinants are widespread, selective pressure changes, such as those due to the introduction of a new antibiotic belonging to the same structural family, render new opportunities for selecting novel resistance determinants. Under these novel conditions, two situations may happen: either the gene present in the population evolves to cope with the new selection landscape, increasing intragenic variability, or alternatively, novel genes conferring resistance to the new type of antimicrobial are recruited and selected, resulting in an increase in the variability of families of resistance genes conferring resistance in the population. The first case may have been the situation concerning TEM-type beta-lactamases. The ancestors of this family of resistance determinants are TEM-1 and TEM-2, which were nearly the unique plasmid-encoded beta-lactamases of *Escherichia coli* and other *Enterobacteriaceae*. The introduction of novel beta-lactams, such as third-generation cephalosporins or combinations of penicillins with TEM beta lactamase inhibitors, resulted in novel selective pressures. As a consequence, there has been an explosive diversification of TEM-1 type mutational variants able to hydrolyze cephalosporins (extended-spectrum TEM beta-lactamases, TEM-ESBL) or to resist the inhibitor's effects (Bush, 2013). However, the ancestor TEM enzymes have not been fully replaced, as the "novel TEM-variants" are less effective in hydrolyzing "old drugs" (as ampicillin), which remain in the market (antagonistic pleiotropy). Mutational diversification of TEM enzymes has probably impeded the emergence of other enzymes providing functions similar to the TEM variants; therefore, TEM enzymes impose the founder effect or "advantage of the first." However, the second case, recruitment of novel genes, requires the capture of genes from commensals or environmental bacteria, as in CTX-M beta-lactamases. It seems that these novel enzymes, with an inactivation profile similar to that of TEM-ESBL, might coexist and eventually replace at least in part these evolved TEM enzymes. This situation might be

explained by the coexistence of different founder effects at different geographical locations or by differences in fitness costs (see following section).

The exclusion of a resistance gene by another one previously present in the population or vice versa may happen only when they share the same geographical location. Epidemic outbreaks by bacteria carrying different genes can occur at different locations if such genes emerge simultaneously among bacterial populations. This indicates that different founder effects may occur at different locations at early stages of the emergence and spread of some antibiotic mechanisms of resistance (Baquero et al., 2013). However, on several occasions a single gene emerging at a specific point enters into epidemic clones and rapidly expands all around the world. A recent example of this situation is the NDM-1 beta-lactamase that emerged at India and is now distributed worldwide (Walsh et al., 2011; Kumarasamy et al., 2010; Yong et al., 2009; Wailan and Paterson, 2014; Dortet et al., 2014). In the case of genes for which acquisition renders substantial fitness costs, sequential events of penetration and extinction of the same gene or allelic forms may happen, a situation that fits well within the category of short-sighted evolution (Levin and Bull, 1994).

## Fitness Costs

It has been generally assumed that the acquisition of antibiotic resistance confers a fitness cost to the resistant microorganisms, reflected in a reduced competitivity of resistant bacteria in comparison with their wild-type, susceptible counterparts (Andersson, 2003; Sander et al., 2002; Levin et al., 2000; Andersson and Levin, 1999; Morris et al., 1998). In principle, this type of cost would consist of a rather nonspecific metabolic burden reflected in a lower growth rate, in any habitat, of the resistant microorganisms in comparison with the wild-type antibiotic susceptible strains. As a consequence, fitness costs are usually estimated either by measuring comparative growth rates or by performing competition assays where the resistant and the susceptible microorganisms grow together (Martinez et al., 2011).

The reasoning about the consequences acquiring resistance in impairing the cell physiology (fitness costs) is two-fold. In the case of mutations in housekeeping genes leading to antibiotic resistance, changes occur in genes that encode elements that are critically involved in bacterial physiology, the basic functions that serve as antibiotic targets and/or the cell transporters involved in the entrance of the antimicrobials. Mutations will de-adapt these elements from the general bacterial metabolism, making them less proficient than the wild-type allele, hence impairing bacterial growth. In the case of genes acquired through horizontal gene transfer, most of the costs will come from the energy resources required for the replication, transcription, and translation of the novel acquired genetic material, and the expression of the novel gene, producing a protein (function) that might collide with the interactive protein network of the cell.

Under these circumstances, it is suitable to think that resistance should disappear in the absence of selection, which would allow the implementation of strategies based on the cyclic use of antibiotics to surpass the problem of antibiotic resistance. Unfortunately, initiatives in this direction have not been successful and resistance remains even in the absence of antibiotics (Sundqvist et al., 2010; Gillespie, 2001). This means that, whereas in some cases measuring growth rates at the laboratory would reflect the fitness costs

(Bottger et al., 2005), in other cases, this is an oversimplification that does not serve to fully understand the effects of resistance in bacterial physiology. Indeed, different works have shown that the fitness costs are specific to the mutation involved, with some of them causing high fitness costs and others that do not clearly challenge the behavior of the resistant microorganism (Balsalobre and de la Campa, 2008). In addition, compensatory mutations or gene amplification events, able to reduce or even suppress fitness costs, are easily selected (Schulz zur Wiesch et al., 2010; Lind et al., 2010; Paulander et al., 2007; Handel et al., 2006). Finally, recent work has shown that the metabolic rewiring of resistant microorganisms may allow them to cope with the potential fitness costs associated with the acquisition of resistance (Olivares et al., 2014; Olivares et al., 2012). In the case of acquisition of resistance genes, some recent works have shown that, on some occasions, there are no clear costs associated with their acquisition (Oggioni et al., 2012), or that the acquisition of a plasmid may increase the fitness of the host (Schaufler et al., 2016), whereas in other cases the costs depend on the allele of the gene that is acquired or on the organism harboring it (Sanchez and Martinez, 2012; Morosini et al., 2000).

This situation where fitness costs are not interchangeable for each gene makes them a relevant bottleneck for the spread of resistance elements. Only those genes for which acquisition renders affordable fitness costs will likely spread in the population. There are, however, some mechanisms that may allow the fixation of a given determinant even when fitness costs are high. One consists of coselection: resistance genes are frequently linked in a gene mobile platform to other resistance determinants or to elements such as siderophores, colonization factors, or toxins that have an ecological value. Any one of these elements may allow coselection of resistance in the absence of antibiotics. The other element consists of the determinants involved in the maintenance of plasmid stability, mainly the toxin/antitoxin systems, that impede the elimination of a plasmid. Once a resistance gene enters into such plasmids, its extinction would be very difficult even when the expression of such resistance gene may confer a high fitness cost.

Learning the fitness costs associated with the acquisition of resistance is then fundamental for predicting the potential spread of a resistance gene from its environmental reservoir to human bacterial pathogens (Martinez et al., 2011). While the analysis of bacterial growth is still useful in some occasions, in-depth analysis of fitness costs require more detailed studies on the effect of resistance in the bacterial physiology, including aspects with relevance for infection such as microbial virulence (Beceiro et al., 2013; Andersson, 2006; Bjorkman et al., 1998). However, despite the fact that natural ecosystems, such as water, are reservoirs and reactors involved in the origin, selection, and spread of antibiotic resistance, the effect of resistance for the survival of the resistant populations in nonclinical settings has been rarely studied (Sanchez et al., 2002). An integrated view of the evolution of antibiotic resistance requires a more in-depth analysis of the effect of resistance for the behavior of resistant populations in natural (nonclinical) ecosystems.

## The Two Ages in the Evolution of Antibiotic Resistance

Antibiotic resistance has evolved in two ages. The first age corresponds to the history of intrinsic resistance determinants, before the introduction of anthropogenic antibiotics. The comparison of available bacterial genomes has shown that intrinsic,

chromosomally encoded resistance genes such as efflux pumps or inactivating enzymes are very well conserved among the members of a given bacterial species. Their finding in all (or most) of the isolates of any of this species indicates that they are very ancient elements that evolved even before the advent of pluricellularity. While resistance determinants present in antibiotic producers have a clear function, either as decontamination determinants or as enzymes belonging to the antibiotic biosynthetic pathway (Benveniste and Davies, 1973), the situation is less clear for nonproducers. One report has shown that producers and nonproducers can coexist in the same habitat (Laskaris et al., 2010), which may allow selection of resistance. However, in other occasions, the forces behind selection of resistance are by far less clear. One example of this situation may be AmpC (Lindberg and Normark, 1986), a beta-lactamase present in most *Enterobacteriaceae*, despite the fact that the gut is not known to harbor microorganisms able to produce beta-lactams. It is unlikely then that the original function of AmpC would be beta-lactam resistance, despite its current relevance for such a role in clinical settings. The most plausible hypothesis is that several of the determinants that today are dubbed as resistance genes are able to render resistance because their original substrate is similar to the antibiotic they inactivate (in the case of antibiotic-inactivating enzymes) or extrude (in the case of multidrug efflux pumps). The term *exaptation* was coined to describe a number of evolutionary processes where the change of function is achieved not through genetic change but because of a change in environment (Gould and Vrba, 1982). We believe that this is the situation with several resistance determinants; they were not previously involved in resistance, but in habitats presenting a high antibiotic load, they can allow bacteria to cope with this deadly selective force.

The second age corresponds to the time in which the microbiosphere has been exposed to the antibiotics produced by human industry. It is worth mentioning that, while intrinsic chromosomally encoded resistance determinants may still keep the original function for which they were selected before the antibiotic era, the situation for genes acquired through horizontal gene transfer is different. These genes are present in a new host that does not have the same ecological habitat or the metabolic and regulatory networks of their original host. Under these circumstances, it would be difficult for mobile resistance genes to maintain the original functions for which they were selected in natural ecosystems, their unique function being conferring resistance to the new host. This second age in the evolution of resistance has allowed the enrichment of a number of resistance genes that are now widespread in clinical and nonclinical ecosystems (Martinez, 2012a). These elements can be considered as autoreplicative genetic pollutants, able to spread between different interconnected environments (Martinez, 2009a; Martinez, 2009b). An analysis of their behavior in such environments is needed to understand the mechanisms behind the acquisition and spread of resistance among the population of bacterial pathogens as well as to implement strategies to reduce such resistance.

## Concluding Remarks

Antibiotic resistance is a relevant problem with potentially severe consequences for human health. For decades, studies of antibiotic resistance have focused on the clinical settings. However, the origin of nearly all antibiotic resistance genes that have been acquired by human pathogens since antibiotics were first used for treating bacterial

infections can be tracked to environmental microorganisms. In recent years, this situation has led to nonclinical ecosystems being more frequently analyzed as potential sources for the emergence, evolution, and spread of antibiotic resistance determinants with clinical relevance. As a consequence, a large number of potential resistance genes, capable of conferring resistance upon their transfer to heterologous bacterial hosts, have been described. These studies indicate that the amount of different potential resistance genes present in nature are several orders of magnitude higher than those that are actually present in human bacterial pathogens. A precise understanding of the bottlenecks mediating such transfer would be highly relevant for the implementation of strategies to decrease the spread of resistance in nonclinical ecosystems. Further, the definition of reservoirs where resistance could emerge, including not only hospitals and other health care facilities but also wastewater treatment plants, farms, and aquaculture sites, is needed to reduce the increase of antibiotic resistance among human pathogens. Possibly, the fight against antibiotic resistance should include eco-evo interventions focused on significant reservoirs (Baquero et al., 2011; Baquero et al., 2015). Of critical importance is progress in developing techniques able to measure the effects of antibiotic resistance in bacterial physiology, ecology, epidemicity, and virulence. It remains essential to be aware that all resistant microorganisms are discharged from treated patients, but also increasingly from contaminated carriers in natural ecosystems, where they can remain. Environmental contamination by bacterial potentially pathogenic organisms and their mobile genetic elements containing successful antibiotic resistance genes has clear consequences for the spread of resistance back into humans, but also provides unlimited possibilities for accessing the environmental gene pool, challenging the future of novel drugs.

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# 3

# One Health

## The Role Wastewater Treatment Plants Play as Reservoirs, Amplifiers, and Transmitters of Antibiotic Resistance Genes and Antibiotic Resistant Bacteria

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## Introduction

Antibiotics now save millions of animal and human lives annually, reduce losses to agricultural and aquaculture, and contribute to increased food productivity. Widespread use of antibiotics over the last 70 years has led to increases in the level of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) isolated in agriculture/acquaculture settings, the environment, and humans (locally, nationally, and internationally) which have, in turn, contributed to thousands of deaths each year. Concerns over the spread of antibiotic resistance have fueled many groups to assess the impacts of ARB/ARGs on human and animal health, agricultural and food production, aquaculture, and human waste management. ARGs have been labeled as emerging pollutants with potential threat to public health worldwide. As the number of antibiotic resistant pathogens increases, the number of antibiotics available for treatment decreases. In 2015, the World Health Assembly adopted a Global Action Plan against antibiotic resistance which recognizes that efforts to reduce ARB/ARGs need to be extended beyond human health (WHO, 2015).

Wastewater treatment plants (WWTPs) and their by-products (biosludge and effluents) have been considered potential reservoirs, amplifiers, and transmitters of ARGs and ARB in a variety of settings (Brechet et al., 2014; Burch et al., 2013; Di Cesare et al., 2016; Dropa et al., 2016; Reinthaler et al., 2003). This is of concern because biosludge is an important by-product of the WWTP process and is now considered a resource. Biosludge has been used for a variety of agricultural purposes including growing food for public consumption, while effluent has been used to recharge aquifers, for water landscaping and agriculture, and as a contributor to drinking water sources (Hayden, 2015; Rice et al., 2013). This suggests that ARGs/ARB found in biosludge and effluent may be transferred to food products, including shellfish, and may contaminate waterways, lakes, rivers, recreational waters, and oceans worldwide. Some studies have also speculated that the wastewater treatment process may increase the proportion of ARB in outlets (Dropa et al., 2016; Silva et al., 2006). Hotspots of ARGs and ARB may be

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found in WWTP outflows where wastewater effluents are discharged from the WWTP into bodies of water. Here, WWTP effluent may contribute to the dissemination of specific ARGs in the natural environment (Amos et al., 2014; Dropa et al., 2016; Proia et al., 2016). Similarly, other studies have shown that use of reclaimed water is a reservoir for ARGs, which increase in the soils after repeated irrigation with reclaimed water. This has potential implications for human health (Fahrenfeld et al., 2013; Broszat and Grohmann, 2018, chapter 11 of this volume).

Residual ARB/ARGs left in the final effluent are normally deposited into bodies of water where they can then be taken up by freshwater and marine wildlife and ultimately cycle back to humans and/or land animals (McLain et al., 2018, chapter 13 of this volume; Prichula et al., 2016; Schroeder et al., 2009). Preliminary data support this hypothesis because high levels of ARGs are detected where WWTP and combined sewer outflows are discharged into Puget Sound in Washington, USA (Dr. L. Rhodes, personal communication). This may be one reason why the Southern Resident Killer Whales carry Gram-negative and Gram-positive resistant and multiresistant bacteria in their respiratory tracts as determined by cultures from exhaled breath samples (Schroeder et al., 2009). Similarly, antibiotic resistant enterococci have been isolated from feces of sea turtles, seabirds, and marine mammals from the southern coast of Brazil (Prichula et al., 2016).

Conventional wastewater treatment does reduce the total number of fecal bacteria but does not necessarily reduce the fraction of ARGs/ARB present. Over 30 years ago, Walter and Vennes (1985) determined that between 0.35% and 5% of the coliforms from a domestic sewage system were resistant to ≥1 antibiotic with ~75% of the multiple resistant strains able to transfer resistance. More recent studies from Gallert et al. (2005), da Costa et al. (2006), and Luczkiewicz et al. (2010) have isolated and characterized multidrug resistant fecal coliforms and/or enterococci from municipal water from multiple geographical areas. To complicate the issue, wastewater effluent is now being considered a resource to be used, especially in drought stricken urban areas for water landscaping and to replenish aquifers (Hayden, 2015; Orange County Water District, 2016).

The wastewater treatment process may increase the abundance of ARGs and the diversity of ARBs, as well as provide selective pressure to increase the diversity of antibiotic resistant phenotypes and transmission of ARGs to new bacterial species. These final products can ultimately contaminate a variety of ecosystems with particular impact on aquaculture, agriculture, the human workers in these industries, and people who consume their products (Fahrenfeld et al., 2013). The potential risk to human and animal health is just now being recognized (Rosenberg Goldstein et al., 2014). This chapter will review the roles that WWTPs and their products play as reservoirs and potential amplifiers of ARBs and ARGs, their role in bacterial diversity and/or transmission through the lens of a One Health perspective, and their influence on the health of humans, animals, and the environment.

## One Health

In the twenty-first century the world is rapidly changing, with more technology and interconnections between people, goods, and food, along with their microbiomes, leading to more rapid transportation around the world. Other issues such as the location of

particular antibiotic resistance genes associated with transposons, a large variety of wide-host range plasmids, and/or the development of particular antibiotic resistant robust clones has allowed global dissemination to occur. One good example of a robust resistant clone is KPC-producing *Klebsiella pneumoniae* ST258 (Bush, 2010).

One Health is a concept that recognizes that the health of humans, animals, and the environment are inextricably linked, and thus a collaborative and integrated multidisciplinary approach is needed to maintain the health of all. The One Health concept is often pictured as a group of three interconnected circles representing the three domains, which interact and respond to changes in any one domain. Interconnecting circles allows us to better visualize that the actions and interventions in any one domain (human, animal or environment) has an impact and influence on the health of all three domains. Thus, problems in human health create problems for animal and environmental health. Unfortunately, most of man's past actions have resulted in negative impacts, which have led to the extinction of animal, plant, and human communities. The climate changes seen today are primarily due to the direct results of human activities, and the associated impacts on infectious disease are just now starting to be appreciated (Lindahl and Grace, 2015). These negative impacts are likely to increase as human and food animal populations increase globally, creating greater challenges and problems (King, 2014).

The One Health approach represents a contrast to traditional practices of human and animal medicine, which have been studied and practiced in isolation rather than as part of a world ecosystem. The environmental contribution to global health has not been generally considered, or if studied, it has rarely been, until recently, in connection with the health of man and/or animals. The world microbial ecosystem includes the microbiomes associated with each domain and the direct and indirect mixing of the different microbiomes which, in some cases, may lead to disease. Human-animal interface is ancient but has expanded with the development of animal and fish farming. It is a continuum of contacts and interactions that allow for barrier breaches of pathogens to occur and an increased driver of infections. This is illustrated by the estimation that ~75% of emerging infectious diseases in humans over the last 20 years have been zoonotic. Zoonotic infection means that the pathogen has spread from animals or insects to people. In some cases, the pathogen becomes established with subsequent spread within humans. However, more commonly, there may be recurrent events of transmission from an animal/insect reservoir to the humans with limited human-to-human transmission, such as the case now occurring with the Zika virus (Lindahl and Grace, 2015; USDA, 2015).

Many bacterial infections such as *E. coli* O157:H7 and enterotoxigenic *E. coli* O114:H4 are acquired from food. *E. coli* O114:H4 caused a huge outbreak in 2011 that, besides causing death and infections, created tensions among EU members along with boycotts of vegetables within the EU (Heymann and Dixon, 2014; Manitz et al., 2014). Dealing with emerging and re-emerging infections that cross species barriers not only impact humans but may impact livestock, pets, wildlife, and crops and may contaminate the environment. The importance of global ecological changes due to human impact on the environment and technological changes in society, along with important changes in how food is produced, processed, and transported around the world have combined to increase the potential risk of disease transmission between animals, food, and humans (Lindahl and Grace, 2015). With environmental contamination as a major by-product of these endeavors, changing the downward spiral of increasing global contamination can only be addressed by improved communications, cooperation, and collaboration across disciplines.

In the mid-twentieth century, antibiotics became the foundation for treating bacterial infectious diseases in both humans and animals. However, ARB and ARGs were recognized within a year of the first use of penicillin (Finland, 1971) in humans, followed shortly by use in agriculture (Feighner and Dashkevicz, 1987). By the 1950s, multidrug-resistant pathogens were identified (Watanabe and Fukasawa, 1961). Since the 1970s, the use and inappropriate use of antibiotics in both humans and animal health has influenced the spread and transmission of ARGs and ARB, which have had major impacts on treatment and health (Marshall and Levy, 2011). Today it is known that antibiotic use in humans and agriculture results in increased antibiotic resistance in all types of bacteria from pathogenic to environmental.

How a particular antibiotic can influence where and how antibiotic resistance and ARB develop and spread from one domain to all three has been illustrated in the literature. One good example of this is the development of vancomycin-resistant *Enterococcus faecium* (VRE) in North America and the rest of the world. In Europe and other parts of the world, the vancomycin-related drug avoparcin was used as a growth promoter in livestock. Over time, VRE developed in chickens, pigs, and swine, and could be detected in their processed meat (Cetinkaya et al., 2000). Once VRE developed in livestock, studies showed that VRE was acquired by farmers and those slaughtering the animals, and then later was found in hospitals (van den Bogaard et al., 2002). Transmission of VRE genes from animals to human bacteria did occur, as did transposon transfer from animals to human bacteria. In contrast, in the United States avoparcin was never used as an additive in livestock food. Early studies suggested that VRE was not found in chickens in the United States, and there was little evidence to suggest that transmission of VRE in healthy adults occurred in the community prior to 2000 (Cetinkaya et al., 2000). In North America, vancomycin was used extensively in the hospital, and VRE emerged as a major nosocomial pathogen within this setting (Rice, 2013). This was due, in part, to the persistence of viable VRE on contaminated surfaces within the hospital for weeks to months, and rooms housing patients colonized or infected with VRE were difficult to clean. Hence, these rooms remained reservoirs for transmission of VRE to new patients (Eckstein et al., 2007). More recently, VRE has been cultured from the general community environment in the United States as illustrated by VRE recovered from recreational beach sand and water and wild crows in North America (Oravcova et al., 2014; Roberts et al., 2009; 2016). What role wastewater and VRE have in transmission of human disease directly is not clear. However, from our recent study, VRE was cultured from >90% of primary and secondary WWTP effluent (Roberts et al., 2016). Other studies have identified VRE in 60% of raw sewage samples from Swedish WWTP and 19% in treated sewage samples, while during that same time frame VRE infections in healthy humans or in Swedish livestock production were uncommon (Sahlstrom et al., 2009).

## Municipal Wastewater and Wastewater Treatment Plant Outputs

Municipal wastewater is a mixture of anything that is flushed down a toilet or washed down a drain. This can include commercial, industrial, hospital, and residential waste in addition to stormwater. The latter is especially important when there are excessive

rain events leading to floods. Such flooding is expected to be become more common as the climate continues to change. Contamination of stormwater into the sewer system may also occur, especially when storm and sanitary sewers are combined. In previous years, the municipal wastewater and biosolids were considered a waste product requiring disposal. However, as drought conditions continue, there has been a paradigm shift, and increasingly municipalities are considering the final wastewater and biosludge produced as resources to be utilized rather than waste products to be disposed of (Hayden, 2015; Reinthaler et al., 2003). This change is occurring throughout the world, though it is not a new idea (Hespanhol, 1997; Stanford Woods Institute for the Environment, 2016).

The primary purpose of the WWTP is to protect the environment from the adverse effects of nutrients such as nitrogen and phosphorus, remove solids, and reduce the number of coliform bacteria before the effluent is deposited into receiving surface waters. The WWTP's primary goal is protecting the receiving surface water quality. A secondary goal is to limit risks to human health as a result of direct exposure to treated wastewater or treated wastewater solids used on commercial and/or agricultural lands. However, WWTPs do not specifically have a goal of reducing the level of ARGs and ARBs in their final waste products (Do et al., 2018, chapter 15 of this volume).

In North America there are ~7.2 million dry tons of biosolids produced annually; ~60% of this material has been applied to the land to renew the soil each year (Yucel et al., 2015). In the USA, wastewater solids that have been processed to "significantly remove pathogens" can be applied to agricultural land used to grow crops not intended for direct human consumption. Wastewater solids that have undergone a "process to further remove pathogens" can be utilized with few restrictions. Rules in other parts of the world differ dramatically, from no processing to extensive processing prior to application onto agricultural land used to grow crops for human consumption. As a result, the potential for transfer of ARGs and ARB from human, animal, and industrial waste varies throughout the world. However, it is clear that both treated wastewater effluent and biosolids are often unappreciated sources of ARGs and ARB that will increase as more communities and countries view effluents and biosolids as a valuable resource to be utilized rather than disposed.

## Pathogens Found in WWTP

Bacterial pathogens (*Salmonella*, *Shigella*, toxigenic *E. coli*, *Giardia*, *Cryptosporidium*, enteric viruses, etc.) that cause diarrheal disease are shed in feces and ultimately end up in the WWTP system. Multiple barrier stages at the WWTP facilities followed by disinfection steps are used to remove and inactivate most of the viruses, bacteria, and protozoa such as *Giardia*. Strict policies on procedures and microbial loads are present throughout North America and the European Union. In general, the protocols do not look for the presence of specific potential pathogens at various steps through WWTP system because of the cost, technical demands, and time; rather, they look for indicator bacteria. In addition, the potential pathogens are normally present in much lower numbers compared to the commensal *E. coli* and/or enterococci making the potential pathogens more difficult to detect. Thus, the U.S. Environmental Protection Agency (EPA) and other countries' agencies have developed alternative methods for predicting

the presence of pathogens and estimations of the risk to human health by limiting the number of fecal bacteria allowed to be discharged (EPA, n.d.).

Recently, a study looked at the microbiome of raw sewage from 71 cities across the United States and compared it with the microbiome of individual human stool samples from the same cities. This study found that the sewage microbiomes reflected what was found in the human stool population. Most interesting, the sewage microbiome had an increased richness and diversity compared to the human stool samples (Newton et al., 2015).

Relatively little is known about the risk to farmers, exposed community members, and WWTP workers from the pathogens, ARGs, and ARB present in WWTP products. In most cases, a link between the presence of WWTP products and human health has not been established. However, one study looking at the reuse of wastewater found higher levels of intestinal parasitic infections among Ugandan farmers than in other people (Fuhrimann et al., 2014). Fenollar et al. (2015) found that WWTP workers were more likely to be colonized with *Tropheryma whipplei*, the cause agent of Whipple's disease, than were nonexposed people. Few other studies have looked at the occupational risk of WWTP products.

Human pathogens, including shiga toxin *E. coli* and enteric viruses, typically die off within a 3-month period in WWTP products, while *Clostridium* spp. can persist over years as dormant resistant endospore forms (Rouch et al., 2011). Spores include those from *C. perfringens* and *C. difficile*, with the majority of the work examining *C. perfringens* (Xu et al., 2015a). A few examples of the human opportunistic pathogens associated with WWTP effluents and biosolids are discussed below.

### *Clostridium difficile*

*C. difficile* is a Gram-positive spore-forming rod and has been considered by the Centers for Disease Control and Prevention (CDC) as an "urgent threat," though at this time drug-resistant *C. difficile* is not a major issue (CDC, 2013). *C. difficile* is one of three pathogens in this urgent threat category. Special protection for people entering hospital rooms with *C. difficile* infections is normally required in countries such as the United State. Because this bacterium produces spores, a quiescent form of the bacteria that is resistant to environmental stress, it is difficult to destroy and is more difficult to remove from the environment and contaminated surfaces than classical vegetative bacterial cells. More hospitals are now treating rooms that have housed *C. difficile*–positive patients with UV light for extra cleaning. Both community-associated and hospital-acquired infections have been documented. There are some differences in community versus hospital diseases. For example, ribotype 078 is more often associated with community versus ribotype 27 for hospital infections. Documented reservoirs for transmission of *C. difficile* have included person-to-person spread, foodborne, waterborne, and zoonotic transmission (Xu et al., 2015a). Toxigenic *C. difficile* have been cultured from raw sewage. Community-associated ribotype 078 spores can survive through the wastewater treatment process and be present in the effluent released into watercourses and/or retained with biosolids (Xu et al., 2014). This may help this particular type spread through communities. A recent publication has examined the potential of *C. difficile* spores to survive wastewater treatment–derived biosolids during land application (Xu et al., 2015a).

This paper found that strains of *C. difficile* that were dominant in the original biosolids were different than the survivors, suggesting that strains differ in their extended dormancy, with *C. difficile* 027 spores predominating in the biosolids. The study concluded that composting of biosolids is a better method of reducing the levels of *C. difficile* than land application. It also brings into question the potential role that biosolids may play in the spread of *C. difficile* in food grown on land treated with WWTP biosolids and/or effluents. Clearly, larger studies are needed to demonstrate if there is a link between WWTP biosolids and/or effluents used in agriculture, contamination of food, and infection with *C. difficile* disease in the community and/or hospital settings.

### Extended-Spectrum β-Lactamase–Producing Enterobacteriaceae (ESBLs)

ESBL enzymes are found mostly in *Enterobacteriaceae* such as *E. coli* and *Klebsiella* spp. ESBL-producing enzymes hydrolyze β-lactam antibiotics including cephalosporin. Cephalosporins are clinically important because they are used extensively as first-line therapies in many severely ill patients with Gram-negative pathogens. ESBL-producing bacteria have been labeled a "serious threat" by CDC (2013). Many ESBL-producing bacteria are multiresistant to non-β-lactam antibiotics, which further reduces treatment options (Blaak et al., 2015). The genes coding for ESBLs producing enzymes are often associated with mobile plasmids and thus can readily spread among different bacteria within an ecosystem and are found all over the world. Blaak et al. (2015) concluded that ESBL-positive bacteria found in municipal wastewater significantly contribute to the presence of these resistant bacteria in local surface water and have the potential to impact public health outcomes. However, there have been extremely limited data published on the actual exposure risk assessment associated with surface waters or recreational waters.

In a 2015 review (Huijbers et al., 2015), there were 23 papers dealing with ESBLs from WWTPs from Africa, Europe, North and South America, and Oceania. All papers had ≥1 sample(s) positive for ESBLs. The samples were taken from untreated influents, secondary effluent and sludge and had levels ranging from $10^2$–$10^6$ CFU/100 mL. There has also been a publication characterizing ESBL-positive *Enterobacteriaceae* emission from WWTP into the ambient air as well as into the river (Korzeniewska and Harnisz, 2013). Of the air samples collected at the WWTP, 23.8% ($n = 10$) were positive for ESBL producers. ESBL-positive bacteria were also identified in the influent, mixed liquor, and effluent. Recent studies of Carbapenemase-producing *Enterobacteriaceae* measured in hospital wastewater have concluded that sources other than clinical isolates (other undetected carriers or colonization of the pipeworks) likely contributed to the concentrations found (White et al., 2016).

Reinthaler et al. (2003) examined five WWTPs and collected 72 sludge samples between January and September 2009. Three of the WWTP biosolids were applied to arable lands, one was used to cap a landfill, and the last one burned the material. The study found that 61% of the samples had ESBL-positive *E. coli*. The two WWTPs that used a lime stabilization method (which is a cost-effective alternative to anaerobic and aerobic digestion) showed increases in the ESBL-producing *E. coli* present. The authors concluded that WWTP biosolids without effective treatment should not be used for agriculture land application.

### *Staphylococcus aureus*/MRSA

*S. aureus* is a Gram-positive cocci able to carry a large number of different antibiotic resistance genes on plasmids or transposons, or integrated into the chromosome. Community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) infections have been increasing throughout the world. They have been labeled a "serious threat" to human health by CDC (2013). MRSA isolates carry the *mecA* gene, which codes for a PBP2a protein that confers drug resistance to all β-lactam antibiotics, eliminating their use in treatment. Both *S. aureus* and MRSA isolates are most commonly associated with mucus membranes and the skin but can be isolated from the feces (Benito et al., 2015). Studies around the world have reported finding both *S. aureus* and MRSA in a variety of WWTP samples (Borjesson et al., 2009; Rosenberg Goldstein et al., 2014); Wan and Chou, 2015). This is interesting since *S. aureus* and MRSA are most often found in nasal mucosa rather than in human intestinal tracts. However, shower and bath water would be expected to have some *S. aureus* and MRSA. Borjesson et al. (2009) reported that they identified *S. aureus*, the *mecA* gene, and MRSA by culture and qPCR assays. This study did not find an obvious seasonal variation between the time of year or sampling sites with the presence of MRSA in the Gothenburg Sweden WWTPs.

Four studies conducted in Europe, N. America, and Oceania identified MRSA from ≥1 samples taken from each study. Samples included untreated secondary and tertiary effluent and biosolids (Huijbers et al., 2015). MRSA has also been identified in wastewater coming from hospitals where they survived their transit into the WWTP and through to the final treated effluent and chlorination stage, suggesting a potential public health risk (Thompson et al., 2012). It is unknown if MRSA-contaminated wastewater represents a potential public health issue to WWTP workers. It is also not clear if MRSA-contaminated effluents/biosolids used for agriculture pose a risk to agricultural workers, or what role it might play as a source of contamination of food grown with contaminated water/biosolids. Clearly as more reclaimed wastewater and biosolids are used in agriculture, more studies will be needed on this issue. Borjesson et al. (2010) suggests that MRSA survives in wastewater. They also imply that the WWTP process may actually select for MRSA that carries more antibiotic resistance genes and the virulence PVL (Panton-Valentine leukocidin) gene. This paper suggested that WWTP products represent an unrecognized human health threat (Borjesson et al., 2010), which is a recurring theme in many of the references in this chapter.

### Vancomycin-Resistant Enterococci (VRE)

Enterococci are opportunistic pathogens which are found in the intestinal tract of man and animals. They are also important nosocomial pathogens and have been labeled a "serious threat" by CDC (2013). Enterococci survive in the environment and are used as water indicators in both marine and fresh water. VRE are a subset of the enterococcal population that carry the *vanA* and/or *vanB* genes conferring vancomycin resistance. VRE is one of the three hospital pathogens (the others are *C. difficile* and MRSA) that require special personnel protection equipment when dealing with an infected patient. Many hospitals do extra cleaning after a VRE, *C. difficile*, or MRSA patient has been discharged to reduce the risk of the next patient becoming infected with these pathogens.

VRE was first identified as a clinical issue in 1986 in Europe and 1989 in the USA. By 1994, *vanA*-positive *Enterococcus* spp., with high-level resistance to vancomycin and teicoplanin, had been isolated from sewage in Europe (Bates, 1997). Since then, VRE has been isolated around the world in sewage systems (Borhani et al., 2014; Harwood et al., 2001; Roberts et al., 2016; Sahlstrom et al., 2009; Varela et al., 2013). VRE has been detected in up to 3% of wastewaters in Portugal and up to 7% of Poland wastewaters (Varela et al., 2013), while Caplin et al. (2008) found 71% VRE positive from raw urban wastewater samples. Roberts et al. (2016) cultured VRE from >90% of primary and secondary effluent samples tested. VRE was isolated from 100% of the 33 WWTPs tested in Asia, Europe, and North America. Samples in these studies were taken from untreated influent, secondary and tertiary effluent, biosolids with levels of VRE that ranged from $10^{-1}$ to $10^{5}$ CFU/100 mL depending on the sample, study, and time of year (Huijbers et al., 2015).

### Enteric Viruses

Enteric viruses have been identified in both raw sewage and treated wastewater and represent an important source of viruses in the environment. A number of studies have been written about enteric viruses that included pathogens such as polio, norovirus, and hepatitis, as well as the relationship between contaminated water, sewage, and human diseases (Asghar et al., 2014; Barril et al., 2015; Hellmer et al., 2014). Low levels of fecal indicator bacteria, which are typically monitored, may not reflect adequately the removal of pathogenic viruses. This was a topic of discussion and concern for the 2016 Olympic Summer Games (Brooks, 2016). Many of the human-specific enteric viruses are found throughout the environment even though man is the only reservoir for them. These viruses can persist wherever the environment has been polluted by human feces and/or sewage (Osulale and Okoh, 2015). Municipal WWTP effluents are one of the main concentrated sources of enteric viruses and represent a large reservoir that can readily contaminate environments and waterways. The presence of the enteric viruses is perceived as a potential risk to human health, but limited studies directly linking human exposure to contaminated WWTP effluents and human disease have been published. However, studies do suggest a linkage between sewage contamination of enteric viruses and viral infections in the community. For example, the rotavirus levels in sewage mirror the infectious status of the population (Myrnel et al., 2006). Examining WWTP for the presence of polio has been part of the global plan to eliminate polio (Asghar et al., 2014). More recently, Hellmer et al., (2014) found that detection of seven pathogenic enteric virus including adenovirus, Aichi virus, astrovirus, hepatitis A, E viruses, norovirus, and rotavirus in sewage provided an early warning of hepatitis A virus and norovirus outbreaks in the community. Most interesting was that some of the same viral strains were found in both the sewage and patient samples. Since many of these viruses are present after completion of a WWTP decontamination process, consideration must be taken when using biosolids and WWTP effluents for agriculture because both may provide a risk to human health. Better human health risk assessment may occur during and/or after the Brazilian 2016 Olympic Summer Games where athletes participating in water sports may be significantly impacted by highly enteric virus contaminated in the water that will be used for many of the events.

### Parasites

*Cryptosporidium parvum* is a protozoan parasite that causes gastrointestinal illness. It is transmitted by ingestion of oocysts from humans or animal feces. Ingestion of 2–10 oocysts or parasites can lead to infection. More than 10 outbreaks due to contaminated water have been documented in the United States since 1988. The largest U.S. outbreak occurred in 1993 with ~403,000 people in greater Milwaukee, Wisconsin, becoming ill due to inadequate removal of oocysts from the municipal water treatment plants (Corso et al., 2003). Untreated and treated wastewaters are both important sources of *Cryptosporidium* and *Giardia* contamination of surface water. In a recent study from Greece (Spanakos et al., 2015), *Cryptosporidium* oocysts and *Giardia* cysts were isolated from both influents and effluents discharged from WWTPs. Other studies from other geographic locations have found high concentrations of *Cryptosporidium* and *Giardia* from reclaimed WWTP water, suggesting the potential risk of exposure to the public (Harwood et al., 2005; Rose et al., 1986; Sykora et al., 1991). More studies are needed in this area.

## Antibiotic Resistant Bacteria (ARB), Antibiotic Resistance Genes (ARGs), and Antibiotic Resistance Plasmids in WWTP

ARGs and ARB have been found throughout the wastewater treatment process from raw influent, primary and secondary effluent, aeration tanks, activated sludge, and residual biosolids (Borjesson et al., 2009; Diallo et al., 2013; Huijbers et al., 2015). The biosolids represent the majority of the biomass and thus the highest concentration of the ARGs and ARB from the treatment process. This material is often used for landscaping and agricultural enrichment, which can lead to environmental contamination of soil, water, and most importantly, the potential to the contamination of food used for consumption by the general public (Burch et al., 2013). However, knowing which specific ARGs are found in which bacterial species and/or genera in WWTP products is critical when selecting specific ARGs for regional, national, and international surveillance studies. Thus, unlike culture methods, which can also lead to bias, when using molecular methods determining which ARGs are found in which specific bacteria is key to the success of future surveillance efforts. The use of whole genome sequencing of WWTP products with emphasis on antibiotic resistance genes would be extremely useful to help determine which suite of ARGs should be examined when screening various components of the WWTP. Few studies concerning metagenome analysis of plasmids have been conducted (Schlüter et al., 2008; Szczepanowski et al., 2009) or have examined the microbiome of human sewage (Cai et al., 2014). More research needs to be done to determine if there are variations by geographical location, seasons, and other factors. As a result, currently most studies looking at specific ARGs and/or resistant plasmids are inherently biased. This should be taken into account when reviewing the literature including the cited studies below.

A recent review on the role of the environment in the transmission of antimicrobial resistance to humans has been published (Huijbers et al., 2015). In this study, 239 datasets were examined for evidence of human exposure to ARB, including extended-spectrum β-lactamases producing *Enterobacteriaceae*, MRSA, and VRE in

exposure-relevant environmental sites. These sites included environmental sources such as recreational areas, drinking water, ambient air, shellfish and fresh produce, wildlife, wastewater, and manure. ARB were detected in all the contaminated wastewater sources ($n = 60$) and molecular characterization supported the transmission of ARB from wastewater to the environment. The review stated that the abundance of the ARB at the various sources suggests a potential risk for human exposure (Huijbers et al., 2015).

Plasmids play an important role in the dissemination of antibiotic resistance and virulence determinates among *Enterobacteriaceae* and other Gram-negative genera. Many of these plasmids are conjugative, which means they can transfer from strain to strain and from one genus to another genus. A significant number of these plasmids are broad host range, which means they are able to transfer between a wide number of different bacterial hosts and genera (Tennstedt et al., 2003). Much of the work on plasmids in wastewater effluent and biosolids has been done with Gram-negative coliforms, *E. coli* and *Pseudomonas* spp. (Akiyama, Asfahl, Savin, 2010; Blazques et al., 1996; Droge, Puhler, Selbistschka, 2000; Szczepanowski et al., 2009; Walter and Vennes, 1985). Walter and Vennes (1985) found that between 0.35% and 5% of the coliforms they isolated were multiresistant, with ~75% having acquired new genes by conjugation. Tennstedt et al. (2003) isolated 97 different multiresistant plasmids from activated sludge within a wastewater treatment plant. Some of the plasmids were transferred to multiple recipients by conjugation. Szczepanowski et al. (2009) identified 140 clinically relevant ARGs within total plasmid metagenome DNA from activated sludge and final WWTP effluents from collected bacteria. The study determined that ~64% of the 192 reference resistance plasmid genes in the study were identified in WWTP effluent discharge, suggesting contamination downstream from the sewage plant was very likely. Evidence for conjugal transfer of Gram-negative antibiotic resistance plasmids in sewage within model systems have been described (Geisenberger et al., 1999), as has Gram-positive *Enterococcus faecalis* gene transfer in WWTP (Marcinek et al., 1998).

A variety of studies have looked for specific ARGs in influent wastewater, after the stages of primary settling, treated effluent, activated sludge, and treated biosolids. Most of these studies select a small subset of the known antibiotic resistance genes characterized by conferring resistance to any one antibiotic class. For example, one study looked at 10 different *tet* genes of 48 known *tet* genes (Roberts and Schwarz, 2016; Roberts, n.d.). The genes included Gram-negative specific efflux genes *tet*(A) through *tet*(E), *tet*(G), and ribosomal protection genes *tet*(M), *tet*(O), *tet*(Q), and *tet*(S) found in both Gram-negative and Gram-positive bacteria (Auerbach et al., 2007) from all 18 samples over a 12-month period. The Gram-negative efflux genes *tet*(A) and *tet*(C) were identified from all samples ($n = 18$). The other Gram-negative efflux genes were isolated from 9 to 16 of the samples, with the least common Gram-negative efflux *tet*(D) identified in 9 of the 18 samples. It is interesting that the most common efflux gene, *tet*(L), which is isolated in similar numbers of Gram-negative ($n = 19$) and Gram-positive ($n = 22$), bacteria was not examined (Roberts and Schwarz, 2016; Roberts, n.d.). This is a common issue with many of the environmental sample studies published. The authors selected tetracycline resistance genes to survey, and the selection was not based on abundance or those most widely distributed among different genera. This provides a significant bias to many environmental studies, including those on WWTP products (Burch et al., 2013; Du et al., 2014; Hong et al., 2014; Naquin et al., 2015). In contrast, among the ribosomal protection genes, which are more often associated with Gram-positive

bacteria, the *tet*(M) and *tet*(O) genes were identified in 17 samples (Burch et al., 2013). This is interesting because the *tet*(M) gene has been identified in 75 different genera, while the *tet*(O) gene is found in 35 different genera and would have been unexpected (Roberts and Schwarz, 2016; Roberts, n.d.).

Integrons were first reported in 1989 (Stokes and Hall, 1989). An integron is defined as the presence of an integrase gene (*intI*) a proximal primary recombination site (*attI*). The most common is the class 1 (*intI1*) genes. Integrons have been identified in ~9% of the bacterial genomes and found in a number of opportunistic and pathogenic Gram-negative species. Studies have used *intI1* genes as a surrogate for identification integrons in a variety of sample types including WWTP products (Du et al., 2014; Xu et al., 2015b). Sulfonamide resistance genes *sul1* and *sul2* are also very commonly carried resistance genes in Gram-negative integrons and have been used to screen for abundance in a variety of environmental samples including WWTP products (Du et al., 2014; Xu et al., 2015b).

Some studies have looked for a variety of different *erm* genes which code for rRNA methylases and conferring resistance to macrolides-lincosamides and streptogramin B ($MLS_B$) (Szczepanowski et al., 2009). There are currently 90 different genes currently identified that code for macrolide-lincosamide-streptogramin resistance (Roberts, n.d.). Distribution of the *erm* genes varies significantly: there are 1 to 36 different genera for *erm* genes, and they are found in 1 to 30 different genera for other MLS genes (Roberts, n.d.). Therefore, it makes sense to include those genes with the broadest host range such as the *erm*(B) gene, found in 36 different genera including both Gram-positive and Gram-negative genera, or the *mef*(A), identified in Gram-positive and Gram-negative genera ($n = 30$). In contrast, 48 (52%) of the MLS genes are found in a single genus and these would not, in general, be of interest for examining WWTP products (Roberts, n.d.).

At the other extreme is the *mecA* gene, which is found only in MRSA and methicillin-resistant coagulase negative *Staphylococcus* spp. (Wielders et al., 2002). Thus, using this gene will only identify a specific subset of *Staphylococcus* spp. and should only be used when looking for MRSA/MRCoNS detection (Soge et al., 2009).

## Conclusion

It is clear that ARB and ARGs are spread between animals, the environment, and humans and from one geographic location to another throughout the world. The environment is clearly an important reservoir for these resistance genes, with WWTP products being an important component as potential amplifiers in causing the spread of ARB and ARGs into the environment. These contaminants not only degrade the local environment but ultimately influence the health of humans and animals associated with that environmental landscape. The environment has provided an increasing number of novel ARGs that have not been found in bacteria traditionally associated with animals or humans (Roberts, n.d.). It is unclear whether these "new genes" will impact the treatment of animal and human infections in the future, but some such as NDM-1 (Walsh et al., 2011) or CTX-M (Canton et al., 2012) have been demonstrated to have this effect. There is also some evidence that WWTP do play a role in the evolution of multidrug-resistant opportunistic and pathogenic bacteria. WWTP is thought to play a

key role in the contamination of environments via effluent in waterways and irrigation, and biosolids in soil and agricultural lands. This is very important as WWTP biosolids and final effluents are considered resources that should be used for agricultural purposes. Thus, it is plausible that there is a human health risk associated with WWTP products, though at this time, the data backing this hypothesis are extremely limited. However, reducing the levels of ARGs/ARB in WWTP by-products before they are recycled is an important component in the multiprong approach to reduce the spread and distribution of ARGs around the world. Advanced wastewater treatments such as ozone, UV, ultrafiltration, chlorination, and membrane bioreactor processes have been shown to be effective in reducing the number of bacteria and may be useful in reducing the level of ARB in effluents and biosolids before they are utilized, thereby reducing the potential risk to humans (Huijbers et al., 2015). However, recent studies report that UV/$H_2O_2$ disinfection processes do not eliminate the possible spread of antimicrobial resistance in the receiving environment (Ferro et al., 2016). Cost effectiveness is another important consideration in application of advanced wastewater treatment options. We need to gain a better understanding of the role that biosolids and effluents play as amplifiers, reservoirs, and transmitters of these bacteria and genes in order to comprehensively assess antimicrobial resistance–related impacts on risks to human health.

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# 4

# Assessing the Impact of Wastewater Treatment Plants on Environmental Levels of Antibiotic Resistance

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The introduction of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) into environmental compartments, such as soil, groundwater, and surface water, has generated a great deal of concern in recent years (Baquero et al., 2008; Berglund, 2015; Lupo et al., 2012; Roca et al., 2015) due to the potential for detrimental effects on the health of humans, animals, and the environment (Ashbolt et al., 2013; Williams-Nguyen et al., 2016a). Research clearly demonstrates the existence of ARB and ARGs in treated and untreated waste from human activities (Bouki et al., 2013; Hong et al., 2013; Rizzo et al., 2013), including in wastewater treatment plant (WWTP) environments, as is discussed further in the chapters of this volume. The magnitude of ARB and ARG dissemination from anthropogenic sources (such as WWTP) into the receiving environment and subsequent exposure of humans and animals are still not well understood despite the numerous observations of ARB and ARG in environmental systems (Baquero et al., 2013; Rizzo et al., 2013; von Wintersdorff et al., 2016). This has prompted calls for environmental monitoring and increased research on the sources of ARB and ARG in water bodies and soil systems (e.g., Ashbolt et al., 2013; Berendonk et al., 2015).

From a public health perspective, such endeavors may be conceived to have two goals: (i) to provide information about the impact of effluent sources on levels of ARB and ARGs in environmental systems and (ii) to provide information about the risk of ARB and ARGs exposure for humans (and perhaps also domesticated animals). The second goal is crucial for efforts to assess health risks posed by dissemination of antibiotics, ARB, and ARGs into the environment (Ashbolt et al., 2013), while a comprehensive understanding of the first is a requirement for successful intervention measures aimed at preventing such contamination. Researchers and policymakers may often wish to design monitoring programs that serve both goals simultaneously, but this may be difficult where study design principles for achieving these two goals differ. In this chapter, we will discuss the basic principles for designing research studies and

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monitoring programs that assess the impact of WWTPs on ARB and ARG in environmental systems (goal 1).

There are several ways to provide evidence for the impact of WWTPs on antibiotic resistance in environmental systems. Both monitoring programs and individual research studies may first seek to determine if anything unexpected or unusual can be identified in a system. For example, a researcher may sample surface water near a freshwater recreational beach and determine if there are any isolates of an indicator species that are resistant to a number of common or important antibiotics (Harwood et al., 2000). A large number of recent studies using indicator species such as *Escherichia coli* provide these kinds of descriptive results (e.g., Blaak et al., 2015; Kappell et al., 2015; Osinska et al., 2016). Regular monitoring programs can add substantial value to this signal-detection effort by improving sampling coverage and cataloging temporal trends in ARB and ARG quantities in environmental systems. These types of efforts provide information on which systems and outcomes may need more intensive research.

Once resistant isolates have been discovered, the research community may then wish to attribute those isolates to a specific source. Microbial source tracking (MST) is one prominent tool used to identify the source of microbes isolated from environmental media, predominantly water. MST-based studies often provide information about whether specific sources or source types are emitting bacteria into the environment (Scott et al., 2002). However, MST also has limitations for efforts to determine the impact of a source on measures of antibiotic resistance (Baquero et al., 2008). For one, MST evidence that an ARB isolated in the environment originates from a specific source does not necessarily indicate that said source has meaningfully increased the amount of that ARB at the environmental site and provides no quantitative estimate of impact. Developments extending MST to estimate the proportion of a target organism associated with a specific source, known as microbial source apportionment, may allow improved application of MST methods to resistance research, such as in situations where some proportion of a clonally spread ARB of interest at a specific location is determined to be attributable to a WWTP (Wang et al., 2013). In this case, intervention measures could be targeted at improvements in the WWTP.

Another limitation of MST for identification of potential sources of antibiotic resistance is reliance on the method of source-specific markers (Harwood et al., 2014). Such markers may be absent or less relevant in studies of antibiotic resistance measures, due to the potential importance of horizontal gene transfer (HGT). WWTPs and other contaminant sources may emit ARB as whole viable cells or cell-free ARG or, more likely, both. Further, ARB that are emitted may persist, decay, or expand clonally depending on environmental conditions. Via HGT, those ARB may also share antimicrobial resistance genes with other bacteria, either native or introduced, within environmental media. This complexity means that source attribution of environmental ARB isolates using tools such as MST with strain-level resolution may represent only a portion of the possible effects of WWTPs and other sources on antibiotic resistance. MST, in its current form, is unable to provide information about the movement of genes and therefore cannot provide information about impacts on antibiotic resistance that are mediated by HGT. A more complete understanding of the impacts of WWTP and other effluent sources on measures of antibiotic resistance in the environment requires other inferential techniques.

Environmental impact assessment includes a set of tools that focuses on the question of whether a source, usually a point source such as a WWTP, will result in a change to a system (compared to the system's usual state) (Downes et al., 2002; Glasson et al., 2013; Krebs, 1998a). These tools have rarely been applied to measures of ARB or ARG. The body of work assessing the impact of point sources of effluent, such as WWTPs, on environmental measures of antibiotic resistance had not been systematically evaluated until recently by our team (Bueno et al., 2017; Williams-Nguyen et al., 2016b). After a thorough screening of more than 5,000 articles that referred to antibiotic resistance in soil, water, air, or wildlife, fewer than 90 studies were finally included. Among them, the majority evaluated WWTPs as the point source, followed by a few articles that evaluated terrestrial agriculture (especially swine farms) and aquaculture facilities. Surface water and associated sediment were the most common type of environment sampled, followed by others (groundwater, soil, biofilm, air, wildlife). One of the main conclusions of the systematic review is that quantitative estimates of the impact of WWTPs and other point sources on the levels of ARB and ARG in receiving rivers or soil systems are few. The spatial and temporal scales investigated were also highly diverse.

The purpose of this chapter is to give interested researchers an overview of the basic principles involved in designing studies or monitoring programs that seek to assess the impact of a WWTP on measures of ARB or ARG in receiving environments. Examples in this chapter will only involve surface water. However, these principles are general and will apply to other environmental compartments (soil, groundwater, air). We also note that while this chapter draws heavily on causal inference theory that was developed in the context of epidemiologic research, these principles are widely useful in observational science and share common themes that have emerged in the disciplines of ecology and environmental impact assessment. We refer the reader to Norton et al. (2014a), Downes et al. (2002), and Krebs (1998a) for a treatment of these concepts using ecological nomenclature.

## Defining the Research Question

> *Improvements in the value of both physicochemical and biological variables to monitoring and assessment will rely as much on asking better questions, which take the relevant scales of variation in time and space into account, as it will on novel chemical analyses or analytical techniques.*
>
> (Downes et al., 2002)

A clearly defined research question is a required first step in any scientific research effort. The validity of any inference drawn from research findings depends on a clearly defined question. As such, research questions should be developed in the planning stages of a study and refined through an iterative process until they are concise, focused, clear, and feasible. How the research question is defined will drive the hypothesis or hypotheses and determine the most appropriate study design, data collection procedures, and data analysis to provide a valid answer for that specific question.

Here we focus on questions about the impact of a point source, such as a WWTP, on environmental levels of antibiotic resistance. The possible ways to define antibiotic

resistance, in environmental systems and elsewhere, are numerous. Where ARB is the focus, one must decide on the antibiotic compound(s) or class(es) of interest, the type of bacteria to be targeted for culture, the method for identifying resistance against the selected antibiotic(s), and the mathematical representation of antibiotic resistance (e.g., proportion of isolates displaying the phenotype or genotype of interest, sites with a positive result in any isolate, etc.). Similarly, where ARG is the focus, specific gene(s) of interest must be selected and a decision made about whether the gene copy number should be normalized against copies of the 16S gene or another measurement. We will refer to each unique definition of antibiotic resistance as an outcome. Similarly, there are many factors to which an environmental medium or system is exposed that could influence development of antibiotic resistance as an outcome. We term each of such factors an exposure.

For example, imagine a situation where a WWTP (Plant A) discharges treated wastewater into a river (River A) that sees frequent downstream use (e.g., water sourced for human or agricultural use). A researcher may wish to understand the impact of the effluent of Plant A on tetracycline resistance in the waterway. This research question can be defined formally using the PECO (or PICO) framework, by selection of the following elements: population (P), exposure (E) (or intervention (I)), comparator (C), and outcome (O) (Parfrey and Ravani, 2009). This framework is a concise way to capture information about the etiological relationship of interest and scope for a particular study. In our example, the researcher could translate the study question into the framework as follows:

P: fecal coliform bacteria in water from River A
E: river locations exposed to Plant A effluent
C: river locations unexposed to Plant A effluent
O: prevalence of phenotypic tetracycline resistance

For established researchers, this exercise may initially feel unnecessary and even trivial. However, on the contrary, explicitly defining a question a priori using this or an equivalent framework is essential for etiologically meaningful observational studies. The following sections will discuss study design considerations and methodological approaches that can enable researchers to arrive at etiologically valid estimates of WWTP impact on antibiotics and antibiotic resistance (ARB and ARG) in environmental systems. But none of these are possible without a specific and clear research question. One way in which the question may be framed is to consider the hypothetical causal relationship between the exposure and the outcome; the use of causal diagrams, as described below, can aid in the precise conceptualization of these relationships (Greenland et al., 1999).

## Causal Diagrams as a Tool for Causal Inference

Causal diagrams, in particular causal directed acyclic graphs (DAGs), are visual and mathematic tools that enable researchers to represent causal relationships among variables of interest in a study or system. In epidemiology, a field that relies heavily on observational evidence, DAGs have become an invaluable tool for encoding and

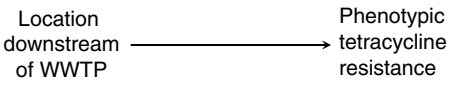


**Figure 4.1** Simple directed acyclic graph depicting the example study question (to understand the impact of the effluent of Plant A on tetracycline resistance in a waterway). Nodes represent factors in the system. Arrows represent causal relationships between factors leading. Arrows point away from a cause toward its effect.

communicating causal assumptions, designing sampling protocols, and aiding analytic judgments (Greenland et al., 1999; Shrier and Platt, 2008). We briefly introduce the concept here to facilitate understanding of how study/program design can uncover or obscure causal knowledge in observational studies of WWTP impact on antibiotics and antibiotic resistance in the environment.

A DAG is composed of nodes that represent variables in a system at a single point in time and causal relationships (represented by arrows) between these nodes. The direction of the arrow between nodes indicates a causal relationship, with the arrow pointing from cause to effect. For example, Figure 4.1 represents the question defined above. Although composed of simple components, the mathematical underpinnings upon which DAGs were developed mean that the arrangements of arrows can reveal whether the causal relationship of interest is estimable from data and under what conditions. This is particularly useful as the causal networks represented become more complex. While it is outside the scope of this chapter to describe the mechanics of DAGs in greater detail, we refer the reader to Foraita et al. (2014) and Hernán and Robins (2016) for additional information.

## Counterfactuals and Causal Effect Estimates

As a starting point for understanding how we may design a study adequate to answer the question defined above, we will use a foundational approach in causal inference known as the potential outcomes model or counterfactual thinking (Greenland, 2005). The potential outcomes model states that there exists only one way to determine causation with absolute certainty. This is to observe the subject of study for the outcome of interest in both the presence and the absence of the exposure in question with everything else being absolutely fixed, including the subject(s) of study, the date and time of observation, and environmental conditions. In other words, it would involve having the same subject or population of subjects exposed and unexposed to exposure stressor at the same time. In our example, we might sample water from various locations on River A downstream of Plant A at 12:00 pm on April 27, 2015. Then we would board our time machine, go back in time, prevent the construction of Plant A and then again sample water from the same locations on River A at 12:00 pm on April 27, 2015. Of course, all climatic and weather conditions as well as any other inputs to River A both at the time of sampling and at all prior times (with the exception of inputs from Plant A) would be identical for both sampling events. See Figure 4.2.

It is quite obvious that, in reality, it is not possible to observe both of these outcomes. One or the other of the outcomes is unobservable and did not actually happen. The outcome of interest can only be observed under the ***factual*** exposure, that is, we can

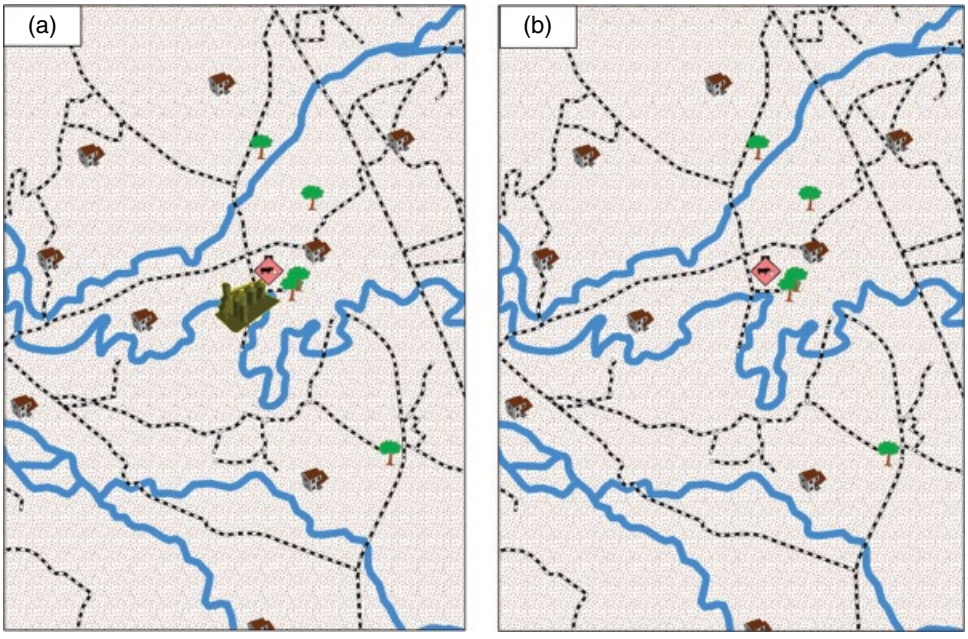


**Figure 4.2** Schematic representing the counterfactual thinking. In (a), a wastewater treatment plant is present. In (b), the wastewater treatment plant is not present, but the rest of the variables (rivers, forests, roads, houses) and other sources (dairy farm) are the same as in (a). We are assuming the time frame is the same as well. These two groups would be exchangeable, and thus valid causal inference would be possible.

only observe the prevalence of tetracycline resistance in the River A in the presence of Plant A. The outcome under the hypothetical alternative is termed the ***counterfactual*** (Hernán, 2004).

Supposing that it were possible to observe the counterfactual, producing a quantitative estimate of the causal effect of exposure (e.g., Plant A) on the outcome (e.g., tetracycline-resistance of fecal coliforms) would be straightforward. We simply compare the value of the target outcome under the two conditions to produce a ***causal effect measure*** or measure of impact. Such an estimate can take a variety of numerical forms. For example, we could simply compare the prevalence of tetracycline resistance in fecal coliforms detected in exposed compared to unexposed samples. Say tetracycline resistant fecal coliforms were detected at 50% of exposed locations but only 20% of (counterfactually) unexposed locations. To determine what is called the absolute ***risk difference*** for this type of resistance due to Plant A, we subtract 20% from 50%. We can thus conclude that Plant A increases the prevalence of tetracycline resistant fecal coliforms in the sampled segment of River A by 30%. Similarly, we could compute the ***relative risk*** by taking the ratio of the prevalence in the exposed group to the prevalence in the unexposed group (i.e., 50%/20%), yielding a relative effect measure of 2.5. We can also conclude that Plant A causes a 2.5-fold increase in the risk of tetracycline resistance in the sampled segment of River A. Additional options for calculating effect measures in ecological impact assessment studies are described by Norton et al. (2014a).

## Producing Causal Effects in the Real World: Exchangeable Groups

You may find yourself wondering about the practical utility of this thought exercise for applied researchers. While it is immediately clear that the counterfactual is, by definition, impossible to observe, counterfactual thinking has immense utility in designing etiologically valuable studies. Counterfactual thinking underlies all scientific endeavors to determine causation, for controlled, observational, laboratory, and field studies alike. Although observing the counterfactual itself is not possible, the ability to infer causality from research studies relies on our ability to identify the next best thing, a comparison group that mimics as closely as possible that counterfactual condition. In other words, we wish to identify an unexposed comparator group that is the same as the exposed group in all important aspects except for the exposure. We term such a comparator exchangeable. If groups are exchangeable, the exposure status of the exposed and unexposed groups could be switched without impacting the results (Dohoo, 2010).

In laboratory science, researchers are careful to ensure that study subjects in experimental and control groups are as similar as possible (e.g., cells coming from a common culture or mice of the same genetic strain), while also carefully controlling environmental conditions to ensure that the only important difference between the groups is the exposure of interest. Similarly, in ecological controlled field trials or biomedical randomized control trials (RCTs), researchers randomly allocate subjects (e.g., individual fields or patients) to treatment or control exposure in an effort to create comparison groups that are as similar as possible to one another except for the treatment. Randomization is the gold standard for creating exchangeable groups because, on average, it will create groups that are equal on both known and unknown factors that may differ between subjects (Akobeng, 2005). In biomedicine, RCTs are placed at the top of the evidence-based hierarchy (Concato et al., 2000).

However, randomization is not possible in all important scientific inquiries. In the example considered here, it is impossible to randomize rivers or river locations to WWTP effluent exposure. Therefore, any investigation of WWTP effluent on landscape-scale environmental systems will be observational by necessity. In observational settings, it is well acknowledged that a statistical correlation between an exposure and an outcome cannot provide evidence that one caused the other (Hernan, 2004). This is because exposed and unexposed comparison groups are often not exchangeable. For this reason, making valid causal inference with observational data is challenging and complex (Hernán and Robins, 2006), and special attention to creating exchangeable comparison groups is required. When we define the comparator (C) in the PECO framework above, we are striving to identify *as nearly as possible* an unexposed but exchangeable group.

## Bias in Estimation of Causal Effects

Selecting or creating exchangeable groups for comparison is a requirement for producing internally valid causal effects from observational data. ***Internal validity*** describes the extent to which an effect measure (or measure of impact) accurately reflects a true causal relationship in the system under study (Trochim, 2006). Where comparison

groups are different on important characteristics (i.e., nonexchangeable), bias in the estimated causal effects is a likely result. In the context of causal inference, bias refers to the under- or overestimation of an estimated effect due to systematic differences in the comparison groups. Such systematic differences can result from a myriad of different scenarios. However, there are three main structural mechanisms by which causal effect estimates derived from observational data can be biased from their true value (Miettinen, 1985). In epidemiology, these are commonly referred to as confounding, selection bias (Hernán et al., 2004), and information bias. However, these three mechanisms of bias in causal effect estimates are general in all observational science, though in other disciplines they may be known by different names.

## Confounding

Confounding in population-level studies typically occurs when a common cause of both the exposure and the outcome induces a noncausal association between the two factors (Hernán et al., 2002), such as in the well-known example of an apparent association between ice cream consumption and drowning deaths at the population level. The confounder in this case is, of course, hot weather, which causes both increased ice cream consumption and more drownings due to increased recreational water use (Figure 4.3a). Such population-level confounding is an important consideration in landscape-scale impact studies that model the relationship of a source type (e.g., WWTPs) with ARB or ARGs in environmental compartments.

In addition to population-level confounding relationships, confounding can arise from incidental spatial proximity of a WWTP to other possible sources of antibiotic resistance such as farms or recreation areas. This type of confounding, sometimes to referred to as spatial confounding (Downes et al., 2002), can occur when a study seeks to estimate the impact of a single source as in the example of Plant A presented here. When a farm and a WWTP just happen to be next to each other, it becomes difficult or impossible to disentangle their separate effects on the adjacent environment for outcomes that are not source specific. Considering Plant A on River A, suppose a researcher

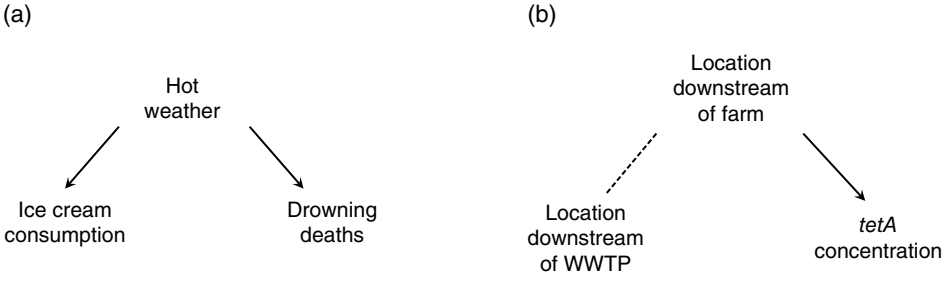


**Figure 4.3** Directed acyclic graphs depicting a situation of potential confounding bias. In each case, the variable on the lower left has no causal effect on the variable on the lower right. Part (a) depicts classical confounding in a population-level study where the association of each variable with a common cause, hot weather, produces an association between ice cream consumption and drowning. Part (b) depicts a variant of confounding that can occur in impact assessment of a single WWTP where the incidental close proximity (shown as a dotted line with no arrowhead) of farm to WWTP causes a spurious association between the WWTP and *tet*(A) concentration in river water.

wishes to determine the impact of Plant A on the Gram-negative tetracycline resistance gene, *tet*(A). Figure 4.2a shows Plant A located adjacent to a dairy farm. In this situation, locations immediately downstream of Plant A are also immediately downstream of the farm. Comparison locations, such as a downstream site, are extremely unlikely to be exchangeable with regard to farm inputs, and thus any estimate of the effect of Plant A on *tet*(A) concentration in river water is confounded by the influence of the dairy.

There are a number of methods available to mitigate the effects of confounding and permit the valid estimation of causal effects in observational research. Careful selection of exchangeable comparison groups is one way to avoid confounding. For studies of a single WWTP, as described in this example, this could mean selecting a WWTP for study that is not subject to spatial confounding. For a WWTP that is spatially confounded by other impacts (e.g., farm effluent), it could mean seeking comparison sites, on the same or other rivers, that are subject to these same impacts (except the WWTP).

### Selection Bias

Collider stratification bias, also known as selection bias, is caused by conditioning on a common effect of either the exposure or one of its causes and the outcome or one of its causes (Cole et al., 2010; Hernán et al., 2004). Typically, this common effect is a factor that influences inclusion of a subject or site in a study. This form of bias is less intuitive than the problem of confounding, yet it can also result in incorrect causal effect estimates. Much work has been devoted to understanding and mitigating the influence of selection bias in observational population health studies, but recent work has also acknowledged its importance for studies in ecological settings (Farrar, 2010; Norton et al., 2014b).

To illustrate how this bias might operate in the estimation of a WWTP's impact on antibiotic resistance in the receiving river, we present an example based on Cole et al. (2010) that has been developed to reflect the present application. We return again to the question of Plant A's impact on tetracycline resistance in coliform bacteria in River A. But let us assume that, in this case, there is no true causal effect of Plant A on tetracycline resistance in the river. Table 4.1 shows this numerically, where an equal prevalence of resistance in isolates sampled at sites downstream (WWTP exposed) and upstream (WWTP unexposed), yielding a null relative risk of 1. However, in this case, the researcher chooses to isolate from water samples only those coliforms that produce extended spectrum β-lactamases (ESBLs) due to the importance of ESBL producers in human clinical settings (Paterson and Bonomo, 2005). It may seem straightforward that one could compare prevalence downstream to upstream as before and obtain a valid effect estimate.

However, let us additionally assume that ESBL-producing coliforms in the river are almost always tetracycline resistant, as might be the case in an agriculturally impacted waterway. But in coliforms released by Plant A, tetracycline resistance does not occur in isolates that produce ESBLs. The causal diagram depicting this situation is presented in Figure 4.4. As shown in the lower portion of Table 4.1, when the researcher compares the prevalence of resistance to tetracycline in isolates from downstream (WWTP-exposed) sites compared to upstream (WWTP-unexposed) sites within the subset of ESBL-producing isolates, the risk ratio indicates that being downstream of the WWTP was associated with an 80% lower risk of tetracycline resistance in river water.

**Table 4.1** Illustration of collider stratification bias (selection bias) on the estimate of WWTP effect on prevalence of tetracycline resistance in coliforms isolated from river water.

| | Resistant to Tetracycline | Total Isolates | Relative Risk |
|---|---|---|---|
| Upstream | 10 (10%) | 100 | 1.0 |
| Downstream | 10 (10%) | 100 | |
| *ESBL:* | | | |
| Upstream | 8 (40%) | 20 | 0.2 |
| Downstream | 8 (9%) | 90 | |
| *Non-ESBL:* | | | |
| Upstream | 2 (3%) | 80 | 8.0 |
| Downstream | 2 (20%) | 10 | |

*Source*: Cole and Platt (2009). Reproduced with permission of Oxford University Press.

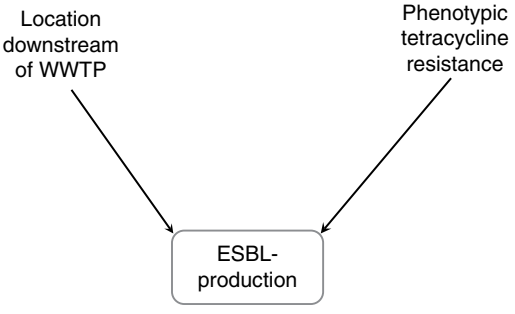


**Figure 4.4** Directed acyclic graph depicting a situation of potential collider stratification bias (selection bias). The box indicates that the variable, ESBL production, has been controlled for by means of restriction, stratification, or regression adjustment. Despite a lack of causal association between WWTP location and tetracycline resistance, a spurious association is created by controlling for ESBL production, a common effect of the two variables.

Alternately, if one were to look at the stratum of isolates lacking ESBL production, results would suggest that being downstream of the WWTP was associated with an 8-fold (800%) increase in risk of tetracycline resistance. Neither of these stratum-specific estimates are accurate representations of the effects due to the WWTP on tetracycline resistance in coliforms. Rather both estimates are biased. In the ESBL stratum, the comparison group has been artificially depleted of tetracycline-susceptible isolates because the majority of these isolates do not produce ESBLs.

It is crucial to point out that whether the stratum-specific estimates presented above are considered to be biased depends on the outcome that one has sought to estimate. Here we have assumed that the researcher was interested in whether the WWTP impacts tetracycline resistance in coliform bacteria, yet he or she estimated this effect in only a subset of this target population. In that case, such a selection process has the potential to lead to bias. However, if the researcher had been specifically interested in the effect of the WWTP

on tetracycline resistance in *ESBL-producing coliform bacteria*, the stratum-specific estimate above would be accurate for that population. The solution to this potential issue lies in the careful construction of the research question and attention to that question through the process of implementation, analysis, and interpretation.

### Information Bias

Information bias, also known as misclassification, results from the use of an effect of the exposure as a surrogate for the exposure itself or, similarly, by the use of an effect of the outcome as a surrogate for the outcome (Shahar, 2009). The use of such surrogates is often necessary, and while it may not cause bias in causal effect estimates, it is possible for the use of these surrogates to lead to unrecognized instances of confounding and collider stratification bias. To show how this can occur, let us return to our example of River A. The researcher now wishes to know about the impact of Plant A on the proportion of bacterial cells in River A sediment samples that contain the gene *tet*(A). This is the true outcome of interest. The best measurement available is the ratio of *tet*(A) and 16S gene copy numbers determined via quantitative polymerase chain reaction (qPCR). However, this method provides an imperfect measure of the true outcome of interest due the propensity for some bacterial taxa to carry multiple copies of the 16Ss gene (Luby et al., 2016; Vetrovsky and Baldrian, 2013). Imagine that sites downstream of Plant A incidentally have a sediment type that favors a microbiome (mixture of bacterial taxa) while sites upstream have sediment that favors a different microbiome. This complex situation is depicted in Figure 4.5. Even in the absence of a true relationship between Plant A and the proportion of sediment bacteria with *tet*(A) (i.e., when the proportion of *tet*(A)-carrying bacteria in the population is identical in downstream and upstream sites), this situation could create a spurious relationship between Plant A and the surrogate outcome (ratio of *tet*(A) copies to 16S copies) due to confounding via sediment type and the associated microbiome.

In the estimation of causal effects from observational data, complete removal or mitigation of all types of bias from a study or analysis is neither possible nor advisable. As will be clear to readers, known and unknown biases will be present to varying degrees

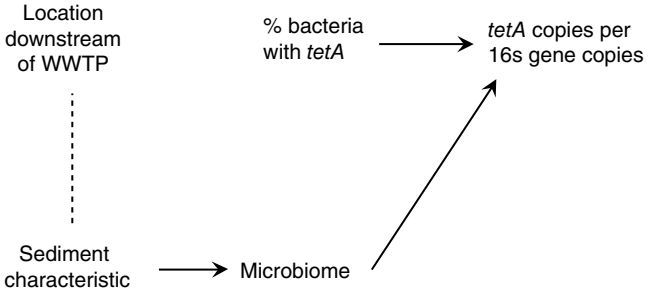


**Figure 4.5** Directed acyclic graph depicting a situation of potential information (or misclassification) bias. An incidental, associational (noncausal) relationship between the location relative to the WWTP and sediment characteristics is shown as a dotted line with no arrowhead. Despite a lack of causal association between % of bacteria with *tet*(A) (or its surrogate), a spurious confounded association is created between WWTP location and *tet*(A) copies per 16 s gene copies.

in any observational research effort. In many cases, such biases, while truly present, will exert little appreciable impact on quantitative effect estimates. It is, however, the responsibility of researchers seeking to uncover etiologic evidence in observational data to think carefully about sources of possible bias and consider them, both in study design and interpretation. We present these illustrations to aid researchers in that effort.

## Study Designs and How These Simulate the Counterfactual

Having identified important potential biases for a specific research question, the foremost method for minimizing the biases described above is through careful study design, in particular defining the comparison group(s). Which study design type is most appropriate will depend on the question and on other considerations, such as feasibility.

For studies assessing the impact of a single WWTP on the receiving river or other flowing water, there are a number of options for defining the comparison, each with benefits and limitations. In most of the examples above, the comparison has been defined as a site downstream of the WWTP compared to a site upstream (comparator). This simple design relies on an assumption that the upstream site is the same as (exchangeable with) the downstream site with the exception of impact from the WWTP. Depending on the specific details of the river and sites in question, this may or may not be a good assumption. If the site selected upstream is different from the downstream site in important ways (e.g., sedimentation), then confounding could result. Taking multiple upstream and downstream sites maybe be one option for improving this design, particularly if upstream and downstream sites are sampled such that confounders are evenly distributed within each group. There is the possibility that no site on the same river might be reasonably assumed to be exchangeable with exposed site(s) of interest (e.g., as in the example shown in Figure 4.2a, where a dairy farm was located adjacent to the WWTP of interest). If so, it may be that better comparison sites are located on another river that resembles the characteristics of the selected exposed locations (e.g., near a dairy farm but lacking the WWTP) (Downes et al., 2002).

Even with careful selection of comparators, it remains possible that the comparison site(s) may differ by chance or due to unmeasured confounding. To mitigate this risk, Downes et al. (2002) recommend the use of the Before-After-Control-Impact (BACI) design for impact assessment. To implement a BACI design, samples are taken at baseline (e.g., before the WWTP is operating) both at the future exposed site and at the comparison site, and then samples are also taken after the WWTP is operating again both at the exposed site of impact and at the comparison site. The researcher can then observe whether the difference between comparison groups differs between the before and after periods. Such a design, though not possible for existing WWTPs, addresses many of the limitations of simpler designs.

Another option that has been pursued by a small number of researchers (Amos et al., 2015; Pruden et al., 2012), and one that might work well for monitoring programs, is a population-based regression modeling approach. In this design, antibiotic resistance measurements are ascertained at a large number of sites in a landscape (e.g., watershed) and the locations and estimates of load are obtained for numerous potential sources. Regression analysis is then used to estimate the effect of WWTPs on the given resistance outcome, typically ARG concentration. We note that the model of Amos et al.

(2015) was intended for use in predictive modeling; however, we believe that a similar model could be used for etiologic inference with an appropriately defined question. Researchers using this approach should report model-based effect estimates along with meaningful interpretation of these estimates.

Studies of soil systems typically focus on land application of treated waste solids (as is discussed elsewhere in this volume). Randomized field trials may be possible for such systems and, where used, have good internal validity though sometimes at the expense of reduced generalizability (see *external validity* below). Where observational research is needed, close attention should be paid to history, hydrology, and other characteristics of the sites to ensure these are as similar as possible. To the best of our knowledge, landscape-scale regression models linking WWTPs or land application of treated waste to antibiotic resistance outcomes in soil have not been attempted, although one study found that nitrogen fertilizer was associated with increased ARG content of soil (Forsberg et al., 2014).

## External Validity

External validity describes the extent to which an effect measure estimated from a given study can be meaningfully applied to other situations or systems (Steckler and McLeroy, 2008). For example, on the basis of our Plant A example, suppose that we know that exposure to Plant A increased the prevalence of resistance in River A by 30%. Can we expect that, given the same spatial and temporal scales, exposure from Plant B on River B would produce the same effect? Can we expect that the effect will be the same if we repeat the study again next year? Can this result, in fact, be extended to understand the effect at other spatial scales, that is, as small as linear feet or as large as hundreds of miles? The answers to these questions can often impact the scientific and societal importance of a study. However, it is important to keep in mind that internal validity must take priority over external validity in nearly all cases. The key reason for this is that an effect estimate that lacks internal validity is not valid for any system including the one under study.

## Special Considerations for Studies in the Environment

Designing studies that involve exposures and outcomes measured in the natural environment (e.g., rivers) have their own suite of challenges, related to the large number of variables and factors that can affect and distort the causal relationship of interest (Singer et al., 2006). For one, it is important to define the spatial scale of study. The larger the scale, the more likely it will be that other alternative point sources (and not the one of interest) may be influencing the quantity of antibiotics, ARB, or ARG in the environment. Thus, selecting appropriate exchangeable comparators becomes more difficult and potential for serious confounding increases. For example, if we choose a spatial scale of 100 kilometers along a waterway, many other potential sources aside the WWTP(s) of interest are likely to be impacting that waterway to varying degrees, including dairy farms, cities, or aquaculture sites. For landscape-scale measurements of numerous individual sources, regression modeling analysis to control for confounding

bias can be used (e.g. Pruden et al., 2012). It is important to note that the exact interpretation of the estimated output parameter will depend on the form and parameterization of the regression model. For a primer on regression analysis, we refer the interested reader to Harrell (2015).

Recording and reporting meta-data about sampling sites, such as temperature, chemical measurements, soil or sediment types, or degree of groundcover, will aid in the understanding of how variability between sites could impact causal effect estimates. For example, collecting geospatial data is potentially useful for implementing spatial analysis, where warranted. Capturing spatial heterogeneity within sampling sites is also an issue of importance that should be considered for the accuracy of causal estimates. Ensuring representativeness of the sample from a site (usually a composite sample) can be expected to reduce potential confounding and improve precision of estimates.

Similarly, because environmental systems can fluctuate over even short periods of time (e.g., changes in water volume or meteorological conditions), sampling within a small temporal window can help to remove temporal confounding and improve precision. However, it is important to note that due to the volatile nature of some systems, effects of WWTP (and other sources) on environmental antibiotic resistance outcomes may change over time, being subject to threshold effects or seasonality. Studies or monitoring programs that feature repeated sampling of the same sites over time will enable researchers to probe these temporal changes or simply provide valuable replication. When using such repeated sampling, it is important to maintain clear definition of comparison groups and incorporate repeated sampling into statistical analysis when appropriate.

Before starting the sampling collection in the field, it is useful to define a sampling protocol to maintain consistency in sample collection and, thus, reduce the potential introduction of biases. Protocols can also include the number of samples needed to detect the effect size of interest (i.e., sample size calculation). For information about determining an appropriate sample size, we refer the reader to Krebs (1998b). Once in the field, deviations to the protocol should be tracked and addressed.

## Considerations for Monitoring Programs

As mentioned in the introduction, increased calls for environmental monitoring programs aimed at tackling the problem of ARB and ARGs in water and soil bring with them the important question of how these programs should be designed. Where the primary goal of such programs is to inform health risk assessments, monitoring should focus on high-use areas and sites that can provide important information for predictions of ARB and ARG fate, transport, and persistence. Alternately, monitoring programs or landscape-scale research studies that have a primary goal of determining the sources of contamination and supporting impact mitigation strategies should focus on including sites that cover a wide range of impacts for which exchangeability can be reasonably assumed or for which detailed data on confounders has been simultaneously collected. As described above, some important potential confounders that should be included are inputs from other sources of antibiotics, biocides (such as metals), ARB, and ARG. When relevant, regression analysis that includes confounder data will be

required to answer questions about source impacts from such landscape-scale data. With careful planning and consideration, it may be possible to serve both of these goals simultaneously.

## Conclusion

In this chapter, we have presented basic principles for the design of research efforts attempting to determine the impact of WWTPs on antibiotic resistance outcomes in receiving environments. Observational data, both a blessing and a curse, holds enormous value for the research community but also requires exceptional care throughout the process of question formulation, study design, analysis, and interpretation. The observational researcher interested in causal knowledge must weigh the likelihood of biases being introduced at each step. When working in field environments, feasibility and logistics can present a challenge despite the best planning. In some cases, it might not be possible to collect information for all the variables required to mitigate potential biases. We note that no study is perfect and all studies, including randomized studies, can be subject to biases. The challenge for us all is reducing these biases to the extent we can and bearing them in mind as we interpret our findings. We present this information as a resource and hope that some find it helpful in the endeavor to understand and mitigate the risks posed by antibiotic resistance in WWTPs and the environment.

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# 5

# Navigating through the Challenges Associated with the Analysis of Antimicrobials and Their Transformation Products in Wastewater

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The presence of antimicrobials in wastewater remains of growing concern due to the increased emergence of antimicrobial resistant bacteria and spread of antimicrobial resistance genes in the environment. Many antimicrobials resist biodegradation; most of them are also very polar, which prevents removal by sludge sorption. Therefore, the ability of municipal wastewater treatment plants (WWTPs) to efficiently remove antimicrobials depends on the compound's physicochemical properties combined with the design and operation of the treatment system. In some cases, treatment leads to partial transformation of antimicrobials into another biologically active compound, many of which have not been identified. In order to assess the role of antimicrobials and their transformation products (TPs) in the emergence of antimicrobial resistance in the environment, it is important to have robust analytical techniques for the quantification and identification of both the parent antimicrobials and their TPs in untreated and treated wastewater. Analysis of antimicrobials in wastewater requires a good understanding of the fundamentals of sample collection, preparation, storage, extraction, and quantification methods that are specifically relevant to antimicrobials. This chapter will discuss advancements and strategies in developing methods of antimicrobial analysis in wastewater, and will include a review of advanced separation and detection techniques, as well as a discussion on how to overcome analytical challenges commonly encountered in the trace analysis of antimicrobials in highly complex matrices, such as wastewater.

## Introduction

Pharmaceutical residues in the environment have been called "emerging contaminants" because of their increasing frequency of detection in the aquatic and terrestrial systems, and their sublethal ecological effects that are just beginning to be uncovered. Both human and veterinary pharmaceuticals are introduced into the environment via many different routes, including discharges from municipal wastewater treatment plants, and via land application of animal manure and biosolids to fertilize croplands. As depicted

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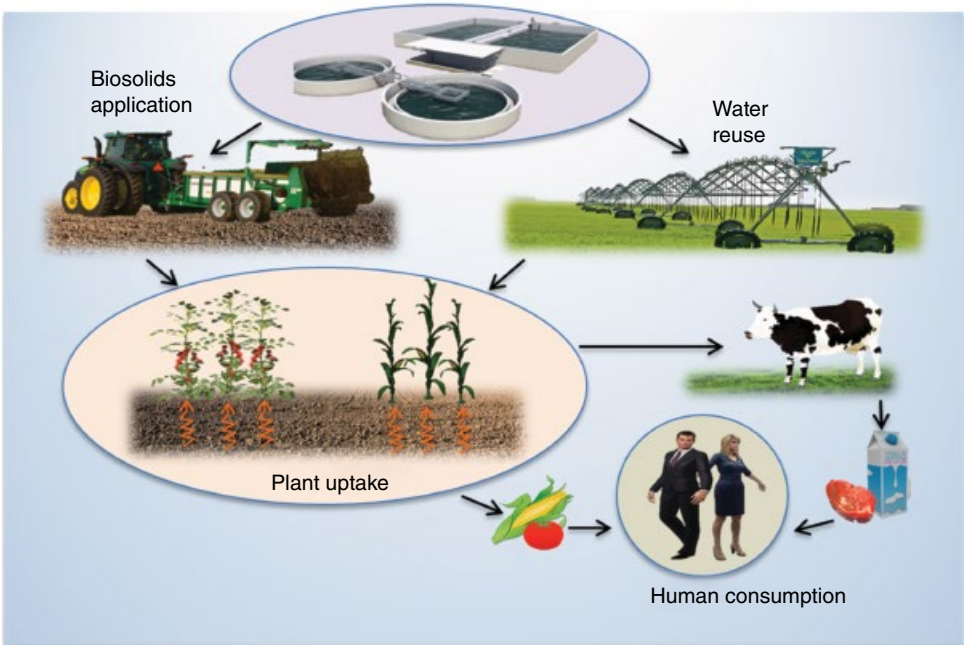


**Figure 5.1** Schematic diagram illustrating how antimicrobials can be introduced into the human food systems. Water reuse, which is a common practice in arid and semiarid regions, uses treated wastewater to irrigate food crops; biosolids are typically land-applied as fertilizer. Both practices can introduce antimicrobial residues into the human food cycle. (*See insert for color representation of the figure.*)

in Figure 5.1, residues of pharmaceuticals, including antimicrobials and their metabolites or transformation products eventually enter the environment through water reuse during irrigation, and through land application of biosolids. These pharmaceuticals not only contaminate terrestrial and aquatic environment, but can potentially be taken up by plants and animals and enter the human food cycle.

Approximately 10 million tons of sewage sludge and manure containing antimicrobial residues are used to fertilize croplands each year. However, it is difficult to determine the actual risks of antimicrobial contamination in the environment because of the high cost of monitoring programs that are necessary to build a comprehensive database for their concentrations in the aquatic and terrestrial systems. Predictive models have been successfully utilized recently to predict environmental occurrence of antimicrobials based upon regional consumption and excretion, with added methodologies to link occurrence and concentrations with the potential for concurrent antimicrobial resistance (Zhang et al., 2015). Where usage data is unreliable or unavailable, strategies of reverse modeling that take advantage of previous monitoring data have also been shown to be effective in calculating predicted environmental concentrations (PECs) that are used in risk assessment (Boxall et al., 2014). However, validation of environmental fate and transport models continues to be a significant challenge. System boundaries, for example, using consumption of antimicrobials or wastewater effluent concentration for the loading parameter, have a large effect on the reliability of PECs (Schwab et al., 2005). Differences between measured environmental concentrations (MECs) and PECs can be

significant and illustrate the complexity of fate and transport modeling, especially when temporal variations are considered (Celle-Jeanton et al., 2014). An important contributor to the differences between MECs and PECs that should not be ignored is the accuracy of the analytical methods used in measuring antimicrobial concentrations in various environmental matrices, including the different compartments of WWTPs.

Most conventional WWTPs cannot fully remove antimicrobials and other pharmaceuticals; thus, advanced water treatment systems are being explored to determine if there are more efficient alternatives. While most active ingredients of drugs are metabolized in the body or removed during wastewater treatment, others remain intact and persist in the environment. Low levels of antimicrobials can eventually end up in finished drinking water and distribution system (tap) water, when using source waters that have been impacted by effluents from WWTPs. Although the human risk associated with chronic exposure to pharmaceuticals in the environment remains unclear, evidence of detrimental ecological impacts is growing. The environmental contamination by pharmaceutical residues, especially antimicrobials, may have profound environmental effects at several levels. The promotion of antimicrobial resistance in pathogenic microorganisms has been the major concern associated with the presence of antimicrobials in the environment. Other issues such as endocrine disruption in fish and wildlife, plant uptake, and phytotoxicity are also significant and warrant discussion.

The importance of identifying and assessing the toxicity of TPs of antimicrobials in WWTPs and under the receiving environments should not be ignored. Recent studies have demonstrated that TPs of emerging contaminants may sometimes exhibit residual toxicity, or in few cases, may even be more potent than the parent compound itself (Sura et al., 2015). It has been suspected that proximity to point sources for antimicrobial residues can contribute to the occurrence of antimicrobial resistance in hotspots and that this may be due in large part to the presence of more persistent and biologically active TPs of antimicrobials.

In recent years, modern analytical instrumentation has advanced significantly, achieving ultra-trace-level detection limits (sub-pg/kg or parts per quadrillion) for some antimicrobials. Nevertheless, difficulties in separating antimicrobials from complex sample matrices still limit our ability to accurately and reproducibly measure antimicrobials and their TPs in wastewater. Even more challenging to assess are the ecological implications of biologically available (or bioavailable) antimicrobial residues at their PECs in soil and water. This chapter aims to summarize and examine the state of the science for the sample preparation, detection, and quantification of antimicrobials and their TPs in the influents and effluents of WWTPs. The challenges of analyzing biosolids are also discussed to highlight the lack of information on the contribution of biosolids as a source of antimicrobial pollution. Finally, advances and issues in the accurate quantification of antimicrobials and their TPs in complex environmental matrices (e.g., wastewater and biosolids) are also included.

## Sample Collection and Storage

While analysts devote a substantial amount of time in optimizing analytical methods for the trace detection of antimicrobials, attention to the role of sample collection or the reliability of results from chemical analysis has been limited. It is crucial that the

samples being analyzed for antimicrobials be a true representation of the population; therefore, sample collection and preparation is of utmost importance. In general, samples are stored in amber glass vials or high-density polyethylene (HDPE) to prevent photodegradation; furthermore, they are kept at a temperature between +4 and −80°C to prevent degradation and microbial activity. The suitability of containers used to collect and store the samples must be considered such that the antimicrobials, especially those that are highly sorptive like the tetracyclines and aminoglycosides, are not lost due to irreversible sorption (Ciarlone et al., 1990; Lam et al., 2010). Often, the containers need to be pretreated to minimize interactions between the target analytes and the surface of the container. For example, amber glass bottles that are used for storing light-sensitive compounds (e.g., tetracyclines) are washed with nitric acid to reduce reactive sites for adsorption; this process removes organic matter and free metal ions on the glassware that the tetracyclines may adsorb to (Tso and Aga, 2010). However, tetracyclines may still adsorb to glass even after soaking in low concentrations of acid (Tso et al., 2011); in this case, a higher concentration of acid is required and the glassware must be soaked for a longer period of time. Alternatively, the surface of the glassware can be modified by methylating the free silanol groups naturally found in silica; while this step can be time-consuming, it is important to prevent analyte loss (Driessen et al., 1978).

Sample preservation to maintain the integrity of analytes is as important as having an accurate analytical method. Analysis can be expensive and time consuming, and if the samples are not preserved properly, the analytical results could be meaningless. Some antimicrobials, such as sulfonamides, are stable for long periods of time (Ferrer and Thurman, 2012), but others will adsorb, oxidize, or degrade rapidly. For instance, studies have shown that tetracyclines should be analyzed within 48 hours of extraction due to their irreversible binding to silanols (Oka et al., 2000; Zhai et al., 2016). Many antimicrobials are oxidized during treatment in WWTPs, but they can also oxidize over time if not stored properly (Peng et al., 2015); hence, if the goal of the study is to assess "removal" efficiencies of WWTPs, it is critical to differentiate whether the oxidation of antimicrobials occurred in the treatment terrain or in the storage container. The pH of the samples must also be considered during storage; while samples from WWTP effluents and surface waters will be around pH 5–8, the pH of aqueous samples is typically lowered to pH 2–3 during sample extraction to increase analyte recoveries. However, some analytes are susceptible to hydrolysis in acidic conditions and therefore the stability of target antimicrobials must be evaluated at the pH used for extraction.

Table 5.1 shows reported stability of antimicrobial classes, both before and after extraction, in the most common storage containers. Notably, fluoroquinolones and tetracyclines are the most unstable classes during storage as aqueous samples, and they degrade even more rapidly in vials after extraction. It is hypothesized that fluoroquinolones and tetracyclines adsorb strongly to natural organic matter (NOM), which tends to stabilize these antimicrobials in water. During extraction most of the NOM is removed, resulting in degradation of these antimicrobial classes (Aristilde and Sposito, 2008; Hellweger, 2013). Macrolides and sulfonamides are shown to be relatively stable; however, since many methods analyze for multiple classes of antimicrobials simultaneously, the storage of samples should be defined based on the stability of the most unstable class of antimicrobials. An analyte is typically considered stable if the concentration remains within 60%–120% of the original concentration at the initial time of storage

**Table 5.1** Stability of different antimicrobial classes in wastewater sludge.

| Antimicrobial Class | Container Type | Stability in Pre-extracted Samples | Stability in Postextracted Samples | Reference |
|---|---|---|---|---|
| Fluoroquinolones | HDPE | <7 days (−18°C) | >72 hours (−80°C) | Fedorova et al. (2014) |
| | Amber glass | <7 days (+4°C) | >48 hours (−40°C) | Göbel et al. (2005) |
| Macrolides | HDPE | >60 days (−18°C) | >14 days (−40°C) | Fedorova et al. (2014) |
| | Amber glass | >30 days (−20°C) | >14 days (−25°C) | Lillenberg et al. (2009) |
| Sulfonamides | HDPE | >120 days (−18°C) | >30 days (−40°C) | Fedorova et al. (2014) |
| | Amber glass | >150 days (−40°C) | >30 days (−20°C) | Huang et al. (2013) |
| Tetracyclines | HDPE | <7 days (−18°C) | >24 hours (4°C) | Fedorova et al. (2014) |
| | Amber glass | <7 days (−40°C) | >48 hours (−80°C) | Li and Zhang (2010) |

(Fedorova et al., 2014). However, the source of signal depletion is not normally defined; it could be due to loss from adsorption, biodegradation, or abiotic transformation of the compound.

## Matrix Effects and Detection Limits

Another important consideration required when analyzing antimicrobials is defining analytical method limits of quantification (MLOQ). The concentrations of most antimicrobials in wastewater influent will be different from those in the effluent. Because the dissolved organic carbon (DOC) content is higher in the influent than the effluent, the problems of matrix effects, interferences, false positives, or false negative detection are more significant in the influent samples. Therefore, designing optimum sample clean up and preconcentration steps for each type of matrix is critical in developing methods for antimicrobial analysis in wastewater.

Table 5.2 shows typical MLOQ in wastewater analyzed by liquid chromatography tandem mass spectrometry (LC/MS). In general, MLOQ are lower in samples that have less complex matrices. For example, in Table 5.2 the lowest MLOQ for several of the antimicrobials were observed in groundwater samples, which is expected because they have the least DOC present. In comparison, wastewater sludge has the highest MLOQ because of its extremely complex nature and high amounts of NOM. Tetracyclines have the highest MLOQ in sludge because tetracyclines tend to adsorb strongly to NOM. To increase extraction efficiency and improve MLOQ, tetracyclines must be desorbed from the sludge; however, overcoming the strong interactions with the various components of the sludge without degrading the tetracyclines is extremely challenging. In fact, a study conducted by O'Connor et al. (2007) on the effect of temperature on the extraction efficiencies of tetracyclines from soil reported that percentage recoveries generally decreased as the temperatures exceeded 40°C. The difficulty of extracting tetracyclines from sludge or biosolids results in the high detection limits of these compounds and is the reason why there are fewer reports of detections of tetracyclines in these matrices relative to their

**Table 5.2** Typical method quantification limits of antimicrobials in wastewater by liquid chromatography with tandem mass spectrometry (LC-MS).

| Antimicrobial Class | Groundwater (ug/L) | WWTP Effluent (ug/L) | WWTP Influent (ug/L) | WWTP Sludge (ng/g) | Reference |
|---|---|---|---|---|---|
| Fluoroquinolones | 0.01 | 0.05 | 0.1 | 1.8 | Lillenberg et al. (2009) |
| Macrolides | 0.02 | 0.05 | 0.07 | 1.3 | Karthikeyan and Meyer (2006) |
| Sulfonamides | 0.01–0.05 | 0.05–0.1 | 0.1 | 0.3 | Radjenovic et al. (2007) |
| Tetracyclines | 0.02–0.05 | 0.05–0.1 | 0.2 | 160 | Li et al. (2013) |

detections in wastewater (O'Connor et al., 2007). Knowing the loading of tetracyclines is important for understanding the impact this has on the proliferation of antimicrobial resistance, though a recent study has shown that tetracycline complexing with NOM or DOC in aqueous solution may change the bioavailability of tetracyclines to bacteria, diminishing the potential for development of antimicrobial resistance (Chen et al., 2015).

## Sample Enrichment and Preparation

Sample extraction requires a fundamental knowledge of the physical and chemical properties of the target analytes in order to design a selective enrichment procedure for the target analytes while efficiently removing matrix interferences. Unfortunately, different classes of antimicrobials have different solubility in organic solvents. The analytes may differ drastically in their chemical stability at different pH conditions, making the decision about which pH to use for extraction very challenging, as well as making it nearly impossible to obtain close to 100% recovery for all analytes. Therefore, when preparing samples for instrumental analysis, several factors must be considered to minimize analyte loss while reducing matrix effects during sample extraction and preconcentration. Unfortunately, an extraction method that is suitable for one class of antimicrobials may not necessarily be optimal for another class. However, as long as the extraction recoveries are reproducible, one common extraction method for all analytes of interest may be acceptable. In other words, even if the recovery is low, as long as the concentrations obtained at the end of the analysis can be related back to the original sample in a reproducible way, and the detection limit is sufficient to allow detection of analytes at trace levels, the extraction method can be acceptable.

Solid phase extraction (SPE) has become the most commonly used method for simultaneous sample extraction, cleanup, and preconcentration of analytes. It is a stepwise chromatography technique designed to partition and/or adsorb one or more components from a liquid phase (sample) onto a stationary phase (sorbent or resin). Separating the target analytes from the other components of the sample matrix using SPE can be achieved in two ways: (i) by adsorbing the analytes on the SPE resin and allowing the unretained components to pass through the SPE cartridge or (ii) by adsorbing the unwanted matrix components on the SPE resin and collecting the unretained target analytes (Figure 5.2). Therefore, the first important step in developing an SPE

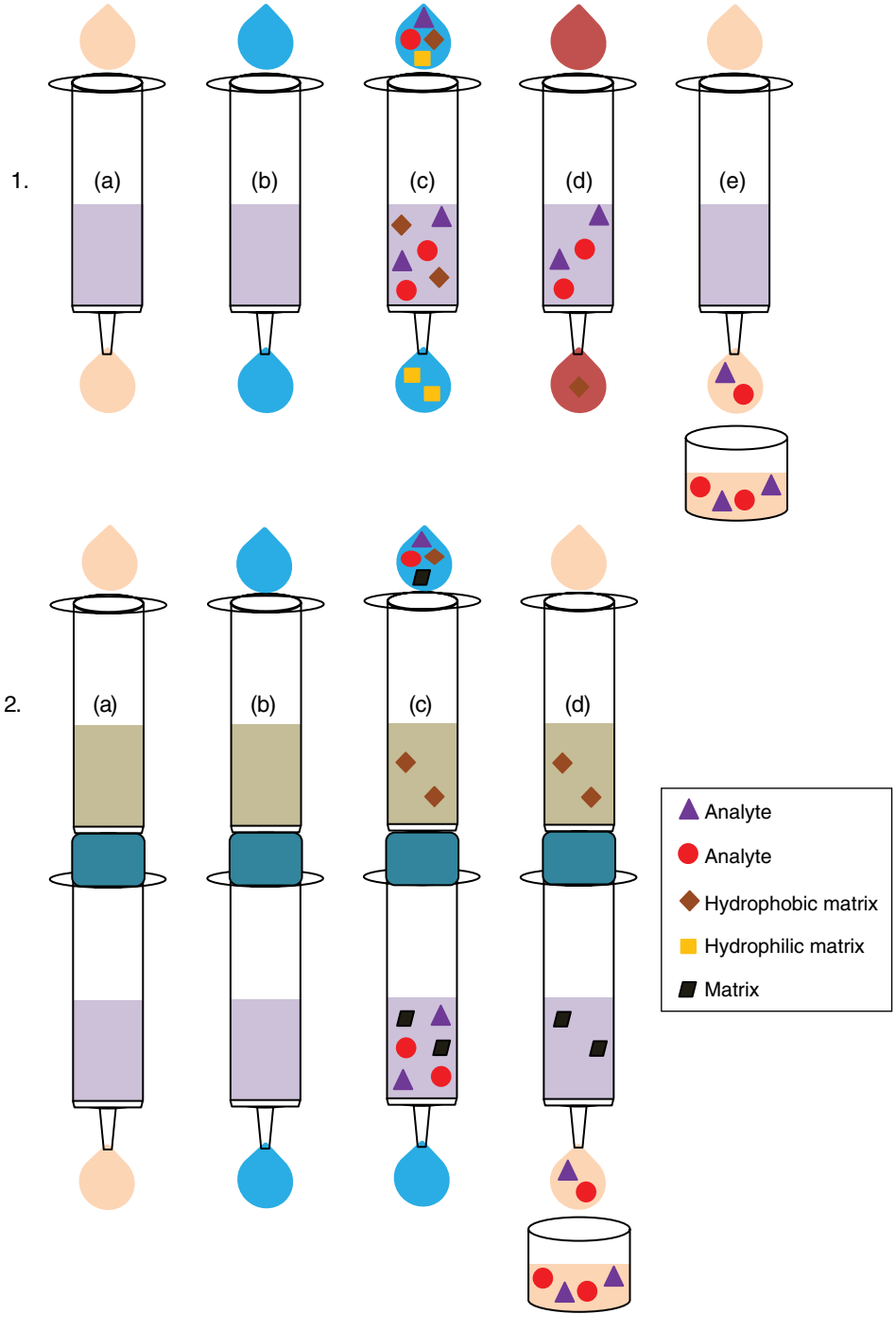


**Figure 5.2** Two different solid phase extraction strategies for the analysis of antimicrobials in aqueous samples. (*See insert for color representation of the figure.*)

method is the selection of the appropriate stationary phase that can discriminate interactions between the analytes and the interferences in the sample matrix.

The use of SPE in the analysis of wastewater influent and effluent has become common due to the ability of SPE to simultaneously isolate and concentrate the analytes without the use of large amounts of organic solvents. Over the years, the availability of SPE stationary phases with various surface chemistries has provided a wider selection of packing materials that can be optimized to simultaneously extract different classes of analytes with varying polarities. Commercially available packing materials for SPE cartridges include hydrophobic sorbents (e.g., C8, C18), hydrophilic sorbents (e.g., cyano, diol), cation and anion exchange resins, and mixed-mode sorbents (containing both ion-exchange and hydrophobic sites). A very popular reversed-phase sorbent for SPE that consists of a mixture of the hydrophilic N-vinylpyrrolidone and the lipophilic divinylbenzene, with the commercial name of Oasis™ HLB (for Hydrophilic-Lipophilic Balance), has been widely used for antimicrobial analysis in water samples because of its ability to extract acidic, neutral, and basic compounds simultaneously.

At the top of Figure 5.2 (Part 1), target analytes are adsorbed onto the SPE stationary phase and unwanted matrix components are either unretained or eluted separately. The steps involve (a) conditioning with an organic solvent (typically the same solvent used to elute the analytes), (b) washing the cartridge with distilled water to prepare for aqueous samples, (c) loading the sample, (d) washing with aqueous solution containing <5% methanol, and (e) elution of the target analytes using appropriate organic solvent.

At the bottom of Figure 5.2 (Part 2), SPE is done in tandem with two different cartridges, where the first is used to collect the matrix and the second is used to collect the analyte. The hydrophobic matrix components are the ones that are adsorbed onto the stationary phase of the first cartridge, while the unretained target analytes pass through. The procedure involves: (a) conditioning with an organic solvent, (b) washing the cartridge with distilled water to prepare for aqueous samples, (c) loading the sample, (d) elution of the target analytes using an appropriate organic solvent.

The former SPE configuration is more commonly performed because it is less complex, labor intensive, or expensive. As shown in Figure 5.2, after loading, the cartridges retain both the analyte and matrix. A suitable solvent is chosen to desorb and elute the target analytes by overcoming the affinity of the analytes with the SPE stationary phase. In order to minimize elution of unwanted species, researchers can also wash the cartridge postloading with a solvent that will elute the matrix but not the analytes. There are several parameters that should be considered when using this technique: the stationary phase and the analyte should have a strong affinity for each other in order to increase retention; however, the sorption of analytes on the stationary phase needs to be reversible. For example, tetracycline binds irreversibly to silanols; many SPE cartridges are packed with a silica-based stationary phase, and depending on the pH of the sample, the stationary phase will have high abundance of silanols that would strongly adsorb tetracyclines. Therefore, knowledge about the acid-base properties of the analytes and their interactions with the SPE stationary phase is crucial in designing SPE methods.

Part 2 of Figure 5.2 illustrates SPE being utilized in tandem, where the first cartridge retains the hydrophobic matrix, while the analytes pass through unretained, and the second cartridge retains the analytes and other matrix components. This is advantageous because when analyzing a wide range of antimicrobials, a cartridge with a high

capacity for multiple compound classes is necessary, but such usage can result in unwanted retention of the matrix. A common example of this is unwanted retention of NOM, consisting of humic and fulvic acids with amine and carboxylic functional groups (Scheel et al., 2008). For instance, an amino ($NH_2$) cartridge can be used in tandem with an HLB cartridge, where the amino cartridge is used to catch the matrix (NOM), whereas the HLB serves to retain the analytes at the concentration step (Wallace and Aga, 2016).

When it comes to analysis of antimicrobials in solids, a simple cleanup method that has been adapted is the so-called "QuEChERS," which stands for "quick, easy, cheap, effective, rugged, and safe," sample preparation procedure (Lehotay et al., 2003). This technique is based on the "salting-out effect" to decrease the solubility of antimicrobials in water while increasing their partitioning into an organic solvent that is added into the sample mixture. The QuEChERS method is performed by first immersing the solid sample in water, and then adding an organic solvent, followed by saturating the water layer with salt. The water and organic solvent become immiscible with each other, and the antimicrobials preferentially partition into the organic layer. This technique typically uses $MgSO_4$ as the salt; however, because tetracyclines chelate to divalent metals, researchers have switched to using sodium salts if tetracyclines are of interest for analysis (White and Cantor, 1971). QuEChERS has been used for sewage sludge samples as a way to remove unwanted matrix, mainly DOC. This technique assists the removal of the DOC prior to SPE (Bourdat-Deschamps et al., 2014).

One of the most popular extraction techniques used to enhance the effectiveness of SPE in preparing wastewater sludge for analysis is pressurized liquid extraction, also known as accelerated solvent extraction. This extraction technique utilizes elevated temperatures and pressures. It has been used successfully for the extraction of antimicrobials from wastewater sludge (Nieto et al., 2010). The use of accelerated solvent extraction has been demonstrated to improve extraction efficiencies for many antimicrobials, including tetracyclines (Ding et al., 2011; Pamreddy et al., 2013) and fluoroquinolones (Golet et al., 2002; Ding et al., 2011).

Ultrasonication-assisted extraction has also been used to enhance the extraction of antimicrobials from wastewater sludge (Pan et al., 2011; Le-Minh et al., 2012). This technique is a popular, straightforward, and cost-effective option because sonicators are widely available in many laboratories. Both accelerated solvent extraction and ultrasonication-assisted extraction have been applied to improve extraction efficiencies for a plethora of antimicrobials; however, the extraction techniques inherently contribute to extraction of NOM or DOC, which can cause significant matrix suppression. Therefore, extracts from solids are typically subjected to an SPE cleanup procedure that will also concentrate the analytes, as discussed above.

## Sample Analysis by Liquid Chromatography/Mass Spectrometry

Two of the most commonly employed techniques for trace analysis of organic contaminants in the environment are gas chromatography and liquid chromatography (LC). However, most antimicrobials are highly water soluble, have very low vapor pressure, and are thermally labile; hence, they are not amenable for gas chromatography analysis.

Due to this limitation, LC has become the method of choice for the trace analysis of antimicrobials in environmental samples such as wastewater. The following section will focus on strategies and considerations when performing analysis of antimicrobials based on LC separations followed by MS detection. One of the main advantages of performing LC separations over doing direct injection during targeted analysis is that chromatography provides an extra layer of selectivity because of retention time information. In addition, increased signal-to-noise ratios are obtained because the instrument can focus on a lower number of analytes per unit of time, and the analyte signal is separated from a significant amount of background signal. A few examples of application of LC separations for antimicrobial analysis in wastewater are shown in Table 5.3.

Mass spectrometry (MS) has become an indispensable tool in answering the most challenging research questions in science and engineering. Different state-of-the-science MS designs are available, and the correct choice of instrument will depend mainly on the research questions being answered and the availability of the instrument in the lab. The less expensive single quadrupole MS and ion-trap MS instruments provide unit-resolution mass data and generally suffer from low signal-to-noise ratios, making it challenging to measure low concentrations of antimicrobials in wastewater. The use of single quadrupole mass analyzers also limits the ability of the analyst to differentiate between two co-eluting isobaric compounds, which are molecules that have the same nominal molecular ions. Isobaric compounds that are not chromatographically resolved may cause either an overestimation or false positive detection of an analyte in environmental samples because one chromatographic peak may be attributed to a single species, when in reality there may be multiple compounds under this one peak. As an example, sulfamethizole (retention time: 12.7 minutes) has been shown to almost co-elute with 10,11-dihydroxycarbamazepine (retention time: 12.9 minutes) (Ferrer and Thurman, 2012). Both compounds have the same molecular ion, *m/z* 271; hence, one may not be able to attribute the molecular ion to sulfamethizole or to 10,11-dihydroxycarbamazepine based on a peak that elutes at ~12.7 to 12.9 minutes. To alleviate this problem, more selective MS detectors can be used. Examples include triple quadrupole MS (QqQ) or high-resolution MS (HRMS) detectors capable of accurate mass measurements, such as the time-of-flight MS (ToF MS), Orbitrap™ MS, or a Fourier transform ion cyclotron resonance (FTICR) MS. The QqQ and HRMS have different ways of differentiating between co-eluting isobaric compounds. In a QqQ MS operated under multiple reaction monitoring (MRM), the precursor ion (e.g., molecular ion) is isolated in the first quadrupole mass filter (Q1), which then undergoes fragmentation in the collision cell (q), and finally the fragmented ions travel onto the second quadrupole mass filter (Q3) where *m/z* selection for specific fragment ions occurs again. A schematic of how MRM takes place inside a QqQ is shown in Figure 5.3. In HRMS, two co-eluting compounds with the same nominal mass of *m/z* 271 can be distinguished from each other because of the higher mass resolving power and ability to provide accurate mass measurements by the instrument; for example, it can distinguish sulfamethizole (*m/z*: 271.0318) and 10,11-dihydroxycarbamazepine (*m/z*: 271.1077). Mass spectrometers with low mass resolving power like the QqQ would not be able to discriminate between these two compounds when analyzed in full scan mode.

Ion-trap mass analyzers are capable of producing mass fragmentation patterns that provide additional selectivity in the analytical method (Batt and Aga, 2005; Gros et al., 2013). However, ion trap mass analyzers generally have lower sensitivity, and this leads

**Table 5.3** Summary of separation and cleanup methods found in literature pertaining to analysis of different antimicrobial residues in wastewater and related matrices.

| Analyte Class | Cleanup/ Enrichment Step | Column Used | Mobile Phase | Reference |
|---|---|---|---|---|
| Antiviral and antiviral metabolites Oseltamivir | 500 mg Oasis HLB | Waters Acquity BEH C18 (2.1 × 50 mm, 1.7 um) | Methanol, 0.1% formic acid | Goncalves et al. (2011) |
| Tetracyclines and macrolides | | Narrow bore BetaBasic C18 RP column (2 × 250 mm) | Acetonitrile: MeOH: 2% formic acid in $H_2O$ (25:12:63) | Snow et al. (2003) |
| Sulfonamides, fluoroquinolones, tetracyclines, macrolides, trimethoprim, and lincosamide | 500 mg Oasis HLB and 1 g tC18 Sep-Pak | BetaBasic C18 (2.1 × 100 mm, 3 um) | Acetonitrile, methanol, 0.3% formic acid | Batt and Aga (2005) |
| Sulfonamides and tetracyclines | Oasis HLB | Betabasic C18 (2.1 × 100 mm, 3 um) | $H_2O$/methanol (96/4 v/v) plus 5 mM $NH_4OH$ (A) and $H_2O$/methanol/acetonitrile (10/10/80 v/v/v) 5 mM $NH_4OH$ (B) | Tso et al. (2011) |
| Sulfonamides | 200 mg Oasis HLB | Xterra MS C18 (2.1 × 50 mm, 2.5 um) | 0.1% Formic Acid in $H_2O$, acetonitrile | Shimizu et al. (2013) |
| Fluoroquinolones | | Review article for fluoroquinolone analysis | | Sturini et al. (2009) |
| Sulfonamides and macrolides | | Betasil C18 (2.1 × 150 mm, 3 um) | 5 mM formic acid / 5 mM ammonium formate (A) ACN (B) | Benotti et al. (2003) |
| Cyromazine, tetracyclines, and sulfonamides | 6 mL, 500 mg Oasis HLB | Agilent C8 (2.1 × 150 mm, 5 um) | 0.1% formic acid in water, 0.1% formic acid in acetonitrile | Wei et al. (2011) |
| Ionophore antimicrobials | | Ascentis RP-amide (2.1 × 150 mm, 3 um) | | Sun et al. (2013) |

(*Continued*)

**Table 5.3** (Continued)

| Analyte Class | Cleanup/ Enrichment Step | Column Used | Mobile Phase | Reference |
|---|---|---|---|---|
| Fluoroquinolones | 3 mL, 500 mg, LC-18 cartridge | Eclipse XDB C18 (4.6 mm × 150 mm, 5 um) | Methanol/phosphoric acid (0.1%v/v) – trimethylamine buffer pH 3.0 (20/80) | Tong et al. (2011) |
| Tetracyclines, sulfonamides, quinolones, and macrolide antimicrobials | Oasis HLB | Intersil ODS-3 column (2.1 × 250 mm, 5 um) | Acetonitrile (A) and 0.3% formic acid in water | Luo et al. (2011) |
| Fluoroquinolones, tetracyclines, and sulfonamides | Oasis MAX | Waters Acquity UPLC BEH C18 Column (2.1 × 100 mm, 1.7 um) | Methanol (A) and ultrapure water with 0.1% formic acid | Zhang et al. (2014) |
| Tetracyclines, sulfonamides, quinolones, phenicols, and macrolides | Oasis HLB | Agilent Eclipse XDB C18 (3 mm × 75 mm, 3.5 um) | 0.01% formic acid (A) and acetonitrile (B) | Pan et al. (2014) |

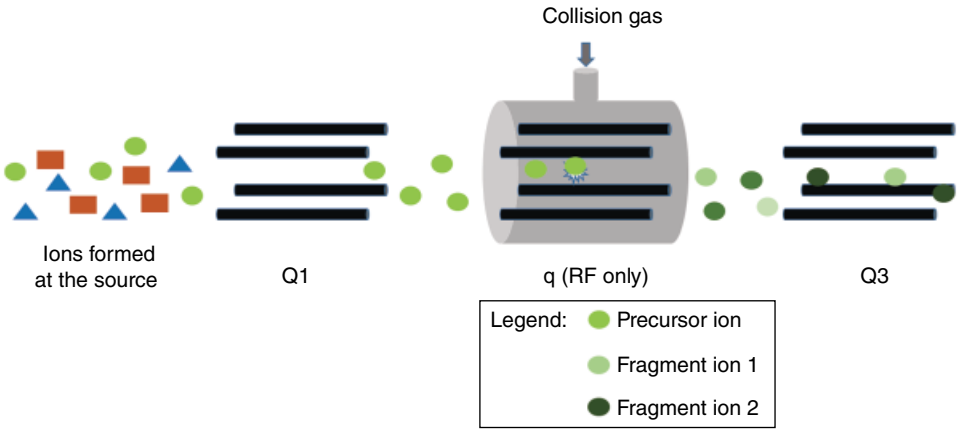


**Figure 5.3** Schematic diagram of how ions are fragmented inside a QqQ. After ions are formed at the source, they are directed to Q1, where the quadrupole filters the masses and sends the precursor ion of interest to q, where collision-induced fragmentation is accomplished by bombarding the precursor ion with a specific collision gas. The fragment ions are sent to Q3 for mass filtering then subsequently sent to the detector. (*See insert for color representation of the figure.*)

to higher limits of detection, compared to QqQ mass analyzers. In two separate works that analyzed for sulfonamides in water, a 100-fold improvement in method limit of detection was observed when analyzed using a triple quadrupole mass spectrometer compared to an ion trap mass spectrometer, even though both works used the same sample amounts (Batt and Aga, 2005; Tso et al., 2011). Triple quadrupole MS is the gold standard in the quantification of antimicrobials in environmental samples because it provides enhanced selectivity, better accuracy, and greater reproducibility, all of which are limited in single quadrupole MS detectors. The software platforms that accompany commercial LC/MS instruments allows the analyst to set defined time segments to increase the number of scans the MS would have to do per analyte in a specified time period. Increased scans allow more data points to be obtained, thus improving the reliability of the collected data set.

Analysis by LC/MS can suffer from ionization suppression due to matrix effects. This has been demonstrated by the observed lower signal intensities of different analytes, including three fluoroquinolones, in sewage water when compared to surface and ground water (Vieno et al., 2006). Another challenge in antimicrobial analysis in wastewater is the potential for false positive detection. Figure 5.4 shows how false positive peaks are observed for sulfamethazine in wastewater even when analyzed using MRM mode. The presence of false positives exemplifies the importance of monitoring for at least two fragment ions per analyte. Simultaneously monitoring for a second mass transition provides an extra layer of selectivity, a feature that is hard to accomplish with other types of MS detectors (though one may argue that high-resolution mass spectrometers in full scan mode have their accurate mass data). In addition to meeting the requirement that both MRM transitions are observed, it is also expected that for a particular analyte, the ratio of the abundance of the quantifying and qualifying ions in the sample matrix be comparable with the ratio of the same ions in a standard solution. Inconsistent fragment ion ratios for analytes when in the sample matrix and when in standards should raise some concerns, and results should be scrutinized thoroughly.

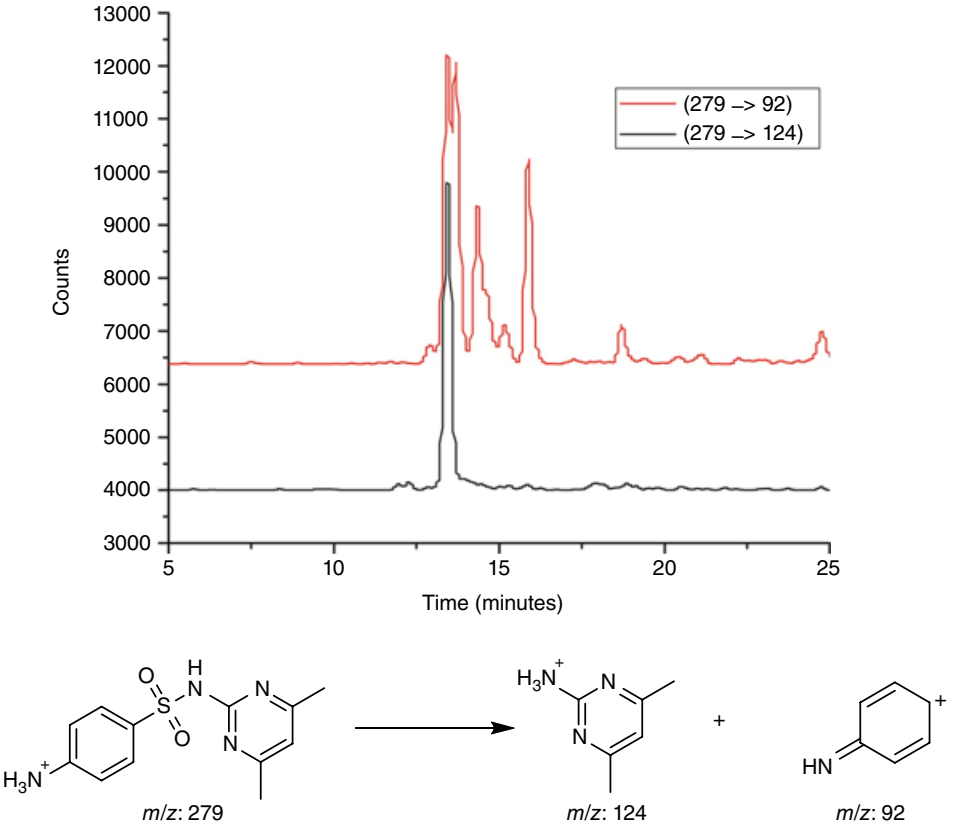


**Figure 5.4** By monitoring two fragments per antimicrobial compound, sulfamethazine in this example, one is able to discriminate between the real peak (overlapping red (top) and black (bottom) trace) and interfering peaks in wastewater matrix. The structure of sulfamethazine and its fragment ions are also shown. (*See insert for color representation of the figure.*)

## Identification of Nontarget Compounds

Three main approaches have been used for the HRMS analysis of emerging contaminants in environmental samples: (i) target analysis, (ii) suspect screening, and (iii) nontarget screening. Targeted compounds are analytes that the experimenter knows or is specifically looking for. Target analysis is performed when reference standards are available to facilitate the identification and quantification of the particular analytes. In contrast, for nontarget screening, there is no previous information about the compounds to be detected. These untargeted compounds pertain to analytes in the sample that may be of significance but on which the experimenter does not apply a bias. Nontarget screening relies on advanced acquisition and processing data software for deconvolution of the large amount of information collected during HRMS analysis. Suspect screening is used when the molecular formula and structure of the suspected molecules can be predicted. *In silico* tools, registration dossiers, and scientific literature are used to assemble the target list.

The HRMS instruments provide accurate mass data that are valuable in the putative identification of targeted and untargeted compounds in water. With accurate mass, the experimenter is able to determine the putative molecular formula of an observed molecule. Using the derived elemental formula, together with the characteristic mass fragmentation pattern, one can facilitate positive identification of an unknown compound without the use of reference standards. With this feature, HRMS instruments have become excellent tools in screening for the presence of emerging pollutants in environmental samples, especially for transformation products (TPs) of emerging contaminants that are formed during wastewater treatment.

Among the various types of HRMS that are used for the identification of nontarget compounds, the FTICR provides the widest mass range, up to 30,000 *m/z*, compared with the Orbitrap's 50 to 6,000 *m/z* and the ToF's 50 to 3000 *m/z* (Ed and Stroobant, 2007). However, the Orbitrap is capable of acquiring more spectra per unit time compared with the FTICR. With a scan rate of up to 12 Hz at resolution setting of 17,500 @ *m/z* 200 achievable by Orbitrap, it is possible to interface this mass analyzer with either a nanoflow or a normal flow LC. Although the FTICR can be coupled with an LC, its advantages are better suited for analysis of compounds by direct infusion to allow longer data acquisition times. By giving the FTICR cell longer analysis time, the effective flight path of the ion is increased, resulting in improved mass data. For an analysis that requires separation, the longer scan time prevents the FTICR from being effectively coupled with LC, or the MS does not have enough time to give an accurate readout between consecutively eluting peaks. While accurate mass data are valuable, a balance between analysis time, sensitivity, and mass accuracy is critical in developing methods for the trace analysis of multiple compounds. The FTICR is not suited for routine quantitative analysis but could be useful in determining the molecular formula of an unknown organic compound as long as the concentration is high enough for detection. For unknown identification of contaminants in environmental samples, ToF and Orbitrap MS have found wider application because of their inherent sensitivity over FTICR. When connected to LC, the ToF and Orbitrap MS detectors allow the recording of full-scan chromatograms with high mass accuracy and resolution that make it possible to selectively search for nontarget compounds or putative TPs and metabolites based on their exact mass.

The Orbitrap has several unique features that offer the high sensitivity and selectivity needed for antimicrobial analysis work. Recent developments include an advanced quadrupole technology that improves precursor ion selection and transmission for more accurate quantitation of low-abundance analytes in complex matrices, like environmental samples. When interfaced with an LC, the Orbitrap's efficiency and sensitivity is improved by the separation of analytes in time. Oftentimes, the accuracy provided by the QToF is enough for most environmental analyses (Ferrer and Thurman, 2012); however, its sensitivity and accuracy is limited to a few orders of magnitude and brings about a limitation that the Orbitrap can overcome with a mass resolving power of up to 100,000 at *m/z* 400 compared to 25,000 for the ToF. A recent review of literature reported that there has been an increase in the utility of the Orbitrap and ToF MS for environmental research, including the analysis of pharmaceuticals and other emerging contaminants in the environment (Richardson and Kimura, 2016). This trend is expected to increase due to the wide array of antimicrobials used in different aspects of society and the increased efforts in understanding the spread and emergence of antimicrobial resistance in nonclinical environments.

Analysis of antimicrobials in wastewater has been primarily performed by targeted approach using QqQ; however, the presence of many other antimicrobials that have not been previously reported in wastewater may be detected by nontarget analysis using HRMS. Once antimicrobials have been identified in wastewater effluents, routine monitoring at trace levels can be performed based on target analysis. When juxtaposed with the QqQ, several studies have shown that the Orbitrap can rival the QqQ in terms of sensitivity (Hamelin et al., 2013; Vanhaecke et al., 2013; Herrero et al., 2014). However, it is the authors' opinion that when comparing instruments of the same age, triple quadrupole MS still achieves lower instrument limits of detection since the sensitivity of the Orbitrap is dependent on the mass resolving power being used, although it sacrifices some of its sensitivity to achieve better selectivity when used at a higher mass resolving power.

## Identification of Transformation Products

The HRMS data are highly beneficial in the discovery of TPs and metabolites during wastewater treatment (Hogenboom et al., 2009; Terzic et al., 2011; Singh et al., 2015). For example, the mass defect may be used in identifying TPs or metabolites that are halogenated in addition to their mass fragmentation patterns. Using ToF MS, the TPs of the X-ray contrast agent iopromide formed during UV/$H_2O_2$ advanced oxidation in wastewater were identified (Singh et al., 2015). Since wastewater analysis using HRMS was done under full scan mode, resulting in an overwhelming amount of data, profiling software that assisted in identifying MS features that significantly changed due to an applied process (e.g., UV/$H_2O_2$ advanced oxidation) proved extremely valuable in processing the data. Using accurate mass measurements provided by a ToF MS (less than 5 ppm error), and the mass defect brought about by the presence of halogens in an organic molecule, TPs were identified with the aid of profiling tools (Singh et al., 2015). The identities of the TPs were verified using MS/MS and the loss of a halogen fragment. In a similar approach, the TPs of the anticancer drug erlotinib during chlorination were identified (Negreira et al., 2015). In both studies, profiling tools were imperative due to the complexity of the matrix; although the examples mentioned are not antimicrobials, the same approach can be used to understand the fate of antimicrobials during wastewater treatment.

One may argue that Nanopure$^{TM}$ water may be spiked with the analyte of interest, then allowed to undergo controlled treatment (to simulate the chemistry that happens during the actual process), followed by identification of TPs that have been formed, but this process fails to account for the analyte transformation effects related to other molecules present in the matrix. With profiling tools, this challenge may be addressed, and thus simulations with actual wastewater can be done to have a better idea of how antimicrobials can transform during treatment. Care must be taken when doing profiling work, and the experimenter is advised to always consider the chemistry of the treatment process. One disadvantage in profiling is that a bias is applied to TPs of higher abundance, and so TPs that are poorly ionized in MS or have low abundance could be missed.

Fragmentation studies are of paramount importance in enabling the structural elucidation of unknown compounds for identification purposes of environmental

pollutants, drug metabolites, and their unique TPs during wastewater treatment processes. In most cases, the key to understanding how TPs will fragment is knowledge of possible fragmentation patterns of the parent molecule. Since treatment is an oxidative process, one must keep in mind the mechanism of oxidation and try to draw probable structures. Since the TP is structurally similar to the parent molecule, understanding the fragments would be easier as they may be oxidized versions of the fragments of the parent molecule.

## Conclusion and Future Directions

The need to understand the fate of antimicrobials and their corresponding metabolites and TPs in WWTPs and the receiving environments cannot be overstressed. With the rapidly advancing technology available in the field of analytical instrumentation, the main challenge in the analysis of antimicrobials in complex environmental samples is minimizing matrix effects to achieve low detection limits and highly reliable results. While it may not be practical to completely eliminate the matrix, there are numerous ways of addressing the sensitivity and reproducibility of the analytical methods using appropriate cleanup steps and by quantification based on isotope dilution MS. With careful planning, proper sample cleanup, use of the appropriate instrumentation based on the purpose of the study, and a more complete understanding of wastewater chemistry, the experimenter should be able to develop a method that will properly answer the research questions at hand. It is expected that with the advent of more sensitive instrumentation, and as more sophisticated *in silico* tools become commonplace, research toward the identification of unknown TPs of antimicrobials in wastewater will advance. Prioritization of which antimicrobials need to be regulated in the environment will become important, and advancements in water treatment systems that prove effective in eliminating these compounds will need to be demonstrated at full scale to prevent further contamination of the environment. The challenges in achieving accurate risk assessment of the impacts of antimicrobials and their TPs from wastewater remain formidable due to various factors, ranging from the accuracy and variability in analytical techniques, to measuring their bioavailability and potency and ability to promote resistance in bacteria. Finally, while advances in analytical instrumentations have facilitated the detection of trace levels of antimicrobials in wastewater, care must be taken in validating methods for specific matrices and applications because the severity of matrix effects can vary substantially between the types of samples (e.g., biosolids, influent and effluent wastewater).

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# 6

# Metagenomic Approaches for Antibiotic Resistance Gene Detection in Wastewater Treatment Plants

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## Concerns about Antibiotic Resistance Genes in Wastewater Treatment Plants

Antibiotic resistance has raised serious concerns in recent years because of the increasing reports about superbugs and the inefficiency of antibiotics in treating life-threatening infections (Amoako et al., 2016; Pruden et al., 2013). The development of antibiotic resistance in bacteria around the world became a matter of special concern that required coordinated actions for decision makers to address (Roca et al., 2015). The science ministers from G8 countries have pledged to a global act for the control of bacterial antibiotic resistance. Since it is assumed that gene carriage can represent resistance potential, one of the methods used to investigate the existence of antibiotic resistance is detecting antibiotic resistance genes (ARGs) in the microbiome samples. Increasing attention is being paid toward the ARGs in the realm of the natural environment in addition to clinical settings. The reason is that the spread of ARGs in the environment might result in the acquisition of antibiotic resistance by the indigenous susceptible bacteria from resistant bacteria through horizontal gene transfer (HGT) of ARGs (Marti and Balcázar, 2013), which largely, although not exclusively, would increase the potential threat for the development of antibiotic resistance in human or animal pathogens.

The emergence of antibiotic resistance "is a complex problem driven by many interconnected factors; single, isolated interventions have little impact," as declared by the World Health Organization (2012). ARGs in the environment may proliferate by the contribution of the constant or even increasing release of various environmental pollutions, such as antibiotics, heavy metals, biocides, disinfectants, or detergents (Roca et al., 2015). The dissemination of ARGs may also be accelerated by increasing global trade and travel. Therefore, improving knowledge on the baseline and background data for antibiotic resistance in the environment is necessary to gain better

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understanding of the environmental behavior of ARGs as well as evaluate the potential risk of ARGs on human health.

To begin with, the profiles of ARGs in pollution sources should be clarified. Wastewater treatment plants (WWTPs) are regarded as significant reservoirs and sources of ARGs in the environment (LaPara et al., 2011; Pruden et al., 2013). They receive wastewater discharged from households and hospitals where antibiotics are commonly used and the level of antibiotic resistant bacteria and ARGs might be elevated. Many pathogens carrying ARGs have been found in WWTP environments, such as antibiotic resistant *Aeromonas* spp. (Figueira et al., 2011), *Acinetobacter* spp. (Kümmerer, 2004), *Enterobacteriaceae* (Zhang et al., 2009a), and *Pseudomonads* (Bouki et al., 2013). Recently, the notorious superbug, methicillin resistant *Staphylococcus aureus*, was found in WWTP effluent in the United States (Goldstein et al., 2012), and *Achromobacter* spp. carrying NDM-1 were identified in effluent from WWTPs located in northern China (Luo et al., 2014). The antibiotic residues or other pollutants in wastewater might create selective pressure for the development of antibiotic resistance. In combination with the dense and diverse microbial communities, these factors might facilitate the proliferation and transfer of ARGs among different microbial communities through HGT. It was reported that HGT is mediated by mobile genetic elements (MGEs), such as plasmids, transposons, integrons, bacteriophages, or combinations of them (Philippe and Douady, 2003; Rizzo et al., 2013). Moreover, WWTPs have considerable impact on the distribution and abundance of ARGs in the environment (Storteboom et al., 2010). Evidence has been presented that effluent discharged from WWTPs was one of the major anthropogenic sources of ARGs in water environments (LaPara et al., 2011; Pruden et al., 2012). ARGs in sludge from WWTPs may also spread into soil and groundwater through the landfill disposal of the treated or untreated sludge (Threedeach et al., 2012). Based on the above reasons, WWTPs are important nodes in the dissemination of ARGs in the environment (Munir et al., 2011). However, the profiles of ARGs in WWTPs remained unclear and the mechanisms by which biological processes used in wastewater treatment influence the dissemination and selection of antibiotic resistant bacteria and ARGs are still poorly understood. Because of the significant role of WWTPs in the dissemination of ARGs in the natural environment, it is necessary to investigate the comprehensive profiles of ARGs in different WWTPs and different wastewater/sludge treatment processes.

## Metagenomic Approaches for ARG Detection

Traditional microorganism studies are based on culture-dependent methods. However, it has been estimated that over 99% of the microorganisms in the environment are unculturable (Hugenholtz, 2002), which means the traditional culture-dependent methods are incapable of fully revealing the composition and the functions of microbial communities in the environment. Therefore, the reservoir of ARGs in the non-cultivatable majority remains relatively unexplored in environmental samples using culture-dependent methods.

A few good molecular biology tools have been adopted for the investigation of ARGs in both culturable and unculturable microbes in the natural environment,

including polymerase chain reaction (PCR), quantitative PCR (qPCR), DNA hybridization, and DNA microarray (Zhang et al., 2009b). Among them, PCR and qPCR are the most widely used methods in the detection of ARGs in pure cultures, as well as mixed environmental samples in which ARG abundance is low. Even though these amplification-based methods are very sensitive, they also suffer from inherent limitations. Due to the difficulty in designing the appropriate primers, PCR or qPCR approaches can only detect well-studied ARGs. Poorly designed primers could cause bias and even false positive/negative results. Furthermore, the throughput of the PCR/qPCR test is low and the number of ARGs examined in one test is very limited. Most of the previous investigations of environmental ARGs using PCR or qPCR approaches examined only selected ARGs (Chen and Zhang, 2013; LaPara et al., 2011; Pruden et al., 2012). Few examinations have increased the number of genes to more than a dozen ARGs in a single study (Zhang and Zhang, 2011). Some improvements have been made to increase the number of target ARGs in environmental samples in one test, such as multiplex PCR (Ng et al., 2001) and high throughput PCR array (Zhu et al., 2013). Nevertheless, the complicated matrices still impede the application of these amplification approaches in detecting ARGs in environmental samples, especially samples from WWTPs. The variety of substances, such as humic acids, organic salts, or other unclear substances from natural or anthropogenic sources in wastewater could inhibit the enzyme activity in the amplification process and thus cause false negative results (Volkmann et al., 2007). DNA microarray is a genomic analysis approach with higher throughput for ARG detection that can perform broader screening of ARGs. However, different probes used in DNA microarray approach might cause cross-talk of gene detection. Coupled with the low sensitivity of the technique, the application of DNA microarray in ARG detection from environmental samples is very limited.

Metagenomic approaches gradually came to the attention of researchers investigating environmental ARGs in recent years. The term *metagenome* was proposed by Handelsman et al. (1998) to describe "the genomes of the total microbiota found in nature." Correspondingly, metagenomics is the study of DNA extracted directly from environmental samples.

Although the concept of "metagenome" and "metagenomics" had been known for nearly 20 years, the application of metagenomics flourished in recent years owing to the rapid advancement and the decreased cost of high throughput sequencing technology. Multiple sequencing platforms have been developed (e.g., Illumina, 454, SOLiD, and Ion Torrent) and the cost of high throughput sequencing can drop to $0.02 per million bases (Di Bella et al., 2013) using the Illumina Hiseq 2000 platform, the throughput of which can reach to 600 G bases in one run.

The metagenomic approach has now proven to be a robust tool in exploring the composition of the diverse microbial communities and grasping their functions in various environments. The microbial compositions and their functional genes in human guts (Qin et al., 2010), cow rumen (Hess et al., 2011), soil (Delmont et al., 2012), sediments (Chen et al., 2013), activated sludge (Ju et al., 2014; Yang et al., 2013), and so on have been well documented using these approaches.

Based on the different experimental design and the objective of investigation, metagenomic approaches can be classified as descriptive and functional metagenomics. In the following, we will discuss these two approaches in detail.

## Descriptive Metagenomics

Descriptive metagenomics is a culture-independent molecular method that relies on metagenomic sequences for the search, annotation, and prediction of genes in environmental samples. To be more specific, DNA is extracted directly from the collected sample. The DNA sample is assumed to represent a random fraction of the total communities in the environmental samples if the extraction biases could be ignored. High throughput sequencing is then performed using the extracted DNA. The generated metagenomic sequences are used to compare directly with a reference database that contains sequences of the known genes for annotation of the target genes (Schmieder and Edwards, 2012) to determine which microorganisms or genes are in the sample. For the detection of ARGs, the sequences from high throughput sequences can be compared with databases composed of ARG sequences. Therefore, this method based on similarity search has the potential to reveal all the known ARGs in the metagenomic data from the sample. The ARG profiles between environments can then be compared. On the other hand, the metagenomic sequences could be used to reconstruct microbial genomes through assembly and binning approaches (Albertsen et al., 2013; Ju and Zhang, 2015; Mao et al., 2014). The sequences from assembly or binning results can then be compared with a reference database for annotation, which will shed new light onto the complex microbial communities and functions from the environmental samples.

Based on the inherent nature of descriptive metagenomics, it has the following advantages in detection ARGs in WWTPs environment. First, since the descriptive metagenomic approach is a sequence-based analysis, the existence of complicated matrices in the environmental samples has little influence on the ARG search in metagenomic data. Therefore, using descriptive metagenomic approaches can avoid false negative results caused by an inhibitor in the sample matrices using the amplification-based method. Moreover, the descriptive metagenomic approach does not rely on the amplification process. The use of primers or probes in PCR or the microarray test is not required. Therefore, descriptive metagenomic approaches would avoid the false positive/negative or cross-talk resulting from the poor design of primers or probes, especially for samples collected from a complex environment like WWTPs.

Second, the nature of descriptive metagenomic analysis enables a much broader scanning of ARGs in environmental samples. Occurrence of all ARGs can be examined in the sample through a single metagenomic analysis, as long as the ARGs sequences are included in the reference database. Furthermore, the metagenomic sequences can be re-examined if new ARGs are identified. Therefore, the application of descriptive metagenomic approaches would greatly advance our knowledge of the existence of various ARGs in WWTPs environments.

Third, besides direct alignment of the ARG-like sequences, more information could be extracted from metagenomic data. For example, sequences of heavy metal resistance genes, plasmids, integrons, or transposons could also be identified using the corresponding reference databases. Moreover, the contiguous sequences generated directly from the high throughput sequencing platform could be assembled into longer sequences (contigs) and the alignment of genes could be investigated. Using this method, researchers can identify the location of ARGs on the DNA sequences and check if the detected ARGs are located on MGEs. Based on the gene alignment results,

the potential for HGT of ARGs can be evaluated and the public health risk of ARGs can be more thoroughly assessed.

Last but not least, metagenomic approaches provide a way to establish a standard method to investigate the profiles of ARGs in WWTPs. As is well recognized, variation in investigation methodologies, data collection, and normalization methods pose serious difficulties in comparing the ARG results from different samples, or even similar samples from different research groups. One recommendation to minimize complications in making comparisons between different samples or research groups is standardizing the investigation methods for ARG monitoring (Wray and Gnanou, 2000). Evaluation of ARGs in environmental samples usually includes DNA extraction, ARGs detection, and data analysis. Among these three parts, ARG detection has the highest variability. The matrices of the different environmental samples and the technical operation of different experiments would have great influence on the qualitative and quantitative results of ARGs using the amplification process. As mentioned above, metagenomic approaches have the capability to avoid the bias induced by amplification approaches, which makes the standardization of ARG detection easier to achieve. Therefore, the profiles of ARGs in different environmental samples can then be compared, leading to a more complete and meaningful interpretation of the ARG profiles in the environment.

## Functional Metagenomics

One of the bases of descriptive metagenomics is the reference database used for metagenomic sequence annotation; thus, descriptive metagenomics can only identify ARGs that have been well documented. On the other hand, functional metagenomics does not require prior knowledge of the ARGs. Therefore, functional metagenomics is a useful tool to identify novel ARGs that involve gene expression from metagenomic clones in surrogate hosts (Mirete et al., 2016; Mullany, 2014).

Functional metagenomics also begins with direct DNA extraction from environmental samples of interest. In the next step, the extracted DNA is fragmented with restriction enzymes or mechanical shearing to obtain the suitable size of the inserted DNA fragment. The fragments are then ligated into a linearized cloning vector. The vector, which includes plasmids, fosmids, cosmids, or bacterial artificial chromosomes, is chosen based on the size of the inserted DNA fragment. The vectors with the inserted DNA fragments are then cloned into the host. Many cloning hosts can be used in functional metagenomics (Wexler and Johnston, 2010). The most commonly used one is *Escherichia coli* because it is amenable to genetic manipulation and many sophisticated genetic modification tools have been developed for it. The review by Mullany (2014) has summarized the vectors and hosts used in the functional metagenomic approach. The target genes can then be identified by an appropriate screening assay. For ARG analysis, the clones are usually plated onto agar containing specific antibiotics with the appropriate concentration to determine the antibiotic resistance of the clones. The function of the insert DNA sequences in the grown clones can be determined and then sequenced.

Functional metagenomics analysis is a very powerful way to discover complete novel ARGs from the unculturable fraction of the microorganisms. Studies using functional

metagenomic approaches have discovered many novel and diverse ARGs in soil collected from Wisconsin and Alaska (Allen et al., 2009; Donato et al., 2010). Forsberg et al. (2012) established the PARFuMS system, which combined functional metagenomics and descriptive metagenomics, for rapid identification of MGEs containing ARGs. Evidence was found and presented for the exchange of ARGs between the indigenous bacteria in soil and clinic-related bacteria. However, functional metagenomics also suffers from inherent limitations, one of which is obtaining expression of all ARGs in the surrogate host. Therefore, choosing the suitable vector and surrogate host is essential for ARG identification and discovery using the functional metagenomic approach.

## Databases and Platforms for ARG Detection Using Metagenomic Approaches

The prevailing application of high throughput sequencing and metagenomics has changed investigation procedures for microbiology and ecology research. The biggest difference between metagenomics and conventional approaches (e.g., bacteria cultures, PCR, etc.) is bioinformatics analysis (Thomas et al., 2012). Details of the principles, methodologies, and up-to-date bioinformatics analysis approaches were introduced in the review by Ju and Zhang (2015). The review by Dudhagara et al. (2015) also thoroughly presented several web sources for metagenomic studies. In this chapter, we will mainly focus on the databases and platforms for ARG detection using metagenomic approaches.

Several databases were constructed to facilitate the sequence-based metagenomic analysis for ARGs, such as the Antibiotic Resistance Genes Database (ARDB) (Liu and Pop, 2009), the Comprehensive Antibiotic Resistance Database (CARD) (McArthur et al., 2013), the expanded version of ARDB (ARDB+) (Port et al., 2012), ResFinder (Zankari et al., 2012), and ARG-ANNOT (Gupta et al., 2013). ResFinder offers ARG detection functions but longer query reads are necessary. To identify sequences of ARG using ResFinder, it required coverage over at least two-fifths of the length of the matching sequence in the database, and the identity of the sequence is no less than 50%. ARG-ANNOT was designed to detect ARGs in bacterial genomes rather than in environmental samples. ARDB and CARD together provide most of the publicly available ARG sequences. They are the two most commonly used reference databases in the investigation of ARGs in environmental samples using metagenomic approaches (Gibson et al., 2015; Looft et al., 2012; Ma et al., 2011; Yang et al., 2013), although ARDB has not been updated since 2009. On the other hand, CARD is continuously and rigorously maintained and provides tools, including BLAST and the Resistance Gene Identifier (RGI) software for prediction of ARG-like sequences (McArthur et al., 2013).

However, neither of the databases can provide detailed ARG profiles for environmental samples, that is, classifications of the identified ARG-like sequences and abundance information for each detected ARG type/subtype, which makes it difficult for users to interpret the search result from the huge metagenomic data using these databases. Yang et al. (2013) have proposed the concept of structured databases for ARDB followed by a further application of this concept for CARD (Ma et al., 2014). Customized scripts were prepared to use the structured database to sort and calculate the abundance of ARG-like sequences. Using this method, the time required for ARG-like sequence

classification after alignment and abundance calculation of different ARG types and subtypes from metagenomic data can be reduced significantly.

ARDB and CARD also provide online analysis, but the capabilities of these platforms are far from sufficient to process the huge amount of data generated by high throughput sequencing for metagenomic analysis. Utilizing the concept of structured database, Yang et al. (2016) have developed an online analysis platform (ARGs-OAP) with a user friendly online interface (http://smile.hku.hk/SARGs) for ARG-like sequence detection from metagenomic data. The two most commonly used databases, ARDB and CARD, were merged and the structured database was then constructed. A hybrid annotation approach was adopted to increase the speed of analysis using ARGs-OAP. This platform can provide results with the classification of the detected ARG-like sequences from metagenomic sequences on both ARG types and subtypes level, which will save a significant amount of time for the users, especially for newcomers to ARG detection using metagenomic approaches.

## Applications of Metagenomics in the Detection of ARGs in WWTPs

Recently, metagenomic approaches have been used in the investigation of ARGs in samples from various environments, such as soil (Donato et al., 2010), rivers (Amos et al., 2014), manure (Ma et al., 2016), and sediments (Chen et al., 2013).

Many of the studies of ARGs in WWTP environments using metagenomic approaches focus on activated sludge for the obvious reason that activated sludge consists of dense and diverse microorganisms. Yang et al. (2013) used the metagenomic approach to determine that the most abundant ARGs in activated sludge collected in a Hong Kong WWTP were the resistance genes of aminoglycoside, tetracycline, and sulfonamide. Seasonal variations were also observed for resistance genes of tetracycline, sulfonamide, and vancomycin in the activated sludge collected from 2007 to 2011. The activated sludge data sets can be distinguished from other types of samples according to the differences of their ARG profiles using statistical analysis, indicating the special feature of ARG composition in activated sludge. Ma et al. (2014) investigated the occurrence and abundance of various kinds of ARGs and MGEs in biofilm, activated sludge, and anaerobic digestion sludge collected in WWTPs along with aquaculture farm sediments and river water. The results from the metagenomic data suggested that ARG abundances were the highest in sediment collected from the fishpond and that ARGs in anaerobic digestion sludge were more diverse than other samples investigated in that study. The abundance of plasmids in these samples was also investigated by the metagenomic approach. A significant correlation was found between diversities of ARGs and plasmids in the investigated samples, indicating the potential contribution of plasmids on the dissemination of ARGs in these environments.

Ju et al. (2016) investigated the fate of ARGs and human bacterial pathogens (HBPs), as well as their co-occurrence in the municipal wastewater sludge digestion process. In total, 323 ARGs and 83 HBPs were identified and the co-occurrence relationship between ARGs and HBPs was investigated through a correlation-based statistical approach as well as network analysis. Results indicated that most of the detected ARGs and a proportion of HBPs could not be effectively removed using anaerobic digestion,

emphasizing the risk of dissemination of ARGs and HBPs from the disposal of the post-digested sludge. The co-occurrence analysis showed that the resistance genes of macrolide-lincosamide-streptogramin and multidrug resistance were more likely to occur with the detected HBPs than with β-lactam resistance genes.

In addition to the ARGs that are found in the total DNA, ARGs in plasmids from activated sludge also attracted great attention. Zhang et al. (2011) studied the ARG profiles in MGEs by plasmid metagenomics, and the results highlighted the prevalence of ARGs as well as MGEs in the microbial community from activated sludge. The comparison of metagenomic data in plasmid and total DNA extracted from the microbiome of influent, activated sludge and anaerobic digestion sludge showed that metagenomic sequences from plasmids had more genes encoding functions of defense mechanisms than in genes such as ARGs and metal resistance genes (Li et al., 2015a).

Apart from the background investigation on the existence of ARGs in samples from WWTPs, efforts have been focused toward optimizing the efficiency of the wastewater treatment process or sludge treatment process in removing ARGs. The investigation comparing the fate of ARGs in wastewater treatment and the sludge treatment process (Yang et al., 2014) showed the existence of 271 ARG subtypes in influent, effluent, activated sludge, and anaerobic digestion sludge. Among them, 78 ARG subtypes persisted through the biological wastewater from influent to effluent and through the anaerobic digestion process from activated sludge to anaerobic digestion sludge. The results suggested that the sewage treatment process resulted in high removal efficiency of total ARGs in wastewater, although the removal efficiency of ARGs in sludge treatment was comparatively smaller than that observed in the sewage treatment (Yang et al., 2014). The composition of microbial communities was also revealed by metagenomic analysis. Moreover, significant correlation between six genera and the distribution of ARGs in the samples from WWTPs were found.

Christgen et al. (2015) compared the efficiency of low-energy anaerobic-aerobic treatment reactors, aerobic reactors, and anaerobic reactors in reducing the amount of ARGs from domestic wastewater. Results suggested that anaerobic-aerobic sequence reactors and aerobic reactors had better performance than anaerobic units in removing ARGs from wastewater. Anaerobic-aerobic sequence reactors and aerobic systems especially reduced the ARGs of aminoglycoside, tetracycline, and β-lactam. However, more than 60 ARG subtypes persisted in all systems, and the treatments had little effect on reducing the abundance of sulfonamide resistance genes and chloramphenicol resistance genes. The major resistance mechanisms of the detected ARGs shifted from target-specific ARGs to ARGs associated with multidrug resistance.

Because of broad scanning capabilities of metagenomic approaches in ARG detection, researchers can find the relationship of ARGs between different environments. For example, Nesme et al. (2014) studied the occurrence and abundance of ARGs in 71 environmental samples. Their results revealed the diversity and abundance of ARGs in different environments and suggested these genes were not randomly distributed. A study by Li et al. (2015b) showed the effects of anthropogenic activities on the distribution of ARGs in 50 samples covering 10 typical environments, including wastewater, river water, drinking water, biofilm, sludge, soil, sediment, and fecal, by using the metagenomic approach. In total, 260 ARG subtypes were identified, which could be classified into 18 ARG types. Results showed that the abundance of ARGs was correlated with the antibiotics extensively used in medical care or veterinary medicine, such

as aminoglycoside, bacitracin, β-lactam, macrolide-lincosamide-streptogramin, chloramphenicol, and so on. Nonmetric multidimensional scaling analysis showed that ARG profiles is quite unique in different samples. Through network analysis, they proposed *tet*(M) and genes coding aminoglycoside resistance as indicators since they may represent other co-occurring ARGs in a quantitative way. Moreover, the co-occurrence patterns between ARGs and microbial taxa revealed interesting information about the possible host of ARGs since ARGs and the microbial taxa carrying those specific ARGs may have similar distribution patterns, as verified by Forsberg et al. (2014). By this method, five bacterial genera and one archaea genus were suspected to be the hosts of ARGs, as shown by the study of Li et al. (2015b) based on the co-occurrence patterns of ARGs and microbial taxa in various environments.

## Challenges and Improvement

Although the metagenomic approach is a powerful tool for ARG detection from environmental samples, there are still several limitations and challenges that need to be addressed. These include the following:

1) *The acquisition of DNA with high quality from environmental samples*. DNA extraction is the basis of metagenomic approaches, especially for functional metagenomics. At present, acquisition of high-quality DNA from environmental materials such as wastewater or sludge remains difficult (Bondarczuk et al., 2016). Evaluation of seven different commercial DNA extraction kits was conducted and FastDNA® SPIN kit for Soil was found to be the most suitable kit for DNA extraction from activated sludge samples (Guo and Zhang, 2012). However, more efforts have to be made to improve the quantity and quality of DNA recovered from environmental samples to smooth the subsequent processes of high throughput sequencing or construction of clone libraries.
2) *The selection of proper sequencing depth*. The metagenomic approach is able to perform the broad scanning of ARG-like sequences in the metagenomic data generated from various high throughput sequencing platforms. However, the sequencing depth may have serious impacts on the completeness of ARG identification using the similarity search method. Tests may be necessary to determine the proper sequencing depth based on the goal of the experiment (to investigate the most dominant ARGs or reveal the comprehensive profile of ARGs) and the complexity of the microbiome in the sample (Di Bella et al., 2013). More detailed discussions about sequencing depths for different purposes of metagenomic approaches can be found from the previous reviews of metagenomics (Hamady and Knight, 2009; Ju and Zhang, 2015).
3) *The record of metadata*. Metadata, which means the data of data, is becoming increasingly important for analysis. Several web sources for metagenomic analysis have required users to fill in metadata before uploading their metagenomic data for analysis. Better records of the metadata would facilitate the comparison of the microbial composition and functional gene profiles in different samples and the ability to see how these differences affect the microbiome findings (Di Bella et al., 2013). Checklists for metadata have been proposed to guide the recording of metadata, such as the Minimum Information about a Metagenome Sequence

(MIMS) checklist, in order to standardize the metadata collected on the samples of interest (Yilmaz et al., 2011).

4) *The timely update and optimization of ARG reference databases and analysis methods*. The quality of the reference database is essential for the application of sequence-based metagenomic analysis in the investigation of environmental ARGs since the reference database of high quality is the basis for obtaining a reliable result from metagenomic analysis. Therefore, the timely update of the reference database would benefit the comprehensive and precise annotation of ARG sequences from metagenomic data. Moreover, the explosive growth of metagenomic data has imposed much higher requirements of computing power for processing data. Other than the cost of high throughput sequencing, computational resources and power have become the bottleneck for metagenomic analysis. Therefore, the development of efficient tools for database search is necessary to tackle with the dramatic increase of metagenomic data and enhance the efficiency of metagenomic analysis for ARGs analysis.
5) *Combination of multiple analysis approaches*. The combination of sequenced-based metagenomic analysis with the traditional PCR and qPCR approaches would be more powerful in studying the fate and behavior of ARGs through wastewater treatment process. Sequenced-based metagenomic analysis provides the broad-spectrum screening of ARGs in samples while PCR and qPCR approaches are more flexible and economical in monitoring the change of specific ARGs through the treatment process.

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# 7

# Antimicrobials and Antimicrobial Resistant Bacteria in Australia

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## Introduction

The prevalence of antimicrobial resistant bacteria (ARB) is a global health issue (WHO, 2001). Through overuse and poor management of antimicrobials, we now find ourselves in the precarious situation where careful management of antimicrobials is essential to combat the widespread emergence of resistant bacteria. While much attention has been directed toward management of antimicrobial use and monitoring the prevalence of bacterial resistance within the community, antimicrobials and ARB in the environment have until recently received comparatively little attention. This is surprising given the reliance on our water resources and the potential for the spread and maintenance of bacterial resistance to antimicrobials in this environment. Antimicrobials have been identified quite ubiquitously in the aquatic environment at subinhibitory concentrations around the globe (Golet et al., 2002; Kolpin et al., 2002; Calamari et al., 2003; Yang and Carlson, 2003; Batt et al., 2006) and Australia is no exception (Costanzo et al., 2005; Watkinson et al., 2006; Watkinson et al., 2009). However, knowledge of subinhibitory effects of antimicrobials on environmental bacteria is scarce and contradictory (Kümmerer, 2004a), despite considerable advancement in this area in recent years.

The presence of antimicrobials in the aquatic environment is governed by a combination of factors including the quantity manufactured, the dosage (amount, frequency, and duration), the excretion efficiency of the parent compound and its metabolites, the adsorption/desorption capacity in varying mediums, the metabolic behaviour of compounds during treatment and transport, and the transport mechanism itself (Hirsch et al., 1999; Diaz-Cruz et al., 2003). Excreted products can be transported to the environment through either point or nonpoint sources. These include waste effluents of manufacturing processes, excreta, disposal of unused or expired drug products, and accidental spills during manufacturing or distribution (Diaz-Cruz et al., 2003). In developed countries, waste management is well established to provide an efficient removal mechanism for waste products. Consequently, this provides a vector for transport of

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antimicrobials to the environment. Developments in waste management have led to the treatment of waste before release to the environment, but elimination of antimicrobials is often incomplete, leading to their incorporation in discharged effluent or presence in sludge material disposed to land.

Antimicrobial resistant bacteria can be considered a contaminant of emerging concern through their regular discharge to the aquatic environment from wastewater treatment plants (WWTPs) (Mezrioui and Baleux, 1994; Guardabassi et al., 1998; Reinthaler et al., 2003; Gallert et al., 2005; Martins da Costa et al., 2006; Watkinson et al., 2007a) and from aquaculture (Toranzo et al., 1984; Bjorklund et al., 1990; Guardabassi et al., 2000; Molina et al., 2002; Chelossi et al., 2003). Studies investigating the prevalence of antimicrobial resistance in the aquatic environment are limited, though over the past decade they appear with increasing frequency (Parveen et al., 1997; Boon et al., 1999; Goni-Urriza et al., 2000; Miranda and Zemelman, 2001; Ash et al., 2002; Harakeh et al., 2006; Barker-Reid et al., 2010; Rathnayake et al., 2011; Stoll et al., 2012). This chapter will focus on the prevalence of antimicrobials and ARB through wastewater treatment and discharge to the environment in an Australian context.

## Antimicrobial Usage in Australia

### Human Consumption

Global antimicrobial consumption has been estimated between 100,000 to 200,000 tons (Wise, 2001). It is somewhat difficult to draw global comparisons due to discrepancies in documentation of usages around the world. In addition, only information regarding human use is generally available, through surveillance programs such as the Pharmaceutical Benefits Scheme in Australia, and it only covers part of the dispensation in the community and very little within hospitals. However, prescription data for the treatment of systemic infection provides a general indication of antimicrobial use within the general community (Figures 7.1 and 7.2).

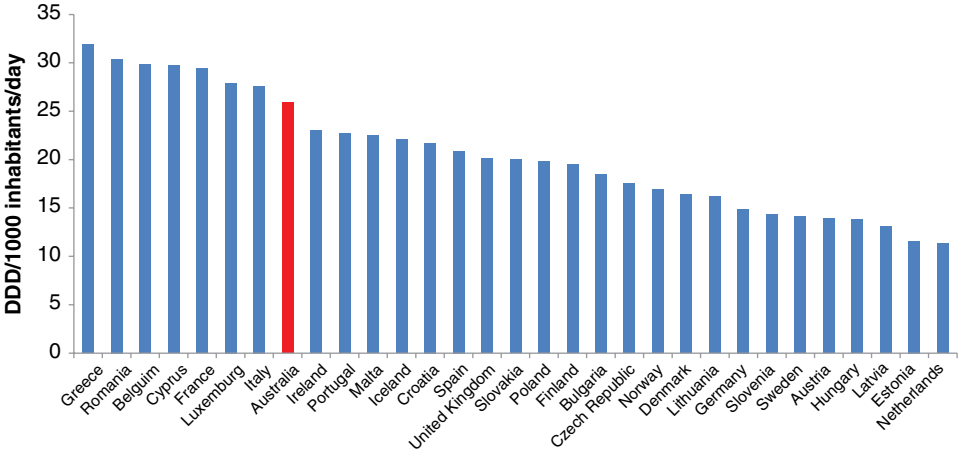


**Figure 7.1** Comparative systemic antimicrobial use in Australia and other countries in 2012. DDD = defined daily doses. Adapted from Control (2014) and Thomas et al. (2015).

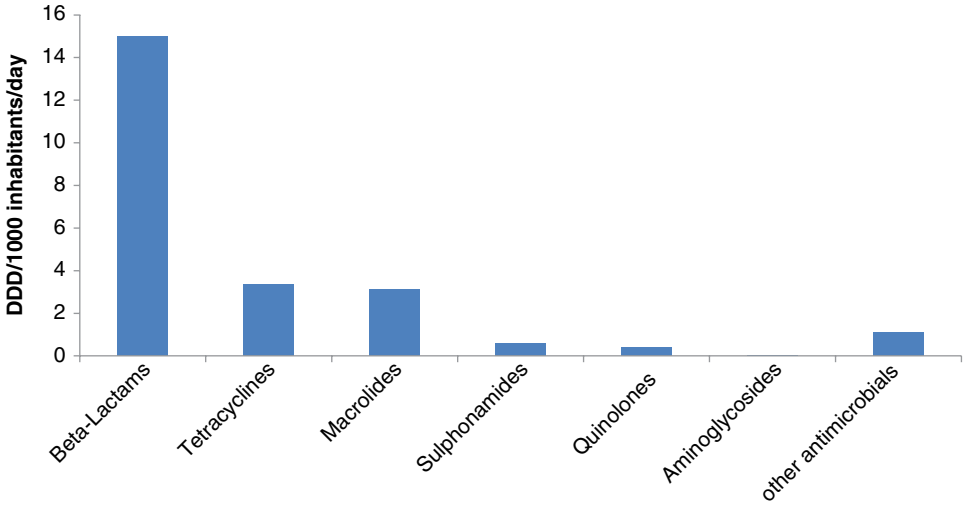


**Figure 7.2** Comparative systemic antimicrobial use in Australia across major antimicrobial groups. Adapted from Mabbott and Storey (2015).

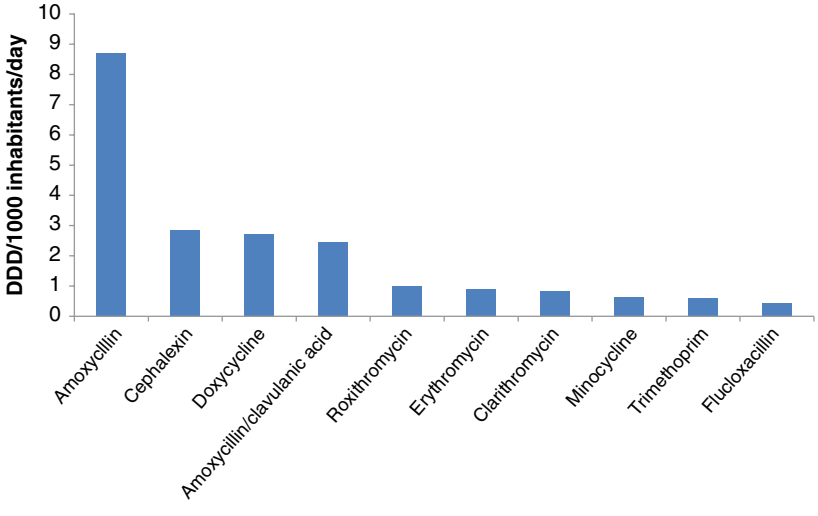


**Figure 7.3** Consumption rates for the top 10 dispensed antimicrobials in Australia, 2014. Adapted from Mabbott and Storey (2015).

Antimicrobial use in the Australian human population is relatively high compared to other countries, with an estimated 25.9 defined daily doses (DDD)/1000 population/day. Of the major antimicrobial groups, the β-Lactams were the most commonly dispensed, followed by the tetracyclines and macrolides (Figure 7.2).

As demonstrated in Figure 7.3, within these antimicrobial groups, amoxycillin and its combination presentations is the most dispensed antimicrobial, followed by cephalexin and doxycycline (Mabbott and Storey, 2015).

## Agricultural Consumption

About 90% of antimicrobials used in agriculture are used as growth-promoting or prophylactic agents, rather than to directly treat infection (Khachatourians, 1998). Veterinary antimicrobials, whether for therapeutic, prophylactic, or stockfeed application, make up the majority of antimicrobials imported into Australia. In the five years from 2005 to 2010, sales averaged 564 metric tons of active antimicrobial compound in Australia for agricultural application (APVMA, 2014). This has increased significantly from the previous reporting period (1998–2003) with an average volume of 438 metric tons sold (TGA, 2003). Figure 7.4 summarizes the trends over the five-year period from 2005 to 2010 for the major antimicrobial groups.

An average 98% of the total antimicrobials sold in Australia for animal use from 2005 to 2010 were used in food animals. The remaining 2% were used in nonfood animals. Of the total quantity of antimicrobials sold for use in food animals, an average 43% were sold for therapeutic or prophylactic purposes (APVMA, 2014).

The Australian Strategic and Technical Advisory Group on Antimicrobial Resistance (ASTAG), providing advice to the Australian Antimicrobial Resistance Prevention and Containment Steering Group, have developed a risk assessment process rating the importance of the various antimicrobial agents available for human use in Australia. While many of these high- and medium-risk antimicrobials are not approved for agricultural use, a number of cephalosporins (subgroup of β-lactams) are used and maintain a high risk rating and warrant close observation.

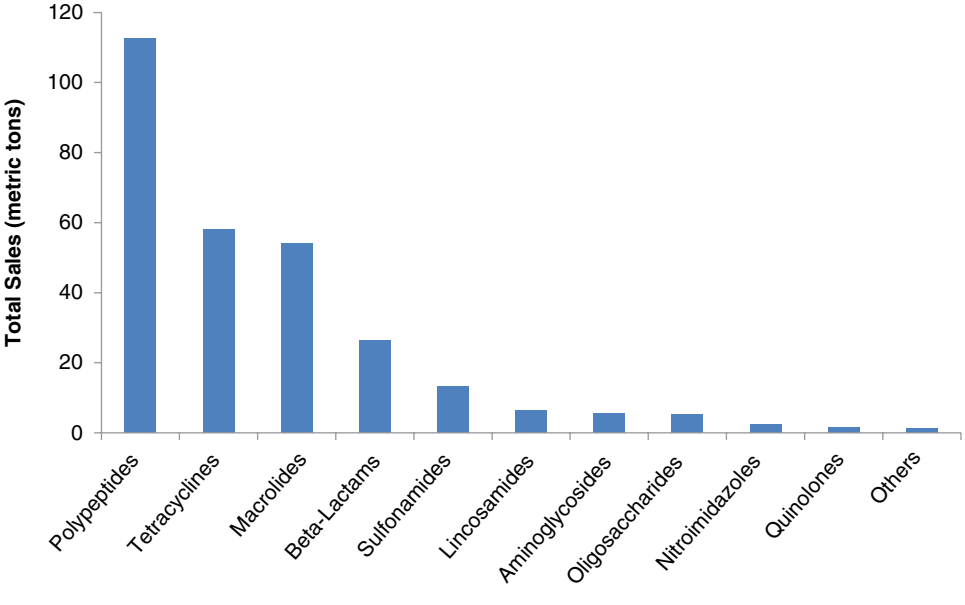


**Figure 7.4** Total sales of veterinary antimicrobials used for therapeutic purposes in food animals in Australia (metric tons of active constituent) by antimicrobial class (July 2005 to June 2010). Source: APVMA (2014).

# Antimicrobials in Australian Conventional Wastewater and the Environment

## Antimicrobials in Australian Wastewater

Few studies have been conducted that look at the prevalence and removal of antimicrobials in Australian wastewater treatment settings. Khan and Ongerth (2004) predicted the fate of the top 50 (by dispensed mass) pharmaceuticals, including antimicrobials, in Australia through sewage generation and treatment using mass balance and fugacity modeling. They predicted 8 (of 13 in total) of the studied antimicrobials to be present in raw sewage above 1 $\mu g\,L^{-1}$ and a further 5 of which would likely be present in secondary effluent above 1 $\mu g\,L^{-1}$.

In related work, the same approach was also used to predict concentrations of these residues in primary and secondary sludge (Khan and Ongerth, 2002). A number of antimicrobials were shown to be present in both primary and secondary sludge in the low $\mu g\ L^{-1}$ range, demonstrating the need for careful consideration of disposal and application of these residues.

Studies into the fate and persistence of antimicrobials in Australia wastewater have largely reflected findings found in other parts of the world. While hospital wastewater has been highlighted as a contributor of antimicrobials to WWTPs (Watkinson et al., 2009), it is evident that other sources are involved as well. For example, the ionophores (monensin and salinomycin) are used solely in agriculture in Australia (Page, 2003), yet traces of these drugs have been found in WWTPs, with waste from meat processing plants and abattoirs discharging to these WWTPs as a possible explanation (Watkinson et al., 2007b; Watkinson et al., 2009). While closed system studies of loads and WWTPs have not been performed, prescription data of community antimicrobial use would also suggest that households are another important contributor of antimicrobials to the waste stream. For example, in 2004, over four million scripts were filled in Australia for amoxycillin and cephalexin (ABS, 2004), which equates (average dose per script ~10 g) (DHA, 2004) to approximately 40 metric tons of these antimicrobials consumed in households per year. Given that many antimicrobials are only partially metabolized and excreted in parent form (Hirsch et al., 1999), a large proportion of this may potentially enter WWTPs. In addition, there is likely some contribution of antimicrobials through the disposal of unused and expired medication (Ruhoy and Daughton, 2007), adding to this burden.

Of the top 10 most used antimicrobials in Australia during the time of this research (TGA, 2003), and still reflective of the major antimicrobials used today (Figure 7.3), 9 have been identified in WWTP influent, including amoxycillin, cefaclor, cephalexin, penicillin V, ciprofloxacin, doxycycline, erythromycin, sulfasalazine, and sulfamethoxazole (Table 7.1). Antimicrobials were found at similar concentrations in Australian WWTP influent to those reported elsewhere in the literature. For example, sulfamethoxazole has been previously reported in WWTP influents with average concentrations of 0.243 $\mu g\,L^{-1}$ (Miao et al., 2004), 0.3 $\mu g\,L^{-1}$ (Karthikeyan and Meyer, 2006), and 1.09 $\mu g\,L^{-1}$ (Yang et al., 2005) compared to a median concentration of 0.25 $\mu g\,L^{-1}$ in the Australian study (Watkinson et al., 2009). Likewise, trimethoprim has been reported at concentrations ranging from 0.18 to 1 $\mu g\,L^{-1}$ in New Mexico influents (Brown et al., 2006), compared with a median concentration of 0.43 $\mu g\,L^{-1}$ in the Australia example (Watkinson et al., 2009).

**Table 7.1** Antimicrobial concentrations and detection frequencies in WWTP influent, WWTP effluent, and environmental surface waters from Australia.

| | WWTP Influents | | | | WWTP Effluents | | | | Environmental Waters | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | freq | med | max | | freq | med | max | | freq | med | max |
| **Antibiotic** | ***n*** | **%** | **μg L$^{-1}$** | **μg L$^{-1}$** | ***n*** | **%** | **μg L$^{-1}$** | **μg L$^{-1}$** | ***n*** | **%** | **μg L$^{-1}$** | **μg L$^{-1}$** |
| **β-lactams** | | | | | | | | | | | | |
| Amoxycillin | 19 | 89 | 1.400 | 6.94 | 23 | 4 | ND | 0.05 | 98 | 30 | ND | 0.20 |
| Cefaclor | 19 | 79 | 0.500 | 6.15 | 23 | 35 | ND | 1.80 | 90 | 28 | ND | 0.20 |
| Penicillin G | 19 | 37 | ND | 0.01 | 23 | 22 | ND | 0.30 | 98 | 28 | ND | 0.25 |
| Penicillin V | 19 | 68 | 0.020 | 13.80 | 23 | 35 | ND | 2.00 | 98 | 15 | ND | 0.01 |
| Cloxacillin | 19 | 26 | ND | 4.60 | 23 | 22 | ND | 0.70 | 98 | 0 | ND | ND |
| Cephalexin | 19 | 100 | 2.800 | 64.00 | 23 | 30 | ND | 0.25 | 98 | 22 | ND | 0.10 |
| **Quinolones** | | | | | | | | | | | | |
| Nalidixic acid | 19 | 47 | ND | 0.20 | 23 | 61 | ND | 0.45 | 98 | 83 | 0.050 | 0.75 |
| Norfloxacin | 19 | 89 | 0.060 | 0.22 | 23 | 87 | 0.025 | 0.25 | 98 | 78 | 0.030 | 1.15 |
| Enrofloxacin | 19 | 47 | ND | 0.04 | 23 | 57 | 0.002 | 0.05 | 97 | 44 | ND | 0.30 |
| Ciprofloxacin | 2 | 50 | 0.600 | 1.10 | 0 | – | – | – | 98 | 30 | ND | 1.30 |
| **Lincosamides** | | | | | | | | | | | | |
| Clindamycin | 17 | 100 | 0.020 | 0.06 | 21 | 81 | 0.005 | 0.07 | 98 | 57 | 0.001 | 0.01 |
| Lincomycin | 19 | 84 | 0.020 | 0.50 | 23 | 70 | 0.003 | 0.30 | 98 | 67 | 0.001 | 0.05 |
| **Macrolides** | | | | | | | | | | | | |
| Erythromycin | 18 | 78 | det.* | det.* | 23 | 83 | det.* | det.* | 98 | 52 | det.* | det.* |
| Erythromycin-$H_20$ | 2 | 0 | ND | ND | 6 | 100 | det.* | det.* | 81 | 83 | det.* | det.* |
| Roxithromycin | 19 | 53 | 0.008 | 0.50 | 23 | 70 | 0.020 | 0.50 | 98 | 63 | 0.009 | 0.35 |
| Oleandomycin | 19 | 11 | ND | 0.005 | 23 | 22 | ND | 0.15 | 98 | 28 | ND | 0.02 |
| Tylosin | 19 | 42 | ND | 0.06 | 23 | 70 | 0.003 | 3.40 | 98 | 81 | 0.001 | 0.06 |
| **Tetracyclines** | | | | | | | | | | | | |
| Chlortetracycline | 19 | 58 | 0.006 | 0.20 | 23 | 61 | 0.005 | 0.25 | 98 | 57 | 0.003 | 0.60 |
| Doxycycline | 19 | 84 | 0.020 | 0.65 | 23 | 78 | 0.010 | 0.15 | 98 | 34 | ND | 0.40 |
| Oxytetracycline | 19 | 11 | ND | 0.35 | 23 | 13 | ND | 0.07 | 98 | 13 | ND | 0.10 |
| Tetracycline | 19 | 21 | ND | 0.10 | 22 | 27 | ND | 0.02 | 98 | 33 | ND | 0.08 |
| **Polyether ionophores** | | | | | | | | | | | | |
| Salinomycin | 19 | 5 | ND | 0.30 | 23 | 0 | ND | ND | 85 | 21 | ND | 0.15 |
| Monensin | 19 | 0 | ND | ND | 23 | 26 | ND | 0.02 | 98 | 94 | 0.002 | 0.15 |
| **Sulphonamides** | | | | | | | | | | | | |
| Sulfamethoxazole | 19 | 89 | 0.250 | 3.00 | 23 | 83 | 0.050 | 0.20 | 98 | 73 | 0.008 | 2.00 |
| Sulfathiazole | 19 | 32 | ND | 0.30 | 23 | 17 | ND | 0.60 | 98 | 21 | ND | 0.04 |
| Sulfasalazine | 19 | 42 | ND | 0.10 | 23 | 52 | 0.004 | 0.15 | 98 | 15 | ND | 0.03 |
| **Others** | | | | | | | | | | | | |
| Trimethoprim | 19 | 100 | 0.430 | 4.30 | 23 | 91 | 0.010 | 0.25 | 98 | 64 | 0.003 | 0.15 |
| Bacitracin | 19 | 0 | ND | ND | 23 | 0 | ND | ND | 98 | 0 | ND | ND |

*Source*: Watkinson et al. (2009). Reproduced with permission of Elsevier.

**Table 7.2** Total concentration of major antibiotic groups and proportion removed through each process during conventional treatment. Total concentrations are represented by the sums of the median values for each antibiotic. Adapted from Watkinson et al. (2007a).

| | INF | POST PST | | POST BRT | | POST FST | | Overall |
|---|---|---|---|---|---|---|---|---|
| | Sub-total ($ng\,L^{-1}$) | Sub-total ($ng\,L^{-1}$) | %PR[a] | Sub-total ($ng\,L^{-1}$) | %PR[a] | Sub-total ($ng\,L^{-1}$) | %PR[a] | %PR[a] |
| **β-lactams** | 5340 | 4615 | 14 | 10 | 100 | 30 | –200 | 99 |
| **Quinolones** | 3980 | 5155 | –30 | 616 | 88 | 680 | –10 | 83 |
| **Lincosamides** | 62 | 57 | 8 | 45 | 21 | 55 | –21 | 11 |
| **Macrolides** | det* | det* | – | det* | – | det* | – | – |
| **Tetracyclines** | 0 | 0 | – | 0 | – | 0 | – | – |
| **Polyether ionophores** | 10 | 5 | 50 | 1 | 88 | 2 | –234 | 81 |
| **Sulphonamides** | 362 | 490 | –35 | 185 | 62 | 270 | –46 | 25 |
| **Other** | 340 | 370 | –9 | 30 | 92 | 50 | –67 | 85 |
| **Overall** | 10093 | 10691 | –6 | 886 | 92 | 1087 | –23 | **89** |

%PR[a]: Proportion removed of previous process
det*: detected, matrix effects prevented quantification
INF = Influent, PST = Primary Settling Tank, BRT = Bioreactor, FST = Final Settling Tank

Overall, the concentration of the investigated antibiotics was reduced by an average of 87% in the liquid phase during conventional treatment (Table 7.2) (Watkinson et al., 2007a). The majority of this removal appeared to occur during biological treatment with large reduction in the concentrations of both β-lactam and quinolone drugs, which dominated the overall antibiotic concentration in the influent of the plant. Large decreases in concentration of all groups are seen, typically greater than 80%, which is similar to that reported elsewhere in the literature (Alder et al., 2001; Giger et al., 2003; McArdell et al., 2003). However, while large decreases in concentration were demonstrated, both quinolones and tetracyclines were found in the WWTP effluent samples, indicating that these processes do not result in complete removal.

Quinolones and β-lactams have been shown to undergo thermal degradation (Fasani et al., 1998), with the latter readily degraded through hydrolytic cleavage and ultimate mineralization to $CO_2$ and water (Hirsch et al., 1999). Due to this, β-lactams are not generally thought to be of concern as environmental pollutants. Low concentrations of a number of β-lactam drugs, however, were sporadically reported in Australian effluent examples (Watkinson et al., 2007c; Watkinson et al., 2009), similar to that reported in the literature (Andreozzi et al., 2004). This may indicate that although these antimicrobials are not considered persistent in the classic sense, a pseudo-persistence may be occurring due to their continual discharge and this phenomenon has previously been recognised in the literature (Ankley et al., 2007).

Degradation of sulphonamides has been reported in WWTPs (Carballa et al., 2004; Perez et al., 2005), and this has been further demonstrated in Australian examples with a decrease in concentration through treatment (Watkinson et al., 2007c; Watkinson

et al., 2009) consistent with other studies (Alder et al., 2001; Ashton et al., 2004; Glassmeyer et al., 2005), indicating that while degradation is occurring, it is incomplete.

Water is a precious commodity in Australia, and its management is critical for preserving the future of this resource. Despite being the driest continent on earth, Australia has one of the highest water consumption rates per capita of any country worldwide (OECD, 1999). Currently, 97% of urban runoff and 86% of effluent discharge is not reused and is discharged into our rivers and coastal areas (CSIRO, 2005). Consequently, wastewater reuse is topical in Australia, as water managers examine the possibilities for utilizing this resource for purposes such as irrigation, aquaculture, indirect potable reuse, and even direct potable reuse, and a number of studies and initiatives to examine the risk of antimicrobials have been undertaken (though many remain unpublished due to sensitivity around the findings and implications within the community).

A study looking at the impact of membrane filtration/reverse osmosis treatment on antimicrobial removal showed even greater removal potential; however, some residues were still present in final water in the low ng $L^{-1}$ range (Watkinson et al., 2007c).

## Antimicrobials in Australian Surface Waters

Antimicrobials have been detected at quantifiable concentrations in more than 50% of studied Australian surface water samples; the antimicrobials include naladixic acid, norfloxacin, clindamycin, lincomycin, roxithromycin, tylosin, chlortetracycline, monensin, sulfamethoxazole, and trimethoprim (Table 7.1). Although the use and management of antimicrobials varies substantially between countries, many similarities can be drawn from the presence of these antimicrobials in surface waters from around the world, with a number of antimicrobials consistently being reported in environmental surface waters (Alder et al., 2001; Lindsey et al., 2001; Zuccato et al., 2001; Kolpin et al., 2002; Yang and Carlson, 2003; Kolpin et al., 2004). This information would suggest that the fate of a particular compound and its persistence through treatment and in the environment is just as important as the volume of that particular compound used for treatment. This has vast implications for many environmental risk assessments based purely on clinical or agricultural usage statistics.

A number of specific antimicrobials were significantly higher ($p < 0.05$) at sites designated as point sources (PSs) compared to nonpoint source (NPS) sites, namely, cephalexin, ciprofloxacin, enrofloxacin, norfloxacin, sulfamethoxazole and trimethoprim in the study (Watkinson et al., 2009). The use of quinolones, such as ciprofloxacin, norfloxacin, and enrofloxacin, has been banned in agriculture in Australia with their application confined solely to human treatment (Unicomb et al., 2006). In addition, sulfamethoxazole and trimethoprim are confined largely to human treatment (TGA, 2003), suggesting a reason for the strong association of these antimicrobials with PS sites. This has implications for the development of microbial source tracking methodology.

Generally, the most frequently detected antimicrobials in WWTP effluent were also the most frequently detected in investigated surface waters, with concentrations decreasing from effluent > PS > NPS, indicating a natural decline in antimicrobial concentration with distance from effluent discharge (Watkinson et al., 2009). Attenuation with distance from an effluent discharge isn't always apparent, and for such compounds, additional sources (e.g., agricultural input) may be contributing additional loadings to streams affecting this apparent trend.

Point source sites also demonstrated a significantly ($p < 0.001$) higher total investigated antibiotic concentration (TIAC) than NPS sites. Additionally, a weak correlation has been shown between TIAC and volume of wastewater discharged within separate river catchments (Figure 7.5), further strengthening the argument that in the limited Australian literature, WWTP discharges are an important source of the targeted antimicrobials to these environmental surface waters (Watkinson et al., 2009). This is not surprising given the large urban population within the studied region and the lack of substantial catchment flow during the study period. Nonpoint sources of antimicrobials can be best assessed during and following substantial rainfall events and may be more influential with distance from urban centers. Additionally, sediments have been shown to be a significant sink of antimicrobials (Capone et al., 1996; Kerry et al., 1996; Kim and Carlson, 2006; Pei et al., 2006), and assessment of the solid phase would also better determine the occurrence and distribution of antimicrobials in surface water systems.

Despite the apparent dominance of WWTP effluent, the agricultural ionophore monensin was the most frequently detected antimicrobial in surface waters. As previously discussed, both ionophores and tylosin were intermittently detected in WWTPs, possibly sourced from meat processing plants or abattoirs; however, this does not fully explain their high frequency in environmental surface waters. Environmental studies indicate that monensin is more likely to partition to soil and has been shown to be biodegradable in manure and soil (Donoho, 1984). However, a study by Kim and Carlson (2006) has reported the presence of ionophores in a watershed, with highest concentration in the sediments, but with substantial concentrations in the surface waters. Additionally, Lissemore et al. (2006) also reported the presence of monensin in surface waters of southern Ontario.

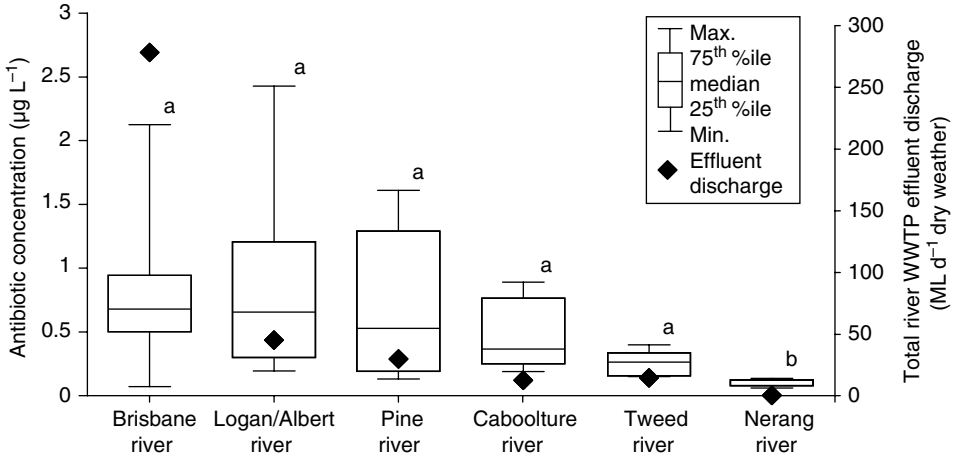


**Figure 7.5** Boxplots of total investigated antimicrobial concentration (TIAC) for each of the studied rivers. Different letters indicate significant differences ($p < 0.05$) between rivers, with similar letters indicating no significant difference ($p > 0.05$). Total WWTP effluent discharged (dry weather flow) within each river system during the study period is displayed (diamonds). Source: Watkinson et al. (2009). Reproduced with permission of Elsevier.

## Antimicrobial Resistant Bacteria in Australian Wastewater and the Environment

While it appears that antibiotics pose little acute environmental toxic risk, based on conventional toxicity tests and environmentally relevant concentrations (Kümmerer, 2004b), the greatest risk of their presence may be their link to antibiotic resistance among bacteria. The increased prevalence of antibiotic resistance with proximity to anthropogenic discharges has been demonstrated repeatedly around the globe, not only restricted to WWTP effluents (Mezrioui and Baleux, 1994; Guardabassi et al., 1998; Goni-Urriza et al., 2000; Guardabassi et al., 2002; Reinthaler et al., 2003; Edge and Stephen, 2005; Gallert et al., 2005; Martins da Costa et al., 2006) but also those of intensive agricultural regions (Aminov et al., 2002; Mathew et al., 2003; Sengelov et al., 2003a; Sengelov et al., 2003b; Burgos et al., 2005; Obeng et al., 2012; Obeng et al., 2013; Barlow et al., 2015) and aquaculture facilities (Smith et al., 1994; Huys et al., 2000; Tendencia and de la Peña, 2001; Chelossi et al., 2003; Hameed et al., 2003; Le et al., 2005; Akinbowale et al., 2006; Sarter et al., 2007) where antibiotics are used as growth promoters and anti-infectives.

Early work in Australia that looked at the incidence of antimicrobial resistance of culturable bacteria found widespread resistance among environmental waste samples (Boon, 1992). Additional work investigated both native and fecal bacteria isolated along a river gradient and found the incidence of resistance to be higher in urban dominated sites versus rural sites (Boon and Cattanach, 1999). Similarly, it has been demonstrated that antibiotic resistance among *Escherichia coli* in environmental surface waters was greatest in locations receiving WWTP discharge (Table 7.3) (Watkinson et al., 2007b; Rathnayake et al., 2011; Watkinson et al., 2007c).

Somewhat surprisingly, incidence and rate of antimicrobial resistance in WWTP *E. coli* were significantly reduced compared to the isolates in adjacent receiving waters

**Table 7.3** Antimicrobial resistance profiles of *E. coli* in the representative river gradient and their major influence. Adapted from Watkinson et al. (2007a).

| | | | Antibiotic Resistance (%) | | | |
|---|---|---|---|---|---|---|
| **Site** | **Influence at site** | **Ave. *E.coli*/100 mls** | **Ampicillin** | **Tetracycline** | **Sulfamethoxazole** | **Ciprofloxacin** |
| 1 | WWTP | 3333 | 12 | 9 | 12 | 1 |
| 2 | WWTP | 364 | 3 | 2 | 4 | 0 |
| 3 | Urban | 300 | 4 | 4 | 6 | 0 |
| 4 | Urban | 197 | 3 | 1 | 0 | 0 |
| 5 | WWTP with Chlorination | 114 | 47 | 24 | 63 | 0 |
| 6 | WWTP with Chlorination | 11 | 0 | 0 | 0 | 0 |
| 7 | Agricultural | 55 | 3 | 7 | 0 | 0 |
| 8 | Agricultural | 40 | 0 | 0 | 0 | 0 |
| 9 | Agricultural | 5 | 9 | 9 | 37 | 0 |

(Table 7.3). Reinthaler et al. (2003) has also demonstrated this to be the case, and it is proposed that treatment selects for more resistant organisms. This can occur through numerous pathways. First, the presence of antimicrobials has been documented within WWTPs (Alder et al., 2001; McArdell et al., 2003; Miao et al., 2004; Batt et al., 2006; Brown et al., 2006) in low concentrations, which could serve as a selective pressure toward resistant organisms. Effluent disinfection, while lowering the total bacterial count, has also been shown to increase the prevalence of antimicrobial resistant bacteria and multiple antimicrobial resistance (Murray et al., 1984). These observations, when combined, could possibly account for the higher incidence and rate of antimicrobial resistance in PS isolates compared to WWTP isolates, due to PS bacteria isolated from areas receiving final effluent, whereas WWTP bacteria include isolates from each stage of sewage treatment, including those from raw effluent that has not yet undergone treatment.

Investigations on the levels of antimicrobial resistance in oyster *E. coli* associated with wastewater discharge demonstrated incidence of resistance was low, with only 10% of all isolates indicating resistance to one antimicrobial (Table 7.3). Limited comparative data is available; however, a study by Cooke (1976a) showed antimicrobial resistance in oyster *E. coli* isolated from WWTP outfalls ranged from 56% to 100%, in complete contrast to this finding. Additionally, incidence of multiple antimicrobial resistance ranged from 11% to 81% (Cooke, 1976b), whereas no multiple antimicrobial resistance was seen in the Australian example (Table 7.4).

A recent study has demonstrated that the prevalence of antibiotic resistance among the fecal bacteria of selected animal populations was directly correlated with their exposure to human contact (Skurnik et al., 2006), which would indicate the primary source of antibiotic resistance is anthropogenic. It is unlikely that antibiotic resistance is developing in WWTP discharges or environmental surface waters, but rather the bacteria in the discharges are either resistant by nature or carrying resistance elements originating from further upstream in the waste catchment (among either human or

**Table 7.4** Percentage of antimicrobial resistant *E. coli* isolates from a variety of sources. Letters indicate significant differences ($p < 0.05$) between sources. Adapted from Watkinson et al. (2007b).

| | **% of isolates with indicated resistance from source type:** | | | | | |
|---|---|---|---|---|---|---|
| **Resistance to** | **WWTP** ***(n = 100)*** | **PS** ***(n = 74)*** | **NPS** ***(n = 88)*** | **ONW5** ***(n = 50)*** | **OAW5** ***(n = 71)*** | **OAW1** ***(n = 79)*** |
| ≥1 antibiotic | **31**[a] | **87**[b] | **62**[c] | **4**[d] | **17**[e] | **4**[d] |
| Ampicillin | **15**[a] | **19**[a] | **7**[b] | NR[c] | **4**[b] | NR[c] |
| Cephalothin | **14**[a] | **41**[b] | **30**[c] | **2**[d] | NR[d] | **3**[d] |
| Naladixic Acid | **5**[a] | **5**[a] | NR[b] | NR[b] | NR[b] | NR[b] |
| Sulfafurazole | **17**[a] | **32**[b] | **20**[a] | NR[c] | **4**[d] | **1**[c] |
| Gentamicin | NR[a] | **8**[b] | NR[a] | **4**[c] | NR[a] | NR[a] |
| Tetracycline | **10**[a] | **51**[b] | **34**[c] | NR[d] | **8**[a] | NR[d] |

WWTP = Wastewater Treatment Plants, PS = Point Source, NPS = Non-point Source, ONW5 = Oysters Native WWTP5, OAW5 = Oysters Active WWTP5, OAW1 = Oysters Active WWTP1, NR = No Resistance

animal populations exposed to antibiotics). Research would suggest WWTPs offer a favourable environment for the transfer and exchange of genetic information and could enhance the development of antibiotic resistance (Mach and Grimes, 1982; Mezrioui and Baleux, 1994; Reinthaler et al., 2003; Inoue et al., 2005). The role that subinhibitory concentrations of antibiotics (such as those identified in this study) play in the development, maintenance, and transfer of this antibiotic resistance is largely unknown; however, it seems reasonable to assume that the presence of antibiotics in wastewater may contribute to the incidence of bacterial resistance within this environment and the associated receiving waters.

Most studies to date of ARB have focused on fecally derived or clinically important organisms when conducting their environmental assessments. This is typically due to their ease of cultivation and clinical relevance, and rather than reflecting the spread and prevalence of antibiotic resistance in the environment, it reflects more the transport and dissemination of resistant organisms from anthropogenic sources. Generally, these organisms are short lived in the aquatic environment (e.g., *E. coli* and *Enterococci* survival has been reported as 0.8 and 2.4 d, respectively, in marine water (Hanes and Fragala, 1967)).

More recent work focusing around genetic and molecular assessments (such as those described later in this book) are really starting to resolve these issues and bring the complete picture to bear. Work to this effect is quite limited in Australia, but a number of key studies have been completed to provide some learning in this space.

An analysis of antibiotic resistance genes in reclaimed water and environmental water samples used for irrigated agriculture found that the presence of these genes in environmental samples from an impacted river system was scarce, but their presence was quite prevalent in reclaimed water samples (Barker-Reid et al., 2010). This has potential impacts for the use and management of water in the area required for irrigated agriculture.

In contrast somewhat, Stoll and colleagues (2012) found widespread distribution of antibiotic resistance genes in surface waters, indicating the variability that can be encountered, likely based around sources, pressures, analytical techniques (detection versus expression for example), and spatial and temporal influences.

## Conclusions

The identification of antibiotic resistant elements within a natural population is an obvious concern, but what are the real implications for public health? Even if we accept the limited evidence that antibiotic resistance is disseminated and maintained within environmental bacterial communities, how does this affect how we monitor and manage antibiotic resistance? Does this evidence suggest a need to change our regulatory framework to combat this risk, real or inferred? It is probably too early to be able to fully address these concerns; however, some light can be shed on the nature and potential risk of antibiotic resistant organisms in the aquatic environment.

The first example is with methicillin resistant *Staphylococcus aureus* (MRSA), which, along with vancomycin resistant *Enterococci* (VRE) are probably the greatest bacterial threats to public health. An Australian survey of *S. aureus* infections from 1999 to 2002 documented 3,129 episodes with MRSA accounting for 40% and 12% of hospital- and community-acquired *S. aureus* infections, respectively (Collignon, 2005). Additionally,

an investigation of wastewater effluent identified genes coding for both methicillin and vancomycin resistance (these drugs are commonly used to treat MRSA infections) (Volkmann et al., 2004), which could potentially be transferred among environmental bacteria. MRSA is relatively short lived (~3–7 days) in environmental waters, so the likelihood of direct exposure would appear to be limited. To put this into perspective, it is estimated that 30% of the population is currently colonized by *S. aureus*, living on the skin and even in the nasal cavity, with a further 3% of the population living unharmed in the presence of MRSA (DH, 2007). So it would appear that there is little direct risk of exposure to these organisms through their limited presence in the aquatic environment. However, the potential for the aquatic environment to become a sink for antibiotic resistant elements that may contribute to the overall antibiotic resistance burden remains an unquantified risk to public health, which leads the authors to the second example of the potential risk of antibiotic resistance in the aquatic environment.

In 1991, the seventh cholera pandemic hit Ecuador and affected an estimated 8000 people (Smith, 2007). This epidemic was highlighted by the presence of a multiresistance strain of *V. cholera*, which had been recently identified in multiple areas around the globe (Smith, 2007). Initial observations declared that this multiresistant bacterium was developed through the use of antibiotics in local shrimp farming. While this has since been discredited, the emergence of multiple antibiotic resistance in a virulent opportunistic pathogen normally found in marine waters highlights the potential risk of the presence of antibiotic resistance in the aquatic environment. The impact of these observations on public health and the potential for the emergence and spread of antibiotic resistance through the aquatic environment demands further investigation to maintain our vigilance against the spread of infectious disease.

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# 8

# The Mobile Resistome in Wastewater Treatment Facilities and Downstream Environments

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Wastewater treatment facilities are often considered to be hotspots for antibiotic resistance as a result of exposure to sublethal concentrations of antibiotics coupled to dense populations of enteric and environmental microbial communities. Consequently, the effluents from these facilities contain high levels of antibiotic resistance genes, which can disseminate to downstream aquatic and soil microbiomes. These microbiomes may be linked to food webs and the water cycles, inevitably contributing to global propagation of antibiotic resistance. The capacity of antibiotic resistance genes to be horizontally transferred across bacterial phyla is a major driving force for the propagation of antibiotic resistance. This phenomenon primarily occurs when antibiotic resistance genes are associated with mobile genetic elements such as plasmids, bacteriophages, and conjugative transposons. Although horizontal gene transfer of antibiotic resistance genes is well studied in clinical environments, relatively few studies have explored this phenomenon within wastewater treatment facilities. Furthermore, the horizontal transfer of mobile genetic elements harboring antibiotic resistance genes from wastewater treatment facilities to downstream microbiomes is almost completely unexplored. This chapter specifically focuses on antibiotic resistance genes from wastewater treatment facilities that are associated with mobile genetic elements. It overviews current understanding of plasmid-, transposon- and integron-associated antibiotic resistance genes in wastewater effluents and biosolids, assesses the fate of these elements in downstream environments, attempts to correlate between resistance genes and specific mobile elements, and underscores crucial knowledge gaps that need to be addressed in order to assesses the epidemiological potential of various wastewater-derived mobile elements within the larger framework of antibiotic resistance.

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## Introduction

Bacteria are the most widespread and ancient life forms, having adapted to nearly all types of habitats on earth (Madigan, 2000). Their adaptive success resides in remarkable genomic plasticity, which is partially associated with their capacity to acquire genetic information by horizontal gene transfer (HGT) (Thomas and Nielsen, 2005). HGT circumvents otherwise slower means of adaptation such as random mutation and intraspecific chromosomal conjugation and is therefore a significant platform for acclimatization to environmental stress. A myriad of studies have demonstrated that the propagation of antibiotic resistance in clinical pathogens is strongly linked to antibiotic resistance genes (ARGs) that are associated with mobile genetic elements (MGEs) (Palmer et al., 2010; van Hoek et al., 2011; Karah et al., 2016). However, the concept that this phenomenon may be a significant driver of antibiotic resistance in the environment has been only recently explored as illustrated by the recent characterization of ARGs as contaminants of emerging concern (CECs) (Pruden et al., 2006).

Wastewater treatment plants (WWTPs) assemble high densities of enteric- and environmentally derived bacteria along with residual concentrations of antibiotics, disinfectants, and heavy metals, which potentially select for antibiotic resistant bacteria (ARB) (Manaia et al., 2016; Seiler and Berendonk, 2012). The wastewater microbiome may therefore select for ARG-harboring MGEs that are eventually discharged within the final effluents to downstream environments (Berendonk et al., 2015; Rizzo et al., 2013). Therefore, while wastewater treatment significantly reduces overall bacterial loads, the relative abundance of ARGs can increase in the course of the wastewater treatment process, leading to their dissemination in wastewater effluents (Ju et al., 2016; Mao et al., 2015).

A myriad of studies have applied culture-based and alternative methods to track antibiotic resistance in WWTPs (LaPara et al., 2011; LaPara et al., 2015; Di Cesare et al., 2016; Li et al., 2015; Parsley et al., 2010; Aydin et al., 2015; Port et al., 2014; Laht et al., 2014; Du et al., 2014; Chen and Zhang, 2013; Tian et al., 2016; Ferro et al., 2016); however, relatively few studies have established a link between ARG abundance and MGE dynamics in WWTP effluents and downstream environments (LaPara et al., 2011; Di Cesare et al., 2016; Li et al., 2015). Dissemination of ARGs along the WWTP, effluent, aquatic, and soil continua is undoubtedly linked to MGE-associated HGT. Therefore, there is an urgent need to investigate the basic mechanisms of HGT in these complex ecosystems, in order to evaluate the contribution of WWTPs to global antibiotic resistance.

This chapter presents a brief overview of HGT in prokaryotes, explores methodologies that can be applied to follow these processes in WWTPs and downstream environments, and reviews current knowledge of MGE-associated ARGs in WWTPs and downstream environments. Finally, it discusses the primary technological gaps that currently hamper the study of ARG HGT within the framework of water reuse and proposes directions for assessing epidemiological risks associated with antibiotic resistance in WWTPs.

## Horizontal Gene Transfer in Prokaryotes

One of the more distinctive traits of microbial genomics is the "horizontal" mobility of genes across species and communities in addition to vertical mobility within populations facilitated by cell division. This unique feature is extensively documented in nature

and is currently recognized as a leading force shaping the evolution of prokaryotes (Thomas and Nielsen, 2005; Frost et al., 2005). In vertical gene transfer, newly acquired genetic elements are maintained and transmitted within a clonal community, whereas HGT (sometimes referred to as lateral gene transfer) can traverse phylogenetic barriers, potentially to an extent in which a given mobilized genetic element is no longer associable with a particular species.

A wide array of gene families is commonly horizontally transferred among prokaryotes. These are generally depicted as "accessory genes" that encode for nonvital functional proteins (Ochman et al., 2000). While the acquisition of genes in itself is believed to be a random process (Von Wintersdorff et al., 2016), selective pressure dictates "the more fitting" pool of gene combinations in a given species within a certain environment. Additionally, genes that enhance the fitness of an organism in a particular niche can also be transferred between niche boundaries, and such HGT might help recipients integrate into new ecological niches (Papke and Gogarten, 2012). Hence, HGT is strongly linked to genome evolution.

Gene exchange in prokaryotes occurs at significantly higher rates than eukaryotes, and this phenomenon has undoubtedly existed in prokaryotes for a considerably longer time, thereby explaining their high level of genetic variability (Soucy et al., 2015). In the past decades, comparative genomics has identified large variation in gene content within individual prokaryotic species, which can only be explained by HGT. For example, an analysis of 61 *Escherichia coli* genomes revealed that only 6% of gene families were shared between all genomes, whereas the remnant gene content consisted of acquired genes (Lukjancenko et al., 2010). The wide variation in genome content in bacteria reflects a continuous process of gene acquisition and loss, highlighting how HGT contributes to the dynamic composition of such genomes.

## Modes of Horizontal Gene Transfer

Three principal mechanisms are responsible for facilitating HGT: conjugation, transduction, and transformation (Thomas and Nielsen, 2005) (Figure 8.1a–c).

Conjugation is a directional donor-to-recipient transfer that involves contact between two cells, which is established through the construction of a conjugative bridge (i.e., sexual pili in Gram negatives). Conjugative transposons and conjugative plasmids (detailed in the following section) carry genetic information required for their excision from a donor cell and the formation of a conjugative bridge with a recipient cell; after which the mobile element is "injected" from the donor into the recipient strain. While conjugation rates can be easily calculated in *in-vitro* experiments, their dynamics in natural environments are still not completely clear. Usually conjugation occurs more frequently between phylogenetically related strains, especially in the case of conjugative plasmids (Smillie et al., 2011).

Transduction is the general transfer of genetic material via phages. In some circumstances, during the packaging of genetic material of a forming phage, host genome segments can be randomly included in the phage capsule; consequently, since the content of the capsule does not affect its functionality, this system may work as a vector for gene transfer to a new host. The advantages of this type of HGT are that (i) the DNA is transferred in a protected capsule that prevents degradation; and (ii) it does not rely on specific conjugative machinery for the transfer. On the other hand, this

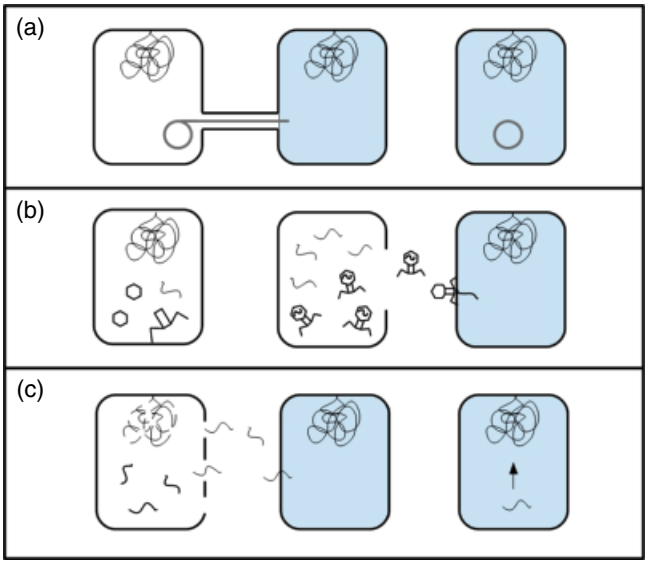


**Figure 8.1** Primary mechanisms of HGT in bacteria. Donor cell: white; recipient cell: gray. (a) Conjugation: transfer of conjugative plasmids or conjugative transposons through direct cell-to-cell link established by the donor cell. (b) Transduction: host genetic material may sometimes be included in the capsule of the forming phages and subsequently be transferred to a new host cell by infection. (c) Transformation: naked DNA is taken up by the cell from its surrounding environment, integrated into the host genome and expressed. (*See insert for color representation of the figure.*)

mechanism is constrained by the length of the DNA that the phage capsule can encompass and by the host tropism range of the phage (Doulatov et al., 2004; Gómez and Buckling, 2011).

The two abovementioned modes of HGT require specialized, protein-mediated mechanisms to occur. In contrast, transformation involves the natural capability of prokaryotes to uptake plasmids or linear DNA fragments that are released to the environment following cell lysis. Despite being the simplest way for bacteria to access external genetic information, for natural transformation to occur, bacterial cells need to be in a regulated physiological state defined as "competence" (Mell et al., 2014). This status enables the cells to facilitate DNA uptake in response to specific environmental conditions related to altered growth conditions, nutrient access, cell density (quorum sensing), or starvation. The proportion of bacteria that develop competence in a population is strongly dependent on the abovementioned conditions and can range from near zero to almost 100% (Mell et al., 2014). Although naked DNA is easily subjected to degradation if released in the environment, there are circumstances in which it can be extremely stable for long time prior to bacterial transformation. For example, it has been shown that sorption of DNA to organic compounds such as humic acids and clay particles in soil can protect the DNA from DNases and physical-chemical degradation, thereby preserving it for a long time (Crecchio and Stotzky, 1998; Saeki et al., 2011; Hong et al., 2013). By application of bench-scale experiments, Domingues et al. (2012) demonstrated how environmental and clinical bacterial strains rapidly acquired antibiotic resistance mechanisms from the environment, showing how strongly transformation is linked to ARG propagation.

## Mobile Genetic Elements as Vectors of Horizontal Gene Transfer

MGEs facilitate the mobilization or rearrangement of genes within a single cell or between genomes from one bacterium to another. Collectively, they have been coined the "mobilome," and they constitute the primary driving force in HGT.

Plasmids are circular double-stranded DNA elements that autonomously replicate inside bacterial cells. Although similar in structure to bacterial chromosomes, they are usually smaller in size, ranging from 2 kb up to 1 Mb, and can be found in low or high copy number within the cells. Considered to be supplemental to the chromosomal genome and generally not required for survival, they do not carry housekeeping genes, which are responsible for the normal function of the cell, but rather accessory genes encoding for a wide array of functions including toxins, virulence factors, metabolic pathways, and protective mechanisms such as resistance to antibiotics and heavy metals (Li et al., 2015; Frost et al., 2005; Bennett, 2008; Hynes and McGregor, 1990; Bhaduri and Smith, 2011). As described above, HGT of conjugational plasmids between donor and recipient bacterial strains is facilitated by a conjugational bridge (McArthur, 2006) (Figure 8.1a). Following conjugation, plasmids depend entirely on host cell protein-based machinery for expression and replication. Similar to certain integrative phages, integrative plasmids can incorporate themselves into prokaryotic chromosomes by means of site-specific recombination, which can be facilitated by self-encoding recombinase genes. Thus, in a particular prokaryotic host they may either be integrated into the chromosome or be maintained episomally in free circular form (Frost et al., 2005).

To date, a myriad of plasmids have been isolated and characterized from both clinical and natural environments. These can be loosely divided in two groups determined by whether they are associated with a narrow range of phylogenetically related hosts, or have a broad host range where they can be harbored by a wider range of phyla (Klumper et al., 2015; Akiyama et al., 2010). The latter plasmids are of particular concern in clinical environments because they can be involved in the propagation of ARGs between different pathogenic strains. Although various molecular-based techniques have been proposed to better classify plasmids associated with these two families, a commonly used classification is based on the stability of plasmids during conjugation, coined plasmid incompatibility (Inc). This is defined as the inability of two plasmids belonging to the same Inc group to be stably inherited (Novick, 1987; Carattoli, 2013). Overall, the host range of a given plasmid is a key factor that controls its ecological distribution and fate (Smillie et al., 2011).

Transposons are structured modular mobile genetic elements, typically composed of two transposable units called insertion sequences (IS), which flank one or more functional genes (McArthur, 2006). The insertion sequence consists of two short inverted repeats (IR), and a gene encoding for a transposase, the site-specific recombinase that facilitates transposition (Figure 8.2). Transposon size varies considerably depending on the size and quantity of associated genes, but they are typically smaller than plasmids (Touchon and Rocha, 2007; Mahillon and Chandler, 1998). Through site-specific integration and excision between chromosomes and plasmids, they can mobilize across an ample range of bacterial hosts (McArthur, 2006). Transposons are often associated with ARGs, and there is growing concern due to the increase in AR-associated transposons in clinical isolates (Berg and Berg, 1983; Liebert et al., 1999; Nordmann et al., 2012; Smet et al., 2010). Similar to conjugative plasmids, conjugative transposons (Rice, 1998) can also mobilize between host and recipient bacteria in three

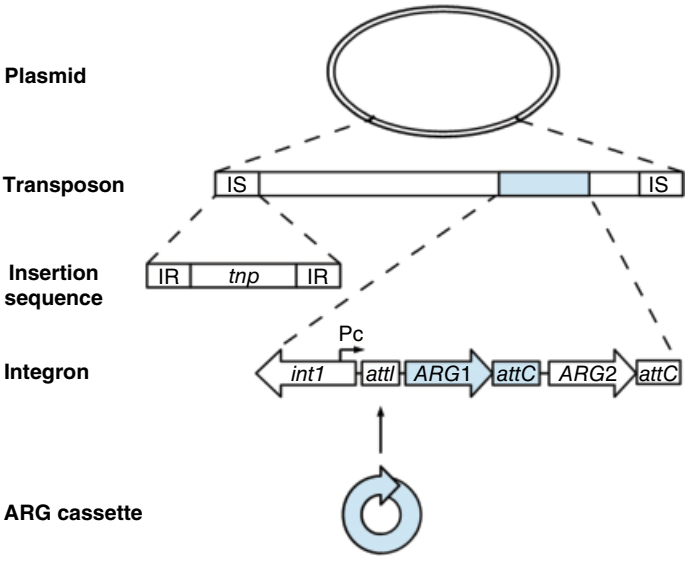


**Figure 8.2** Schematic representation of MGEs in hypothetical hierarchical configuration. In transposons, the insertion sequence (IS) consists of two inverted repeats (IRs) flanking a transposase (*tnp*) gene; these sequences guide mobilization and integration of the whole transposon. In Class 1 integrons, GCs (in this case an ARG-harbouring cassette) consist of a functional genetic element (e.g., ARG1 or ARG2) and an *attC* site. Integrase1, encoded by *int1*, promotes integration of GCs by facilitating recombination between *attC* and *attI* sites. The *Pc* promoter, located in the *int1* sequences, promotes the expression of the acquired GCs. (*See insert for color representation of the figure.*)

steps: (i) excision from the host genetic element, (ii) transfer into a new host, and (iii) reintegration into the recipient genetic element (Rice, 1998; Burrus et al., 2002). Additionally, depending on the type of transposon, this process may also encompass a replicative step that leaves a copy of the transposon-associated gene in the donor. These elements differ from conjugative plasmids in that they cannot be isolated as circular self-replicative molecules. Once integrated in the new host, transposons replicate with the chromosome or plasmid they are part of, contributing to the ecovariance of the host bacterial strain.

Integrons are two-component genetic elements composed of a gene encoding for an integrase (Int recombinase) and an integration site coined *attI* that is proximal to a *Pc* promoter. This simple structure can acquire and express gene cassettes (GCs) by facilitating site – specific recombination between an *attC* site on the GC and the *attI* site of the integron (Figure 8.2) (Cambray et al., 2010). The inserted genes can be taken from the mosaic-like gene cassette within the integron, or they can be acquired from other cassettes in the genome of the host cell. In this manner, integrons can acquire and express multiple gene cassettes. The closer a given gene is to the *Pc* promoter in the cassette, the higher its expression. Integrons are not capable of autonomous mobilization and therefore cannot be strictly defined as MGEs; however, since they are often associated with plasmids and transposons, they are considered part of the mobilome. Although they can be found in Gram positives, they are predominantly associated with Gram negative bacteria (Partridge et al., 2009; Grape et al., 2005).

In clinically associated bacteria, integron GCs frequently harbour ARGs, and they are strongly associated with multidrug resistance due to their capacity to incorporate multiple GCs encoding for different antibiotic resistance mechanisms (Grape et al., 2005; Fluit and Schmitz, 2004; Ribeiro et al., 2011). Two major groups classify these genetic elements: chomosomal integrons and mobile integrons (Cambray et al., 2010). Chromosomal integrons carry accessory genes (up to 200 mostly of unknown function) that are strongly associated with the evolutionary history of Gram-negative bacteria, whereas mobile integrons contain a limited number of GCs frequently encoding for antibiotic resistance that are sometimes referred to as resistance integrons or multiresistance integrons (Boucher et al., 2007; Stalder et al., 2012).

Gene transfer agents (GTAs) are phage-like gene delivery systems integrated to the genome of a bacterial host that are capable of carrying and delivering genes encoding random traits from a donor to a nearby recipient genome by means of capsid proteins. GTAs evolved from prophages that lost their ability to target and pack their own DNA. In contrast to phage mediated transduction however, GTAs cannot transfer all the genetic information required for self-replication in the new host (Lang et al., 2012). The rate of occurrence and the impact on bacterial population of this newly described mechanism is not yet understood and therefore will be not be discussed in detail in this chapter.

A prerequisite for the functionality of all of the abovementioned MGEs following HGT is their compatibility with the transcriptional machinery of the recipient cell. This is, in fact, a *conditio sine qua non* for the access to the genetic content of these mobile elements and may limit the extent of HGT.

## Barriers to HGT

Despite the wide array of mechanisms described above, several factors may hamper the spread of genetic material among prokaryotes, thus circumscribing HGT events; these barriers can be briefly classified as genomic, physical, and functional (Popa and Dagan, 2011).

GC content and sequence similarity were shown to relegate HGT events to closely related taxonomic groups (Popa and Dagan, 2011; Majewski and Cohan, 1999). Acquisition of integrative genetic material (such as integrative plasmids) is based on a homologous recombination mechanism that is more likely to occur between high similarity sequences shared between phylogenetically related strains (Frost et al., 2005). Similarly, phylogeny may limit HGT in transduction due to the narrow host range tropism of bacteriophages (Doulatov et al., 2004).

Ecological and physical barriers can also shape networks of gene exchange and can restrict gene transfer to a specific habitat or niche. Close proximity of donor and recipient strains is a prerequisite for conjugation, as well as for transformation of unstable naked DNA (e.g., after a cell lysis). Additional mechanisms may also impose ecological restrictions of bacterial gene exchange; however, these are still not always completely understood (Smillie et al., 2011; Domingues et al., 2012; Nielsen et al., 2013). HGT may also be constrained by functional properties of the host strain and the acquired MGE. This may include low levels of MGE-host promoter compatibility as well as differences in codon usage (Popa and Dagan, 2011). Finally, maintenance of an acquired MGE is highly dependent on selective pressure; therefore, it should provide contextualized ecological benefits that outweigh the fitness cost of the additional bulk of DNA (Kümmerer, 2009).

### Correlations Between MGEs and ARGs: The Mobile Resistome

MGEs carrying ARGs have been comprehensively described in a multitude of environmental and clinical microbiomes. Nonetheless, a global understanding of the dynamics of these elements within and between clinical and natural environments is to a great extent an enigma. There is significant evidence supporting the "resistome" hypothesis, which postulates that natural environments provide an immense and only partially discovered pool of ARGs that under the proper conditions can be acquired and spread among clinically associated bacteria (D'Costa et al., 2006). This hypothesis is corroborated by two major observations: (i) the worldwide distribution of the most common ARGs is generally correlated with the chronological introduction of antibiotics in clinics; the most recent the molecule used to treat bacterial infections, the less abundant the diffused resistance and vice versa (van Hoek et al., 2011; Davies and Davies, 2010; Graham et al., 2016); (ii) mechanisms of genetic resistance to all known classes of antibiotics were found to be associated with one or more of the above-described MGEs (Carattoli, 2013; Stalder et al., 2012; Stokes et al., 2007; Martinez et al., 2015).

Several studies have provided evidence for HGT of MGE-associated ARGs from anthropogenically associated bacteria to bacteria from natural environments (Berendonk et al., 2015), and circumstantial evidence also indicates mobilization of these elements from more pristine to anthropogenic microbiomes (Forsberg et al., 2014). While the dynamics of HGT within and across natural (nonclinical) environments is poorly understood, some evidence suggests that these events are rare; for example, Forsberg et al. (2014) showed that in soil microbiomes, ARGs were less associated with MGEs than in clinical environments, leading to the hypothesis that HGT frequencies are significantly higher in clinical bacteria than in soil. While environmental boundaries may significantly constrain HGT of mobile ARGs, once acquired, these elements can rapidly disseminate across the globe (Nordmann et al., 2012), highlighting the magnitude of this phenomenon and the need for a deep understanding of its basis.

## Potential Mobilization of Antibiotic Resistance Genes in Wastewater Treatment Plants

Horizontal gene transfer is strongly implicated in the spread of antibiotic resistance from and to anthropogenic environments (Gillings and Stokes, 2012). However, pinpointing specific loci and factors fostering these events from a global perspective is extremely challenging. Recently, increasing attention has been given to the urban water cycle, with WWTPs being tagged as primary hotspots of ARB and MGEs. These elements are discharged with effluents into aquatic ecosystems or used for irrigation, where they can be transmitted through food and water webs to the human microbiome. The urban water cycle is considered to be "promiscuous" for using the human environment both as a provider and receiver of ARB/ARGs (Manaia et al., 2016; Berendonk et al., 2015; Rizzo et al., 2013; LaPara et al., 2011).

WWTPs receive high daily loads of sewage from diverse anthropogenic environments, including residential areas, hospitals, farms, and industry. These sources contain residual concentrations of active antibiotic compounds (ACs) as well as human and animal pathogens and commensals, many of which are resistant to antibiotics (Rizzo

et al., 2013; Caucci et al., 2016). Consequently, several studies conducted over the last decades have concentrated on measuring ACs and the diversity and abundance of ARB/ARGs in influents and effluents of WWTPs, while others have assessed the impact of different wastewater treatment approaches on elements associated with antibiotic resistance (Caucci et al., 2016; Munck et al., 2015; Szczepanowski et al., 2009; Guardabassi et al., 2002). Although a unifying and global comprehension of these complex systems is still missing, several studies support the current hypothesis that WWTPs are hotspots for antibiotic resistance. Nonetheless, additional studies must be conducted in this direction to determine the potential impact of WWTP effluents on the global propagation of antibiotic resistance.

Two factors in the wastewater treatment process that are generally indicated as the primary drivers of antibiotic resistance are: (i) the presence of persistent and transitory sublethal antibiotic concentrations (Le-Minh et al., 2010), which can select for antibiotic resistance determinants, and (ii) the profuse abundance and diversity of bacteria that are suspended in the water or localized in biofilms and flocs (Andersson et al., 2008). Although one of the aims of WWTPs is to significantly reduce bacterial loads from influent sewage, during the initial steps of the treatment indigenous WWTP biofilm- and floc-associated bacterial abundance actually increases. For instance, 10 g of activated sludge is estimated to host over 700 bacterial genera and 3000 species (based on operational taxonomy units at a 97% similarity cutoff) (Zhang et al., 2012; Grady et al., 2011).

## Sublethal Concentrations of Antibiotic Compounds and Potential Selection for Antibiotic Resistance Determinants

In spite of being commonly associated with clinical use, antibiotics may have much wider implications in natural ecosystems where they are detected at concentrations far below those used in clinics. This has led to the notion that, in some cases, they act as signaling or regulatory compounds that are involved in mediating competition (Aminov, 2009). This hypothesis is supported by the fact that at sublethal concentrations certain antibiotics can indeed trigger transcriptional responses that are responsible for adaptive benefits (Fajardo and Martinez, 2008; Martinez, 2009; Yim et al., 2007). Thus, we can discern between anthropogenically derived antibiotics, which can have lethal/inhibitory concentrations, as opposed to environmentally derived antibiotics that, at sublethal concentrations, have different implications.

Active ACs administered in animals and humans are discharged into sewage, where they are diluted to concentrations that are several hundred-fold below clinical minimal inhibitory concentrations (Karthikeyan and Meyer, 2006). Once in the WWTP, an antibiotic can remain in solution or can adhere to organic solid interfaces by sorption, depending on the chemical properties of the molecule (Zhang et al., 2014). Although significantly below minimal inhibitory concentration levels, these sublethal antibiotic levels can still impose a positive selection for resistance (Gullberg et al., 2014). For instance, Gullberg et al. (2011) showed that given the appropriate number of generations, subinhibitory concentrations of antibiotics create a selective advantage for resistance within a bacterial population, enabling a small initial fraction of resistant strains to out-compete nonresistant ones. The antibiotic concentrations used in these experiments were similar to those found in WWTPs and in certain anthropogenically

impacted soil and aquatic environments (Kümmerer, 2009), suggesting that selection of resistance strains can occur in these environments as well. In a recent two-year survey in the city of Dresden (Germany), Caucci et al. (2016) found that the overall burden of seasonal prescribed antibiotics for outpatients follows the same pattern of ARG abundance in effluent of a WWTP. Although this was shown in a single surveyed plant, this study provides a direct link between the quantity of antibiotics discharged in sewage and the magnitude of AR within WWTPs. Additionally, it supports the hypothesis that for a resistance-conferring MGE to be maintained in a given host population, there should be a minimum selective concentration of the related antibiotic, which depends on host genetic and environmental conditions (Kümmerer, 2009).

Several studies have shown that integrons are enriched during wastewater treatment (Di Cesare et al., 2016; Karkman et al., 2016). Sublethal concentrations of antibiotics may also facilitate resistance due to stimulation of recombination rates. A wide range of stresses including sublethal levels of antibiotics can induce the SOS response in bacteria that is known to stimulate integrase-mediated recombination in integrons (Guerin et al., 2009). Hence, as a consequence of antibiotic exposure, new GCs can be acquired and existing GCs can be rearranged within integrons, leading to increased levels of antibiotic resistance (Cambray et al., 2011).

## Cross-Resistance and Coselection: Two Potential Drivers of Antibiotic Resistance

Several studies have evaluated ARG abundance and diversity in tandem with concentrations of associated ACs; however, a 1:1 correspondence between an antimicrobial molecule and its resistance mechanism seems to be the exception rather than the rule (Martinez et al., 2015). This discrepancy can be potentially explained by either cross-resistance, whereby resistance mechanisms expand their specificity to different classes of chemical molecules (Martinez, 2009) or coselection, where exposure to one AC can facilitate resistance to another due to localization of multiple resistance mechanism on a single MGE.

Cross-resistance has been documented for extended-spectrum beta-lactamases, multidrug efflux pumps, and aminoglycoside-acetyltransferases (Sun et al., 2014; Shaikh et al., 2015; Casin et al., 2003). The multitude of chemical compounds in WWTPs (including an array of sublethal concentrations of antibiotics) is believed to stimulate cross-resistance through the evolution of bacterial resistance mechanisms, whereby they expand their capacity to a broader range of compounds.

MGEs such as integrons, transposons, and plasmids often contain different genetic elements (not necessarily resistance linked) in a mosaic-like structure, and each of them can be subjected to different selective pressures. Any positive selection stimulated by one of these genetic elements automatically and indirectly selects for the whole MGE and thus for the other genes. Hence, the selection of multiresistance MGE can be theoretically mediated by any of the selective agents (Di Cesare et al., 2016; Li et al., 2015). Therefore, coselection of antibiotic resistance determinants can be exerted from compounds other than antibiotics. For example, WWTPs often contain significant concentrations of heavy metals and disinfectants (quaternary ammonium compounds) (Seiler and Berendonk, 2012). Metal resistance genes are often colocalized on MGEs together with ARGs, and therefore the presence of heavy metals can select for

antibiotic resistance even in the absence of a particular antibiotic (Di Cesare et al., 2016; Li et al., 2015; Rosewarne et al., 2010). Not surprisingly, antibiotic-resistant bacteria are enriched at locations contaminated with heavy metals (Zhu et al., 2013; Hölzel et al., 2012; Stepanauskas et al., 2006). Standard WWTPs lack efficient systems for removal of heavy metals (Fu and Wang, 2011), and these pollutants may contribute to making WWTPs hotspots for resistance. In addition, the presence of heavy metals may have a synergistic effect on antibiotic resistance. For instance, Gullberg et al. (2014) found that a combination of very low concentrations of antibiotics and heavy metals has a substantially greater effect on resistance selection than the single compounds alone in bacteria harbouring a multiresistance plasmid. This finding suggests that low levels of mixed ACs, such those found in WWTPs, can be more than enough to select for multiple resistance genetic elements, contributing to their propagation in downstream environments.

## Approaches to Study Mobile Genetic Element–Associated Antibiotic Resistance Genes in Wastewater Treatment Plants

During the past decade, an array of methods has been applied to specifically target MGE-associated ARGs in WWTPs. These methods include culture-based and molecular techniques that both independently or combined provide useful insight into the dynamics of the associated antibiotic resistance determinants.

Culture-based assessments of bacteria in WWTPs are generally limited to selected commensal and pathogenic genera, specifically those associated with the human gut such as *Enterococcus* and *Enterobacteriaceae* (including *Escherichia* and *Klebsiella*), that can be isolated using selective media (Rizzo et al., 2013; Cheng et al., 2012; Kaplan et al., 2013). Because these methods can only be applied to a small fraction of cultivable bacteria, they cannot provide a comprehensive overview of environmental microbiomes (Daniel, 2005; McNamara et al., 2002). Nonetheless, these approaches can be combined with molecular-based techniques and robust sequencing to detect and monitor strains carrying ARG-harbouring MGEs, to subsequently elucidate mobilome dynamics within specific populations. For example, Kaplan et al. (2015) extracted and sequenced seven plasmids from WWTP sludge-derived multidrug resistant *Klebsiella* strains and found that while some of the plasmids appear to have originated in clinical environments, others seem to be indigenous to WWTP environments.

Quantitative polymerase chain reaction (qPCR) is currently a gold standard for monitoring ARG and MGE abundance in WWTPs. This pivotal technique can also be applied to track MGEs in complex environments in order to estimate the potential of HGT. For instance, qPCR targeting the transposon specific gene *tnpA* and the integron specific gene *int1* was used to evaluate possible enrichment of these MGEs in urban parks irrigated with recycled wastewater (Han et al., 2016). Similarly, in other studies, the abundance of transposon- and integron-associated genes was tracked in WWTPs to evaluate the possible enrichment of clinically associated class 1 integrons and transposons (Di Cesare et al., 2016; Karkman et al., 2016). The *int1* gene in particular is important in these and other related studies as it is indicated as a marker for anthropogenic pollution (Gillings et al., 2015; Bondarczuk et al., 2016).

Metagenomics, the sequencing of whole genomes from complex microbial communities, circumvents the limitations of culture-based methods (Hugenholtz and Tyson, 2008;

Sharpton, 2014) and therefore can significantly contribute to the capacity for evaluation of the fate of MGEs and ARGs in WWTPs in a more inclusive manner (Schmieder and Edwards, 2011). Interpretation of metagenomic data requires exhaustive bioinformatic analysis, necessitating expertise and a robust computer infrastructure. Nonetheless, metagenomics has revolutionized understanding of environmental microbiomes and has been successfully applied to WWTPs, where it has elucidated bacterial community structures (Munck et al., 2015), indicated the presence of pathogens (Ye and Zhang, 2011), and helped with the identification of ARGs and MGEs (Zhang et al., 2011). Screening metagenomic data to elucidate WWTP mobilomes can provide a valuable snapshot of the prominent MGE-associated ARGs present and may be useful to monitor the effects of effluent discharge on downstream environments.

Different platforms and technologies are available for sequencing metagenomes, including 454, Illumina, SOLiD, Ion Torrent, and Pacific Biosciences (Hodkinson and Grice, 2015). These sequencing platforms differ in output read length and number, coverage, amount of starting DNA required, and costs. The length of output reads is pivotal for reassembling metagenomes into contigs, as shorter reads require higher computational efforts. To date, the longest read lengths (up to 10 kb) are generated by the Pacific Biosciences (PacBio) platform, which conversely requires a high amount of DNA per run (0.5–5 μg) (Buermans and den Dunnen, 2014). Newly emerging long-read sequencing platforms are pivotal in elucidating MGEs from complex metagenomes because these often contain repetitive sequences and are difficult to assemble.

Plasmids have been subjected to intense analysis in WWTPs and downstream environments due to their high genetic content and their extended mobility potential (Gatica and Cytryn, 2013; Rahube et al., 2014; Jechalke et al., 2015). However, in contrast to integrons and transposons, plasmids lack unique representative genetic sequences that universally characterize them. The host range varies from plasmid to plasmid and they are not generally physically linked with the bacterial chromosome (Carattoli, 2013). Additionally, they vary in genomic content and size, and therefore, their study has more constraints and requires customized approaches according to the scope of the analysis. Metagenomics is a valid approach for acquiring a comprehensive picture of plasmid composition in complex environments such as WWTPs (coined the plasmidome). However, it requires an unbiased and representative extraction of plasmids from the analyzed community. Different-size plasmids facilitate differential extraction efficiencies, and therefore the extraction of both small and large size plasmids from environmental matrices may require different methods (Norman et al., 2014). Protocols for plasmid extraction directly from complex environmental bacterial communities without cultivation steps have recently been developed. During plasmid isolation, a Plasmid Safe DNase treatment is applied to reduce genomic contamination and the plasmids are subsequently amplified (Zhang et al., 2011; Kav et al., 2012). These methods significantly enrich plasmid DNA and enable acquisition of both small and large plasmids from complex environments (Norman et al., 2014; Kav et al., 2012).

Metagenomic-based analyses have recently been applied to elucidate WWTP plasmidomes. For example, Li et al. (2015) compared the plasmid and whole metagenomes of two urban WWTPs. The retrieved plasmidomes predominantly contributed to the resistome of the bacterial communities at different stages of the treatment and showed a diverse ARG profile in both diversity and abundance (as compared to the resistomes of the total metagenomes). From these plasmidomes, 323 ARG subtypes were

identified, with tetracycline, β-lactam, and quinolone resistance genes being the most abundant. Interestingly, plasmid metagenomes from WWTP influents contained substantially more ARGs than those from activated sludge, suggesting that many plasmids may not persist in the WWTP process. Sixteen novel plasmids with a complete circular sequence were assembled and annotated, five of which were predicted to contain ARGs (Li et al., 2015).

Metagenomic approaches detect functional genes by aligning generated sequencing data with annotated databases. It is therefore possible that many of the ARGs identified in WWTPs using this *in-silico* approach may not encode for active antibiotic resistance mechanisms. Furthermore, functional genes that were not previously annotated cannot be computed and will be overlooked.

When a whole community is analyzed using metagenomics, the analyzed DNA is fragmented to relatively short sequences whose size depends on the platform used (Sharpton, 2014); thus, the assignment of transposons and integrons equally distributed between two or more phylogenetically distant host strains could be difficult in computational analysis. This becomes impossible when it comes to establishing a linkage between a host cell and a single plasmid, as the latter is physically separated from the chromosome. To cope with this, a novel PCR-based method called Emulsion Paired Isolation and Concatenation PCR (epicPCR) was recently developed (Spencer et al., 2016). This technique allows the amplification of two genes within single cells that are encapsulated in polyacrylamide beads. These can include the 16S rRNA gene for phylogenetic identification (Sharpton, 2014) of the encapsulated bacterium, and any other functional gene of known sequence, such as those encoding for specific ARGs, transposases, integrases, and plasmid incompatibility determinants. The system is devised in a way such that two initially separated sequences are amplified, producing one single amplicon that can be subsequently purified and sequenced. This approach could be highly promising for HGT tracking in WWTP samples and in downstream environments, as it can be applied on whole bacterial communities. Its limitation is that only one gene per time can be sorted besides the 16S rRNA gene marker. For example, if the chosen gene identified a class of MGE, such as a transposase-encoding gene, the content of that MGE would remain unknown. Conversely, associating one single ARG with a given host strain would not tell whether its genomic location is on one MGE or another. Nevertheless, epicPCR could be combined with the other methods described above to elucidate HGT dynamics in WWTPs.

As described above, PCR- and metagenome-based approaches do not enable functional assessment of identified ARGs. This obstacle can be circumvented by applying functional-based approaches that express environmental ARGs in vectors. Transposon-aided capture (TRACA) and functional metagenomics are two such approaches that have been applied for functional characterization of MGE-associated ARGs in WWTPs (Parsley et al., 2010; Zhang et al., 2011). Both of these high-throughput methods are based on cloning DNA fragments in expression vectors, transforming them to competent antibiotic sensitive hosts, and testing the transformants for resistance on selective antibiotic-containing media. Afterward, the cloned insert is retrieved and sequenced to identify the gene that confers resistance. These methods are generally time consuming but are suitable for identifying novel ARGs with a functional approach, and they often enable the detection of flanking MGEs involved in the transfer of these genes. They are also limited by the host strain, which may not possess mechanisms required for

expression of the captured ARGs. Zhang et al. (2011) combined TRACA and metegenomic analyses to pinpoint plasmids from activated sludge in a WWTP in Hong Kong. They were able to identify multiple plasmids that were previously annotated, as well as class 1, 2, and 3 integrons and several transposons. Additionally, two novel plasmids were acquired and 35 ARGs and several virulence factors were identified in the retrieved plasmidome (Zhang et al., 2011).

Each of the methods described above has limitations and therefore, a comprehensive understanding of MGE dynamics in WWTP undoubtedly requires integrated approaches that combine more than one method thereby obviating the limitations of individual different techniques. Recent studies showed that the combination of metagenomics and qPCR might provide a more comprehensive and quantitative insight into the dynamics of microbial communities and ARGs during wastewater and sludge treatment (Ju et al., 2016; Aydin et al., 2015). Additional data from a larger number of WWTPs is necessary to validate the specificity and sensitivity of each technique, and standardization of these methods is required for routine monitoring. Finally, whenever possible culture-based techniques should be used to support molecular-based techniques in order to associate genotype and phenotype in to a unified set of data.

A concerted effort is required to translate data generated on the abundance and diversity of ARGs and MREs to parameters that can be applied for risk assessment. Such an approach was eloquently demonstrated in a comprehensive analysis of aquatic metagenomes along an environmental gradient ranging from strongly anthropogenically impacted aquatic sources (WWTP effluents) to pristine marine and freshwater ecosystems (Port et al., 2014). The metagenomes were screened for ARGs, and an AR index that quantifies antibiotic resistance potential was established for each ecosystem. Applying this methodology, the authors established a unique measure for screening different types of environments with a single approach, generating an AR propagation potential index for each of them.

## Effects of Wastewater Treatment on the Fate of Mobile Genetic Elements Associated With Antibiotic Resistance

Wastewater treatment effectively reduces bacterial load, but it is still not clear whether this reduction affects ARB and ARG abundance equally or if there is a preferential advantage for resistance determinants. This discrepancy is mainly due to a limited number of studies and the lack of standardized methods for evaluating ARB and ARG abundance (Manaia et al., 2016). The ultimate fate of ARGs in WWTPs seems to vary between facilities in different studies, and a common pattern of abundance cannot be easily elucidated. Furthermore, the rate of HGT events that occur during the treatment cannot be accurately established using currently available technologies, and the mere comparison between influent and effluent ARG abundance provides only partial understanding of these events. Despite the above limitations, a number of common trends observed in recent studies shed light on ARG dynamics in WWTPs.

Studies targeting single WWTPs from Laht et al. (2014) and Karkman et al. (2016) concluded that overall, wastewater treatment does not significantly impact ARG abundance; however, Karkman et al. found that the relative abundance of transposon- and class 1 integron-associated genes increased in effluents. Increased abundance of Class 1 integrons in WWTP effluents has been shown to be strongly linked to heavy-metal resistance genes as well as specific ARGs (i.e., *sulI*), providing a snapshot of HGT

dynamics in WWTPs and posing questions regarding the risk associated with the discharge of these MGEs in downstream environments (Di Cesare et al., 2016; Du et al., 2014; Chen and Zhang, 2013).

In contrast to the concerning scenarios described above, more optimistic studies recently suggested limited levels of ARG and HGT dissemination from WWTPs. Munck et al. (2015) characterized a unique "core resistome" in WWTPs with little similarity to clinically defined resistance mechanisms or to resistomes of downstream environments. They discovered that less than 10% of this core resistome is detected outside of the plants, equating the risk associated with final effluents of WWTPs with that of the native soil resistome (Munck et al., 2015). Kaplan et al. (2015) reported preferential phylogenetic associations between a set quinolone-resistance plasmids in *Klebsiella* strain hosts, isolated from a large WWTP in Israel, suggesting that many WWTP-associated MGEs may be constrained by phylogenetic boundaries, further limiting their capacity to propagate in downstream environments.

Despite these optimistic scenarios, inter- and intraenvironmental transmission of ARGs by HGT may occur at very low rates and ranges and therefore should not be taken for granted given the presence of MGEs. These studies suggest that quantitative analyses alone may not be sufficient to infer the risks associated with the abundance of ARG. Parameters other than the simple abundance of an MGE and/or ARG should be evaluated to assess risk potential, and caution should be taken when making inferences regarding the dissemination of these elements to downstream environments.

Several studies have assessed the impact of specific WWTP processes and disinfection technologies on ARB and ARG abundance, and some of these have specifically focused on MGEs. For example, Tian et al. (2016) speculated that both vertical and horizontal gene transfer is significantly restricted in anaerobic digestion processes, and therefore they evaluated the resistomes and mobilomes of mesophilic (35°C) and thermophilic (55°C) sludge using metagenome sequencing and quantitative PCR. Following a single-step temperature increment, the thermophilic sludge harboured a significantly smaller resistome and a condensed mobilome (including plasmids, insertion sequences, and integrons) relative to the mesophilic sludge. Additionally, the abundance of specific bacterial taxa postulated to carry the analyzed ARGs also decreased. Based on these results, the authors concluded that HGT frequency decreases under thermophilic conditions (Tian et al., 2016). This evidence suggests that this type of intervention may be introduced in industrial scale systems to limit HGT events in secondary treatments, thus limiting ARGs release in effluents.

Ferro et al. (2016) monitored bacterial growth and ARG abundance in WWTP effluent that underwent UV/$H_2O_2$ disinfection processes. Although the analyzed bacteria were clearly inactivated by the disinfection, the ARGs were not effectively removed from the effluent (Ferro et al., 2016). In the presented scenario, it is not clear whether these genes are still active and, if so, whether they can be horizontally transferred to microbiomes in downstream environments.

## Antibiotic Resistance Genes and Mobile Genetic Elements in Effluents and Downstream Environments

WWTP effluents can be recycled for agriculture or discharged into aquatic environments. In this context, it has been postulated that these effluents are significant point

sources of ARGs and that they can contribute to the propagation of antibiotic resistance in surface waters and other downstream environments (LaPara et al., 2011; Berglund et al., 2015; Rodriguez-Mozaz et al., 2015; Czekalski et al., 2014). Despite the immense global scope of WWTP discharge, little is known about the potential and the frequency of HGT of ARGs from effluents to these downstream environments, or about the abundance of MGEs such as plasmids and integrons.

Berglund et al. (2015) evaluated the abundance of seven ARGs encoding resistance to six classes of antibiotics, and the *int1* gene for class 1 integrons, in sediments from a river receiving WWTP effluents. The authors observed a ten-fold increase in the relative abundance (gene copies/16S rRNA gene copies) of the analyzed ARGs and *int1* in the sediments downstream from the WWTP relative to those upstream. Notably, the abundance of integron integrases was relatively uniform up to 2.5 km downstream to the discharging point. The level of related ACs was evaluated before and after the point of discharge; however, all tested compounds were below detection levels, suggesting that selective pressure was not a factor in these sediments (Berglund et al., 2015).

LaPara et al. (2015) examined the effect of 14 WWTP effluents on the abundance of ARGs (conferring resistance to tetracycline, sulfonamides, macrolides, and fluoroquinolones), and *int1* and genes associated with plasmids from incompatibility group A/C in the upper Mississippi river water column. This qPCR-based survey revealed that the abundance of the analyzed elements was clearly higher in WWTP effluents than in the receiving river, and this was not correlated to a statistically significant difference in bacterial abundance (based on 16S rRNA gene analysis). Increased abundance of *int1* and the ARGs was consistent for the relatively long stretch probed (>600 miles). IncA/C plasmid markers were detected in effluents but not in the receiving river, suggesting that these MGEs do not persist in downstream environments (LaPara et al., 2015). Although effluents are regarded as a significant point source of antibiotic resistance given the high volume of WWTP discharge, the authors concluded that the huge dilution effect coupled with the natural decay of the incoming antibiotic resistance determinants buffered the potential risk of the effluents, at least in the aqueous phase of the river.

Few studies have assessed the contribution of bacteriophages in transduction of ARGs in WWTPs, such that current knowledge is highly obscure. Nevertheless, recent metagenome-based analyses of hospital wastewater (Subirats et al., 2016) revealed that the relative abundance of ARGs in phage DNA was significantly higher than bacterial genomic DNA, indicating that this HGT mechanism may have a greater role in the propagation of antibiotic resistance than previously thought.

The effect of treated wastewater (TWW) discharge on aquatic environments might thus change depending on the environmental characteristics of the sample analyzed. However, in aqueous environments, given the consensus that aquatic bodies receiving WWTP effluents predominantly have an overall higher concentration of antibiotic resistance determinants than that of the receiving surface water, it seems realistic to postulate that a risk of AR propagation exists. Further surveys are still needed to better understand the contribution of MGEs to HGT of ARGs in water environments (Pruden et al., 2013).

A different scenario has been observed for wastewater reuse in agriculture. Treated wastewater irrigation and the application of biosolids from WWTPs as fertilizers are commonly practiced worldwide, especially in arid or semiarid regions, characterized by limited access to freshwater. The considerable amount of active ACs as well as ARGs

and ARB in TWW and biosolids from WWTPs poses questions about clinical risks associated with dissemination of AR in food and water webs. The ultimate risk is associated with acquisition of antibiotic resistance determinants from pathogenic strains that can threaten human health, a phenomenon strongly demonstrated in the 2011 Germany *E. coli* O104:H4 outbreak (Frank et al., 2011; Mellmann et al., 2011).

Several studies have explored the effect of treated wastewater irrigation and biosolid application on the soil resistome in an attempt to evaluate trends in ARGs and MGEs. Differences in physicochemical composition and bacterial load of aqueous and soil matrices complicates identification of common trends, as does the presence of significant levels of background resistance in soil microbiomes (Negreanu et al., 2012). Nonetheless, an immediate increase in ARG abundance following treated wastewater irrigation seems to be a common trend in agriculture (Han et al., 2016; Jechalke et al., 2015; Wang et al., 2014a; Wang et al., 2014b). Moreover, a few studies have also found a positive association between ARG abundance and the abundance of mobilome-associated gene markers (mainly class 1 integrons and clinically relevant transposons), indicating a potential involvement of HGT in the spreading of AR to the receiving soils (Wang et al., 2014a; Wang et al., 2014b). Despite temporal increases in AR, several studies have revealed that stimulation of AR increase following TWW/biosolids application is highly transient (Gatica and Cytryn, 2013; Negreanu et al., 2012; Riber et al., 2014). This observation can be explained by the capacity of the soil microbiome to outcompete effluent-derived ARBs, or by a reduction in selective pressure due to reduction in antibiotic concentrations between the effluent and soil (Gatica and Cytryn, 2013; Riber et al., 2014).

Despite the above, other studies suggest that anthropogenic amendments can result in extended propagation of soil resistomes. For example, a recent study conducted in Denmark analyzed archived soil samples subjected to manure application or inorganic fertilization (as a control) over an 87-year time span by applying qPCR to quantify four β-lactam ARGs (*bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{OXA}}$, and *bla*$_{\text{CTX-M}}$) and the class 1 integron integrase (int1) (Graham et al., 2016). Remarkably, the abundance of these genes over time was roughly correlated with the historical appearance of clinical isolates in Denmark. Additionally, there was a chronological reduction in the abundance of *bla*$_{\text{CTX-M}}$ following the banning of nontherapeutic use of antibiotics in Denmark, suggesting that suspension of manure application could lead to a partial reinstatement of ARG abundance to a baseline level. Interestingly, the *int1* gene was the only one that continuously increased over time only in manure amended soils, possibly due to exposure to a different selective pressure (such as the presence of heavy metals). This finding suggests that *int1* can be used as a robust proxy for manure application. Moreover, the persistence of such MGEs even in the absence of selective pressure directly imposed from ACs could imply higher HGT potential; in other words, an integron-rich community is likely to more easily uptake ARG cassettes and more easily adapt to changing conditions.

ARG abundance on edible vegetables from manure amended soils was assessed by Rahube et al. (2014). Since the consumption of vegetables from manure-amended or TWW-irrigated soils represents a path by which ARB can threaten human welfare, the authors screened various edible vegetables harvested from manure-amended and nonamended soils for the presence of 46 gene targets associated with plasmid incompatibility groups, integrons, and ARGs. Enumeration of culturable bacteria on selective

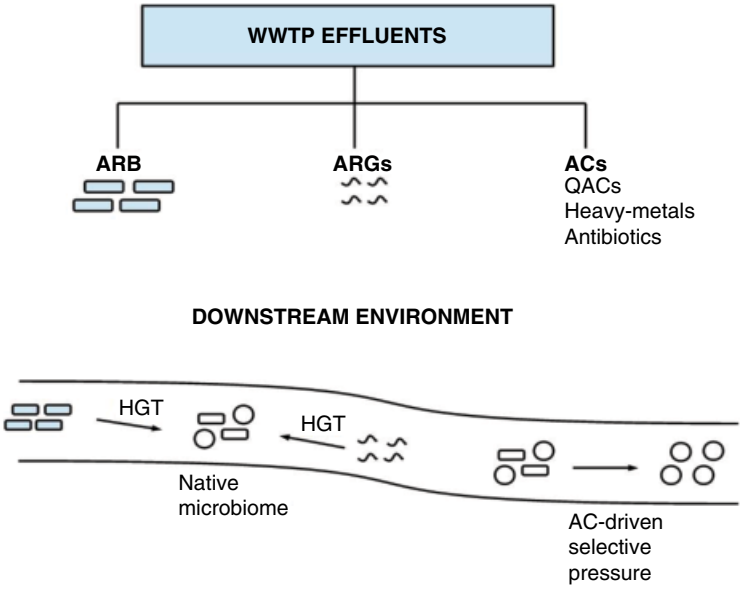


**Figure 8.3** Hypothetical scenario depicting the impact of WWTP effluents on downstream environments. Bacteria from native aquatic and terrestrial microbiomes can acquire ARGs by HGT of effluent-derived ARB or ARGs, by conjugation or transformation, respectively. Additionally, ACs in effluents can exhert selective pressure on the native microbiome, enhancing soil- or water-born ARG abundance. (*See insert for color representation of the figure.*)

media showed no differences in antibiotic resistance between the two soils. Nevertheless, in a qPCR-based screening, antibiotic resistance determinants (including markers for plasmid incompatibility group) were only detected in vegetables from manure-applied soils before harvest and not in controls (Rahube et al., 2014). Although similar experiments have not been conducted to validate these results, this work is consistent with the posed question of AR spreading through agricultural routes. Moreover, it demonstrated that even preharvest manure application may be enough to pose a substantial public health risk.

A critical observation for all the above mentioned studies is that they were not always able to link a given antibiotic resistance determinant to a phylogenetically defined set of host strains, which considerably hinders the understanding of horizontal transfer dynamics. The mere detection of an ARG could be linked to different sources, including (i) discharge of ARB from WWTP effluents that eventually take part of the new ecosystem composition, (ii) HGT of effluent-derived MGEs having the downstream bacterial ecosystems as receiver of new ARGs, and (iii) acquired AR selection in native aquatic and soil microbiomes imposed from a perturbed chemical composition (Karah et al., 2016; Czekalski et al., 2014; Wang et al., 2014) (Figure 8.3); additionally, a combination of these factors cannot be excluded.

## Conclusions and Future Directions

Microbial genomes are characterized by rapid evolutionary rates that are stimulated by HGT, which serves as a flexible means of adaptation. Their capacity to acquire and donate MGEs is a fundamental trigger in propagation of antibiotic resistance. The water

cycle connects urban communities, WWTPs, and downstream environments in a promiscuous circle in which every step potentially plays a role in fostering ARG enrichment and spread through a complex combination of positive selective pressure and optimal conditions for HGT. Although a clear picture of these dynamics is far from being achieved, evidence that WWTPs are sources for propagation of antibiotic resistance in downstream environments necessitates further research targeting these environments that can be used to establish true epidemiological risk assessments. Consolidated approaches applying several of the methods described above should be implemented, which may include the following:

1) Application of culture-based methods to identify keystone bacterial strains that show elevated frequencies of antibiotic resistant determinants associated with HGT.
2) Application of complex molecular methods (such as PCR-based technologies, functional metagenomics, and metagenomics) in order to establish a comprehensive toolbox of genetic proxies that can be applied for tracking effluent-associated ARGs and MGEs in downstream environments. These genes should be scarce in downstream environments (background) and profuse in WWTP effluents.
3) Development of inclusive MGE transmission models based on microcosm experiments that integrate culture-based and molecular analyses described above. These models can be applied to evaluate HGT potential in diverse environmental niches within and downstream of WWTPs (e.g., biofilms, flocs in aqueous phase effluent pipes, and sediments or aqueous phase in receiving surface water).
4) Assessment of the efficiency of various WWTP treatment technologies in the removal of MGE-associated ARG dynamics, by applying the above-described tools in both bench-scale microcosm and full-scale systems. This can divulge the most suitable treatment methods for neutralizing or limiting HGT and customize the best technology according to different types of influents in WWTPs.
5) Development and application of easily applied routine methods for "quality control assessments" of MGE-associated ARGs. These methods can be transferred to stakeholders and should be included among the other biological risk assessments in order to better direct the possible uses of TWW effluents.

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# 9

# Bacterial Diversity and Antibiotic Resistance Genes in Wastewater Treatment Plant Influents and Effluents

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Due to the worldwide health impacts of antibiotic resistant pathogens, scientists are increasingly interested in the role of wastewater treatment plants (WWTPs) as a sink and source for antibiotic resistant bacteria and their genes. To date, the dynamics of resistant bacteria and associated genes in municipal WWTPs remains relatively unexplored, but there is clear evidence that antibiotic resistant organisms and genes are released with WWTP effluents to receiving environments. Studies have demonstrated that the absolute quantity of antibiotic resistance gene copies are reduced during conventional wastewater treatment, but it is also apparent that relative abundances of key resistance genes normalized by 16S rRNA copy numbers frequently show no significant reduction and sometimes even increase. This pattern appears to be WWTP specific, however, and there are also cases where the relative abundances of some resistance genes have reportedly decreased from WWTP influent to effluent. In this context it is also important to investigate the microbial composition of WWTP influents and effluents, and our review of the existing data suggests that the rarer species of WWTP microbial communities may have a relatively large impact on the abundance of antibiotic resistance genes. In this chapter, we summarize these results and discuss their implications for the spread of resistance genes within the wider environment. A discussion of methodological considerations relevant to the study of antibiotic resistance in complex microbial communities is also included.

## Introduction

Modern sanitation and wastewater treatment are intended to protect downstream environments and prevent the spread of infectious diseases, and fortunately, as effluent monitoring and epidemiological data attest, contemporary treatment technologies go a long way toward achieving these goals. Nevertheless, the chemical and microbiological contaminants in wastewater are continually changing as a result of

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social, medical, commercial, and industrial changes. This constant flux brings unavoidable uncertainty to the task of water regulation and makes ongoing assessment of wastewater treatment efficacy for contemporary conditions essential. New challenges include a diverse group of influent constituents loosely referred to as "contaminants of emerging concern." Pharmaceuticals, including antibiotics, are a key subgroup within this category (Sauvé and Desrosiers, 2014; Petrie et al., 2015), and the term is also increasingly used in reference to biological pollutants such as antibiotic resistant bacteria and their genes (ARB and ARGs) (Rysz and Alvarez, 2004; Gao et al., 2012; Berendonk et al., 2015).

Understanding the dynamics of ARB and ARGs in the urban water cycle is an increasingly important goal as antibiotic resistance is recognized as one of the greatest human health challenges of the twenty-first century (WHO, 2012) and wastewater is suspected to be a major hotspot for the evolution and propagation of antibiotic resistant microorganisms (Rizzo et al., 2013; Berendonk et al., 2015). ARB and ARGs present particularly complex challenges for risk assessment and environmental management because they are both highly dynamic and subject to significant knowledge gaps and technical measurement difficulties. New combinations of resistance genes are continually evolving via genetic mutation and recombination, and genetic exchange across phylogenetic barriers (i.e., horizontal gene transfer) can also occur (Berendonk et al., 2015). Conventional microbiological monitoring and regulatory approaches for wastewater effluent management do not specifically address or manage ARB and ARG related risks, and an increasing amount of research is therefore being conducted to address this important gap. Efforts to communicate the implications of widespread antibiotic resistance to politicians and the broader public are also increasing as the impacts of resistant pathogens on human health become more evident (WHO, 2012; McKennan, 2013).

Municipal wastewater treatment plants (WWTPs) were originally designed to remove solids and nutrients from wastewater and to reduce the microbial load. Effective removal of metals and other industrial pollutants was also desirable, and subsequent research has focused on optimizing these processes. Research is now being directed toward enhancing the removal of micropollutants such as antibiotic residues and antibiotic resistant bacterial strains and mobile genetic elements. Although these are present in low concentrations relative to many other wastewater pollutants, it is possible that their impacts may greatly outweigh their relative concentrations, and as ARB and ARGs were not among the pollutants that WWTPs were originally designed to remove, it is not surprising to find that current treatment techniques may be suboptimal when it comes to this challenge (Batt et al., 2006; Munir et al., 2011). Ironically, it is even possible that biological treatment, disinfection, and advanced oxidation processes designed to improve effluent quality and remove recalcitrant pollutants could potentially be adding to the risk of ARB and ARG selection and propagation by selecting for highly robust microbial communities that are inherently resistant to key physical and chemical stressors, including chlorine, UV radiation, antibiotics, and other coselection factors (e.g., metals). As documented later in this chapter, all treatment plant effluents contain some residual microbial load; therefore, it is unavoidable that some of these robust bacteria, including ARB and ARGs, will be emitted into downstream environments and reuse networks. Understanding and mitigating the associated risks is rapidly becoming a high-priority research topic.

## Bacterial Community Structure in Wastewater Treatment Plants

WWTPs are considered hot spots of microbial diversity and resistance because they receive contaminated wastewater from diverse sources and contain a variety of different environments (e.g., settling tanks, activated sludge systems, anaerobic digesters, etc.) with dense bacterial loads (Schlüter et al., 2007a). Bacteria are present throughout the entire wastewater collection and treatment network, that is, from sewer to effluent, and play a key role in secondary treatment processes such as activated sludge treatment and aerobic and anaerobic digestion. The bacteria participating in these secondary treatment processes have frequently received detailed study, both to understand their relative abundance and their specific functions and/or functional redundancy (e.g., Siripong and Rittmann, 2007; Wells et al., 2011; Wang et al., 2016). By contrast, very few studies published to date have reported specifically on the identity and diversity of microbial communities in WWTP influents and effluents, although this has started to change as the demand for (safe) recycled water sources increases and culture-independent diversity analysis approaches are progressively adopted throughout the research community.

Generally speaking, municipal wastewater has been found to maintain highly diverse microbial communities, including both pathogenic and nonpathogenic species, human and animal commensal species, and environmental bacteria. These include species and strains that are both resistant and susceptible to antibiotics and other chemical and abiotic stressors. Clearly, as the influent stream passes through different treatment stages and environments, the structure of the microbial community will change as some species may be outcompeted, unable to survive or reproduce, or simply encyst or form endospores until more favourable conditions arise. Some of these organisms will be released into the wider environment with WWTP effluents, including both pathogenic bacteria and antibiotic resistant/multiresistant bacteria that may or may not be virulent (Varela and Manaia, 2013).

## Bacterial Communities in WWTP Influents and Effluents

Until relatively recently, bacterial screening of WWTP influents and effluents usually focused on specific bacterial taxa, especially medically relevant pathogens such as *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Acinetobacter* spp., *Aeromonas* spp., etc. (e.g., Goni-Urriza et al., 2000; Boczek et al., 2007; Zhang et al. 2009). The relatively narrow range of species that were typically examined (considering the broad diversity of wastewater bacterial communities) was largely due to methodological constraints as earlier studies relied on culture-based techniques. A detailed discussion of methodological considerations pertinent to this topic is provided toward the end of this chapter, but it is important to note that method bias did result in much of the wastewater microbial community remaining unrecognized/undetected during earlier monitoring studies. This situation has changed significantly in recent years as culture-independent methods (e.g., DGGE, qPCR, 16S rRNA high throughput sequencing, and metagenomics) have been increasingly developed and applied. Comparative assessment of influent and effluent microbial communities is now becoming increasingly common as researchers strive to determine which bacteria and genes are preferentially selected

or removed during wastewater treatment, and whether this differs according to geographic location or other abiotic factors such as contaminant loading.

Differences in local industry, climate, population loads, and demographics all have an impact on influent wastewater characteristics. For instance, some locations may be affected by extreme temperatures or by high salinity caused by coastal groundwater infiltration or industrial effluents. Moreover, treatment regimens also vary on a local scale, ranging from simple primary treatment systems to sophisticated tertiary treatment and reuse schemes. Given this inherent variability in influent characteristics and treatment, it is not surprising that influent and effluent bacterial communities differ among WWTPs, or that temporal variations within individual WWTPs are observed. For example, McLellan et al. (2010) found greater similarity between samples collected on the same date from two WWTPs in the same metropolitan area (Milwaukee, USA) than between samples collected from the same WWTP on different dates, and VandeWalle et al. (2012) reported inverse seasonal patterns in two dominant *Acinetobacter* spp. within the same Milwaukee sewer catchment. Novo et al. (2013) also reported temporal variations in influent and effluent community profiles. Our own research in Germany only indicated minor seasonal changes in influent community composition; these were not statistically significant and were linked to extreme rainfall events and flooding (Caucci et al., 2016). Interestingly, McLellan et al. (2010) reported that microbial communities in the Milwaukee sewer infrastructure (a combined sewage/stormwater system) were dominated by microbes of nonfecal origin, indicating that a large proportion of WWTP influent bacteria are of environmental origin.

At higher taxonomic levels, or phyla, the most abundant taxa tend to be similar across different WWTPs. For example, a number of studies have reported WWTP influent communities dominated by *Proteobacteria*, although the relative contribution varies. McLellan et al. (2010) and Ye and Zhang (2013) reported that *Proteobacteria* composed close to or more than 60% of the total influent bacterial community in WWTPs in the USA and Hong Kong, respectively. Yang et al. (2014) also reported *Proteobacteria* to be the dominant phylum in influent at the same Hong Kong WWTP but with a lower percentage contribution (40.5%). Among the *Proteobacteria,* McLellan et al. (2010) found the *Gamma-* and *Betaproteobacteria* to be the most abundant in influent communities and *Epsilonproteobacteria* the least abundant. The relative abundance of *Gammaproteobacteria* and *Betaproteobacteria* in influents is not consistent between WWTPs, however, with some studies reporting a predominance of *Gammaproteobacteria* (McLellan et al., 2010; Ye and Zhang, 2013) and others reporting a greater abundance of *Betaproteobacteria* (Novo et al., 2013). These variations may be partly related to methodological differences. *Proteobacteria* also appear to be a dominant phylum in effluent communities, with Ye and Zhang (2013) and Yang et al. (2014) reporting that *Proteobacteria* made up 60% and 50.8%, respectively, of the effluent community at Shatin WWTP (treating saline sewage) in Hong Kong.

On the basis of the current literature, *Proteobacteria* are followed in relative abundance in wastewater influents by *Actinobacteria*, *Bacteroidetes*, and *Firmicutes*. The percentage contributions of these phyla vary, but according to McLellan et al. (2010) they composed <40% of the total influent community. Ye and Zhang (2013) reported smaller proportions of *Actinobacteria*, *Bacteroidetes*, and *Firmicutes* in the Shatin WWTP influent, although they were still the dominant phyla after *Proteobacteria*. In total, Ye and Zhang counted 19 different phyla in the influent samples and 23 in the

effluent samples from the same plant but noted that rarefaction curves indicated that greater sequencing depth would yield additional species from these samples. Yang et al. (2014) also reported a large proportion (40.9%) of *Firmicutes* in the influent, but this fell to 4.43% in the effluent. In their study, *Actinobacteria* and *Bacteroidetes* were more abundant in the effluent than in the influent, but the general findings that these four phyla are the most numerous still held.

The results from Ye and Zhang (2013) showed that the most dominant orders in the influent sample were *Desulfobacterales*, *Clostridiales*, *Desulfovibrionales*, *Lactobacillales*, and *Bifidobacteriales*. This differed from the dominant effluent populations, which included *Vibrio*, *Mycobacterium*, and *Pseudomonas*. Our recent work in Germany (Caucci et al., 2016) also demonstrates differences in influent and effluent communities, with the effluent sample communities showing greater divergence than the influent samples. Figure 9.1 presents an example from our recent work in Australia, showing the dominant taxa in the final treatment stages of two colocated WWTPs. These data demonstrate that effluent community structure is WWTP dependent; they also demonstrate clear and consistent effects of disinfection processes on effluent microbial communities with pre- and postdisinfection communities forming separate clusters. The microbial response to disinfection is complex. For example, Figure 9.2 presents a breakdown of the relative abundance of *Firmicutes* in these two WWTPs before and after disinfection and shows a significant decrease in *Firmicutes* from 10% pre-UV to 0.4% post-UV (including a decrease of *Clostridium XI* from 3% to 0.3%). This is in stark contrast to an increase in the relative abundance of *Firmicutes* from 5% prechlorination to 11% postchlorination (including an increase in *Clostridium XI* from 2% pre-Cl to 6% post-Cl). *Clostridium X1* includes a number of a spore forming bacteria, including *Clostridium difficile* (Collins et al., 1994).

Differences in the bacterial communities of different WWTPs were also demonstrated in the sequencing-based studies by McLellan et al. (2010) and Ye and Zhang (2011, 2013). The former reported higher diversity in the influent community (>3000 operational taxonomic units (OTUs)), whereas Ye and Zhang recorded just over half that, with 1667 OTUs. McLellan et al. (2010) did not investigate the effluent

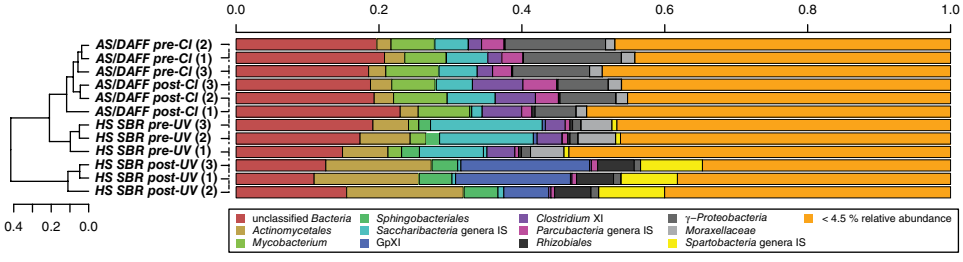


**Figure 9.1** Taxonomic annotation-based hierarchical clustering of dominant taxa (>4.5% of the total community) in pre- and postdisinfection effluent samples from two colocated wastewater treatment plants in Australia. AS/DAFF indicates activated sludge (AS) treatment followed by dissolved air flotation and filtration (DAFF). HS/SBR is a sequencing batch reactor (SBR) treating high salinity (HS) influent. Results are based on total DNA extraction from 1L water samples (0.22 µm filtered) and Illumina MiSeq partial 16S rRNA gene sequencing. This figure shows triplicate samples from a single sampling date for each site, including pre- and post- chlorination at the AS/DAFF plant and pre- and post-UV disinfection at the HS/SBR plant. (*See insert for color representation of the figure.*)

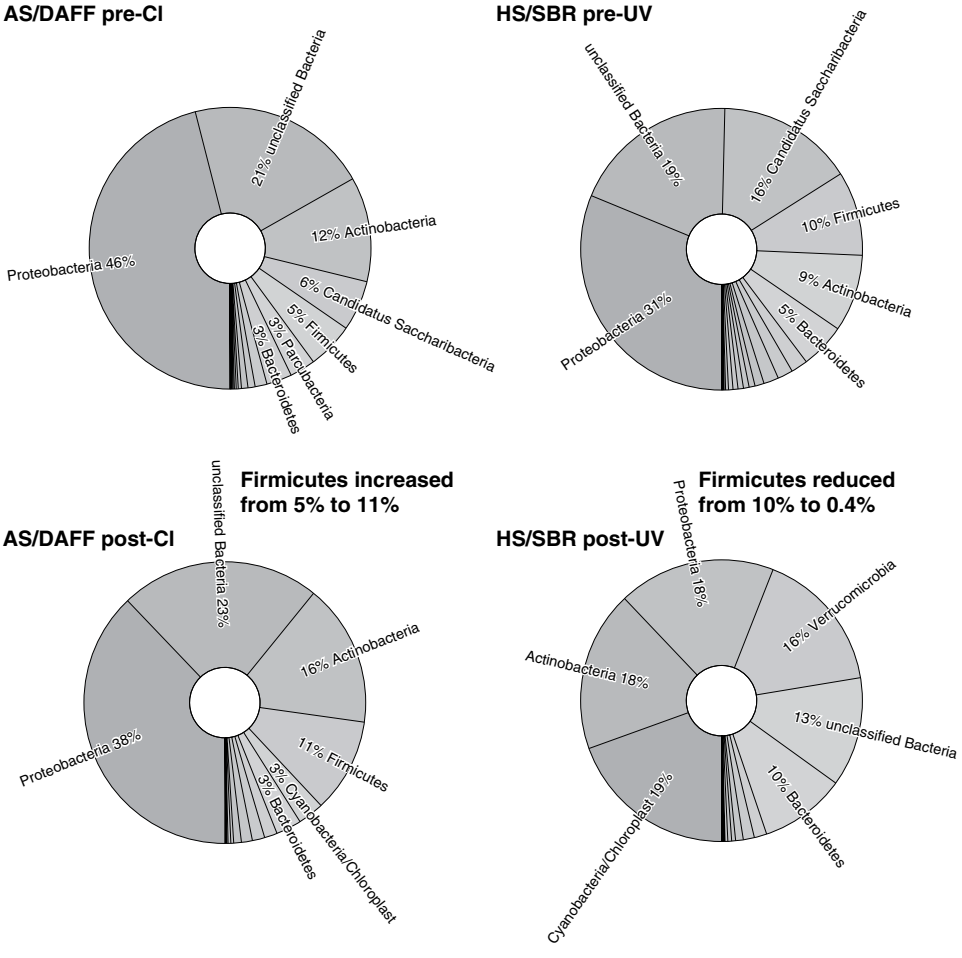


**Figure 9.2** Relative abundance of Firmicutes pre- and postdisinfection (UV vs. chlorination (Cl)) at two colocated wastewater treatment plants in Australia. AS/DAFF indicates activated sludge (AS) treatment followed by dissolved air flotation and filtration (DAFF). HS/SBR is a sequencing batch reactor (SBR) treating high salinity (HS) influent. Results are based on total DNA extraction from 1L water samples (0.22 μm filtered) and Illumina MiSeq partial 16S rRNA gene sequencing.

community, but Ye and Zhang (2013) found greater diversity (1932 OTUs) in the effluent than in the influent. When Ye and Zhang (2011) analyzed the proportion of pathogenic bacteria in the influent sample they identified 0.16% of the sequences as pathogenic, with 99% identity to known pathogens. A further 1.14% shared 97%–99% similarity with known pathogens. The relative abundance of pathogens in the overall community changes in response to treatment just as the rest of the community structure does. Ye and Zhang (2013) reported that some pathogen-related sequences were significantly reduced in the effluent, but there were also some pathogens that increased in relative abundance, such as *Aeromonas hydrophila*. Such trends are likely to be WWTP dependent, and results of this type also depend on the database the sequences are compared to and on the sequencing depth. Some pathogens will be below the

detection limit if they are relatively rare community members and the sequencing depth is not deep enough to detect them. It should be noted that both McLellan et al. (2010) and Ye and Zhang (2013) reported that the sequencing depth achieved in their studies was not deep enough to describe the whole diversity in the analyzed samples, but as explained in the section on methodological considerations further ahead, these kinds of analyses are improving all the time along with advances in high throughput sequencing technologies. Future research on this topic will certainly benefit from increased sequencing depth.

## Resistance Genes in WWTP Influents and Effluents

While it is clearly important to investigate the fate and abundance of medically relevant bacteria throughout the wastewater treatment process, a narrow focus on specific phylotypes significantly restricts the overall knowledge gained about ARB and ARGs in WWTPs because resistance genes are also commonly carried by nonpathogenic bacteria. Currently, the full diversity of the wastewater resistome, and the potential of ARB and ARGs to persist, recombine, and spread to other environments is uncertain, but previous research has shown that ARGs are frequently carried on mobile genetic elements such as plasmids, transposons, and integrons (Davies, 1994; Bennett et al., 1999) and that the wastewater resistome is extremely diverse (Moura et al., 2010; Szczepanowski et al., 2009). Evolution of new combinations of resistance genes may occur during wastewater treatment as high-density, high-diversity bacterial communities are expected to be conducive to complex horizontal gene transfer events (Schlüter et al., 2007b). Evidence of this has previously been demonstrated in model systems (e.g., Courvalin, 1994; Geisenberger et al., 1999; Marcinek et al., 1998).

For more than a decade, the presence of resistance genes in WWTP microbial communities has been studied and has since been widely observed and reported. One relevant study, particularly notable for its large scale, is that by Szczepanowski et al. (2009) in which ARB from activated sludge and WWTP effluent were selected by culturing. Total plasmid DNA was then extracted and scanned for resistance genes using 192 resistance-gene-specific PCR primer pairs, which were designed to target genes providing resistance to medically important groups of antibiotics. This approach supported the detection of 123 different resistance genes in the combined effluent sample and 140 in the activated sludge sample, with subsequent amplicon sequencing confirming close similarity to the target reference genes used for primer design (indicating clinical relevance).

In recent resistance gene research, culture-based approaches have often been set aside in favor of gene quantification by quantitative PCR (qPCR), or for metagenomics analyses which enable one to interrogate the structure of the bacterial community and the number of resistance genes in the same sample. Using transposon-aided plasmid capture, high-throughput sequencing, and metagenomics to investigate an activated sludge plasmidome, Zhang et al. (2011) found that the most abundant resistance genes identified were coding for tetracycline, macrolide, and multidrug resistance. Genes conferring resistance to tetracycline and its derivatives have previously been targeted for quantification in wastewater and downstream environments because tetracyclines are one of the most widely used classes of antibiotics in human and veterinary medicine.

Unfortunately, because of the large number (>38) of known tetracycline resistance genes (Roberts, 2005), the same genes have not always been targeted for qPCR in different studies. In addition, variations in DNA extraction and qPCR conditions and standards mean that results cannot easily be compared across different studies. As documented in Table 9.1, reported values for individual *tet* genes in international WWTP influents range from $10^3$ to $10^{10}$ copies per mL, and the order of relative abundance of the different *tet* genes is not consistent between different studies and WWTPs.

Other resistance genes that have been quantified in WWTP research include the *sul1* and *sul2* genes. These confer resistance against sulfonamides, which have been in use since the 1930s and are now consistently detected in wastewater environments. *Sul1* is reported to be closely linked to class 1 integrons and is often located together with other resistance genes on these mobile elements (Antunes et al., 2005). This makes *sul1* a popular target for research because it is not only usually quantifiable in wastewater environments, it also provides an indication of the importance of horizontal gene transfer (HGT). *Sul2* is reportedly hosted on both small nonconjugative plasmids and large multiresistant conjugative plasmids (Antunes et al., 2005; Rodriguez-Mozaz et al., 2015). As shown in Table 9.1, reported values for *sul1* and *sul2* in international WWTP influents range from $10^4$ to $10^{10}$ copies per mL. Effluent *sul* gene copy numbers tend to be one or two orders of magnitude lower than in the influent, but this is not always the case (Table 9.1).

Sulfonomide and tetracycline genes are among the most commonly studied ARGs in WWTPs to date, but they are not the only genes in focus. Genes conferring resistance to more recently introduced and "last resort" antibiotics of high medical relevance are increasingly in the spotlight. For example, Berendonk et al. (2015) suggested the use of *vanA* and *mecA* (conferring resistance to vancomycin and methicillin, respectively) as suitable candidate genes for a more standardized approach to wastewater resistome research, as these antibiotics are particularly important in current health care. Several genes belonging to the *bla* family (e.g., $bla_{\text{NDM-1}}$, $bla_{\text{CTX-M}}$, $bla_{\text{SHV}}$, and $bla_{\text{TEM}}$) that confer resistance to beta-lactam and carbapenemase antibiotics have also been highlighted for future monitoring (Berendonk et al., 2015). Whereas *sul* and *tet* resistance genes are typically found to be present or above the detection limit in all of the influent and effluent samples assessed, some of the more medically relevant resistance genes that have not been in use for such a long time are not always detectable. Caucci et al. (2016) reported the average *vanA* concentration over two years and all seasons in a German WWTP to be $1.38\text{x}10^4$ – $6.51\text{x}10^4$ $\text{mL}^{-1}$ (Table 9.1). Börjesson et al. (2009) reported *mecA* copy numbers in a Swedish WWTP ranging from $10^1$ – $10^2$ $\text{mL}^{-1}$, and in some of their samples *mecA* was either not present or below detection. These studies show that genes conferring resistance to last resort antibiotics are less abundant in WWTP environments than genes conferring resistant to more widely and broadly consumed antibiotics. Laht et al. (2014) suggested that the higher abundance of sulfonamide and tetracycline resistance genes compared to beta-lactamase genes may be related to their longer and broader consumption history.

Reductions in the absolute number of ARGs in wastewater effluent relative to influents have frequently been reported and are in line with the anticipated decrease in bacterial loads from influent to effluent. The number of 16S rRNA copy numbers (indicating the magnitude of the bacterial load), generally decreases by several orders of magnitude from influent to effluent in secondary treatment plants (e.g., Gao et al., 2012; Laht et al.,

**Table 9.1** Resistance gene copy numbers per mL in WWTP inflow and outflow.

| Gene | Inflow/Raw Sewage (copies per mL) | Outflow (copies per mL) | Plant Loads (cubic meters/day) | Reference | Comment |
|---|---|---|---|---|---|
| *tet*(A) | 1.60E + 03 ± 1.90E + 03 | 3.50E + 02 ± 3.10E + 02 | 55,000 | Makowska et al. (2016) | |
| | 2.82E + 04 – 2.19E + 05[a] | 1.10E + 03 – 1.20E + 05[a] | 100,000; 150,000; 35,000 | Wen et al. (2016) | |
| | 6.03E + 07 – 1.58E + 08[a] | 2.40E + 04 – 8.00E + 03[a] | 150,000 | Zhang et al. (2009) | |
| | 6.31E + 09 | 3.98E + 07 | 400,000 | Li et al. (2015) | from figure |
| *tet*(B) | 1.90E + 03 ± 1.40E + 03 | 1.80E + 01 ± 2.20E + 01 | 55,000 | Makowska et al. (2016) | |
| | 1.58E + 10 | 1.26E + 06 | 400,000 | Li et al. (2015) | from figure |
| *tet*(M) | 1.00E + 03 ± 1.70E + 03 | 2.50E + 01 ± 4.90E + 01 | 55,000 | Makowska et al. (2016) | |
| | 1.00E + 09 ± 1.00E + 09 | 4.00E + 07 ± 8.00E + 07 | 120,000 | Laht et al. (2014) | |
| | 1.00E + 09 ± 3.00E + 08 | 4.00E + 07 ± 4.00E + 07 | 25,000 | Laht et al. (2014) | |
| | 4.00E + 08 ± 8.00E + 08 | 4.00E + 06 ± 8.00E + 06 | 270,000 | Laht et al. (2014) | |
| | 7.94E + 08 | 1.58E + 06 | 400,000 | Li et al. (2015) | from figure |
| | 4.20E + 06 ± 1.10E + 07 | 2.60E + 06 ± 1.50E + 07 | 140,600 | Caucci et al. (2016) | |
| *tet*(W) | 2.63E + 06 – 1.00E + 07[a] | 4.27E + 04 – 6.31E + 06[a] | 100,000; 150,000; 35,000 | Wen et al. (2016) | |
| | 1.80E + 05 ± 1.50E + 05 | 4.40E + 02 ± 4.40E + 02 | 19,700 | Al-Jassim et al. (2015) | |
| | 2.34E + 05 – 2.51E + 07[a] | ND – 4.27E + 03[a] | Not reported | Munir et al. (2011) | |
| | 3.16E + 07 | 1.26E + 03 | Not reported | Gao et al. (2012) | from figure |
| | 3.16E + 10 | 1.02E + 06 | 400,000 | Li et al. (2015) | from figure |
| *tet*(O) | 4.57E + 04 – 2.82E + 05[a] | 3.55E + 02 – 1.78E + 05[a] | 100,000; 150,000; 35,000 | Wen et al. (2016) | |
| | 9.70E + 04 ± 1.10E + 05[a] | 2.50E + 02 ± 2.40E + 02[a] | 19,700 | Al-Jassim et al. (2015) | |
| | 3.24E + 05 – 4.07E + 07[a] | ND – 9.12E + 03[a] | Not reported | Munir et al. (2011) | |
| | 1.58E + 07 | 2.00E + 03 | Not reported | Gao et al. (2012) | from figure |
| | 7.94E + 08 | 6.31E + 06 | 400,000 | Li et al. (2015) | from figure |

(*Continued*)

**Table 9.1** (Continued)

| Gene | Inflow/Raw Sewage (copies per mL) | Outflow (copies per mL) | Plant Loads (cubic meters/ day) | Reference | Comment |
|---|---|---|---|---|---|
| *tet*(Q) | 8.70E + 04 ± 1.00E + 05 | 1.60E + 02 ± 1.50E + 02 | 19,700 | Al-Jassim et al. (2015) | |
| | 1.58E + 07 – 1.00E + 09[a] | 6.31E + 03 – 1.58E + 06[a] | Not reported | Auerbach et al. (2007) | from Munir et al. ((2011) |
| | 6.31E + 09 | 1.26E + 07 | 400,000 | Li et al. (2015) | from figure |
| *sul*I | 7.60E + 04 ± 6.60E + 04 | 1.50E + 04 ± 1.20E + 04 | 55,000 | Makowska et al. (2016) | |
| | 2.69E + 05 – 9.33E + 05[a] | 1.45E + 04 – 6.17E + 05[a] | 100,000; 150,000; 35,000 | Wen et al. (2016) | |
| | 2.88E + 05 – 3.47E + 07[a] | 2.34E + 04 – 5.62E + 06[a] | Not reported | Munir et al. (2011) | |
| | 3.00E + 08 ± 2.00E + 08 | 2.00E + 07 ± 4.00E + 07 | 120,000 | Laht et al. (2014) | |
| | 4.00E + 08 ± 4.00E + 08 | 4.00E + 07 ± 1.00E + 07 | 25,000 | Laht et al. (2014) | |
| | 2.00E + 08 ± 3.00E + 08 | 2.00E + 06 ± 2.00E + 06 | 270,000 | Laht et al. (2014) | |
| | 3.16E + 06 | 3.98E + 03 | Not reported | Gao et al. (2012) | from figure |
| | 5.01E + 10 | 3.98E + 08 | 400,000 | Li et al. (2015) | from figure |
| | 8.90E + 07 ± 1.50E + 08 | 2.70E + 06 ± 7.10E + 06 | 140,600 | Caucci et al. (2016) | |
| *sul*II | 2.60E + 04 ± 3.30E + 04 | 6.20E + 03 ± 5.30E + 03 | 55,000 | Makowska et al. (2016) | |
| | 5.62E + 06 – 2.24E + 07[a] | 3.39E + 05 – 1.66E + 07[a] | 100,000; 150,000; 35,000 | Wen et al. (2016) | |
| | 8.00E + 08 ± 1.00E + 09 | 1.00E + 07 ± 2.00E + 07 | 120,000 | Laht et al. (2014) | |
| | 1.00E + 10 ± 1.00E + 09 | 2.00E + 08 ± 2.00E + 08 | 25,000 | Laht et al. (2014) | |
| | 1.00E + 09 ± 2.00E + 09 | 2.00E + 07 ± 3.00E + 07 | 270,000 | Laht et al. (2014) | |
| | 3.16E + 09 | 9.55E + 07 | 400,000 | Li et al. (2015) | from figure |
| | 6.70E + 06 ± 2.00E + 07 | 1.70E + 05 ± 4.80E + 05 | 140,600 | Caucci et al. (2016) | |

| | | | | | |
|---|---|---|---|---|---|
| *bla*-ctx32 | 1.00E + 10 ± 3.00E + 10 | 2.00E + 05 ± 2.00E + 05 | 120,000 | Laht et al. (2014) | |
| | 5.00E + 06 ± 7.00E + 06 | 2.00E + 05 ± 2.00E + 05 | 25,000 | Laht et al. (2014) | |
| | 6.00E + 06 ± 1.00E + 07 | 3.00E + 04 ± 7.00E + 04 | 270,000 | Laht et al. (2014) | |
| | 2.70E + 05 ± 6.30E + 05 | 9.90E + 03 ± 1.00E + 04 | 140,600 | Caucci et al. (2016) | |
| *bla*-oxa58 | 2.00E + 08 ± 3.00E + 08 | 9.00E + 06 ± 2.00E + 07 | 120,000 | Laht et al. (2014) | |
| | 4.00E + 07 ± 3.00E + 07 | 3.00E + 06 ± 3.00E + 06 | 25,000 | Laht et al. (2014) | |
| | 1.00E + 08 ± 2.00E + 09 | 5.00E + 05 ± 1.00E + 06 | 270,000 | Laht et al. (2014) | |
| | 1.30E + 06 ± 2.50E + 06 | 7.00E + 03 ± 9.60E + 03 | 140,600 | Caucci et al. (2016) | |
| *bla*-shv34 | 4.00E + 07 ± 5.00E + 07 | 7.00E + 06 ± 7.00E + 06 | 120,000 | Laht et al. (2014) | |
| | 5.00E + 08 ± 7.00E + 08 | 1.00E + 08 ± 1.00E + 09 | 25,000 | Laht et al. (2014) | |
| | 1.00E + 07 ± 3.00E + 07 | 4.00E + 06 ± 1.00E + 07 | 270,000 | Laht et al. (2014) | |
| | 4.60E + 05 ± 9.90E + 05 | 2.40E + 05 ± 4.50E + 05 | 140,600 | Caucci et al. (2016) | |
| *van*A | 4.10E + 03 | 1.90E + 02 | 1,100,000 | Narciso-da-Rocha et al. (2014) | Approx. average calculated from figure |
| | 6.51E + 04 ± 7.77E + 04 | 1.38E + 04 ± 1.54E + 04 | 140,600 | Caucci et al. (2016) | |

[a] a range of values; minimum and maximum.

2014; Makowska et al., 2016). In several cases, the numbers of resistance genes have also been reported to decrease, but in other cases researchers have observed that the relative abundance (normalized by 16S rRNA copies) of specific resistance genes actually increased in the effluent. This indicates positive selection of resistant microorganisms (Laht et al., 2014). Using culture-based approaches, researchers have also observed positive selection of antimicrobial resistant isolates as an effect of wastewater treatment. For instance, Łuczkiewicz et al. (2010) documented increased resistance to fluoroquinolones. Varying trends have been observed overall. For example, Caucci et al. (2016) used qPCR to quantify 11 different resistance genes before and after treatment, including *sul1*, *sul2*, *tetM*, *qnrB*, $bla_{shv\text{-}34}$, $bla_{ctx\text{-}m\text{-}32}$, $bla_{oxa\text{-}58}$, *vanA*, $bla_{kpc\text{-}3}$, *MecA*, and *dfrA1*, but only found evidence of positive selection for *vanA*. Alexander et al. (2015) also demonstrated *vanA* enrichment in treated wastewater. This gives cause for concern as vancomycin is typically considered a drug of last resort, having proven useful in treating multiple drug–resistant infections.

On a normalized basis (resistance gene copies per 16S rRNA copies), there is now an increasing amount of research demonstrating that many resistance genes do not show any significant reduction in relative abundance as a result of wastewater treatment; in other words, similar proportions of the influent and effluent community/population are carrying ARGs. However, as pointed out in Caucci et al. (2016), this could also be the result of significant changes in the population structure during the treatment process, as changes in community structure may also obscure resistance-related trends. Yang et al. (2014) used a high-throughput sequencing-based metagenomics approach to look for the presence and abundance of ARGs in a WWTP in Hong Kong and identified 263 ARG subtypes in the influent. By contrast, the diversity identified in the effluent was far less (155 subtypes). Most of the resistance gene subtypes evidently originated from the influent as 150 subtypes were present in both the influent and effluent. The most abundant resistance genes in the influent conferred tetracycline resistance (23.1%) and aminoglycoside resistance (14.8%), but multidrug-resistant gene subtypes were also abundant (20.2%). The same types of resistance were also abundant in the effluents, where the relative abundance of tetracycline, aminoglycosides, and multidrug resistance was 9.12%, 12.7%, and 18.5%, respectively. Genes for sulfonamides showed the greatest relative increase in the effluent: 19.5% in the effluent compared to 1.79% in the influent.

In general, the removal efficiency of ARGs during treatment processes has been reported to be close to 99% (about two orders of magnitude) and more, except in the case of sulfonamide resistance genes, for which the removal efficiency was 98.09% (Yang et al., 2014). This greater abundance and persistence of sulfonamide resistance in WWTP effluents was also indicated by Rowe et al. (2015) using a metagenomics approach. The abundance of resistance genes in influents and WWTPs may fluctuate throughout the year depending on the season. For example, in Caucci et al. (2016), we observed a seasonal trend with higher ARG abundances in autumn and winter than in other seasons. This also coincided with higher outpatient antibiotic consumption. Other studies have only detected minor or no differences between seasons (Laht et al. 2014; Karkman et al., 2016). As pointed out above, however, seasonal trends may be obscured by climatic events, particularly in the case of combined sewer systems where storm water surges can drastically dilute the influent wastewater and may result in untreated overflows.

## Bacteria Carrying Antibiotic Resistance Genes

Although the conditions in WWTPs are thought to be ideal for acquiring ARGs and selecting for ARB, not all bacteria in WWTPs are antibiotic resistant. Some bacteria are resistant to more than one antibiotic from different classes and some are resistant to a single antibiotic, but many carry no resistance to any antibiotics. Makowska et al. (2016) reported the number of heterotrophic bacteria in influent and effluent samples respectively to range from $1.6 \times 10^6$ to $2.4 \times 10^7$ and $1.6 \times 10^4$ to $1.0 \times 10^5$. The percentage of tetracycline resistant heterotrophs was in the range of 0.1%–0.3% and 0.3%–0.5% in the influent and effluent, respectively, while the proportion of sulfonamide resistant heterotrophic bacteria was comparatively higher, with 0.1%–2.7% in the influent and 4.8%–8.1% in the effluent (Makowska et al., 2016). Similar abundances for total heterotrophs and tetracycline and sulfonamide resistant heterotrophic bacteria were observed by Munir et al. (2011), Gao et al. (2012), and Li et al. (2015). Novo et al. (2013) reported temporal changes in the proportion of resistant heterotrophs, *Enterobacteria*, and *Enterococci* in both influents and effluents, with relatively more resistant bacteria in the spring than autumn. A greater proportion (20%–42%) of heterotrophs and *Enterobacteria* (23%–61%) were amoxicillin resistant compared to *Enterococci* (0.3%–1.2%). The opposite pattern was seen in the case of tetracycline, where 15%–23% of *Enterococci* were tetracycline resistant; this was ~10 times higher than the proportion of tetracycline resistance heterotrophs. More than 60% of the *Enterococci* populations were resistant to sulfonamides, while for the *Enterobacteria* and total heterotrophs, sulfonamide resistance did not exceed 15%. In most cases, the treatment processes did not decrease the proportion of the bacterial populations that were resistant (Novo et al., 2013).

In Caucci et al. (2016) we analyzed the relationship between bacterial community changes and ARG abundances but did not uncover any significant link for most of the 11 genes examined, although it was surmised that the consistent increase from influent to effluent of *vanA* may have been linked to a relative increase in abundance of *vanA*-carrying taxa (possibly Enterococcus). Yang et al. (2014) analyzed the bacterial community and ARGs in WWTP inflow and outflow using metagenomics and used multiparametric analysis to assess the impact of microbial communities on ARG distribution. They found that 6 out of 283 genera used in the analysis showed significant correlations with ARG distributions. These six genera were *Flavobacterium*, *Poriferibacter*, *Bacteroides*, *Acinetobacter*, *Actinobaculum*, and *Streptococcus*. Some of the 78 resistance gene subtypes used in the analysis were influenced by several of these genera, and some by all, but the majority of resistance genes were predominantly influenced by *Bacteroides*, *Acinetobacter*, *Actinobaculum*, and *Streptococcus* (Yang et al., 2014).

## Wastewater Treatment Efficiency and Antibiotic Resistance

As reported above, the removal efficiency of bacteria and resistance genes in conventional WWTPs can be close to or even greater than 99%. For a conventional contaminant this would be regarded as very high removal efficiency, indicating effective pollution mitigation and management. However, due to the uncertain and potentially very large risks associated with the propagation of ARB and ARGs, researchers and

managers must look beyond the simple statistics of removal efficiencies to determine more precisely how to effectively minimize harmful ARB and ARG effects on downstream environments and human and animal health. To look only at removal efficiencies can be misleading, as high removal efficiency does not necessarily mean that very few bacteria and/or resistance genes will be released. In reality, because of the continual influent inputs, a large number of bacteria and resistance genes will be released with effluents over time. As demonstrated throughout this chapter, the absolute and relative quantities of resistance genes released in effluents varies widely and can be as high as $10^8$ per mL per gene (Table 9.1). There are fewer data available for important antibiotics such as the beta-lactam antibiotics and vancomycin, but the published data indicate that they are currently much less abundant than *tet* and *sul* genes. The WWTP processing rates documented in Table 9.1 can also be used to estimate the copy numbers released by these plants. The processing rates are reported in cubic meters per day, and the resistance gene quantities are per mL, which means that to estimate the average daily release of a resistance gene we need to multiply the copy numbers per mL by $10^{10} – 10^{11}$, depending on the size of the plant. Although the treatment plants may have removed up to 99% of the resistance gene load in the influent, this simple calculation starts to put the scale of the remaining 1% into perspective.

The fate and effects of ARB and ARGs emitted to downstream environments in WWTP effluents remain largely underdetermined, but research on this topic has shown that absolute quantities of ARGs are more abundant downstream from WWTPs than they are upstream (Marti et al., 2013; Czekalski et al., 2014; Narciso-da-Rocha et al., 2014; Proia et al., 2015). Proia et al. (2015) reported that in comparison to resistance gene loads upstream of a WWTP, some resistance genes (*ermB* and *qnrS*) were significantly elevated in river biofilms as far as 1 km downstream from the WWTP. In contrast, LaPara et al. (2015) reported that WWTP outflows on the Upper Mississippi River did not impact the river environment, as ARG copies were not elevated in the river, although there were more copies in the effluent than in the river samples (78- to 831-fold higher in the effluent than in the river). This study was conducted along a stretch of the Mississippi River including 14 different WWTPs, and the authors suggested that one of the reasons why no increase in resistance gene abundance was observed may have been due to the large dilution effect; the Mississippi is one of the largest rivers in the world (LaPara et al., 2015). The outcome of a similar study could well be very different for a smaller river, as it is not uncommon in smaller rivers for WWTP effluent volumes to significantly impact river flows.

## Methodological Considerations for Understanding WWTP Microbial Communities and Potential Downstream Effects

Although some culture-based protocols have been developed into highly reliable, standardized techniques for effluent monitoring, and used to produce valuable data about bacterial dynamics in a wide range of wastewater treatment scenarios, the use of culture-based approaches for exploring the grand questions of microbial diversity and ecology is inherently biased and limited. This is because they are restricted to the analysis of culturable species, when, in fact, the vast majority of environmental bacteria remain uncultivable (Amann et al., 1995; Zengler et al., 2002; Nichols

et al., 2010). Furthermore, axenic phenotypes differ from those found in real environments such as wastewater because they lack the effects of microbial interactions. A very clear demonstration of phenotypic differences between axenic cultures and those where microorganisms are interacting was recently provided by Tyc et al. (2014), who showed that out of 146 environmental isolates, 42% of them demonstrated antimicrobial activity only when interacting with a competing species. Culture-based approaches still offer some important benefits, such as the possibility to isolate novel microorganisms that can be used for the production of antibiotics, pathogen control, and bioremediation; however, they are increasingly set aside in favor of culture-independent approaches (e.g., metagenomics) that circumvent key shortcomings of culture-based approaches.

In recent decades, breakthrough technological advances have turned high-throughput sequencing and metagenomics into a "Swiss Army knife" for microbial ecology with many different options available. Sequence-based approaches focusing on the study of a single marker gene through a group of microorganisms (e.g., a phylogenetic marker gene or resistance gene) are particularly popular at present, but studies that aim to sample or even fully screen the complete metagenome through shotgun sequencing are also being published more frequently (e.g., Zhou et al., 2015).

Currently, PCR-based approaches combined with high-throughput sequencing facilitate in-depth analysis of marker gene diversity throughout multiple samples within very short time periods and at a relatively low per-sample cost (Vasileiadis et al., 2013; Herbold et al., 2015). Typically, DNA is extracted directly from the environmental samples, the marker gene of interest is amplified by PCR, and the multiplexed product is sequenced. However, all these steps entail biases that are sample specific and need to be considered during experimental design, data analysis, and interpretation. For example, methods using a filtering process to concentrate bacterial cells from water samples for DNA extraction (Staley et al., 2015) may induce a size-dependent selection bias (Portillo et al., 2013) and DNA extract purity, primer-design strategy, PCR processes, and sample multiplexing strategies can also bias gene survey outcomes (Kreader, 1996; Hamady et al., 2008; Bartram et al., 2011; Berry et al., 2011; Caporaso et al., 2012; Vasileiadis et al., 2012; Herbold et al., 2015). Bioinformatics and data analysis strategies (Ramette, 2007; Larsen et al., 2012; Buttigieg and Ramette, 2014), including the selection of databases for comparative purposes, also have an impact on the overall results and must be considered particularly carefully (Werner et al., 2012).

When using marker gene targeted approaches in microbial ecology, gene quantification is usually performed using intercalating dyes such as SYBR green or fluorescently labeled (TaqMan) probes (Kim et al., 2013). Aside from the aforementioned PCR-related biases, both of these approaches have specific pros and cons when compared with each other, particularly in relation to assay specificity (Kim et al., 2013). Probe (TaqMan)-based approaches usually ensure greater assay specificity, which is often considered as a positive; however, in the event that there is variation in sequences of the same gene derived from different organisms (as is likely in environmental microorganisms), a TaqMan probe–based approach may significantly restrict the breadth of the target group that can be assayed, in which case an intercalating dye assay (e.g., SYBR green) may be considered preferable. One of the advantages of the probe-based approach compared with the intercalating dye approach is the possibility to multiplex reactions using different fluorophores; this allows the abundance of the gene(s) of interest to be

directly expressed in both absolute counts and in terms of relative abundance to a "housekeeping" target (DeCoste et al., 2011). As some resistance genes may be quite rare in target environments, it must be anticipated that quantification using qPCR will not always be possible using standard real-time PCR instruments, but newly available digital PCR instruments are expected to help lower detection limits. Digital PCR uses the same primers and probes as qPCR but provides absolute quantification of target DNA/RNA molecules and does not require the use of standard curves (Baker, 2012). Eliminating the need for standard curves reduces error and improves precision, enabling day-to-day and lab-to-lab reproducibility.

Shotgun sequencing of environmental DNA extracts can potentially circumvent the biases and issues (e.g., amplification/primer-designing biases) of PCR-based approaches. Although still expensive, it is becoming increasingly feasible as sequencing technologies continue to improve and become cheaper. Shotgun sequencing offers great advantages, as the complete microbial community can potentially be surveyed simultaneously for both phylogenetic and functional gene targets. Significantly, the taxonomical boundary issues that create a challenge for PCR primer designing are not a problem for shotgun approaches, meaning that quantitative information can be obtained for all life domains (Ranjan et al., 2016). Common strategies for shotgun sequencing include nucleic acid extraction, followed by the preparation of shotgun libraries, which are sequenced and analyzed. This is done either by mapping the reads against sequence databases or by assembling them into larger contiguous sequences (ideally containing phylogenetic marker sequences), which are then used to predict genomic features and coded protein functions. Currently, the major issues limiting adoption of this approach for analyzing highly complex environments is one of cost, as sequencing to an adequate screening depth can be prohibitively expensive (Di Bella et al., 2013). As rule of thumb, 5–10x coverage of the dominant community member numbers is suggested in order to support adequate sequence assembly and genome reconstruction of those members (Kunin et al., 2008). As an example, in current technology terms, this translates into ~5–10 million 250 bp paired-end reads of ~400 bp shotgun library inserts (DNA fragments) being required to interrogate a dominant community comprising 100 genomes (assuming an average genome size of 3 Mbp and assuming the community is mainly prokaryotic). However, one must consider that the usual number of dominant OTUs found in wastewater samples is larger than 100, with each OTU comprising several genomes. Potentially, the "effective species number" described by Jost (2006) could serve as an indication of the number of dominant OTUs in a given environment and thereby support the estimation of sequencing effort needed to adequately interrogate shotgun metagenomes. Sufficient sequencing effort and proper experimental design can result in the assembly of contigs and their allocations into taxonomical bins, thus succeeding in answering both core questions of metagenomics of "who is there?" and "what are they doing?" (Handelsman, 2004; Sangwan et al., 2016). Even if the sequencing effort is not sufficient for the construction of robust contigs, the sequence reads can be submitted to existing short read, web-based annotation pipelines which can provide the phylogenetic and functional profile of the analyzed samples (Dudhagara et al., 2015). If limited to shallow sequencing, however, key findings may currently be better obtained using phylogenetic marker surveys combined with functional prediction (Langille et al., 2013).

## Conclusions

Given the potential impacts of antibiotic resistant bacteria on human and animal health, the need for further research to support robust risk assessment and management of ARB and ARGs in wastewater environments is clear. To date, the dynamics of resistant bacteria and associated genes in municipal WWTPs remains relatively unexplored, but there is clear evidence that ARGs and resistant organisms are released with WWTP effluents to receiving environments. Studies have demonstrated that the absolute quantity of ARG copies are reduced during conventional wastewater treatment, but it is also apparent that the relative abundance (normalized to 16S rRNA copy numbers) of key resistance genes often shows no significant reduction. This pattern appears to be WWTP specific however, and there are also some cases where the relative abundance of resistance genes decreases from WWTP influent to effluent. Importantly, our review of the existing data suggests that the rarer species of WWTP microbial communities may have a high impact on the relative abundance of ARGs in wastewater. This raises the specter of methodological issues affecting the study of antibiotic resistance in complex microbial communities. Fortunately, ongoing advances in nucleotide sequencing–based technologies and methods are opening the door to increasingly powerful methods for ARB and ARG research.

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# 10

# The Effect of Advanced Treatment Technologies on the Removal of Antibiotic Resistance

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## Introduction

The declaration of antibiotic resistance (AR) by the World Health Organization (WHO) as "a complex problem driven by many interconnected factors, with single isolated interventions having little impact" (WHO, 2012) has put AR in the spotlight in the last few years. Soil and water environments have been characterized as recipients, reservoirs, and sources of antibiotic resistance genes (ARGs) of clinical concern, with inputs of residual antibiotics and antimicrobials acting as ARG amplifiers (Heuer et al., 2011). Observed AR determinants previously found in clinical environments have also been employed by environmentally relevant, nonpathogenic bacteria (Zhang et al., 2009). Treated effluents from urban wastewater treatment plants (WWTPs) have been characterized as hot spots of AR, as AR determinants have been detected in these effluents, ending up in soil and aquatic environmental compartments, spreading and potentially accumulating AR among environmentally relevant bacteria (Rizzo et al., 2013b). Consequently, the release of AR determinants into the environment through WWTP effluents is suspected to be among the main anthropogenic sources of antibiotics, antibiotic resistant bacteria (ARB), and ARGs. Moreover, the conventional biological treatment creates a potentially ideal environment for the development of AR, as ARB and antibiotic susceptible bacteria in the WWTP environment are continuously mixed with subinhibitory concentrations of antibiotics and other ARB in close proximity, increasing the spread and prevalence of AR (Lupo et al., 2012; Michael et al., 2012). Various studies have been conducted in the context of the presence of AR in treated wastewater effluents by conventional wastewater processes, that is, conventional activated sludge (CAS) treatment, showing that ARB and ARGs are not effectively removed by

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conventional wastewater treatment processes already in place (Figueira et al., 2011; Lupo et al., 2012; Novo et al., 2013; Czekalski et al., 2014; Alexander et al., 2015; Rodriguez-Mozaz et al., 2015). The residual concentrations of ARB and ARGs end up as a result in environmental compartments such as soil and surface water environments through the reuse of the treated effluents (Finley et al., 2013).

Currently, treated wastewater is widely reused to compensate for diminishing water supplies, as it has been recognized to be a reliable alternative source of irrigation water, making wastewater reuse a practice of utmost importance (Kalavrouziotis et al., 2013; Fatta-Kassinos et al., 2015). Presently, wastewater reuse recommendations established by the State of California, the US Environmental Protection Agency (EPA), and World Health Organization (WHO, 2006) form the background of the majority of legal guidelines proposed in countries such as the United States, Portugal, Spain, Italy, and Cyprus (Becerra-Castro et al., 2015). These recommendations, although inclusive of important physicochemical parameters of wastewater such as turbidity, suspended solids (SS), pH, salinity, conductivity, biochemical oxygen demand (BOD) and chemical oxygen demand (COD), lack the inclusion of important microbiological parameters, as they are mainly focused on cultivable fecal indicators and nematode eggs (EPA, 2012). As a result, the majority of pathogenic microorganisms that may be present in treated wastewater due to the inability to remove them by CAS treatment are excluded from currently available guidelines, and the presence of ARB and ARGs is overlooked.

The oversight of ARB and ARGs in existing regulations regarding treated wastewater reuse worldwide poses a potential risk of microbiological contamination of ground and surface water, wildlife, and food chains, as shown in studies on their inadequate removal during CAS treatment (Becerra-Castro et al., 2015). Consequently, the available knowledge regarding AR in wastewater effluents calls for the need to apply the "precautionary principle," in other words, take remedial action to avoid or mitigate potential harm, even when complete knowledge is not available (Schäfer and Beder, 2006). As a result, active stewardship must be applied in wastewater treatment practices in order to prevent the selection pressure, flow, and spread of AR determinants such as antibiotic residues, ARG, and ARB to and from environmental reservoirs (Pruden et al., 2013). The optimization and incorporation in the conventional urban wastewater treatment of new, alternative, and advanced wastewater processes is one important step for the mitigation of the spread and accumulation of AR determinants.

Such alternate treatments to conventional wastewater processes include membrane processes such as microfiltration; ultrafiltration; and reverse osmosis; as well as biological and natural processes, which utilize biological material such as biosolids to degrade incoming organic pollutants and include processes such as the membrane bioreactor (MBR); and chemical oxidation processes, namely, advanced oxidation processes (AOPs), which make use of nonselective and highly reactive free hydroxyl radicals ($HO^{\bullet}$) to oxidize and subsequently degrade organic compounds. These processes have been investigated only in the last few years, with the majority of research so far focusing on the removal of antibiotic residues and ARB from urban wastewater effluents. This chapter will focus on the use of biological and natural processes, as well as AOPs, to remove ARB and ARGs.

## Biological and Natural Treatment Technologies for the Removal of ARB and ARGs

Due to the increasing concern surrounding AR in aquatic ecosystems, several studies have been conducted that assess the release, emergence, and potential spread of ARB and ARGs into the environment through CAS-treated wastewater effluents (Pruden et al., 2006; LaPara et al., 2011; Munir et al., 2011; Zhang et al., 2011; Marti and Balcázar, 2013; Sidrach-Cardona et al., 2014), in order to gain better understanding into their occurrence and behavior in aquatic ecosystems. However, studies investigating the removal of these microcontaminants from wastewater effluents are limited and the evidence available only covers a limited number of cultivable ARB and a limited amount of ARGs. As a result, this chapter is an effort to provide a comprehensive review of the available studies on wastewater treatments that aim to replace the CAS treatment for removing ARB and ARGs. Most studies covered in this chapter include the examination of both ARB and ARGs, so these will be analyzed together for each included study, when appropriate.

### Membrane Bioreactor

Few studies have focused on the removal of ARB or ARGs by alternative biological treatment processes of urban wastewater. These processes make use of various types of biological treatment of the influent wastewater, through the degradation of organic microcontaminants by microorganisms, such as the combination of biological treatment with physical separation of the solids through membranes, producing a highly clarified effluent. The MBR technology is currently widely accepted as an unconventional technology to substitute for CAS treatment for the recovery of highly clarified wastewater effluent (Judd, 2015). Its wide use has been attributed to its notable advantages in comparison with the CAS process, including high biodegradation capacity and efficiency, low sludge production, low cost, and simplicity of construction.

In a study by Munir et al. (2011), the occurrence of ARB and ARGs was monitored in raw wastewater and pre- and post-disinfected effluents by different wastewater treatments, including an MBR process. Monitoring of tetracycline resistance genes (*tet*(W) and *tet*(O)), sulfonamide resistance (*sul*1), and tetracycline and sulfonamide resistant total heterotrophic bacteria, was performed. The MBR facility was shown to provide high treatment efficiency for most ARGs (6-log removal for *tet*(W), 7-log removal for *tet*(O), 2.5-log removal for *sul*I). A 3-log removal of tetracycline-resistant heterotrophic bacteria and a 4.6-log removal of sulfonamide-resistant bacteria were observed after the MBR treatment.

In a study by Du et al. (2015), the variation of five ARGs, namely *tet*(G), *tet*(W), *tet*(X), *Intl*1 and *sul*1 was investigated in a full-scale municipal WWTP with a sequential anaerobic/anoxic/aerobic-MBR system (hydraulic retention time, HRT = 9.8 hours, solid retention time, SRT = 21 days, membrane type = polyvinylidene fluoride (PVDF) hollow fiber membranes, nominal pore size = 0.1–0.4 μm). A reduction in the copy number of the examined ARGs (copies $mL^{-1}$) in the MBR effluent compared to the influent was observed, with *tet*(G), *tet*(W), *tet*(X) and *sul*1 effluent concentrations being 0.67, 1.88, 1.71, and 1.36 $\log_{10}$ copies $mL^{-1}$, compared to the respective influent concentrations

(3.11, 4.61, 4.73, and 3.84 $\log_{10}$ copies $mL^{-1}$). The authors argued that although there was a decrease in ARG quantities over the treatment process, there was still a high concentration discharged into the effluent. Moreover, a study by Xia et al. (2012) investigated the removal of antibiotics and selected ARGs in a lab-scale anoxic/aerobic MBR of 6 L capacity (HRT = 6–24 hours, SRT = 3–60 days, membrane type = PVDF hollow fiber membranes, nominal pore size = 0.02 μm) using artificial wastewater containing raw wastewater spiked with antibiotics (tetracycline, oxytetracycline, chlortetracycline, sulfamethoxazole, sulfadiazine, ampicillin) and the ARGs *tet*(C), *tet*(E), *sul*1, and *sul*2. The results of this study showed that a longer SRT (above 30 days) produced a high antibiotic removal (>90%), while *sul*1 and *sul*2 resistance genes were found in higher concentrations as SRT increased, unlike *tet*(C) and *tet*(E) genes. In a different study (Yang et al., 2013), the horizontal gene transfer of ARG was investigated in a lab-scale MBR reactor of 10 L capacity (membrane type = PVDF hollow fiber membranes, nominal pore size = 0.22 μm) using secondary treated wastewater. For the purposes of this study, the inoculated donor strain was rifampicin (Rif)-resistant *Escherichia coli* K12 harboring the plasmid RP4, which was grown (and resistant) in media containing kanamycin, ampicillin, tetracycline, and rifampicin. The results of this study have shown that the average transfer frequency of the RP4 plasmid from the donor strain to cultivable bacteria in activated sludge was $2.76 \times 10^{-5}$ per recipient. Moreover, the abundance of RP4 remained high and stable at $10^4$ copies $mg^{-1}$ of biosolids, indicating a transfer of ARG from donor strains to sludge bacteria.

Overall, it was shown that the MBR process favors the removal of ARB and ARGs, as there is a removal of examined ARGs in the studies available, of more than 3 logs per sample volume. The removal of ARGs has not been shown to be attributed to biodegradation according to Breazeal et al. (2013), but the main mechanism of removal of ARGs is potentially the membrane filtration of the influent wastewater mixed with the biological material found inside the reactor where biodegradation takes place, also known as mixed liquor suspended solids (MLSS). However, higher SRTs may contribute to a high transfer potential of ARGs through horizontal gene transfer mechanisms in bacterial communities in close proximity to each other. The potential for ARG transfer may add to the risk of AR spreading and developing in new ecosystems.

Despite the shown efficiency of the MBR, the effect of the operating conditions on the removal of ARB and on the total DNA as well as the total number of ARGs has not been examined in the available scientific literature. The available studies investigate the effect of the MBR on total bacterial populations and on a limited number of ARGs, overlooking the effect of the combination of the most important operating conditions, including SRT, HRT, SS, dissolved organic carbon (DOC), and wastewater colloids on the removal of ARB and ARGs. Moreover, the effect of different levels of these conditions in real urban wastewater has not been examined so far.

### Constructed Wetlands

The constructed wetland is another example of an alternative process to CAS. It is a natural wastewater treatment that has been around since the 1960s due to its low operation and maintenance costs. This configuration of a semi-aquatic ecosystem has

been proven to remove more than 70% of BOD and SS (Strauch, 2007) in incoming wastewater. The upgrading of constructed wetlands over the last few decades has enabled the increased removal of organic compounds and nutrients from wastewater. Their function relies on the large variety of microorganism communities, which proliferate and produce various physical and chemical reactions. Depending on their wastewater flow, wetlands can be divided into three types: (i) free surface flow constructed wetlands, (ii) horizontal subsurface flow wetlands, and (iii) vertical subsurface flow wetlands. According to Sharma et al. (2016), the efficiency of constructed wetlands in removing ARGs has been related to the flow pattern present. According to Liu et al. (2014), there was a high removal of ARGs in a surface flow pattern constructed wetland, which was attributed to the higher oxidation state of the aqueous phase. In other studies, ARGs were not efficiently removed by vertical subsurface flow or horizontal subsurface flow wetlands (Chen et al., 2014; Liu et al., 2014). However, in a study by Huang et al. (2015) 45%–99% of tetracycline resistance genes in swine wastewater were reduced by vertical upflow wetlands and by a conventional vertical flow wetland.

Chen and Zhang (2013) investigated the effect of different biological processes (biological aerated filters and constructed wetlands) and of ultraviolet (UV) disinfection (UV transmittance = 45%, total power = 900 kW, light intensity ≥ 1 $mW/mm^2$) on the prevalence of the *tet*(M), *tet*(O), *tet*(Q), *tet*(W), *sul*1, and *sul*2 ARGs in wastewater and biosolid samples of three full-scale WWTPs. The constructed wetland contained four stabilization ponds and one horizontal subsurface flow wetland with an HRT of 1.6 days and a total surface area of 3675 $m^2$, while the biological aerated filters had an HRT of 3 hours and a total area of 1500 $m^2$. The results of the study showed a decrease of ARGs by 1–3 orders of magnitude after treatment in the constructed wetlands, while a reduction of 0.6–1.2 orders of magnitude was observed after treatment with the biological aerated filter. A smaller reduction in the examined ARGs was observed after UV disinfection, with 0.5–0.7 orders of magnitude for *tet* genes and 0.3 orders of magnitude for *sul* genes. Next, Sidrach-Cardona and Bécares (2013) investigated the fate of cultivable amoxicillin + clavulanic acid, azithromycin- and doxycycline-resistant total coliforms, *E. coli*, and *Enterococcus* during treatment of real wastewater in a pilot-scale constructed wetland system, comparing this to an urban WWTP treating the same influent. The results showed no significant differences in the ARB proportions among the constructed wetlands (free water surface flow, subsurface flow, and hydroponics). Moreover, no difference was found in the proportion of the ARB between the constructed wetlands and the WWTP effluent. However, hydraulic design and plant presence were important in reducing total bacteria in the wetlands at a higher degree than the WWTP.

Overall, constructed wetlands have been shown to achieve a high degree of removal of ARGs, always in relation to the present flow patterns, compared to the conventional WWTP treatment. Moreover, horizontal flow constructed wetlands were shown to achieve a higher reduction than biological aerated filters, as well as vertical flow constructed wetlands. However, no complete removal of the examined ARGs was observed in the conducted studies, highlighting the need for a more targeted and optimized approach toward the removal of such microcontaminants.

## Advanced Oxidation Processes for the Removal of ARB and ARGs

Advanced oxidation processes (AOPs) are another group of alternative treatment methods; they have been defined as "water treatment processes which are performed at room temperature and normal pressure, based on the *in situ* generation of a powerful oxidizing agent, such as $HO^{\bullet}$ at a sufficient concentration to effectively decontaminate water matrices" (Glaze et al., 1987).

The production of $HO^{\bullet}$ has been examined through the application of various types of AOPs, among which are chemical and photochemical reactions (Oturan and Aaron, 2014). In this chapter the most prevalent chemical and photochemical AOPs in the scientific literature will be examined in respect to their application for the removal of ARB and ARGs. A summary of the main findings regarding the examined AOPs, their operational parameters, the targeted ARB and ARGs, and the antibiotic resistance removal result by each AOP is shown in Table 10.1. The mechanism of action by each AOP examined in this chapter on ARB and ARGs is shown in Table 10.2.

### Ozonation

Ozonation is a chemical AOP that involves the degradation of organic contaminants by molecular ozone ($O_3$) and $HO^{\bullet}$ radicals. The reactions that take place in aqueous matrices during the application of ozonation are shown next (Equations 10.1–10.5).

$$O_3 + OH^- \rightarrow HO_2^- + O_2 \tag{10.1}$$

$$O_3 + HO_2^- \rightarrow O_2 + HO^- + O_2^{-\bullet} \tag{10.2}$$

$$O_3 + O_2^{\bullet -} \rightarrow O_3^{\bullet -} + O_2 \tag{10.3}$$

$$HO_3^{\bullet} \rightarrow HO^{\bullet} + O_2 \tag{10.4}$$

$$HO^{\bullet} + O_3 \rightarrow HO_2^{\bullet} + O_2 \tag{10.5}$$

More specifically, ozonation at a high pH (>8) exhibits an enhanced generation of $HO^{\bullet}$ (Beltran, 2003). The strong oxidation potential of both ozone and $HO^{\bullet}$ (2.07 and 2.8 V, respectively) indicates their high oxidative capacity (Ikehata et al., 2006). The difference between the two types of reaction taking place during ozonation is that molecular ozone reactions are selective, specifically attacking organic molecules having nucleophilic moieties such as carbon double bonds, aromatic rings, and functional groups containing sulfur, phosphorus, nitrogen, and oxygen while $HO^{\bullet}$ reactions are nonselective, attacking organic and inorganic compounds through hydrogen abstraction, radical-radical reactions, electrophilic addition, and electron transfer reactions, eventually leading to complete mineralization of organic compounds (Oppenländer, 2003).

The action of $O_3$ against microorganisms for effective inactivation of ARB has been attributed to its reactivity with various organic functional groups, such as amines, activated aromatics, and reduced sulfur moieties, within the cellular membrane in Gram-positive bacteria and in the cell wall/membrane in Gram-negative bacteria (von Gunten,

**Table 10.1** Summary of the studies conducted on the effect of most frequently examined advanced oxidation processes (AOPs) on antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs).

| Experimental Conditions | Target ARB/ARG | Antibiotic Resistance Removal | Reference |
|---|---|---|---|
| ***Ozonation*** | | | |
| • Bench-scale system<br>• 2 L CAS effluent, spiked with erythromycin at 100 $\mu g\,L^{-1}$<br>• $O_3$ dose: 0.3 $mg\,L^{-1}$ | *E. coli* resistant to:<br>• erythromycin | • Complete inactivation of the bacterial population within 15 min | Michael-Kordatou et al. (2017) |
| • Bench-scale system<br>• Solution spiked with tetracycline at 10 $mg\,L^{-1}$<br>• $O_3$ dose: 3 and 7 $mg\,L^{-1}$ | • Transfer of pB10 plasmid | • With the presence of ppb level of residual tetracycline after ozone oxidation, higher facilitation of gene transfer was observed | Oh et al. (2015) |
| • Bench-scale system<br>• 6 L CAS effluent<br>• $O_3$ dose: 177.6 $mg\,L^{-1}$ | • *sul*1<br>• *tet*(G)<br>• *intI*1<br>• 16S rDNA | • Relative abundance of ARGs (normalized to 16S rDNA) increased during ozonation<br>• Reduction of the total abundance of 16S rDNA<br>• 1.68–2.55 log reduction of ARG | Zhuang et al. (2015) |
| • Bench-scale system<br>• 1 L CAS effluent<br>• $O_3$ dose: constant flow rate of 150 $cm^3\,min^{-1}$ | • Enterobacteria<br>• Total heterotrophs<br>• Enterococci<br>• 16S rRNA<br>• *intI*1<br>• *van*A<br>• *bla*TEM<br>• *sul*1<br>• *qnr*S | • 4-log reduction in all types of cultivable bacteria<br>• 2-log reduction for 16S rRNA<br>• 2-log reduction for *intI*1<br>• ARG abundance to LOQ after a contact time of 30 min | Sousa et al. (2017) |

(*Continued*)

**Table 10.1** (Continued)

| Experimental Conditions | Target ARB/ARG | Antibiotic Resistance Removal | Reference |
|---|---|---|---|
| • Full-scale ozonation system<br>• CAS effluent of the WWTP<br>• $O_3$ dose: 0.9 g of $O_3$/1 g DOC | • Enterococci<br>• *P. aeruginosa*<br>• Staphylococci<br>• Enterobacteria<br>• *van*A<br>• *bla*VIM<br>• *erm*B<br>• *amp*C | • Enterococci and *P. aeruginosa* little affected after treatment<br>• 2-log reduction of *erm*B<br>• *van*A and *bla*VIM abundance increased within the surviving wastewater population | Alexander et al. (2016) |
| • Pilot-scale system<br>• Ozonation to CAS effluent<br>• Contact time 20 min with 0.73 mg $O_3$/1 mg DOC<br>• Sand filtration or granulated activated charcoal<br>• Adsorption or a combination of both techniques | *E. coli*, enterococci and staphylococci resistant to:<br>• ampicillin<br>• chloramphenicol<br>• ciprofloxacin<br>• erythromycin<br>• vancomycin | • Reduction in the concentrations of total and antibiotic resistant enterococci and staphylococci (<LOD)<br>• Antibiotic resistant *E. coli* and staphylococci survived ozone treatment | Lüddeke et al. (2015) |
| ***UV/$H_2O_2$ photolysis*** | | | |
| • Bench-scale sunlight/$H_2O_2$ system<br>• 250 mL CAS effluent<br>• Spiking with multidrug resistant *E. coli* strain at $10^6$ CFU mL$^{-1}$<br>• [$H_2O_2$]: 10, 20, and 50 mg L$^{-1}$ | *E. coli* resistant to:<br>• ampicillin<br>• ciprofloxacin<br>• tetracycline | • Complete inactivation (<LOD) was achieved in all experiments with $Q_{UV}$ = 30, 18, and 8 kJ L$^{-1}$, respectively<br>• The antibiotic resistance pattern was not affected | Fiorentino et al. (2015) |
| • Pilot-scale sunlight/$H_2O_2$ system<br>• 8.5 L autoclaved CAS effluent<br>• Spiking with multidrug resistant *E. coli* strain at $10^5$ CFU mL$^{-1}$<br>• [$H_2O_2$]: 0.588, 1.470, and 2.205 mM | *E. coli* resistant to:<br>• ampicillin<br>• ciprofloxacin<br>• tetracycline | • Complete inactivation (<LOD) was reached in 150, 120, and 120 minutes of treatment with $Q_{UV}$ = 7.92, 6.75, and 5.93 kJ L$^{-1}$, respectively<br>• The investigated process did not affect antibiotic resistance pattern exhibited by the survived colonies | Ferro et al. (2015) |

| | | | |
|---|---|---|---|
| • Pilot-scale sunlight/$H_2O_2$ system<br>• 8.5 L autoclaved CAS effluent<br>• Spiking with multidrug resistant *E. coli* and *E. faecalis* strains at $10^5$ CFU mL$^{-1}$<br>• Spiking with carbamazepine, flumequine and thiabendazole at 100 μg L$^{-1}$<br>• [$H_2O_2$]: 20 mg L$^{-1}$ | *E. coli* and enterococci resistant to:<br>• ampicillin<br>• ciprofloxacin<br>• tetracycline | • LOD of AR *E. coli* was reached after an average dose $Q_{UV}$ of 6.29 kJ L$^{-1}$, after 120 min<br>• AR *E. faecalis* was found to be more resistant as the LOD was achieved at a higher $Q_{UV}$ dose (14.86 kJ L$^{-1}$) and treatment time (240 min) | Ferro et al. (2015) |
| ***Solar Fenton oxidation*** | | | |
| • Pilot-scale solar Fenton system<br>• 250 L CAS effluent<br>• [$Fe^{2+}$] = 5 mg L$^{-1}$<br>• [$H_2O_2$] = 75 mg L$^{-1}$<br>• pH = 2.8–2.9<br>• [ofloxacin, trimethoprim] = 100 μg L$^{-1}$ | *Enterococcus* resistant to:<br>• ofloxacin<br>• trimethoprim | • Decrease in the ofloxacin and trimethoprim resistance percentage of enterococci after solar photo-Fenton treatment | Michael et al. (2012) |
| • Pilot-scale solar Fenton system<br>• 60 L CAS effluent<br>• [$Fe^{2+}$] = 5 mg L$^{-1}$<br>• [$H_2O_2$] = 50 mg L$^{-1}$<br>• pH = 4<br>• [clarithromycin, sulfamethoxazole] = 100 μg L$^{-1}$ | *Enterococcus* resistant to:<br>• clarithromycin<br>• sulfamethoxazole | • Decrease in the prevalence of clarithromycin and sulfamethoxazole-resistant *Enterococcus* as treatment time increased | Karaolia et al. (2014) |
| • Bench-scale solar Fenton system<br>• 250 mL CAS effluent<br>• Spiking with multidrug resistant *E. coli* strain at $10^6$ CFU mL$^{-1}$<br>• [$Fe^{2+}$] =2 0 mg L$^{-1}$<br>• [$H_2O_2$] = 40 mg L$^{-1}$<br>• pH = natural | *E. coli* resistant to:<br>• ampicillin<br>• ciprofloxacin<br>• tetracycline | • LOD of AR *E. coli* was reached after an average dose $Q_{UV}$ of 15.34 kJ L$^{-1}$<br>• Complete (<LOD) inactivation<br>process did not affect the antibiotic resistance prevalence in survived colonies | Fiorentino et al. (2015) |

(*Continued*)

**Table 10.1** (Continued)

| Experimental Conditions | Target ARB/ARG | Antibiotic Resistance Removal | Reference |
|---|---|---|---|
| • Pilot-scale solar Fenton system<br>• 8.5 L autoclaved CAS effluent<br>• Spiking with multidrug resistant *E. coli* strain at $10^5$ CFU $mL^{-1}$<br>• $[Fe^{2+}]$ = 0.09, 0.179, 0.358 mM<br>• $[H_2O_2]$ = 0.294, 0.588, 1.176 mM<br>• pH = natural | *E. coli* resistant to:<br>• ampicillin<br>• ciprofloxacin<br>• tetracycline | • Complete inactivation (<LOD) was reached in 240 min of treatment with $Q_{UV}$ = 15.34 kJ $L^{-1}$ (0.090/0.294 mM of $Fe^{2+}/H_2O_2$)<br>• No effect on antibiotic resistance pattern exhibited by the survived colonies | Ferro et al. (2015) |
| • Bench-scale solar Fenton-like system (waste iron shavings from machining processes were used as a metallic iron)<br>• 300 mL of CAS effluent<br>• $[Fe^{2+}]$ = 10 M<br>• $[H_2O_2]$ = 0.023 M<br>• pH = 3 | Total coliforms resistant to:<br>• ampicillin<br>• ciprofloxacin<br>• gentamicin<br>• tetracycline<br>• chloramphenicol | • Total elimination of the examined ARB | Mackuľak et al. (2015) |
| • Bench-scale solar Fenton system<br>• 500 mL CAS effluent<br>• $[Fe^{2+}]$ = 0.001 M<br>• $[H_2O_2]$ = 0.01 M<br>• pH = 3 | • *sul*1<br>• *tet*(X)<br>• *tet*(G)<br>• 16S rRNA | • 2.58–3.79 log removal of the ARG | Zhang et al. (2016) |
| • Pilot-scale solar Fenton<br>• 60 L of MBR effluent<br>• [erythromycin, clarithromycin and sulfamethoxazole]$_0$ = 100 μg $L^{-1}$<br>• $[Fe^{2+}]$ = 5 mg $L^{-1}$<br>• $[H_2O_2]$ = 50 mg $L^{-1}$<br>• pH = 2.8 | *E. coli*, *P. aeruginosa* and Klebsiella spp. resistant to:<br>• erythromycin<br>• clarithromycin<br>• sulfamethoxazole<br>• *erm*B<br>• *sul*1<br>• *mec*A<br>• *amp*C<br>• *enc*<br>• *ecf*X | • Complete removal of bacteria after the process<br>• Low level of *P. aeruginosa* repair (2 CFU 100 $mL^{-1}$, 24 hours after treatment)<br>• Total DNA concentration was reduced by 97% after the integrated process<br>• *sul*1 and *erm*B were still present after treatment | Karaolia et al. (2017) |

| | | | |
|---|---|---|---|
| ***Heterogeneous $TiO_2$ photocatalysis*** | | | |
| • Bench-scale $TiO_2$ photocatalysis<br>• $TiO_2$ - Degussa P 25<br>• UV-A irradiation<br>• 4 scenarios of exposure:<br>a) 0.0625 mg mL$^{-1}$ $TiO_2$ × 400 μW cm$^{-2}$ UV-A<br>b) 0.0625 mg mL$^{-1}$ $TiO_2$ × 800 μW cm$^{-2}$ UV-A<br>c) 0.125 mg mL$^{-1}$ $TiO_2$ × 400 μW cm$^{-2}$ UV-A<br>d) 0.125 mg mL$^{-1}$ $TiO_2$ × 800 μW cm$^{-2}$ UV-A | • Methicillin-resistant *S. aureus* (MRSA)<br>• Multidrug-resistant *Acinetabacter baumannii* (MDRAB)<br>• Vancomycin-resistant *Enterococcus faecalis* (VRE) | • Exponential decrease of bacteria with increasing $TiO_2$ dose<br>• The results obtained using the strains of MDRAB and VRE suggested that the altered resistance to $TiO_2$ photocatalysis might be related to the acquisition of genetic material in the form of plasmids | Tsai et al. (2010) |
| • Bench-scale $TiO_2$ photocatalysis<br>• $TiO_2$- coated crystallizing dish served as the supporting container for water samples irradiated with UV-A/LED lamps<br>• UV intensities tested: 6, 7, and 8 mW cm$^{-2}$ | *E. coli* resistant to:<br>• ampicillin<br>• streptomycin | • 3-log removal of the bacterium<br>• Removal of ARB decreased with circle time in the studied range, while it increased with duty circle | Xiong and Hu (2013) |
| • Bench-scale $TiO_2$ photocatalysis<br>• Sunlight/$TiO_2$:[$TiO_2$] = 100 mg L$^{-1}$<br>• Sunlight/$H_2O_2$/$TiO_2$: two different ratios $TiO_2$:$H_2O_2$ were investigated (10:100 and 50:100 mg L$^{-1}$) | *E. coli* resistant to:<br>• ampicillin<br>• ciprofloxacin<br>• tetracycline | • Sunlight/$TiO_2$: inactivation down to the LOD was achieved in 150 min of treatment with a $Q_{UV}$ of 20 kJ L$^{-1}$<br>• Sunlight/$H_2O_2$/$TiO_2$: inactivation down to the LOD was achieved in 60 min with a $Q_{UV}$ of 3-5 kJ L$^{-1}$ | Fiorentino et al. (2015) |
| • Pilot-scale $TiO_2$ photocatalysis<br>• 8.5 L autoclaved CAS effluent<br>• Spiking with multidrug resistant *E. coli* strain at $10^5$ CFU mL$^{-1}$<br>• Solar irradiation<br>• [$TiO_2$] = 50 mg L$^{-1}$<br>• [$H_2O_2$] = 0.147 and 0.588 mM | *E. coli* resistant to:<br>• ampicillin<br>• ciprofloxacin<br>• tetracycline | • At 50 mg L$^{-1}$ of $TiO_2$: inactivation down to the LOD was achieved in 150 min of solar treatment with a $Q_{UV}$ of 7.88 kJ L$^{-1}$<br>• At 100 mg L$^{-1}$ of $TiO_2$: inactivation down to the LOD was achieved in 180 min with a $Q_{UV}$ of 9.94 kJ L$^{-1}$ | Ferro et al. (2015) |

(*Continued*)

**Table 10.1** (Continued)

| Experimental Conditions | Target ARB/ARG | Antibiotic Resistance Removal | Reference |
|---|---|---|---|
| • Bench-scale $TiO_2$ photocatalysis<br>• 500 mL CAS effluent<br>• Simulated solar irradiation<br>• $[TiO_2] = 0.2\,g\,L^{-1}$ | *E. coli* resistant to:<br>• ciprofloxacin<br>• cefuroxime<br>• tetracycline<br>• vancomycin | • Inactivation of bacteria down to the LOD | Rizzo et al. (2014) |
| • Bench-scale $TiO_2$ photocatalysis<br>• 200 mL distilled water and autoclaved secondary effluent<br>• Immobilized $TiO_2$ stirred tank reactor | *E. coli* resistant to:<br>• rifampicin<br>• chloramphenicol | • 2.5-log decrease in viable cell numbers ($CFU\,mL^{-1}$) within 180 min of treatment<br>• Recovery of the two strains of ARB back to their original numbers, after post-treatment incubation as the sublethally injured ARB employed repair mechanisms for damage reversal and regrowth | Dunlop et al. (2015) |

CAS = conventional activated sludge; LOQ = limit of quantification; WWTP = wastewater treatment plant; DOC = dissolved organic carbon; LOD = limit of detection; CFU = colony-forming units; AR = antibiotic resistant.

**Table 10.2** Summary of the mechanisms of action of each AOP on ARB and ARGs.

| AOP | Mechanism of Action | Reference |
|---|---|---|
| Ozonation | • $O_3$ reacts with unsaturated bonds within the membrane-bound phospholipids and lipopolysaccharides<br>• Damage of the cell wall/membrane<br>• Increased permeability of the cell membrane occurs, leading to leakage of cellular components to the outside of the cell and cell lysis<br>• The inner molecular components such as ARG are less affected by $O_3$ as they may remain within cell debris after ARB loss of viability | Dodd (2012), Pryor et al. (1991) |
| UV/$H_2O_2$ photolysis | • Combined action of the UV radiation with the enhanced production of radicals by $H_2O_2$ due to received radiation energy<br>• Interaction of UV radiation with target moieties in bacterial cells by light absorption from certain chromophores such as the L-tryptophan<br>• Photochemical inactivation reactions occur inside the cell<br>• Three mechanisms of UV action on pyrimidines:<br>I) pyrimidine-pyrimidine dimerization<br>II) pyrimidine-pyrimidone coupling between two adjacent pyrimidines<br>III) protein-DNA cross-linking<br>• The damaged impact of $H_2O_2$ in exposed bacteria includes high mutation rates, growth defects, and death | Dodd (2012), Mishra and Imlay (2012) |
| Solar Fenton oxidation | • During solar Fenton process, iron and $H_2O_2$ are released and penetrate cells, affecting the cell metabolism<br>• Once the cell membrane selective permeability and structural integrity have been compromised by $O_3$, the cell interior becomes exposed to the external environmental conditions. More specifically, after the oxidative damage on the cell wall/membrane, increased permeability occurs, leading to leakage of cellular components to the outside of the cell<br>• Cellular damage through the production of ROS<br>• The main damaging molecules to cell components are the reduced forms of molecular oxygen in $H_2O_2$, namely ROS<br>• The resulting presence of ROS produces oxidation of loose ferrous iron inside the cell, producing the Fenton oxidation and further production of ROS<br>• As the $H_2O_2$ reaction constants with iron centers are very high, even submicromolar concentrations of $H_2O_2$ can inactivate enzymes | Dodd (2012), Malato et al. (2009), Park et al. (2005) |
| Heterogeneous $TiO_2$ photocatalysis | • Conferred cell damage produced by ROS<br>• Modification and destruction of the structure of the cell membrane as a result of lipid peroxidation<br>• Initial bacterial cell membrane damage in the outer lipopolysaccharide and peptidoglycan wall<br>• Once external component damage and increased membrane permeability is achieved, further oxidative attack on the cell inner components is allowed, leading to cell inactivation | Alrousan et al. (2009), Malato et al. (2009), Bandala and Bustos (2015), Byrne et al. (2015) |

ROS = reactive oxygen species.

2003). Initially, on the surface of the bacterial cell, $O_3$ reacts rapidly with unsaturated bonds within the membrane-bound phospholipids and lipopolysaccharides (Pryor et al., 1991). Moreover, it was shown to be relatively unreactive toward amide groups of peptide-bound amino acids, making it reactive only toward N-terminal amino acids, free amines, aromatic and/or organosulfur side chains of tryptophan, tyrosine, histidine, lysine, methionine, and cysteine in cellular proteins and peptidoglycan layer (Dodd, 2012).

Once the cell membrane selective permeability and structural integrity have been compromised by $O_3$, the cell interior becomes exposed to the external environmental conditions. More specifically, after the oxidative damage on the cell wall/membrane, increased permeability occurs, leading to leakage of cellular components to the outside of the cell (Dodd, 2012). In a study by Hunt and Mariñas (1999) using transmission electron microscopy, it was shown that cellular inactivation by $O_3$ was produced before the extensive degradation of cellular components, showcasing that oxidation of the membrane is a primary step to the lethal action of $O_3$. More precisely, the electron micrographs showed a similar cellular content of *E. coli* cells exposed to 0.0826 mg $L^{-1}$ $s^{-1}$ $O_3$ at pH 7.2 and *E. coli* cells in controls (no $O_3$). Despite this observation, there was 5-log reduction in cultivable *E. coli* cells after exposure to $O_3$. Despite the remarkable damage induced on the cell membrane by $O_3$, in a study by Cho et al. (2010) it was shown that internal component damage is less intense when most damage takes place on the outer cell components. As a result, the inner molecular components such as ARGs may be less affected by such oxidants, as they may remain within cell debris after ARB loss of viability. The studies conducted on the inactivation of ARGs by $O_3$ showcased this finding, as ARB were shown to be successfully inactivated while the abundance of ARGs was less effectively affected by the applied ozonation treatment, as they are still present even after the treatment.

The molecular action of $O_3$ on nucleic acids has been investigated by Ishizaki et al. (1984). The mononucleotides thymidine monophosphate (TMP) and deoxyguanosine monophosphate (dGMP) were shown to react faster with $O_3$ than deoxycytidine monophosphate (dCMP) and deoxyadenosine monophosphate (dAMP) at a neutral pH environment, making the first two mononucleotide reactions with $O_3$ a limiting factor in the extension of DNA and thus in cell structural modifications. The double strand DNA (dsDNA) reactivity with $O_3$ was shown to be lower compared with the $O_3$ reaction rates on each individual nucleobase, a phenomenon which has been attributed to the protection of dsDNA reactive sites by H-bonding between the individual DNA strands (Theruvathu et al., 2001). The most pertinent mechanisms of disinfection on ARB and ARG destruction are given in Table 10.2.

Few studies have dealt with the potential of the ozonation process to remove antibiotic-related microcontaminants, including ARB and ARGs, and these are shown in Table 10.1. Despite the important findings of these studies, there is still a lack of research on the separation of the role of molecular $O_3$ action from the secondary reactions taking place in solution by the produced $HO^{\bullet}$, which is continuously generated during aqueous decomposition of $O_3$.

### UV/$H_2O_2$

The photolysis of $H_2O_2$ by UV radiation, which is absorbed at 200–300 nm wavelengths, produces a homolytic scission of the O-O bond of the molecule and leads to the

formation of $HO^{\bullet}$ radicals, which in turn contribute to $H_2O_2$ decomposition by secondary reactions (Liao and Gurol, 1995). The reactions taking place for the formation of $HO^{\bullet}$ radicals according to Oturan and Aaron (2014) are shown in Equations 10.6–10.12.

$$H_2O_2 + h\nu \rightarrow 2HO^{\bullet} \tag{10.6}$$

$$HO^{\bullet} + H_2O_2 \rightarrow H_2O + HO_2^{\bullet} \tag{10.7}$$

$$HO_2^{\bullet} + H_2O_2 \rightarrow HO^{\bullet} + H_2O + O_2 \tag{10.8}$$

$$HO^{\bullet} + HO_2^{-} \rightarrow HO_2^{\bullet} + HO^{-} \tag{10.9}$$

$$2HO_2^{\bullet} \rightarrow H_2O_2 + O_2 \tag{10.10}$$

$$HO^{\bullet} + HO_2^{\bullet} \rightarrow H_2O + O_2 \tag{10.11}$$

$$2^{\bullet}OH \rightarrow H_2O_2 \tag{10.12}$$

The reaction rate during $H_2O_2$/UV photolysis is higher in alkaline media (pH > 10), a fact that may be attributed to the fact that the $HO_2^-$ anion resulting from the ionization of $H_2O_2$ can strongly absorb UV radiation and produce the reactive oxygen species (ROS) $HO^{\bullet}$, the superoxide radical anion $HO_2^{\bullet}$, and the singlet oxygen $O_2^*$

The disinfection action of $H_2O_2$/UV photolysis and the inactivation of ARB and ARGs may be credited to the combined action of the UV radiation with the enhanced production of radicals by $H_2O_2$ due to received radiation energy (Table 10.2). More specifically, UV radiation alone exhibits its own mode of action on pathogenic microorganisms as well as biological material found in receiving wastewater matrices. It has been shown that UV radiation interacts with target moieties in bacterial cells by physical processes first, such as light absorption by certain chromophores such as the L-tryptophan, which subsequently leads to photochemical inactivation reactions inside the cell. Due to the observed high absorption capacity by nucleic acids, UV radiation is highly suited for inactivation of ARG (Eischeid et al., 2009). The pyrimidines (TMP, UMP, and dCMP) are most susceptible to UV-induced damage due to three mechanisms of UV action on their structure according to Dodd (2012): (i) pyrimidine-pyrimidine dimerization where two pyrimidine bases are located next to each other on single stranded DNA, (ii) pyrimidine (6-4) pyrimidone coupling between two adjacent pyrimidines, and (iii) protein-DNA cross-linking. Microorganisms receiving a sublethal dose of UV radiation may become resistant to induced oxidative stress, leading to partial recovery of damaged defense mechanisms and adaptations to oxidative stress. The effect of UV radiation was also shown to be exhibited through UV-A oxidative stress, which is damaging to internal cell components, causing lipid peroxidation and DNA rupture generating single strand breaks and nucleic acid modifications, leading to mutagenesis or lethal damage.

Regardless of the studies made on the effect of UV radiation on the quantification of extracellular ARGs through molecular analyses, only limited studies have been made on the effect of UV radiation in vivo on bacterial DNA, and more specifically on the potential changes on specific ARGs inside living cells. These may include behavioral changes and changes in replication, growth, and other survival mechanisms. In detail, Hoelzer and Michod (1991) investigated the effect of UV radiation on tryptophan- and methionine-prototrophic *Bacillus subtilis* cells, producing 20%–40% reduction in the ability of the intracellular DNA to transform auxotrophic recipient bacteria to prototrophic ones.

Moreover, limited work has been done on examining the ability of UV-irradiated bacteria to undergo light or dark repair, thus restoring the activity of DNA. DNA repair may take place in ARB that were exposed to UV or within competent recipient cells that have received UV-damaged DNA from ARB donors. Remarkably, Michod et al. (2008) suggests that UV-induced damage in genetic material may bring about recombinational repair of damaged DNA, which may substantially benefit pathogenic bacteria through oxidative defense. In the long term, the reproduction of cells with repaired genetic material may help get rid of mutations, increase the rate of adaptation of the population, and in pathogens, may infrequently create new infective strains.

The damaging impact of $H_2O_2$ in exposed bacteria includes high mutation rates, growth defects, and death (Mishra and Imlay, 2012), while increased levels of environmental oxygen concentrations can exacerbate this impact. $H_2O_2$ has the ability to penetrate the lipid bilayer in the cell membrane, as it has a permeability coefficient similar to the one for water. As molecular oxygen in $H_2O_2$ cannot react with biomolecules, the reduced forms of it, namely, ROS, are the main damaging molecules to cell components. The resulting presence of ROS produces oxidation of loose ferrous iron inside the cell that is not protein bound, producing the Fenton oxidation and further production of ROS (Park et al., 2005). The Fenton oxidation is also utilized during wastewater treatment for the same purpose, namely, DNA damage and bacterial inactivation and/or death ("Solar Fenton Oxidation" section). As the $H_2O_2$ reaction constants with iron centers are very high ($1000–50000\,M^{-1}\ s^{-1}$), even submicromolar concentrations of $H_2O_2$ can inactivate enzymes in a very short time. Therefore, the key is to keep $H_2O_2$ concentrations below harmful levels. Employing extremely active peroxidases and catalases accomplishes this, that is, ROS scavenging enzymes that reduce and degrade $H_2O_2$ down to physiological levels inside cells, bringing about bacterial survival and repair in the presence of $H_2O_2$. ROS scavengers include β-carotene (singlet oxygen scavenger) and superoxide dismutase, which protect bacteria from radiation and oxidation.

The use of $H_2O_2$/UV photolysis has not been extensively examined in the existing scientific literature so far, with only a few studies existing on the inactivation of total bacteria and ARB in wastewater effluents (Table 10.1). Overall, there is very limited literature examining the effect of $H_2O_2$/UV photolysis on the removal of total DNA, ARB, and ARGs. The few available studies have shown the efficiency of the process for overall disinfection of a limited number of both total bacteria and ARB, highlighting the current gaps in research such as the investigation of a large variety of ARB, and of ARGs using molecular techniques, in real wastewater effluents after secondary treatment. Moreover, the mechanisms of action that cause disinfecting effects and the degradation of ARGs that are present in treated wastewater effluents, as well as the bacterial reactivation potential after UV-induced damage, need to be further examined for the optimization of the process and production of safer wastewater effluents, which safeguard human and environmental health.

### Solar Fenton Oxidation

Solar Fenton oxidation is a highly efficient homogeneous treatment that involves the catalytic breakdown of hydrogen peroxide ($H_2O_2$) in reaction with ferrous or ferric iron ($Fe^{2+}$ or $Fe^{3+}$) in an acidic medium to form active transitory species such as $HO^{\bullet}$, in the presence of UV-Vis sunlight (Ruppert et al., 1993). Coupling the Fenton process with

solar irradiation has resulted in added advantages to this type of photochemical AOP, as the process is simple, clean, relatively inexpensive, and more efficient than chemical AOP. As UV radiation is coupled with a powerful oxidant, $H_2O_2$, and a catalyst, ferrous iron, the degradation or destruction/inactivation of microcontaminants through the processes of photodecomposition, excitation and degradation, direct $H_2O_2$ oxidation, and photocatalytic oxidation through the production of $HO^{\bullet}$ radicals is enabled. The reactions are summarized in Equations 10.13 and 10.14 (Rodríguez-Chueca et al., 2014).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO^{\bullet} \tag{10.13}$$

$$Fe(HO)^{2+} + h\nu \rightarrow Fe^{2+} + HO^{\bullet} \tag{10.14}$$

During the Fenton treatment, the catalytic regeneration of ferrous iron from ferric iron is a rate-limiting step in the cycle of iron catalysis, leading to the dependence of the reaction on the iron concentration in solution. In addition to this issue, due to the low solubility of the ferric iron, precipitation occurs when pH is not acidic, depending on the iron concentration and temperature, making the acidification of the aqueous solution necessary for the photo-Fenton treatment. In experimental settings, this challenge can also be troublesome for working with microorganisms, since at very low pH around 3 the investigated microorganisms are not viable, without the need for Fenton treatment. Also, the competition between the pollutants and dissolved iron kept in solution for $HO^{\bullet}$ radicals may pose a challenge to the efficiency of the Fenton process.

The action of the solar Fenton process on the cell metabolism depends on the concentrations of iron and $H_2O_2$, which are released during the photocatalytic process and penetrate cells (Table 10.2). The result is cellular damage through the production of ROS, which is described in the sections on ozonation and UV/$H_2O_2$. Very few studies have dealt with the inactivation potential of solar Fenton oxidation for ARB and AR, and these are shown in Table 10.1.

Generally, it has been shown that the solar Fenton oxidation is effective in the inactivation of cultivable ARB to various examined antibiotics in available literature, as at the end of the treatment the number of cultivable bacteria is reduced to the limit of detection (LOD) in all studies conducted. Despite this efficiency, the removal of ARG has not been examined in depth, as the limited number of available studies only examines a limited number of ARGs. Furthermore, the correlation of the affected ARB to the present ARG and total DNA concentrations, especially those resistant to oxidation treatment, is yet to be determined. Moreover, there is a gap in research regarding the effect of the solar Fenton oxidation on the functional characteristics of a wide range of cultivable ARB, in relation to the phenotypic expression of resistance determinants, on a wide range of commonly encountered ARGs and on total DNA in real effluents. More research is needed in the abovementioned voids in research, as well as into the reactivation potential of the inactivated ARB, after the oxidation is over and treated effluents are intended for reuse and disposal into aquatic ecosystems.

### Heterogeneous $TiO_2$ Photocatalysis

Heterogeneous photocatalysis is another widely investigated AOP, during which the photocatalyst is present as a solid, with the reactions taking place at the interface between solid-liquid or solid-gas. The main reactions are electrochemical redox

reactions that involve hole and electron transfer from the photo-excited semiconductor. More specifically, the semiconductor ($TiO_2$) gets excited by the absorption of radiation, which has energy equal to or greater than the band gap energy ($E_{bg}$). The electron band of the conduction band may be passed to an electron acceptor with a more positive reduction potential than the conduction band, and the valence band hole may accept electrons from donor species with a smaller electrochemical reduction potential than the valence band potential. The potential difference generated is close to the band gap energy of the semiconductor, leading to the generation of ROS on the particle-solution interface (Byrne et al., 2015).

Heterogeneous photocatalytic disinfection with the use of $TiO_2$ is among the most widely investigated AOP. However, it is still an emerging field of research, which has given rise to the design and synthesis of novel nanostructured photocatalytic materials, which possess novel properties for efficient application in wastewater disinfection. Among the newly investigated photocatalytic materials are zinc oxide with different sizes and morphologies as well as graphene composites (Alarcón et al., 2011; Baruah et al., 2012). $TiO_2$ action only occurs when it is irradiated with UV light ($\lambda < 400$ nm), thereby limiting the sensitivity of the material to only a small part of the solar spectrum (about 5%). Various authors have investigated modifications to $TiO_2$ to extend its absorption to the visible part of the spectrum, while improving its photosensitivity and quantum yield, as well as reducing the band gap energy requirements for photocatalytic activation (Popa et al., 2008; Pelaez et al., 2009; Rizzo et al., 2013a). Among the doped materials examined for the enhancement of $TiO_2$ photocatalytic efficiency, silver, vanadium, iron, and palladium-modified and nitrogen-doped $TiO_2$ have been shown to enhance photocatalytic performance as to the inactivation of Gram-negative bacteria such as *E. coli*, *P. aeruginosa*, and *Prevotella intermedia*, as well as of some Gram-positive bacteria, including *Staphylococcus epidermis*, *S. pyogenes*, *S. saprophyticus*, and *Streptococcus aureus* (Mo et al., 2007; Sheel et al., 2008; Nair et al., 2012; Uboncholakate et al., 2012). Photocatalytic disinfection has been investigated using suspensions of $TiO_2$ and their immobilized form, with the latter possessing the advantage of cost reduction, decreased material loss, and avoidance of catalyst recovery at the end of the process (Gelover et al., 2004). So far, the most efficient form of $TiO_2$ is Aeroxide P25, as immobilized forms of variations of $TiO_2$ present equal efficiency with a suspension of Aeroxide P25 (Bandala and Bustos, 2015).

As has been previously mentioned, the main inactivation mechanism of microorganisms during AOP is the conferred cell damage produced by ROS, which are able to modify and destroy the structure of the cell membrane as a result of lipid peroxidation (Alrousan et al., 2009; Malato et al., 2009). Initial bacterial cell membrane damage is brought about in the outer lipopolysaccharide and peptidoglycan wall, followed by lipid peroxidation and protein and polysaccharide oxidation, which bring about negative impacts on the regulatory function of cell membrane regarding its capacity for internal and external interchange (Dalrymple et al., 2010) (Table 10.2). Once external component damage and increased membrane permeability is achieved, further oxidative attack on the cell inner components is allowed, leading to cell inactivation (Bandala and Bustos, 2015).

Various studies have dealt with the inactivation of pathogenic microorganisms in wastewater matrices, but very few have investigated the removal of the total DNA and the complete range of ARB and ARGs that are present in such matrices (Table 10.1), as only a limited number of these have been examined so far. It should be noted that

while heterogeneous photocatalysis has great potential as a biocidal for pathogenic microorganisms including ARB, its effect on ARGs has not been investigated so far. Moreover, caution must be exercised when conducting photocatalytic disinfection, as organisms have the potential to recover from sublethal ROS dose stress, leading to repair and regrowth. Some studies in fact, have reported this phenomenon (Rincón and Pulgarin, 2004; Dunlop et al., 2010), highlighting the need for complete microorganism inactivation and further in-depth analyses to ensure the prevention of further subsequent repair and regrowth.

The mineralization potential exhibited by the described processes is affected by various factors, including wastewater-inherent characteristics such as dissolved solids and SS, alkalinity, water pH, and temperature of the water matrix. For example, SS may hinder the reaction rate of photochemical reactions by light absorption, and light scattering may be reduced as a result. The performance may thus be impaired. Moreover, carbonates, bicarbonates, chloride ions, and natural organic matter may act as radical scavengers, competing with target pollutants for the produced radicals, increasing oxidant demand, and lowering the treatment efficiency, as well as adding financial cost in materials and equipment. As a result, AOPs may be implemented most efficiently as polishing treatments during wastewater treatment, following a process that removes SS or a large fraction of the natural organic matter, such as the MBR process.

## Integration of Processes

For the assurance of a high-quality effluent that meets current standards and is safe for disposal into natural aquatic environments, a "multiple-barrier approach" (Neoh et al., 2016) may be adopted, with an alternative biological treatment such as MBR as the first barrier in the integrated treatment train. This approach can be realized through the integration of the biological technology with other post-treatments that can polish the produced effluent by removing contaminants of emerging concern, such as ARGs and ARB, which may not be removed by the alternative biological treatment. These post-treatments are AOPs, and they may mitigate the risk of contaminated effluent once disposed into natural environments (Gimeno et al., 2016).

Such integration of technologies also aims to gain the side benefit of cost reduction and targeting a larger variety of microcontaminants than a single technology would do, improving the quality of the final treated effluent, compared with conventionally treated effluent. The high SS removal (>99%) from an MBR effluent leaves this treated wastewater almost free from scavengers of $HO^{\bullet}$ formed during the AOP operation, which enhances the AOP efficiency and improves the post-treatment effluent quality (Dialynas and Diamadopoulos, 2009). The production of turbidity by SS containing various types of scavengers ($Cl^{-}$, $SO_4^{-2}$, $CO_3^{2-}$, $HCO_3^{-}$) in a water matrix may react with the $HO^{\bullet}$ during solar-driven chemical oxidation, influencing the microcontaminant mineralization potential of the process (Klamerth et al., 2010; Michael-Kordatou et al., 2015). SS may also weaken the light transmission rate during solar-driven processes due to the absorbance of light by these solids, and they may deposit in the solar reactor, blocking the entrance of solar light (Scheible, 1987). So far, very few studies exist in the framework of an integrated urban advanced, alternative wastewater treatment approach, examining the removal of ARB and ARGs.

Moreira et al. (2016) evaluated the removal efficiency of photocatalytic ozonation using immobilized $TiO_2$ with light-emitting diodes (LEDs) for selected housekeeping genes (16S rRNA), selected ARGs (*bla*TEM, *qnr*S, *sul*1), and different ARB (total heterotrophs, enterobacteria, and enterococci) resistant to ciprofloxacin, gentamicin, and meropenem. Although the ARG abundance was successfully reduced to levels close or below the LOD, after storage of the treated wastewater the total heterotrophs and examined ARB and 16S rRNA recovered to pretreatment levels. In another study, Lüddeke et al. (2015) applied ozonation in combination with charcoal or slow sand filtration at a pilot scale to determine the elimination of *E. coli*, enterococci, and staphylococci, and their resistance against ampicillin, chloramphenicol, ciprofloxacin, erythromycin, and vancomycin. The combined process reduced the concentrations of total and antibiotic resistant *E. coli*, enterococci, and staphylococci below the LOD. However, antibiotic resistant *E. coli* and staphylococci survived ozone treatment. In a pilot-scale study (Karaolia et al., 2017) the removal of selected ARB (*E. coli*, *P. aeruginosa*, and *Klebsiella* spp.) along with selected ARGs (*erm*B, *sul*1, *mec*A, *amp*C, *enc* and *ecf*X) was investigated in an integrated MBR–solar Fenton oxidation process. The results of this study showed a complete removal of the examined bacteria after the process, although there was a low level of *P. aeruginosa* repair (2 CFU $100\,mL^{-1}$, 24 hours after treatment). The total DNA concentration was reduced by 97% after the integrated process, while *sul*1 and *erm*B were still present after treatment, indicating the further challenge of their removal.

## Conclusions

This chapter is a preliminary attempt to provide a literature review regarding the removal of ARB and ARGs using alternative biological and natural processes, as well as AOP or a combination of these. The investigation of the application of such processes aims at providing a means of replacing or enhancing the conventionally applied wastewater treatment processes, which have been shown to be inefficient in the removal of ARB and ARG. Throughout this chapter, the efficiency of the alternative processes, the operational parameters examined in each study, and the resulting AR removal have been reported.

More specifically, MBR has been shown to be effective in removing different cultivable ARB (3-log up to 4.6-log removal) and various ARGs (2.5-log up to 7-log removal), mainly due to the filtration step of the hybrid biological/filtration process. Also, constructed wetlands have shown a high removal of ARB and ARGs compared to the CAS process, but the removal is always related to the flow pattern in place, as horizontal flow constructed wetlands have been shown to be more efficient in the removal of AR compared to the vertical flow ones. Despite the reported efficiency of the abovementioned processes, only a few operational parameters have been investigated for the optimum removal efficiency, while there is a gap in knowledge regarding the effect of each operational parameter such as solid and hydraulic retention times and the combination of the most important ones, in the removal efficiency of ARB and ARGs, in real wastewater matrices.

AOP is a group of treatment processes that make use of $HO^{\bullet}$, a powerful oxidizing agent for the decontamination and disinfection of water matrices. The most widely

examined AOPs include ozonation, UV/$H_2O_2$ photolysis, solar Fenton oxidation, and heterogeneous $TiO_2$ photocatalysis.

Ozonation is a highly efficient chemical AOP that has been shown to achieve a 2-log reduction of ARG, while ARB have shown variable responses to ozonation in studies ranging from bench-scale application to full-scale ozonation systems. Moreover, a high ARG transfer and abundance has been observed within surviving wastewater bacterial populations. As a result, further research is required for the complete comprehension of the role of ozone in the inactivation of ARB and destruction of ARGs, for the further optimization of the process. The currently available data on the disinfection mechanisms by ozone on ARB and ARGs are not adequate to decipher the complete role of both $O_3$ and produced $HO^{\bullet}$.

UV/$H_2O_2$ photolysis combines the action of UV radiation with the production of $HO^{\bullet}$ after the addition of a powerful oxidant, $H_2O_2$. Very few studies have been done on the removal of ARB and ARGs by UV/$H_2O_2$ photolysis, indicating the gap in knowledge regarding the effect of this type of treatment on these microcontaminants. Moreover, the mechanisms of action that produce an adequate disinfecting effect for complete inactivation of ARB while eliminating the reactivation potential of ARB have not yet been determined in real wastewater effluents.

There are various studies investigating the effect of solar Fenton oxidation, at bench-scale and pilot-scale applications on the removal of ARB and ARGs. Overall, the examined ARB are reduced to the LOD in studies examining solar Fenton disinfection, while the few that examined ARGs have shown less removal efficiency, as they are still present in treated effluents after the solar Fenton treatment. The correlation of the affected/inactivated ARB to the complete array of the present ARG and total DNA concentration in treated effluents remains to be examined, especially of those that are resistant to oxidation. In addition, the effect of solar Fenton oxidation on the functional characteristics of unaffected ARB by the treatment in relation to the phenotypic expression of the corresponding resistance determinants is yet to be determined. Furthermore, optimum operational conditions for complete bacterial and other pathogenic microorganism inactivation must be explored, as well as the conditions that favor the reactivation of such pathogens.

The last examined AOP, namely, the heterogeneous $TiO_2$ photocatalysis, makes use of a solid catalyst on whose surface redox reactions take place with the ultimate goal of production of $HO^{\bullet}$. Despite the efficiency of this process on the inactivation of ARB, the potential for the removal of ARGs has not yet been explored. Moreover, the recovery of bacteria from the sublethal ROS stress exerted by the photocatalytic treatment may arise due to repair and regrowth, as reported previously. This indicates the need for further optimization of the catalytic materials exploited, as well as further investigation of the operational parameters to ensure the prevention of the observed repair and regrowth.

Finally, this chapter investigated the few studies conducted that combine two or more alternative wastewater treatments, with the ultimate goal of the assurance of an up-to-par effluent that is safe to dispose in aquatic ecosystems. The few conducted studies show a reactivation of ARB after the integrated treatment is over, as well as survival of ARB in the presence of ozone. The optimization of integrated process may form a promising process train for the removal of ARB and ARGs, but extended studies are needed to discover the most effective combination of processes for this purpose.

The consideration and in-depth investigation of these alternative processes can assist in closing gaps in knowledge, which may be the key to mitigating the spread and accumulation of antibiotic resistance determinants in urban wastewater effluents.

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# 11

# Antimicrobial Resistance Spread Mediated by Wastewater Irrigation

## The Mezquital Valley Case Study

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The Mezquital Valley, in the north of the metropolitan area of Mexico City, is one of the world´s largest sewage fields. For more than four years, we studied the effect of untreated wastewater irrigation on the input of pathogenic bacteria, antibiotics, antibiotic resistance determinants, and their spread by mobile genetic elements in the affected soils and water bodies. The study was enabled by the availability of a soil chronosequence: soils that have been under wastewater irrigation for 1 year up to more than 100 years were compared with soils that have never been in contact with wastewater. The analyses were performed by quantitative real-time PCR (qPCR) in combination with cultivation approaches to characterize the microbial community in the affected soils. Moreover, 16S rDNA amplicon sequencing of the bacterial soil communities was performed, revealing a shift of the bacterial populations toward γ-*Proteobacteria*, including potential human pathogens in soils that have been under long-term wastewater irrigation. Data on occurrence and potential spread of antibiotic resistance will be presented, in correlation with accumulation of antimicrobials and heavy metals in these soils. The data will be critically assessed and compared with those of other studies. Perspectives on future investigations in the area will be given, which will study the impact of changing the irrigation regime from raw wastewater to treated wastewater.

## Introduction

Wastewater (WW) reuse for agriculture purposes provides benefits and risks at the same time (Siebe and Cifuentes, 1995). In some areas, for example, semiarid and arid areas, this practice or using partially treated WW for irrigation is the only way to ensure that farming can continue over the course of the entire year (Siebe and Cifuentes, 1995; Frenk et al., 2013; Negreanu et al., 2012; Elifantz et al., 2011; Jueschke et al., 2008). In addition, the WW provides the necessary nutrients (carbon, phosphorus, nitrogen) for the cultivation of plants, making fertilizer application unnecessary (Siebe and Cifuentes, 1995). However, the release of pathogenic bacteria, pharmaceuticals (e.g., antibiotics),

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antibiotic resistance genes, and other contaminants (e.g., xenobiotics and heavy metals) into the environment by WW irrigation or other anthropogenic activities (e.g., application of manure) might promote their accumulation and possibly also a dissemination of multiresistant bacteria (Figure 11.1).

A series of studies have been conducted to assess the impact of WW irrigation or treated WW irrigation on different aspects of soil quality, presence of biological contaminants in soils, and plant uptake of biological contaminants by crops grown in the irrigated fields (Mok and Hamilton, 2014; Hong et al., 2013; Becerra-Castro et al., 2015; Norton-Brandão et al., 2013; Carey et al., 2016; Gatica and Cytryn, 2013). In addition, a few studies investigated the impact of the irrigation scheme on the indigenous microbial soil community (a timely review on the topic is presented in Becerra-Castro et al. (2015)). However, most of the studies concentrated on only one or two of these aspects.

Thus, we conducted a comprehensive investigation of the major biological factors potentially impacted by WW irrigation in the Mezquital Valley in Mexico. We analyzed

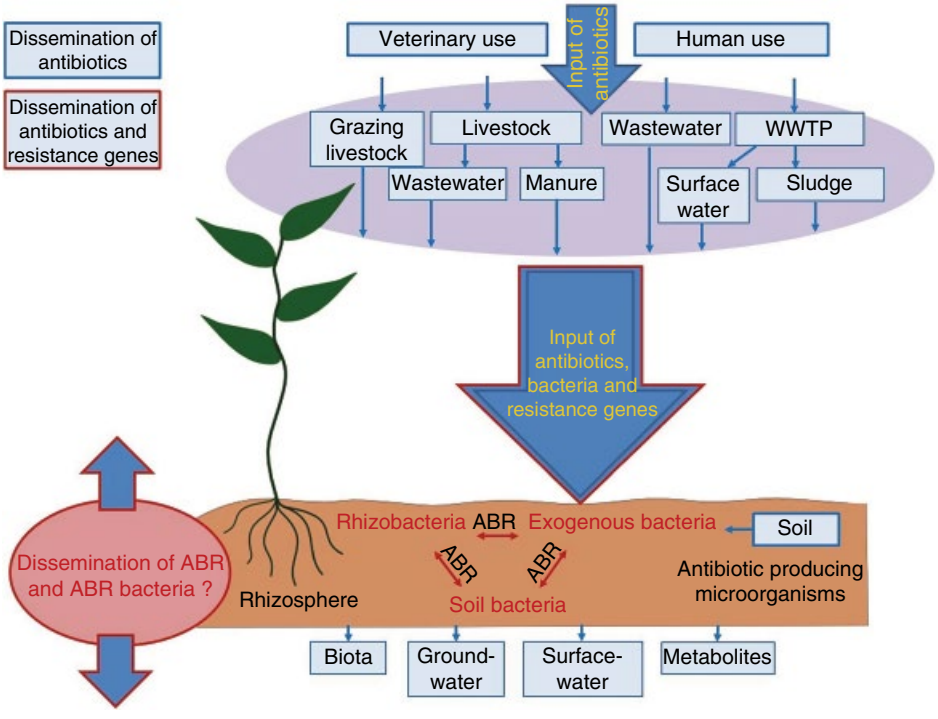


**Figure 11.1** Spread of antibiotics, bacteria, and resistance genes in the environment. By anthropogenic activities (e.g., manure application and wastewater irrigation), antibiotics (and other selectors for resistance genes), bacteria and antibiotic resistance genes are released into the environment. This might promote their accumulation and dissemination in the soil (transfer of antibiotic resistance genes from exogenous to soil bacteria and rhizobacteria) and might lead to the distribution of resistant bacteria via biological and physical forces (e.g., by wildlife, wind, water, or by consumption of near-ground plants). ABR = antibiotic resistance/antibiotic resistant; WWTP = wastewater treatment plant. (*See insert for color representation of the figure.*)

the occurrence of antibiotic resistance genes and fecal indicators, such as enterococci in different environmental compartments (WW, reclaimed water, soil, and plants) as well as the impact of irrigation with untreated WW on the bacterial soil community.

## Effect of Untreated Wastewater on Antibiotic Resistance Genes and Microbial Populations in Soil

Wastewater reuse for irrigation is widely practiced in agriculture to alleviate water shortages. Irrigation with unpurified WW can allow large numbers of antibiotic resistance genes and nosocomial pathogens to be released with the WW into the soils (Khan et al., 2013; Böckelmann et al., 2008; Levantesi et al., 2010). The Mezquital Valley is the world´s largest WW irrigation field (Raschid-Sally and Jayakody, 2009). Here, WW irrigation has been practiced for more than 100 years (Jimenez and Chávez, 2004). Over the past century, the irrigated area in the Mezquital Valley has increased due to the expansion of the Mexico City Metropolitan Area, providing unique conditions in which fields irrigated with WW for different time periods (a so-called chronosequence) could be sampled for our studies. In WW-irrigated soils from the Mezquital Valley, a significant increase of *sul* resistance genes (encoding sulfonamide resistance) of about two orders of magnitude (normalized to the copies of bacterial 16S rDNA), compared to rain-fed soils from the same area was observed (Dalkmann et al., 2012). An increase in the number of enterococci, typical fecal indicators, was also detected (approximately five times more in WW-irrigated soils than in rain-fed soils) (Dalkmann et al., 2012).

In German soils fertilized with swine manure containing high concentrations of the sulfonamide antibiotic sulfadiazine, an increase of the concentration of *sul* genes related to the sulfonamide concentration was also observed (Heuer et al., 2011). In contrast, there was no increase in the abundance of *sul* genes found in Israeli soils irrigated with treated WW containing much smaller sulfonamide concentrations (Negreanu et al., 2012).

Furthermore, WW irrigation can have a large impact on the bacterial soil community. In the Mezquital Valley WW-irrigated soils, a significant increase of *Proteobacteria* (up to 30% more), especially of γ-*Proteobacteria*, was observed by 16S rDNA amplicon sequencing compared to rain-fed soils from the same area (Broszat et al., 2014). An increase in γ-*Proteobacteria* was also detected in soils irrigated with treated WW in Israel (Frenk et al., 2013) and could be found in manured soil (Ding et al., 2014), suggesting that the abundance of this class of *Proteobacteria* increases in nutrient-rich environments. Additionally, 16S rDNA amplicon sequencing of total DNA from the Mezquital Valley soils revealed potentially pathogenic organisms, for example, *Stenotrophomonas* and *Pseudomonas*, which were not detectable in rain-fed soils. These findings were confirmed by a cultivation approach of soil bacteria from the WW-irrigated soils. Phylogenetic affiliation of the isolates by 16S rDNA sequencing showed an increased occurrence of γ-*Proteobacteria* (39% *Stenotrophomonas* and 5% *Pseudomonas*) among the isolates from WW-irrigated soils, which could not be isolated from rain-fed soils. Resistance analyses of the isolates showed a greater incidence of antibiotic resistances, especially of multiresistances (up to six different antibiotic resistances) in the isolates from WW-irrigated soils compared to the isolates from the rain-fed soils (Broszat et al., 2014).

## Wastewater Pathway and Efficacy of Soil Aquifer Treatment (SAT)

In arid and semiarid areas, WW irrigation reduces the pressure on other water sources (Chávez et al., 2011). Furthermore, soil-reclaimed and chlorinated water may be a potential drinking water source and an option to meet the increasing water demand of megacities like Mexico City (Jimenez and Chávez, 2004). Mexico City generates over 52 $m^3$/s of raw WW, which is mostly discharged to the WW-irrigation fields in the Mezquital Valley without treatment (Chávez et al., 2011). We followed the pathway of the WW from Mexico City to the Mezquital, beginning with the WW effluent of two hospitals in Mexico City. In addition, we took samples from one of the major WW channels, which transports the WW out of the city to the Mezquital, from several junctions of the WW with surface water of differing hygienic quality and finally from the reclaimed water after passing through the soil. We selected five antibiotic resistance genes (*sul*1, *sul*2, *qnr*A, *qnr*B and *qnr*S) that mediate resistance against sulfonamides (*sul* genes) or fluoroquinolones (*qnr* genes) for measurement. Furthermore, $bla_{SHV5}$ and $bla_{CTX\text{-}M}$ genes that encode extended-spectrum beta-lactamases and are frequently found in bacterial isolates from patients in Mexican hospitals were also assessed. In addition, we determined the concentration of *Enterococcus* spp. as a fecal indicator and the total bacterial concentration. All of the resistance genes were present in high copy numbers ($10^4$–$10^{10}$ gene copies/100 mL water) in the WW samples. The concentrations of all antibiotic resistance genes were largely reduced (reduction between 3 and 5 orders of magnitude) after passing through soil and transport in the aquifers, but the *sul* genes were still found in relatively large concentrations in the reclaimed water ($10^4$ *sul*1 gene copies/100 mL water, Figure 11.2). Enterococci were likewise still present in large concentrations in the reclaimed water (up to $10^4$ *Enterococcus* spp. 23S rDNA gene copies/100 mL water). However, *sul* genes as well as enterococci were determined below the detection limit after chlorination of the reclaimed water.

Therefore, WW purified by SAT and chlorine treatment could be suitable as a drinking water source and thus should be considered as a means to meet the increasing water demand of the population of Mexico City (Broszat, 2013; C. Siebe et al., unpublished data).

## Effect of Input of Antibiotics on Resistance Genes, Mobile Genetic Elements, and Heavy Metals in Soil

Together with organic waste (such as WW and manure), antibiotics, multiresistant bacteria, antibiotic resistance genes, and heavy metals are introduced into the soil (Dalkmann et al., 2012; Qadir et al., 2010; Jechalke et al., 2015). Wastewater from Mexico City contains low levels of antibiotics, but it is applied frequently (approximately once per month) by flood irrigation onto the soils in the Mezquital Valley (Dalkmann et al., 2012; Broszat, 2013). The concentration of the soil-adsorbed antibiotics depends on several factors including the soil composition (e.g., clay content, pH) and the sorption coefficient of the different antibiotics (Dalkmann et al., 2012). The results from the Mezquital Valley soil chronosequence (soils that have been irrigated from zero to

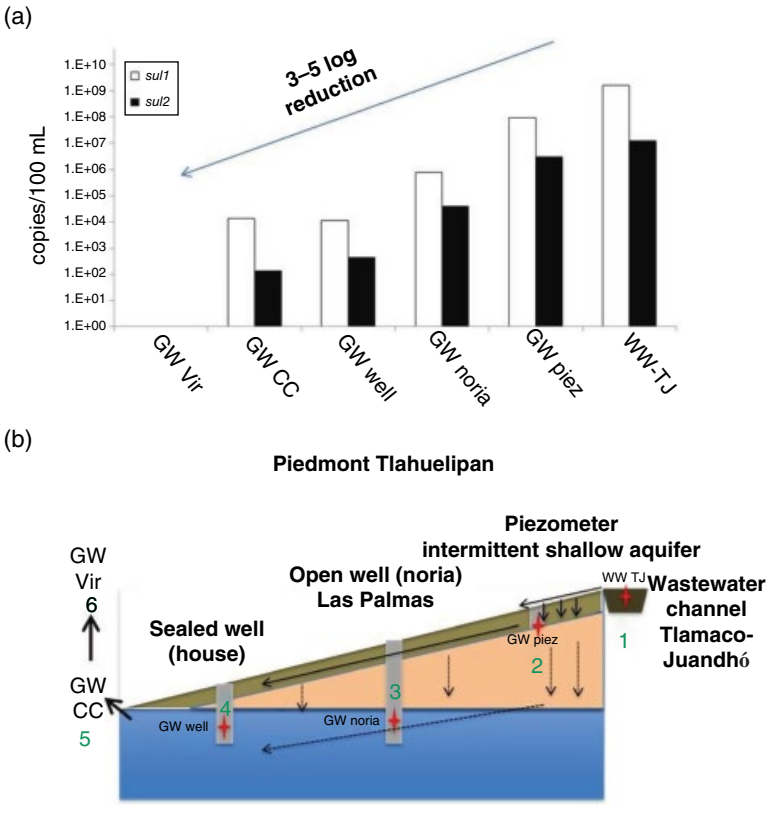


**Figure 11.2** Reduction of concentrations of antibiotic resistance genes by SAT (modified from Broszat, 2013). (a) Reduction of *sul* genes during SAT. GW Vir = spring water after chlorination; GW CC = spring recharged by SAT-treated WW; GW well = sealed well; GW noria = open well; GW piez = piezometer installed in 3 m depth; WW-TJ = WW channel. (b) Sampling sites 1: WW channel, 2: piezometer in 3 m depth, 3: open well, 4: sealed well, 5: spring, 6: chlorinated spring water. WW = wastewater; GW = groundwater. (*See insert for color representation of the figure.*)

100 years) have shown an increase in the concentrations of sulfamethoxazole and ciprofloxacin with the duration of irrigation, which could not be observed for the relative abundance of the respective resistance genes (Dalkmann et al., 2012).

Heavy metal resistance genes are frequently found on mobile genetic elements, such as transposons and plasmids, which also carry integrons and antibiotic resistance genes. Exposure to heavy metals can therefore lead to a coselection of antibiotic resistance genes. Jechalke et al. (2015) showed that WW irrigation in the Mezquital Valley led to an increase of bacterial biomass in the irrigated soils and, accordingly, to an increase in absolute abundance of class 1 integron integrase gene (*intI*1), quaternary ammonium compound resistance genes (*qac*E/*qac*EΔ1), streptomycin resistance gene (*aad*A), tetracycline resistance gene (*tet*(W)), and IncP-1 plasmids signature gene, *kor*B (Jechalke et al., 2015). In parallel, concentrations of the heavy metals zinc, copper, lead, nickel, and chromium, as well as of phosphorous and sulphur significantly increased. In terms of the relative abundance of the selected genes, no increase was observed, indicating no

enrichment in the soil bacterial community due to repeated WW irrigation or to a potential coselection by increasing concentrations of heavy metals. Nevertheless, in both studies by Dalkmann et al. (2012) and Jechalke et al. (2015), an increase in gene copy numbers per gram of soil for all analyzed targets was found along with extended duration of irrigation, which might lead to a higher exposure of field crops and farmers to resistant bacteria, antibiotic resistance genes, and mobile genetic elements (Dalkmann et al., 2012; Broszat, 2013; Jechalke et al., 2015).

To study the distribution of antibiotics and antibiotic resistance genes within the soil (rhizosphere and bulk soil), column experiments were performed (K. Lueneberg et al., unpublished data). Two types of soil (WW-irrigated soil and rain-fed soil) were irrigated with WW amended with different concentrations of sulfamethoxazole and ciprofloxacin. It was shown that preferential water flow paths emerged within the soil columns, which acted as hot spots for accumulation of antibiotics and *sul* genes. Unexpectedly, the effect in rain-fed soil was larger when compared to soil with a long-term history of WW irrigation, suggesting that the soil bacterial community in the WW-irrigated soil may have adapted to WW and the antibiotics therein. The presence of the *sul*1 resistance gene increased in the rain-fed soil after WW and sulfamethoxazole application. The relatively small bioavailable concentrations of ciprofloxacin did not show an effect on the abundance of *qnr*B and *qnr*S resistance genes. These column experiment results coupled with the occurrence of preferential flow in the soil of this area demonstrate the potential risk of groundwater contamination with antibiotics and resistance genes by WW irrigation (K. Lueneberg et al., unpublished data).

Increased abundance of *sul*1 genes could be also observed in soil mesocosm experiments after application of manure containing sulfadiazine (Ding et al., 2014). Atoyan and coworkers (2007) investigated the effects of short-term tetracycline amendment (10 days) on antibiotic resistance and removal of fecal indicator bacteria in aerated and unaerated leachfield mesocosms. They found that short-term addition of tetracycline at environmentally relevant concentrations only minimally increased pathogen removal from WW and development of antibiotic resistance among pathogenic bacteria in leachfield soil (Atoyan et al., 2007).

## Health Risks for Consumers

Wastewater-irrigated soils and crops grown on fields irrigated with untreated or partially treated WW are a potential reservoir for multiresistant bacteria, which might pose risks to field workers and consumers of the agricultural products. Recently, several studies have been performed to quantify diverse pathogenic bacteria and antibiotic resistance genes in irrigation water: Here, the study of Carey and coworkers (2016) is reported as an interesting example. They investigated the occurrence, concentrations, and antimicrobial resistance patterns of vancomycin-resistant enterococci (VRE) and vancomycin-susceptible enterococci at three different spray irrigation sites in the United States using reclaimed municipal WW originating from treatment plants that employed different standard technologies. Total enterococci and VRE were detected in 71% and 4% of reclaimed water samples, respectively. *Enterococcus faecalis* was the most common species identified. At one of the spray irrigation sites, UV radiation decreased total enterococci to undetectable levels; however, subsequent storage in

an open-air pond at the site led to bacterial regrowth, resulting in increased concentrations of enterococci (Carey et al., 2016). Carey et al. concluded that enterococci resistant to one of the last resort antibiotics, vancomycin, were only present in small numbers in reclaimed water at the spray irrigation sites, while resistance to other antimicrobial classes, such as quinupristin/dalfopristin (52% of isolates), tetracycline (13%), and ciprofloxacin (17%) were more prevalent, in particular among non–*E. faecalis* isolates (Carey et al., 2016).

A few studies have been performed to evaluate the occurrence of pathogens on plant surfaces that have been subjected to WW or treated WW irrigation to assess the health risks for the consumers of these crops (Carey et al., 2016; Blaustein et al., 2016). Mok and Hamilton (2014) chose an innovative approach to assess the exposure factors for WW-irrigated Asian vegetables. They determined the volume of water left on different Asian vegetables and lettuce after overhead sprinkler irrigation. Water capturing capability differed significantly between the vegetables tested, with lettuce showing the least water remaining on the leaves. Then, Quantitative Microbial Risk Assessment (QMRA) studies were designed to estimate the rotavirus disease burden (an important causative agent of diarrheal disease) from consumption of WW-irrigated Asian vegetables. Results demonstrated that estimated risks from these reuse scenarios exceed WHO guideline thresholds for acceptable disease burden for WW reuse, indicating that reduction of pathogen concentration or stricter risk management is required for safe reuse (Mok and Hamilton, 2014).

Only very few investigations determined plant uptake of pathogens or antibiotic resistance genes from recycled water. To assess the potential health risks for consumers of the crops from the Mezquital Valley WW-irrigation fields, we examined total DNA from near-ground plants (herbs that are eaten raw as salads) collected from irrigation fields for the presence of enterococci and antibiotic resistance genes by PCR. We have shown that the main health risks stem from bacteria on the plant surfaces, as no plant uptake of the resistance determinants and fecal indicators tested (*sul*1, *sul*2, *qnr*A, *qnr*B, *qnr*S, $bla_{\text{SHV-5}}$, $bla_{\text{CTX-M}}$, *mec*A, and *E. faecalis*) was observed (Blasi, 2013). Furthermore, it was demonstrated that after long-term irrigation with untreated WW, the concentrations of heavy metals in alfalfa and maize in the Mezquital Valley did not exceed the permitted values (Siebe and Cifuentes, 1995; Dalkmann et al., 2012; Siebe, 1994a, 1994b).

These studies have convincingly shown the high filtration capacities of the soil, but on the other hand Castro-Rosas and coworkers demonstrated that lettuce from the Mezquital Valley WW-irrigation fields was highly contaminated with fecal coliforms (found on the surfaces of 99% of the 130 tested lettuce samples) (Castro-Rosas et al., 2012). Therefore, these results confirm that near-ground crops should not be grown on fields irrigated with untreated WW, as is recommended by the WHO (Blumenthal et al., 2000).

These data are in line with the findings of Wu et al. (2015), who collected recent research data on plant uptake of pharmaceutical and personal care products from recycled water. The data provided clear evidence that pharmaceutical and personal care products can transfer from soil to plants when treated WW is used for irrigation (Wu et al., 2015). For pharmaceutical and personal care products with relatively high bioaccumulation factors in roots (e.g., triclosan and carbamazepine), high concentrations of residues can be found in tuber vegetables such as carrot and radish. On the other hand,

the presence of pharmaceutical and personal care products with high translocation potential and accumulation tendency in leaves/stems (e.g., carbamazepine, diclofenac, triclosan, and chloramphenicol) can lead to relatively high levels in leafy vegetables such as lettuce, spinach, and cabbage and may further transfer to fruits (Wu et al., 2015).

Human health and ecological risks of crops contaminated with low levels of pharmaceutical and personal care products are still not clear. However, based on the adverse effects of pharmaceutical and personal care products on nontarget organisms such as aquatic organisms, potential risks exist through consumption of crops contaminated with pharmaceutical and personal care products by humans or animals, and thus, uptake of these compounds by plants should be explored more thoroughly (Wu et al., 2015).

## Conclusions and Perspectives

In summary, the analyses have shown that the main risks of WW irrigation for humans are associated with direct contact with the WW and by consumption of insufficiently washed crops originating from WW irrigation fields. These risks can be relatively simply reduced by wearing protective masks and gloves when handling WW and by avoiding the consumption of near-ground plants such as herbs or lettuce, and/or by thoroughly washing the crops prior to consumption with a germ reducing agent. Additionally, it was shown that WW purified by SAT and chlorine treatment may be suitable as drinking water and thus could be a way to meet the increasing water demand of megacities, such as the metropolitan area of Mexico City.

In our opinion, even more promising options to provide the area with water of acceptable hygienic quality would be (i) the rehabilitation of an urban river, the Magdalena-Eslava river system, as a water source for Mexico City, as proposed by a group of scientists from the UNAM (National Autonomous University of Mexico) (Mazari-Hirart et al., 2014) and (ii) the proper operation of a very large high-standard WW treatment plant recently constructed in the Mezquital Valley.

The Magdalena-Eslava river system is the last urban river in the vicinity of Mexico City supplying water to the urban area. Physicochemical parameters showed significant differences between the natural area, where the river originates, and the urban area, where it receives untreated WW (Maziri-Hiriart et al., 2014). Mazari-Hiriart et al. (2014) found that nutrient concentrations and conductivity in the river were similar to domestic WW. Fecal indicators and various pathogens were found in elevated concentrations, presenting a health threat to the population living near the river (Mazari-Hiriart et al., 2014). This urban river should be rehabilitated as a sustainability practice; due to the public health issues and in view of the population exposure where the contaminated river flows through the city, its water should be treated to allow its ecosystem services to recover. The river represents an iconic case for the city as it connects natural and urban areas in a socioecological system that can likely provide clean water for human consumption (Mazari-Hiriart et al., 2014). The contaminated water could be treated and reused for irrigation; thus, Mazari-Hiriart et al. consider the construction of two new WW treatment plants and improved operation of the existing ones as urgent priorities that could result in better, more sustainable water use in Mexico City (Mazari-Hiriart et al., 2014).

Another option to provide the Mezquital Valley with clean irrigation water would be the prompt operation of the world´s largest WW treatment plant, which was very recently constructed (construction finished at the end of the year 2015) in Atotonilco, a village on the border between the state of Mexico and the state of Hidalgo. It has a treatment capacity of 35 $m^3$ WW/s, which would result in a total of 3 million $m^3$ of treated WW daily, thereby transforming a major portion of the WW generated in Mexico City into clean water for crop irrigation (CONAGUA, 2011).

In the very near future, together with collaborators from the UNAM, we aim to initiate a large interdisciplinary research project to investigate the effects of changing the irrigation regime in the Mezquital Valley from untreated WW irrigation for decades to clean water irrigation in terms of potential mobilization of heavy metals, pharmaceuticals, and pathogenic bacteria and their respective antibiotic resistance genes from soil as well as potential population shifts of microbial communities in the affected soils. Our objectives will focus on calculating benefits and potential disadvantages of changing a long-term agricultural practice in terms of ecological effects on soil, groundwater, surface water, and crops as well as on the people living in the area due to reduction of the disease burden by avoiding contact with WW and consumption of WW-irrigated crops. These initiatives are directed toward meeting overarching goals of sustainable food security and adequate provision of safe drinking water to all citizens in the region.

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# 12

# Antimicrobial Resistance Related to Agricultural Wastewater and Biosolids

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## Key Points

- Current on-farm systems for treating agricultural wastewater have shown varied degrees of effectiveness in reducing some measures of resistance.
- Of the studies to date, swine production systems are the most frequently studied, but there is insufficient data to discern any commodity-specific trends in resistance.
- A greater focus on isolate-based measures of antimicrobial resistance (as opposed to the commonly used gene-based methods and DNA community screening) is recommended to provide valuable information on multidrug resistance and fit the current paradigm in water quality of using indicator organisms.

Unlike the fairly streamlined human wastewater treatment process, there is considerable variety in the processing of agricultural wastewaters. The food animal production sector produces large amounts of manure and process wastewater, which can be introduced into the environment via grazing, production area runoff, seepage from storages and treatment systems, or via land application as a nutrient source to growing crops. While wastewater and biosolids from treated human wastewater streams are sometimes applied to agricultural land, it is comparatively much more common for untreated livestock manures and wastewaters to be land applied. A second, less obvious source of agricultural wastewater comes from food processing operations. These may include vegetable washing and packing operations, meat processing facilities, and other systems that process agricultural products. In some instances, these waste streams are handled by municipal wastewater treatment operations; in others, food processing wastes are managed on-site at the processing facility. This chapter outlines common types of treated and untreated wastewaters and biosolids

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| | |
|---|---|
| ***Process wastewater*** | Fresh water that has been in contact with manure, litter, mortalities, or composted manure and mortalities. |
| ***Anaerobic lagoon*** | A man-made earthen basin constructed with a compacted clay or synthetic liner designed to contain manure, process wastewater, and precipitation; it is sized based on the volatile solids loading rate of manure in order to facilitate anaerobic decomposition of manure solids. |
| ***Earthen manure storage basin*** | A man-made earthen basin designed to contain manure, process wastewater, and precipitation, but not sized to promote any treatment of the manure; normally smaller than anaerobic lagoons. |
| ***Litter*** | A mixture of dry bedding material (e.g., rice hulls, sawdust, etc.), poultry manure, feathers, and spilled feed produced in poultry production houses. |
| ***Biosolids*** | Separated solids, settled lagoon sludge, scraped feedlot manure, or other solid manure products. |
| ***Production area*** | The area on a livestock farm that includes the animal confinement area, the manure storage area, the raw materials (e.g., feed) storage area, and the waste containment areas. |
| ***Feedlot*** | A dirt lot where livestock (most commonly beef cattle) are confined, fed, and watered. |
| ***Livestock housing*** | A roofed structure where livestock are housed and maintained such that the animals and accumulated manure are not exposed to precipitation (poultry houses, swine barns, dairy freestall buildings, etc.) |
| ***Pasture*** | A fenced area where livestock (most commonly beef cattle, dairy cows, horses, goats, and sheep) are contained and allowed to graze at a stocking density that does not decimate the vegetation. |

from agricultural food animal production, compares the use and definition of the term *antibiotic resistance* in clinical and environmental settings, summarizes research results related to antibiotic resistance in agricultural wastewaters and biosolids, and discusses emerging strategies for managing agricultural wastewaters, including on-site treatment methods similar to human wastewater treatment plants. Existing knowledge gaps and needs for future research are proposed.

## Introduction

Antibiotic resistant bacteria and antibiotic resistance genes (ARB/Gs) are ubiquitous in soil and water regardless of whether or not antibiotic drugs have been used for human or animal care in the immediate vicinity (D'Costa et al., 2006; Bhullar et al., 2012; Durso et al., 2012; Durso et al., 2016). The question, therefore, is not whether there are ARB/Gs in agricultural waste streams, but which ARB/Gs are present and in what way does their

presence relate to ARB/Gs in the environment? Also of interest is how concentrations of ARB/Gs in agricultural systems compare to those in nonagricultural systems, and how management practices impact the types and amounts of ARB/Gs present in agricultural systems (Pruden et al., 2006; Durso et al., 2012; Durso and Cook, 2014; Agga et al., 2015).

Recent research is demonstrating that the ARB/Gs present in any particular sample are a function of the underlying microbial community structure of the sample (Durso et al., 2012; Fosberg et al., 2014; Peng et al., 2016), which responds to a variety of factors including antibiotic drugs (Udikovic-Kolic et al., 2014; Fosberg et al., 2014). Likewise, multiple studies have shown that the number of antibiotic resistance genes is positively correlated with the total bacterial density in a sample (Durso et al., 2012; Sui et al., 2015). The structure of the microbial community also impacts horizontal gene transfer since some bacterial species may be more likely than others to acquire or transfer antibiotic resistance genes from other species (Subbiah et al., 2016). Additionally, some bacteria (e.g., spore formers such as Clostridia) may be more capable than others of surviving in animal production settings, thereby increasing the opportunities for the genes they carry to survive in waste streams and be transmitted through the environment (Durso et al., 2012). Consequently, any treatment or management practice that alters the microbial community is likely to also impact the types and concentrations of ARB/Gs in agricultural manures, wastewater, and the environment where these waste streams are present.

## What Are Agricultural Wastewater and Biosolids?

Agricultural wastewater is any wastewater that is produced during agricultural activities that has been in contact with livestock manure, mortalities, or other nutrient sources. Agricultural biosolids may include dry or solid manure, poultry litter, composted manure, composted animal mortalities, or treated sludge (e.g., anaerobic lagoon sludge, anaerobic digester solids, etc.). This chapter will focus primarily on these waste streams, which may comprise urine and feces excreted directly from the animals, or excreta mixed with flush water, wash water, or storm water. Examples include fresh water used to flush manure out of a production area, storm water falling on a lot where animals are present, and liquid leachate from a manure or mortality composting system, among others. Wastewater can also include runoff or leachate from solid manure piles, compost, manure-amended soils, feed materials, mortalities, and land-applied wastes (Table 12.1). Food processing wastewaters, including those from meat, egg, dairy, and vegetable processing systems, and crop processing wastewaters, including those from ethanol and biodiesel, that are not handled by municipal treatment systems are also considered agricultural wastewater. Antibiotic resistance associated with the use of antibiotics on crops and in aquaculture is reviewed elsewhere (Stockwell and Duffy, 2012; Cabello et al., 2013; Done et al., 2015; Williams-Nguyen et al., 2016), as is the general topic of antibiotic resistance in water (Zhang et al., 2009).

Wastewaters from agricultural livestock production are similar to municipal wastewater in that they generally include large amounts of excreted urine and fecal material that contain nutrients, microorganisms, and natural and synthetic chemicals. In general, however, agricultural wastewaters have a greater organic load and microbial concentration than municipal wastewaters and contain more solids per unit of liquid volume, principally because less fresh water is utilized in livestock production systems than in municipal systems. The physical, chemical, and microbiological characteristics

**Table 12.1** Sources of agricultural waste summarized by their potential for direct or indirect inputs to surface water.

| Potential for Direct Inputs into Surface Water | Potential for Indirect Inputs into Surface Water |
|---|---|
| Animal contact with surface water | Anaerobic treatment lagoons* |
| Runoff from open lot production area | Earthen manure slurry basins* |
| Discharge/overflow from manure storage structures | Land-applied manure, litter, compost, biosolids, etc. |
| Leachate runoff from manure and litter stockpiles | Leachate from burial of mortalities |
| Erosion and runoff from fields receiving manure | Land-applied aquaculture sediments |
| "Spills" during manure handling activities | Land-applied food processing wastes |
| Aquaculture effluents | |
| Discharge of food processing wastewaters | |

*If not properly designed, can contaminate via seepage or leakage (Chee-Sanford et al., 2001); proper design of anaerobic lagoons with compacted clay liners allows for a maximum permeability rate of no more than $1 \times 10^{-6}$ cm/sec.

of agricultural wastewater differ not only from municipal systems (Pruden et al., 2006; Agga et al., 2015) but also among commodities (i.e., beef wastes vs. poultry wastes) and within individual commodity systems (Koike et al., 2007). For example, Brooks et al. (2014) describe differences in lagoon effluent microbial communities and antibiotic resistance associated with multiple stages of swine production, with finisher farms having less diverse communities and fewer ARGs compared with nursery or sow operations. Koike et al. (2010) noted differences between their results and those of Chen et al. (2007) for *erm*(B), *erm*(F), and *erm*(C), although they both examined swine lagoons.

While municipal wastewater typically undergoes treatment through one or more physical, chemical, or biological processes, agricultural wastes may be treated (i.e., via anaerobic lagoons, anaerobic digestion, composting, or other methods) or left untreated. For this reason, agricultural wastes are thought to impose a greater microbial public-health risk than municipal wastes that may be subjected to multiple treatment regimens. However, their comparative risks regarding ARB/Gs remain largely unknown. Studies performed to date show differences in the types and concentrations of ARB/Gs in agricultural and nonagricultural samples (Pruden et al., 2006; Durso et al., 2012; Ibekwe et al., 2016), but the potential impact of these differences on human health risk has not been determined.

In the United States, a variety of federal and state regulations govern the management of agricultural wastewaters. The Clean Water Act National Pollutant Discharge Elimination System (NPDES) is administered at the federal level by the Environmental Protection Agency (EPA) and, in most states, is implemented by a state regulatory agency. An exception is states where a regional EPA office administers the NPDES program at the state level. Although the primary focus of these regulations is controlling the release of manure-associated nutrients to surface waters, on a functional level they limit, to some degree, the off-site transport of other manure-borne contaminants, including antibiotic drugs, resistant bacteria, and resistance genes. The structure of the agricultural wastewater industry in the developed world is fundamentally different from the structure of the municipal wastewater systems. Unlike human wastewater treatment systems, which are generally funded by local and regional governments from

tax dollars, the costs associated with agricultural wastewaters in the United States are generally borne by individual producers and private or corporate entities. This funding structure has a direct impact on the types, scale, and practicality of wastewater treatment in agricultural production systems. Primary treatment of liquid wastes to remove suspended solids is common, as are a variety of secondary biologically based treatments. However, the endpoints for both of these processes are different from their respective counterparts in human wastewater treatment. A key factor contributing to this variability in treatment goals is that municipal waste streams are treated to achieve required "discharge standards" prior to releasing wastewater to receiving streams, whereas agricultural waste systems are designated as "no discharge" systems. Essentially, the discharge of agricultural wastewater is illegal regardless of the degree of treatment.

## What is Antibiotic Resistance?

Antibiotic resistance (AR) is a broad term, used specifically by clinicians to refer to infectious disease treatment failure. Although this term is commonly used by scientists, public health officials, and policy makers when referring to agricultural and environmental systems, most measurements taken in these systems are not associated with immediate infectious disease treatment failure. We will use the term *resistance* in this chapter with the caveat that there is no standard definition of resistance for environmental bacteria, and an acknowledgement that agricultural and environmental resistance is not equivalent to clinical resistance.

There are two main parameters that are measured to determine AR in agricultural settings. First, antibiotic resistance genes (ARGs) are the pieces of DNA that code for resistance. Normally, ARGs are found within and carried by a bacterial host. However, ARGs can persist in the environment even if the host cell is dead and these genes can be transferred or "traded" between bacteria. Second are the antibiotic resistant bacteria (ARB), which are living microorganisms. In clinical settings, they are the infectious disease agents. However, all bacteria, not just pathogenic bacteria, have the potential to be resistant. In the agricultural and environmental literature, a specific bacterium is considered resistant when it displays a reduced susceptibility to a specific drug or when it harbors a specific ARG.

There are many different ways to measure resistance. It is becoming clear that individual ARGs have distinct ecologies (Chee-Sanford et al., 2001; Knapp et al., 2010; Storteboom et al., 2010a; Cook et al., 2014; Durso et al., 2016) and that not all types of resistance are equally important when it comes to the potential human and animal health impacts of antibiotic drug use in food animal species (Durso and Cook, 2014).

It has become increasingly common to conceptualize ARGs as environmental pollutants or contaminants, and this vocabulary is frequently used when discussing AR associated with agricultural wastewater (Storteboom et al., 2010a). However, unlike pathogens or toxic chemicals, the potential threat posed by ARGs is indirect (Williams-Nguyen et al., 2016). This means that the mere presence of the gene, regardless of the concentration, is not enough to pose a health risk. Identifying and quantifying the risk of agriculturally generated ARB/Gs to human health remains one of the primary knowledge gaps in the field.

Antibiotic drugs are widely thought to be the main driver enriching ARB/Gs in agricultural and environmental samples, and the general assumption is that the presence and concentration of drugs should correlate with the presence and concentration of resistant bacteria and resistance genes. However, the empirical evidence suggests that

in applied field situations the relationship between the antibiotic drug and the specific resistance as measured by presence of ARB/Gs is much more complex than this (Williams-Nguyen et al., 2016; Jindal et al., 2006; Hong et al., 2013; Stanton et al., 2011). Also, a number of other compounds, such as heavy metals, have been shown to selectively enrich ARB/Gs and even practices such as the application of antibiotic-free manure has been shown to increase the number of ARB/Gs in the soil (Udikovic-Kolic et al., 2014).

## Summary of Research Results

The summation of research results that follows is focused only on ARB/Gs from agricultural sources. ARB/Gs from municipal waste applied to agricultural fields are covered elsewhere in this book and will not be discussed here.

The entry of ARB/Gs to the environment from agricultural operations, particularly in feces and manure, is thought to be the predominant contributor to ARB/Gs in surface waters, compared to contributions from the enrichment of native bacteria (Storteboom et al., 2010a). The most direct input of ARB/Gs into surface waters comes from the deposition of fecal material into surface waters via grazing of animals or in-stream aquaculture. For example, a study at four U.S. aquaculture facilities demonstrated that although the same types of tetracycline resistance genes were found in both the influent and effluent of the facilities, their concentrations were greater in the effluent (Seyfried et al., 2010). ARB/Gs can also enter surface waters via runoff from pastures or feedlots (Harmel et al., 2013), unintended "spills" of manure during conveyance or land application, runoff and erosion from fields receiving application of manure, and direct discharge from a manure storage structure.

For groundwater, unintended seepage from manure containment systems and leaching of liquids from mortality burial pits are the primary mechanisms contributing to ARB/Gs from agricultural settings. Clay-lined anaerobic treatment lagoons are quite common on livestock operations that generate liquid manure (e.g., swine farms, dairy farms, and some poultry operations). Manure lagoons and other storage facilities are specifically designed to contain the waste; design standards dictate the procedure for constructing the bottom and sides of a clay-lined lagoon (ANSI/ASAE EP403.3, 2010), and a permeability test is typically required for a finished structure prior to any manure being introduced to the system. However, reports of surface and groundwater contamination from swine, cattle, and poultry production and manure storage facilities prove that these systems can sometimes fail (Chee-Sanford et al., 2001; Jindal et al., 2006; Mackie et al., 2006; He et al., 2014; Hsu et al., 2014; Jahne et al., 2015). Maintaining the integrity of lagoon liners is important to prevent contamination of surface and groundwater in the vicinity of manure storage facilities (Hong et al., 2013), as is proper design and management to contain waste during excessive rainfall events (Jahne et al., 2015). Groundwater monitoring studies using multiple wells to track accidental contamination demonstrated spatial patterns that strongly suggest individual ARB/Gs can be transported in the subsurface, similar to other contaminants (Koike et al., 2007). However, studies by the same group, also sampling groundwater wells, reveal an indigenous tetracycline resistance gene pool in groundwater as well (Koike et al., 2007). Likewise, some types of ARB/Gs are commonly found in soils, including soils that have

not been manured, or that have no agricultural inputs (Schmitt et al., 2006; Durso et al., 2016), highlighting the need to account for baseline or background levels of the target, particularly when measuring impacts of specific management practices (Rothrock et al., 2016).

### Land Application

An important way in which AR from animals is introduced into the environment is via the application of solid and liquid manures, litter, compost, and wastewaters to cropland (Williams-Nguyen et al., 2016; Marti et al., 2014). Thus, knowing the types of ARB/Gs in land-applied manures and other animal waste products is important for predicting ARB/Gs in runoff. The land application of manure as a fertilizer has been practiced since Neolithic times (Bogaard et al., 2013); however, the advent of widely available chemically synthesized fertilizers, combined with changes in the concentration and scale of animal production operations in North America, has fundamentally changed the role of animal manures as fertilizers. Although manures supply essential crop nutrients and provide benefits to soil health, runoff from manure-amended fields is an important source of ARB/G-rich surface water inputs. In the context of AR, manures applied to soil can provide a vehicle for manure-borne bacteria and manure-associated genes to enter the environment. Agronomic management practices can impact the survival of land-applied manure-borne bacteria and the persistence of manure-borne genes. For example, Cook et al. (2014) observed differences in the persistence of *sul*I and *str*B between conventionally tilled and no-till soils that had been amended with poultry litter, with the genes persisting longer in the no-till soils. The addition of narrow grass hedges on fields, traditionally used to control runoff of nutrients, has been shown to also reduce runoff of ARGs (Soni et al., 2015). The timing of wastewater application to fields, with respect to precipitation, is known to affect runoff of nutrients, and avoiding winter applications has also been recommended as a way to minimize runoff of ARB/Gs (Pei et al., 2007; Peak et al., 2007). Animal management practices, such as diet modifications, have also been suggested to impact survival of ARB in soil (Merchant et al., 2012).

It is commonly assumed that the land application of manure significantly increases the concentration of ARGs in the soil (Heuer et al., 2011); however, a retrospective study examining 100 years of human wastewater application to a Mexican soil found no enrichment of ARGs (Jechalke et al., 2015). In the short term, application of ARB/G-rich manures to soil increases the total load of ARB/Gs at the site of application, and the presence of manure on fields increases the potential for runoff that contains fecal bacteria, including ARB/Gs (Durso et al., 2011a; Joy et al., 2014; Ruuskanen et al., 2016). ARB/Gs can persist in fields (Alexander et al., 2011), but their concentrations generally decrease over time and with depth in soil (Garder et al., 2014; Jechalke et al., 2015; Tang et al., 2015). The soil microbial community has demonstrated great ability to remediate resistance from manure incorporated into agricultural soils (Sengelov et al., 2003; Zhou et al., 2010; Jechalke et al., 2015). This process is not immediate, taking up to one year (Marti et al., 2014). However, one greenhouse study reports an increase in the relative abundance of ARGs in soil over the course of a 12-year study (Fang et al., 2015). Concerns remain that ARB/Gs introduced via land application of manures can travel via dust or contaminate surface and ground waters via runoff and seepage before they

are remediated by the soil (Hong et al., 2013). However, in a field-scale study examining transport of tetracycline, sulfonamide, and erythromycin resistance genes, subsurface transport was not observed (Farhenfeld et al., 2014). These concerns, paired with limited data, illuminate the urgent need for more research in this area.

The spatial and temporal distribution of specific ARB/Gs in various manure-based wastewaters remains a knowledge gap. A small number of studies examining these dynamics have been performed, and results suggest that there is great variability from year to year, even when the exact same measure is used (Koike et al., 2007; Sui et al., 2015). Koike et al. (2007) sampled two swine lagoons over three years and found that the measurements for each of seven tetracycline resistance genes in each of the two lagoons were significantly different over the three-year sampling period, suggesting that unidentified factors, possibly including stochastic factors, contribute to the number of gene copies detected. However, there are reports where the survival or persistence of ARB/Gs does appear to be similar from year to year. In a two-year study by Cook et al. (2014), the general persistence of three ARGs in poultry litter–amended soils was similar in both years of the study.

## Types of Resistances Measured in Agricultural Wastes

It is important to know the types and concentrations of ARB/Gs in source manures and the resulting agricultural wastewaters to evaluate the efficacy of best management practices. While a growing body of work documenting—and sometimes quantifying—various ARB/Gs in agricultural manure-management systems exists, significant knowledge gaps remain. Livestock waste is generally described as having high concentrations of ARGs (Peak et al., 2007; Pei et al., 2007); however, "high" is rarely defined. In a summary of 40 recent publications on the topic, AR was frequently quantified using gene-based measures, with gene copies per milliliter or gram of sample, or gene copies per copy of 16S rRNA as the primary outcomes reported (Tables 12.2–12.4). The greatest reported value of a single ARG from these studies was $5.8 \times 10^{11}$ copies of *tet*(X) per milliliter of swine manure. The lowest value reported for *tet*(X) was $2.0 \times 10^{5}$ copies per gram of control soil. So, for example, in absence of experimental control samples to measure background or baseline levels of *tet*(X) in the particular system being studied, results to date suggest that measurements in the $10^{5}$ or $10^{6}$ range would be considered low for absolute numbers of *tet*(X) copies per gram or milliliter of sample. Many studies normalize data to the 16S rRNA gene copy number in order to correct for the total number of bacteria present in a sample. As an example of the range of normalized values commonly reported for *tet*(X) in agricultural wastewater samples, of the 40 papers reviewed for this chapter, the lowest value was $10^{-5}$ for cattle storage pond sludge (Zhang et al., 2013), and the highest value was in the $10^{-1}$ range for soil amended with free-range poultry litter (He et al., 2014). Looking across all genes assayed, the lowest value reported for 16S normalized data was in the $10^{-8}$ range, representing quantification of *qnr*B from compost (Wang et al., 2015). Most normalized values fell in the $10^{-2}$ to $10^{-3}$ range, nominally representing one ARB for every 100 to 1000 bacterial cells in the agricultural waste samples, respectively.

Since some bacteria carry multiple copies of the 16S gene, normalized ARG values can be impacted by community composition. *Escherichia coli* carries seven copies of the 16S gene, while members of Actinobacteria carry an average of three, Alphaproteobacteria

**Table 12.2** Ranges of reported tetracycline resistance genes in agricultural wastes and wastewaters. Values are means of studies quantifying gene copy numbers in food animal wastes and wastewaters. If available, measured values were used. All other values were estimates based on published graphs.

| Gene | Num of Studies[1] | Measure[2] | Value | Sample and Citation |
|---|---|---|---|---|
| ***tet*(A)** | *n* = 4 | Min abs | 1.3E + 05 | Swine effluent, Tao et al., 2014 |
| efflux | | Max abs | 1.0E + 07 | Swine nursery manure, Brooks et al., 2014 |
| | *n* = 4 | Min 16S | 1.0E–06 | Dairy manure, Sandberg and LaPara, 2016 |
| | | Max 16S | 2.6E–02 | Swine effluent, Tao et al., 2014 |
| ***tet*(B)** | *n* = 3 | Min abs | 9.0E + 01 | No use lagoon, Peak et al., 2007 |
| efflux | | Max abs | 7.0E + 06 | Cattle fed CTC, Alexander et al., 2011 |
| | *n* = 3 | Min 16S | 4.0E–06 | No use lagoon, Peak et al., 2007 |
| | | Max 16S | 7.1E–01 | Stockpiled beef manure, Xu et al., 2015 |
| ***tet*(C)** | *n* = 3 | Min abs | 1.9E + 04 | Groundwater, Koike et al., 2007 |
| efflux | | Max abs | 3.8E + 08 | Soil with free range poultry litter, He et al., 2014 |
| | *n* = 3 | Min 16S | 1.2E–05 | Swine manure, Cheng et al., 2016 |
| | | Max 16S | 7.2E–01 | Stockpiled beef manure, Xu et al., 2015 |
| ***tet*(G)** | *n* = 3 | Min abs | 3.0E + 06 | Swine waste liquid, Chen et al., 2010 |
| efflux | | Max abs | 2.7E + 10 | Summer swine effluent, Sui et al., 2015 |
| | *n* = 4 | Min 16S | 1.5E–05 | Mixed manure slurry, Fahrenfeld et al., 2014 |
| | | Max 16S | 1.2E–01 | Soil with free range poultry litter, He et al., 2014 |
| ***tet*(H)** | *n* = 2 | Min abs | 4.7E + 02 | Swine lagoons—3 years ave., Koike et al., 2007 |
| efflux | | Max abs | 2.6E + 09 | Soil with broiler manure, He et al., 2014 |
| | *n* = 1 | Min 16S | 3.3E–03 | Indoor broiler manure, He et al., 2014 |
| | | Max 16S | 1.1E–01 | Free range poultry litter, He et al., 2014 |
| ***tet*(L)** | *n* = 2 | Min abs | 4.0E + 00 | No use lagoon, Peak et al., 2007 |
| efflux | | Max abs | 8.0E + 07 | Cattle fed CTC and SMZ D0, Alexander et al., 2015 |
| | *n* = 3 | Min 16S | 4.0E–07 | No use lagoon, Peak et al., 2007 |
| | | Max 16S | 8.3E–01 | Manure from CTC fed animal & compost, Xu et al., 2015 |
| ***tet*(M)** | *n* = 5 | Min abs | 1.0E + 03 | Groundwater, Koike et al., 2007 |
| r'some | | Max abs | 1.6E + 11 | Winter swine manure input 2, Sui et al., 2015 |

(*Continued*)

**Table 12.2** (Continued)

| Gene | Num of Studies[1] | Measure[2] | Value | Sample and Citation |
|---|---|---|---|---|
| protect | *n* = 9 | Min 16S | 2.0E–06 | No use lagoon, Peak et al., 2007 |
| | | Max 16S | 8.6E–01 | Manure from animal fed CTC SMZ, Xu et al., 2015 |
| ***tet*(O)** | *n* = 4 | Min abs | 3.9E + 02 | Groundwater, Koike et al., 2007 |
| r'some | | Max abs | 5.9E + 08 | Free range poultry litter, He et al., 2014 |
| protect | *n* = 13 | Min 16S | 5.5E–08 | Pristine water, Pruden et al., 2006 |
| | | Max 16S | 3.0E–02 | Dry stacked manure, Fahrenfeld et al., 2014 |
| ***tet*(Q)** | *n* = 4 | Min abs | 1.9E + 03 | Groundwater, Koike et al., 2008 |
| r'some | | Max abs | 1.4E + 09 | Broiler litter, He et al., 2014 |
| protect | *n* = 9 | Min 16S | 2.0E–06 | No use lagoon, Peak et al., 2007 |
| | | Max 16S | 2.0E + 00 | Simulated swine manure storage D1, Joy et al., 2014 |
| ***tet*(T)** | *n* = 1 | Min abs | 4.9E + 05 | Soil with free range poultry litter, He et al., 2014 |
| r'some | | Max abs | 1.7E + 07 | Indoor broilers, He et al., 2014 |
| protect | *n* = 3 | Min 16S | 3.9E–05 | Soil with indoor broiler manure, He et al., 2014 |
| | | Max 16S | 3.3E–04 | Soil with free-range poultry litter, He et al., 2014 |
| ***tet*(W)** | *n* = 8 | Min abs | 9.0E + 01 | No use cattle lagoon, Peak et al., 2007 |
| r'some | | Max abs | 9.0E + 10 | Cattle manure tylosin animals, Alexander et al., 2011 |
| protect | *n* = 15 | Min 16S | 6.0E–08 | Pristine water, Pruden et al., 2006 |
| | | Max 16S | 8.9E–01 | Control beef manure, Xu et al., 2015 |
| ***tet*(X)** | *n* = 3 | Min abs | 2.0E + 05 | Control soil microcosm, Sandberg and LaPara, 2016 |
| enzymatic | | Max abs | 5.8E + 11 | Swine manure input 1 winter, Sui et al., 2015 |
| | *n* = 5 | Min 16S | 4.0E–05 | Cattle storage pond sludge, Zhang et al., 2013 |
| | | Max 16S | 2.0E–01 | Soil with free range litter, He et al., 2014 |

[1] Number of studies used to determine estimates for the listed tetracycline resistance gene. Some studies reported data in only one format, some studies provided data in both formats (absolute and normalized). If data were provided in both formats, it was used and counted for both, and so is represented twice in the table.

[2] Minimum and maximum values for: (abs) absolute quantification, reported as target gene copies/mL, gene copies/g, gene copies/g dry weight, or (16S) target gene copies/16S rRNA gene copies.

**Table 12.3** Ranges of reported sulfonamide and macrolide resistance genes in agricultural wastes and wastewaters. Values are means of studies quantifying gene copy numbers in food animal wastes and wastewaters. If available, measured values were used. All other values were estimates based on published bar or line graphs.

| Gene | Num of Studies[1] | Measure[2] | Value | Sample and Citation |
|---|---|---|---|---|
| *sul*I | *n* = 4 | Min abs | 3.3E + 04 | Pre-app soil, Cook et al., 2014 |
| | | Max abs | 5.0E + 10 | Free range poultry, He et al., 2014 |
| | *n* = 14 | Min 16S | 3.0E−06 | Pristine water, Pruden et al., 2006 |
| | | Max 16S | 2.2E + 00 | Soil amended with free range poultry, He et al., 2014 |
| *sul*II | *n* = 4 | Min abs | 2.1E + 06 | Swine effluent, Tao et al., 2014 |
| | | Max abs | 1.3E + 09 | Soil amended with free-range manure, He et al., 2014 |
| | *n* = 13 | Min 16S | 1.6E−06 | Heavy ag impacted water, Pruden et al., 2006 |
| | | Max 16S | 9.2E−01 | Manure from CTC SMZ fed animals, Xu et al., 2015 |
| *erm*B | *n* = 8 | Min abs | 2.0E + 00 | Swine slurry mesocosum D22 shade, Knapp et al., 2010 |
| | | Max abs | 2.9E + 11 | Swine manure input 1, Sui et al., 2015 |
| | *n* = 4 | Min 16S | 3.0E−07 | Dairy manure, Sandberg and LaPara, 2016 |
| | | Max 16S | 7.9E−01 | Stockpiled beef manure, Xu et al., 2015 |
| *erm*F | *n* = 6 | Min abs | 1.0E + 00 | Swine slurry mesocosm D22 dark, Knapp et al., 2010 |
| | | Max abs | 9.0E + 11 | Swine manure ameded soil, Garder et al., 2014 |
| | *n* = 3 | Min 16S | 1.0E−05 | Mixed manure slurry, Fahrenfeld et al., 2014 |
| | | Max 16S | 8.3E−01 | Control and stockpiled manure, Xu et al., 2015 |
| *mef*A | *n* = 14 | Min abs | 1.5E + 08 | Swine treatment effluent 2 summer, Sui et al., 2015 |
| | | Max abs | 1.9E + 11 | Swine manure input 2 winter, Sui et al., 2015 |

[1] Number of studies used to determine estimates for the listed tetracycline resistance gene. Some studies reported data in only one format, some studies provided data in both formats (absolute and normalized). If data were provided in both formats, it was used and counted for both, and so is represented twice in the table.

[2] Minimum and maximum values for: (abs) absolute quantification, reported as target gene copies/mL, gene copies/g, gene copies/g dry weight, or (16S) target gene copies/16S rRNA gene copies.

an average of two, and Acidobacteria an average of one (Větrovský and Baldrian, 2013). When combined with the knowledge that some increases or reductions in ARGs are driven by underlying changes in the microbial community structure, care is warranted when evaluating modest differences between samples using normalized 16S data.

**Table 12.4** Mean concentrations of ARGs in agricultural wastes. Values are means of studies quantifying gene copy numbers in food animal wastes and wastewaters. If available, measured values were used. All other values were estimates based on published bar or line graphs.

| | ARG copies/g or mL | | ARG copies/16S | | 1 ARG per X 16S copies[3] | |
|---|---|---|---|---|---|---|
| Gene | Ave[1] | Ave C/B[2] | Ave | Ave C/B | Ave | Ave C/B |
| *tet*(A) | 2.3E + 06 | 3.0E + 05 | 7.1E–03 | 2.5E–04 | 140 | 4000 |
| *tet*(B) | 1.1E + 06 | 2.3E + 05 | 2.8E–01 | 3.6E–01 | 4 | 3 |
| *tet*(c) | 5.3E + 07 | 4.7E + 05 | 2.4E–01 | 7.1E–01 | 4 | 1 |
| *tet*(G) | 5.2E + 09 | none | 2.0E–02 | 4.4E–02 | 51 | 23 |
| *tet*(H) | 8.6E + 08 | 6.6E + 02 | 4.3E–02 | none | 24 | none |
| *tet*(L) | 1.3E + 07 | 1.2E + 06 | 3.3E–01 | 4.1E–01 | 3 | 2 |
| *tet*(M) | 1.6E + 10 | 4.5E + 08 | 1.1E–01 | 2.8E–01 | 9 | 4 |
| *tet*(O) | 1.1E + 08 | 6.0E + 02 | 4.1E–03 | 7.3E–05 | 244 | 13,790 |
| *tet*(Q) | 3.4E + 08 | 2.0E + 03 | 1.6E–01 | 2.3E–06 | 6 | 444,444 |
| *tet*(T) | 7.7E + 06 | none | 1.5E–04 | none | 6,668 | none |
| *tet*(W) | 1.3E + 10 | 5.1E + 09 | 8.7E–02 | 2.2E–01 | 11 | 5 |
| *tet*(X) | 5.7E + 10 | 2.0E + 05 | 2.0E–02 | none | 51 | none |
| *sul*I | 7.3E + 09 | 1.9E + 08 | 1.6E–01 | 8.5E–03 | 6 | 117 |
| *sul*II | 3.9E + 08 | 1.0E + 08 | 1.4E–01 | 6.8E–05 | 7 | 14,815 |
| *erm*B | 7.5E + 08 | 5.0E + 07 | 2.7E–01 | 3.9E–01 | 4 | 3 |
| *erm*F | 5.5E + 10 | 8.0E + 07 | 3.6E–01 | 8.3E–01 | 3 | 1 |
| *mef*A | 3.6E + 10 | none | none | none | none | none |

[1] Mean of all samples with reported data in each reporting format. Excludes control and baseline samples.
[2] Mean of control or baseline samples.
[3] Calculated by dividing 1 by the value of ARG copies/16S.

Ideally, studies quantifying resistance genes would report and evaluate both absolute numbers (copies/g) and normalized (copies/16S) data (Peak et al., 2007; He et al., 2014; Tao et al., 2014; Sandberg and LaPara, 2016).

Tetracycline resistance is common in soils and manures (Schmitt et al., 2006) and is one of the most commonly studied resistances in environmental and agricultural samples. Tetracycline resistance has been documented in beef, dairy, swine, poultry, and aquaculture production systems (Wu et al., 2010; Ji et al., 2012; Fahrenfeld et al., 2014; Agga et al., 2015; Ruuskanen et al., 2016). It has been measured in cattle manure (Alexander et al., 2011), compost (Xu et al., 2015), storage ponds (Zhang et al., 2013), and lagoons (Smith et al., 2004; Pei et al., 2007; Peak et al., 2007), as well as organic livestock operations (Stanton et al., 2011; McKinney et al., 2010). Tetracycline resistant bacteria and genes are common in swine production wastes and wastewaters and have been found in every stage of swine waste management systems examined so far.

This includes swine manures and manure slurries (Cotta et al., 2003; Chen et al., 2010; Brooks et al., 2014; Wang et al., 2015; Whitehead et al., 2013, 2016; Cheng et al., 2016), standard and deep pit manure storages (Joy et al., 2014; Whitehead and Cotta, 2016), anaerobic treatment lagoons (Chee-Sanford et al., 2001; Koike et al., 2007; Zhang et al., 2013), anaerobic digesters (Tao et al., 2014; Sui et al., 2015), biofilters (Sui et al., 2015), manure-amended soils (Sengelov et al., 2003; Jindal et al., 2006), effluents (Jia et al., 2014), and tile drainage systems, both before and after manure application (Frey et al., 2015). Tetracycline resistant bacteria and genes have also been detected in organic swine production systems (McKinney et al., 2010); organic, free-range, and conventional poultry systems (Brooks et al., 2010; McKinney et al., 2010; Merchant et al., 2012; Cook et al., 2014; He et al., 2014); and aquaculture systems (Seyfried et al., 2010; Storteboom et al., 2010b; Gao et al., 2012; Shah et al., 2012). Absolute numbers of any specific tetracycline resistance gene per gram or milliliter of soil range from $10^4$, reported in control soil, to $10^{11}$ copies in some swine manure samples (Sui et al., 2015; Sandberg and LaPara, 2016). In some instances, absolute numbers of ARGs in control samples can reach the high end of this range. For example, control cattle manure and manure from animals fed chlortetracycline had $3 \times 10^{10}$ and $8 \times 10^{10}$ copies of *tet*(W) per gram dry weight, respectively (Alexander et al., 2011). Table 12.3 summarizes concentrations of tetracycline, sulfonamide, and macrolide resistance genes found in agricultural manures and wastewaters.

The ultimate concern over antibiotic resistance in agricultural wastewaters is the potential impact of these bacteria and genes on human clinical outcomes. A variety of other measures of resistance, including drug classes commonly used in human medicine, have been evaluated in agricultural wastewaters. The number of studies including these data, however, remains small. The majority of the information gathered to date is from phenotyping of isolates. Limited gene-based quantitative work has been done, although the metagenomic sequencing and an 84 target AR qPCR-based assay have been applied to characterize ARB/Gs in food animal manures and wastewaters (Durso et al., 2011b; Li et al., 2013; Agga et al., 2015; Fang et al., 2015; Ma et al., 2016). In general, results indicate that regardless of the resistance type examined, it can be found in agricultural wastes. Resistance to multiple classes of β-lactam antibiotics has been evaluated in agricultural solid and liquid wastes by a number of researchers (Knapp et al., 2010; Merchant et al., 2012; Agga et al., 2015). Brooks et al. (2009a, 2009b, 2014) have examined cephalosporin resistance in swine and poultry isolates, and Udikovic-Kolic et al. (2014) measured cephalothin-resistance in cattle manure. The *bla* gene has been quantified throughout swine wastewater treatment by Tao et al. (2014), who detected $10^7$ copies/mL in influent and $10^5$ copies/mL in effluent, and *bla* genes have been detected in fields amended with both cattle and swine manures (Ruuskanen et al., 2016).

Brooks et al. (2009a, 2009b, 2014) also collected data on aminoglycoside, methicillin, and quinolone resistance, as well as intl1 in both swine and poultry operations. Limited additional information on aminoglycoside resistance in isolates from poultry houses is available (Lee et al., 2002; Merchant et al., 2012) and shows that bacteria, including bacteria carrying aminoglycoside resistance, can survive for extended periods of time in agricultural production settings.

## Efficacy of On-Farm Manure Treatments

The two main strategies for treating manure on farms are containment and biological processing (Pruden et al., 2013) (Figure 12.1). Because the number of ARGs is generally correlated to the concentration of bacteria in a sample, on-farm treatments that reduce the total bacterial load are likely to also reduce the number of antibiotic resistant bacteria in a sample. Depending on the nature of the treatment, ARGs may also be degraded, though it is possible for them to persist even after their host bacterium dies. Containment of manure has been used effectively to manage nutrients from agricultural wastewaters, and the underlying principle is valid for ARB/Gs as well. Engineered systems are designed and constructed to sequester and store wastes, preventing the contaminants from directly entering ground and surface waters. Although ARB/Gs can and do enter ground and surface waters when these systems fail, when designed and operated correctly, they are able to retain manures and the associated ARB/Gs within the structure.

The major biological processes by which agricultural waste is traditionally treated on site at animal production facilities are composting, aerobic and anaerobic lagoons, and anaerobic digesters. Other less common systems include constructed wetlands, algal ponds, and biofiltration systems (Ibekwe et al., 2016). Some novel, niche-based systems, such as vermicomposting using fly larvae (Wang et al., 2015), have also been proposed

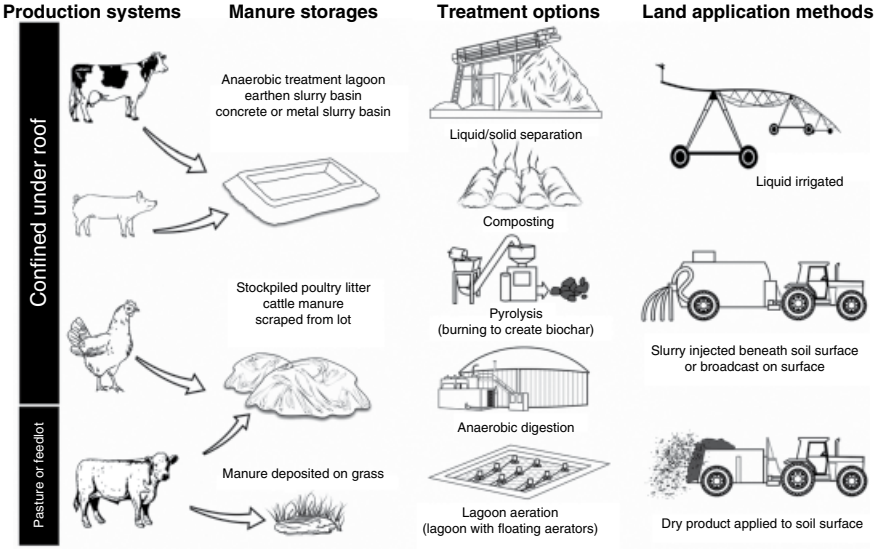


**Figure 12.1 Common manure collection, storage, treatment, and utilization practices on U.S. livestock farms**. Pigs, dairy cows, and chicken are typically raised in a structure with a roof, though pastured or open lot systems do exist for raising these animals. Swine and dairy manure are often collected in a pit as a slurry, or flushed to a lagoon where they become more dilute, while poultry excretions are deposited on a floor covered in bedding material (forming "litter") or in a pit below the floor. Beef cattle may be confined to an open lot (feedlot) where manure is scraped into piles, or on pasture where manure is deposited on the ground during grazing. For all systems that confine animals, collected manure may be further processed or treated prior to land application, though direct application to land as a fertilizer for agricultural crops is the most common practice.

as a way to remediate ARB/Gs in food animal production settings. The evidence examining the efficacy of any of these systems for eliminating ARB/Gs is mixed (Youngquist et al., 2016). In some instances, the measured ARB/Gs targets are reduced following these treatments (Pei et al., 2007; McKinney et al., 2010; Jia et al., 2014; Tao et al., 2014; Xu et al., 2015; Garder et al., 2012), while in other instances no reduction, or even increases, are observed (Chen et al., 2010; McKinney et al., 2010). A recent review of anaerobic digestion and composting found that the only treatments that appear to consistently reduce ARB involve thermophilic processes, but even these processes do not routinely degrade ARGs (Youngquist et. al., 2016). As with land application of wastes and wastewaters, time is an important component contributing to the efficacy of these treatment systems.

### Resistance in *E. coli* and *Enterococcus*

*E. coli* and *Enterococcus* are important water quality indicators, with current wastewater regulations and monitoring activities relying on the enumeration of these organisms. Because of its central role in regulatory actions, the antibiotic resistance profiles of *E. coli* from agricultural systems is of particular interest, and it has been suggested that *E. coli* and fecal enterococci be used as an indicators for phenotypically and genotypically measured antibiotic resistance (Berndonk et al., 2015). Although labor-intensive compared to gene-based community DNA screening, the culture and resistance profiling of *E. coli* and *Enterococcus* allows for the collection of data on the presence of multidrug resistance and aligns data from agricultural wastewaters with antibiotic resistance information being collected on these organisms from human and animal health care settings. The two largest U.S. surveillance programs for antimicrobial resistance, the National Antimicrobial Resistance Monitoring System (NARMS) and the National Animal Health Monitoring System (NAHMS), focus primarily on measuring AR in foodborne pathogens, such as *Salmonella*; however, some data is collected on AR in *E. coli* and *Enterococcus*.

## Discussion

There is disagreement within the scientific community regarding which AR targets should be measured, both from the perspective of discerning the relative contribution of agricultural production systems to the overall AR burden in the environment, as well as from the perspective of measuring targets that are most likely to contribute to adverse human health outcomes (Allen and Stanton, 2014; Durso and Cook, 2014). Some researchers argue that tetracycline and sulfonamide resistance is less relevant, in terms of human health, than resistance to other classes of drugs (Allen and Stanton, 2014). The food safety and public health communities tend to be guided by the CDC threat report (CDC, 2013) and the WHO priority list (AGISAR, 2012), while the environmental quality community tends to be guided by drug usage on farms. Looking at what types of resistance have been described in agricultural wastewaters, the most frequently studied resistance class to date is tetracyclines, with 33 of the 40 papers summarized in Table 12.2 assaying for phenotypic or genotypic evidence of tetracycline resistance. Sulfonamides and macrolides are the next most frequent target, with 20 and 15 studies,

respectively, choosing these classes of resistance for their study. Beta-lactam resistance was measured in five studies, while fluoroquinolone and chloramphenicol resistance were each examined in two. Metagenomic studies hold promise for evaluating a broad suite of genes, but to date the total number of samples evaluated using this method remains small (Li et al., 2015; Ma et al., 2016). There remain large data gaps for individual ARGs, and this lack of data hinders the ability to draw any commodity-based conclusions regarding ARB/Gs.

It is becoming clear that the individual types of antibiotic resistance and individual ARGs each have their own ecologies, and the conclusions regarding the amount of AR in agricultural wastewaters depends, to some degree, on which target is measured (Durso et al., 2016). As an example, tetracycline resistance is coded for by 48 genes and has been found in 126 bacterial genera (Roberts and Schwarz, 2016). Most studies to date will assay for only a small number of these genes when characterizing agricultural wastewater samples. In the 33 studies from Table 12.2 that measured tetracycline resistance genes, the mean number measured was 3, with a range from 1 to 11. The *tet*(W) gene is the most frequent choice (58% of agricultural wastewater AR papers in Table 12.2), followed by *tet*(O) (50%) and *tet*(M) (45%). All three code for ribosomal protection proteins but are carried by different groups of bacteria. The *tet*(W), *tet*(O), and *tet*(M) genes have been identified in 32, 35, and 75 bacterial genera, respectively. All three have been identified in both Gram negative and Gram positive bacteria, but only *tet*(M) has been identified in both *E. coli* and *Enterococcus*, two important fecal indicator organisms (Roberts and Schwarz, 2016). A recent metagenomic analysis suggests *tet*(M) be used as an indicator gene, based on its co-occurrence with other ARGs in the study (Li et al., 2015), and there is a voluntary effort based in Europe for researchers worldwide to collect quantitative data on *tet*(M) (Thanner et al., 2016). Among the tetracycline resistance efflux pump genes, *tet*(C), *tet*(B), and *tet*(A) are the most frequently assayed (9%, 8%, and 7%, respectively) based on studies in Table 12.2. To date, these three genes have only been identified in Gram negative bacteria. The *tet*(L) gene, also coding for efflux pump, has been identified in 41 Gram negative and Gram positive genera to date, including both *E. coli* and *Enterococcus* isolates. Thus, for tetracycline resistance, it is clear that the taxonomic composition of the bacterial community can impact which tetracycline resistance genes are detected, a fact that must be considered when evaluating AR in agricultural wastewaters.

In conclusion, studies of AR in agricultural wastewaters are dominated by gene-based methods and screening of community DNA. Isolate-based measures, while more labor intensive, provide valuable information on multidrug resistance and fit the current water quality paradigm of using indicator organisms. Tetracycline, sulfonamide, and macrolide resistance are, to date, the most thoroughly characterized AR types in agricultural wastewaters. Limited data is available for aminoglycoside, quinolone, and methicillin resistance in manures and manure-impacted soil and water. Swine production areas are the most frequently studied, but there is not yet enough data to discern any commodity-specific trends in resistance. Farm-based wastewater treatments have been shown to be effective in reducing some measures of resistance, but there remains variability within and between systems, and the drivers of both phenotypic and genotypic measures of resistance remain largely unidentified.

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# 13

# Environmental Antibiotic Resistance Associated with Land Application of Biosolids

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As of October 2014 there were 14,780 municipal wastewater treatment plants in the United States treating an average of 32,345 million gallons of wastewater per day (Shen et al., 2015). Treatment of wastewater results in the production of 6 to 7 million dry tons of sewage sludge per year. With growing populations, developing nations build modern sewer systems and centralized water treatment works based on biological nutrient removal (Bibby et al., 2010) and thus, this waste stream continues to grow on a global scale as urban populations increase. The early stages of biological removal involve placement of wastewater into aerobic holding tanks, where sludge largely results from the conversion of soluble organic matter into bacterial biomass.

In the United States, approximately 60% of municipal biosolid waste is applied to agricultural land (USEPA, 2000; NRC, 2002). Biosolids contain a broad variety of micro- and macronutrients; about 60% of the solids content in biosolids is organic matter (USEPA, 1989). Land application of biosolids combines inexpensive disposal of these abundant materials with the return of valuable nutrients to the soil, which may enhance soil properties and plant yield (McLaughlin et al., 2007). Alhough the majority of biosolids are applied to croplands, land application on rangelands, especially semiarid rangelands, is particularly attractive because the climate of these ecosystems typically allows for year-around application. In addition, distance from urban areas and large acreages in private holdings make rangelands particularly suitable for land application (Wester et al., 2003, 2011).

Despite the benefits to soils and crops related to their nutrient richness, questions abound regarding potential detrimental effects of biosolids on the environment. Such questions often relate to the potential for biosolids to spread antibiotics and antibiotic resistance. Research has demonstrated that certain pharmaceuticals can attach to suspended particles in wastewater and accumulate in biosolids during wastewater treatment (Heidler and Halden 2008; Jones-Lepp and Stevens, 2006; Ternes et al., 2004). As a result, pharmaceutical compounds have been detected in biosolids, with typical concentrations ranging from the lower micrograms per kg to a few mg per kg

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(Wu et al., 2010; Kinney et al., 2006). It has been proposed that terraccumulation of sorbed compounds via biosolids application provides a reservoir of pollutants that can mobilize in soil and leach to groundwater or be carried by seasonal erosion to surface water (Rooklidge, 2004; Pedersen et al., 2003).

In addition to the potential for direct pollution of soil and water, trace pharmaceuticals contained in biosolids can remain biologically active and may therefore select for antibiotic resistance in bacteria within the biosolids and in soil (Chander et al., 2005). Enteric bacterial, viral, and protozoan pathogens become incorporated into biosolids during the wastewater treatment process, with the result that antibiotic resistant organisms can be released to the environment with biosolids application. The long-term implications of pathogen application to soils is a matter of some conjecture, as enteric organisms are generally poorly adapted to survival in the natural environment, and pathogens that are introduced to soil are influenced by both climatic (e.g., moisture, temperature, sunlight, competitive organisms, nutrients, and type of soil) and agronomic variables (e.g., the methods and timing of biosolids application) (Lang et al., 2003). In addition to concerns regarding the environmental fate of living microorganisms in biosolids, it has been proposed that antibiotic resistance genes themselves pose a contamination threat. Bacterial genes encoding for antibiotic resistance are often carried on mobile genetic elements, such as plasmids, integrons, and transposons. While some experiments have shown that elevated levels of integrons harboring antibiotic resistance genes in manure-amended soils persist for long periods of time (Byrne-Bailey et al., 2011), others have suggested that increased resistance in these soils is transient and that most soils return to baseline resistance levels within a few months after application (Zhou et al., 2010).

The extent to which human activities in general and the application of biosolids to agricultural lands in particular, contribute to the maintenance of environmental reservoirs of antibiotic resistance is poorly understood. Concerns are fueled by a lack of knowledge of the effects of trace antibiotics on ecosystem processes, by questions related to the survival of enteric bacteria in the soil, and by an absence of critical information regarding the movement of resistance genes within and between microbial populations in the environment. The following chapter summarizes the known connections between biosolids application and antibiotic resistance, and highlights multiple areas in which additional research is needed.

## Regulations for Biosolids Application in the United States

In the United States, the use and disposal of sewage sludge is regulated under 40 CFR (Code of Federal Regulations) Part 503, established by the Environmental Protection Agency in 1993 (Federal Register, 1993). The Part 503 rule sets criteria for levels of metals and pathogens that are safe to humans and other animals when biosolids are used for land application (USEPA, 1994). This regulation also established requirements for controlling the ability of sludge to attract vectors (insects, rodents, and birds) that may be involved in pathogen transmission. Sludge to be used for agricultural purposes must first be treated to reduce odor, vector attraction, and pathogens present, and treatment must conform to the intended use of the biosolids, with Class A biosolids considered free from microbial risks and able to be used without any restrictions. Class B

**Table 13.1** Part 503 Pathogen and Indicator Density Limits (Adapted from USEPA, 2000, and Zaleski et al., 2005)

| Biosolids Class | Pathogen or Indicator | Standard Density Limit (dry weight) |
|---|---|---|
| Class A | *Salmonella* | <3 MPN* per 4 grams total solids |
| | | OR |
| | Fecal coliforms | $<10^3$ MPN per gram total solids AND |
| | Enteric viruses | <1 PFU** per 4 grams total solids AND |
| | Viable helminth ova | <1 viable ova per 4 grams total solids |
| Class B | Fecal coliforms | $<2 \times 10^6$ MPN per gram total solids |

* MPN = most probable number, an estimate of viable bacteria within a given sample. MPN is derived statistically by assessing presence/absence of bacteria in multiple subdivisions of that sample.
** PFU = plaque forming units, a measure of the number of infectious virus particles, derived from a cultural assay.

biosolids are not considered pathogen free and certain restrictions are used to control access to sites where the biosolids are land applied (Table 13.1).

If the treated biosolids will be applied on crops that may be eaten raw, come into contact with the public, or are marketed, the sludge must be treated to Class A standards such that pathogenic microorganisms are reduced below detection limits (Table 13.1). Class A biosolids are produced by thermophilic digestion, composting where temperatures of greater than 55°C for 3 days must be reached. Alternative treatment methods include heat treatment (260°C for 30 minutes) or pasteurization and lime treatment with a pH of greater than 12 for 2 hours (Gerba and Smith, 2005).

The importance of composting conditions (including time, and interior and ambient temperatures) are of critical importance in biosolids treatment. Miller et al. (2014) observed increased levels of antibiotic resistance genes in sludge that was stored at 4°C for up to 6 weeks, and hypothesized that reduced ambient temperatures impacted horizontal gene transfer through mechanisms hypothesized to be enhanced cell competence or improvement in genetic recombination and transfer. Regardless of the mechanism inducing increased gene presence, this finding could have important implications for biosolids storage prior to land application.

Class B biosolids may be spread onto agricultural fields but cannot be used on crops that are to be consumed raw. Class B biosolids are produced by anaerobic (mesophilic) or aerobic treatment, lime stabilization, and/or air drying and low temperature composting. These treatments significantly reduce the level of pathogens, but some pathogenic microorganisms, including *Salmonella* and enteric viruses,may still be present. Pepper et al. (2010) reported that *Salmonella* concentrations averaged $40 \pm 118$ MPN (most probable number) per 4 grams dry weight of Class B biosolids following mesophilic digestion, similar to pathogen numbers reported by Farrah and Bitton (1984) following aerobic digestion. For this reason, application of Class B biosolids requires a permit from local regulatory authorities that includes restrictions on the amount of biosolids that can be applied per acre. Class B biosolids must also be treated to control vector attraction or incorporated into the soil by injection after application. In addition, public access is not allowed to the site for 30 days on private farmland, or 1 year on land

with a high potential of public exposure (such as parkland). Food crops with harvested parts that have the potential to come in contact with biosolids and are totally above ground cannot be harvested until 14 months after application. This time is extended to 20 months for food crops grown below the soil surface. All of these restrictions are based on conservative risk assessments of transmission of enteric pathogens via contract with the soil and food chain (USEPA, 1993).

## Linkages between Biosolids Application and Antibiotic Resistance

### Antibiotic Residue in Biosolids

It has been well documented that pharmaceuticals are adsorbed on sewage sludge particles during wastewater treatment. The rate of this process depends on drug chemical structure and its mobility, hydrophobicity, biodegradation, and the nature of the sludge itself (Bondarczuk et al., 2010), and it is certainly affected by the wastewater treatment train. For example, although treatment might eliminate 80% of fluoroquinolones or tetracyclines, removal of other antibiotic classes from sewage sludge, notably the macrolides, is less efficient (Sukul and Spiteller, 2007). Antibiotics such as beta-lactams or trimethoprim can be degraded by chlorination alone (Dodd and Huang, 2007; Gulkowska et al., 2008), and treatments including ozonation, water softening and coagulation (Adams et al., 2002), membrane filtration (Koyuncu et al., 2008), ionic treatment (Choi et al., 2007), and adsorption to activated carbon (Ternes et al., 2002) have been shown to be effective in removal of some percentage of pharmaceutical residue.

Antimicrobial accumulation in biosolids and subsequent transfer into the environment has been assessed through research focused on the widely used compounds triclocarban (3,4,4'-trichlorocarbanilide) and triclosan (5-chloro-2-(2,4-dichlorophenoxy)-phenol). Digested municipal sludge accumulates approximately 50 μg triclocarban and 30 μg triclosan per gram dry sludge (Heidler and Halden, 2007). Following municipal application of biosolids to agricultural fields, accumulation of these compounds ranged from 1.2 to 65 $ng\,kg^{-1}$ for triclocarban and 0.16 to 1.0 $ng\,kg^{-1}$ for triclosan (Cha and Cupples, 2009). Once introduced into the environment, the compounds sorb to soils or sediments and are not predicted to readily degrade (Halden and Paull, 2005; Ying et al., 2007). Furthermore, field experiments showed detectable triclocarban and triclosan in roots, stems, and leaves of pumpkin, zucchini, and switch grass, though potential human exposure to these compounds was two orders of magnitude less than exposure from using products containing triclocarban (Aryal and Reinhold, 2011).

An important point is that modern treatment of water from human activity is far less common in developing countries, where antibiotic-polluted biosolids and other residues are released into the environment without further processing in many cases (Martinez, 2009).

### Antibiotic Resistant Bacteria and Antibiotic Resistance Genes in Biosolids

Multiple studies have demonstrated amplification of antibiotic resistance genes during biological wastewater treatment. Kim et al. (2006) evaluated the fate of resistant bacteria during treatment processes and demonstrated that an increase in organic loading

resulted in amplification of tetracycline resistance, indicating that resistance development is affected significantly by changes in wastewater treatment plant operating conditions. This was further supported by the findings of Munir et al. (2011), who monitored Michigan biosolids for the tetracycline resistance gene *tet*(W); they reported that concentrations were lowest in lime-stabilized biosolid samples ($10^7$ to $10^8$ per gram), but no significant treatment differences in *tet*(W) gene levels were observed in biosolids produced using advanced (membrane bioreactor) and conventional water treatment (activated sludge, oxidative ditch) methods. Findings of resistance amplification are not surprising, given that biological wastewater treatment relies on the maintenance of conditions conducive to bacterial activity. Not only do bacteria flourish in the presence of elevated carbon and energy sources, but conditions within biological reactors are also conducive to the transfer of mobile genetic elements harboring resistance genes between bacteria on both an inter- and intraspecies level (Cytryn, 2013).

Although it is clear that biosolids contain elevated levels of antibiotic resistant bacteria and resistance genes, the effect of biosolids application on the resistome of biosolids-amended soils is less certain. Riber et al. (2014) determined the prevalence of cultivable tetracycline and gentamicin-resistant pseudomonads in biosolids-amended soils and found that, though the number of resistant isolates increased significantly in treated soils directly after fertilization, these numbers decreased rapidly over time, suggesting that resistant bacteria were not proliferating in soils. Reported survival of fecal indicator bacteria following biosolids application is highly variable. Pourcher et al. (2007) studied the survival of enteric microorganisms in sewage sludge following direct land spreading and reported that enteroviruses were not detected 2 weeks after spreading on the soil, whereas the concentration of fecal indicators (*E. coli* and enterococci) fell slowly with an observed decrease of between 1.2 and 1.8 log over 2 months. While Gibbs et al. (1997) and Jones (1986) found *Salmonella* in the soil nearly 9 months after spreading sewage sludge, others have observed the disappearance of these same bacteria in less than 6 weeks (Nicholson et al., 2005; Gessel et al. 2004). Given the numerous environmental parameters influencing the survival of microorganisms and the complexity of their interactions, it is not surprising that the results obtained by different studies do not always agree. The varying survival times confirm the necessity to include several indicators (including both bacteria and viruses) to estimate any public health risk related to biosolids spreading.

Results of studies examining relative survival of resistant microorganisms are similar to those above in that elevation in gene presence and resistant bacteria in soils following biosolids application appear to be short-lived. Riber et al. (2014) demonstrated a higher frequency of horizontal gene transfer in biosolids-amended soil compared to an untreated control only in the first week after fertilization, while Rahube et al. (2014) showed that even though an increase in the variety and abundance of target genes was observed in the season of biosolids application, it diminished to no observable effect after 15 months. These findings suggest that following biosolids land application, the lack of selective pressure for antibiotic resistance may induce the loss of the plasmid carrying the resistance genes, leading to lower recovery of resistance genes over time (Smith and Bidochka, 1998).

Of all of the confounding factors contributing to variability in both reported survival of antibiotic-resistant bacteria and presence of antibiotic resistance genes following biosolids application to soil, none has been as critical, yet under-reported, as native soil

antibiotic resistance. It is traditionally assumed that anthropogenic activities result in proliferation of soil antibiotic resistance, yet recent work indicates that the soil microbiome is characterized by high levels of native antibiotic resistance, and that antibiotic resistant bacteria and resistance genes related to wastewater or biosolids application generally are not persistent in the soil environment (Negreanu et al., 2012; Munir and Xagoraraki, 2011). Zerzghi et al. (2010) evaluated the influence of 20 years of Class B biosolid application to Arizona soils and found no statistically significant differences between treatments with respect to concentrations of antibiotic resistant bacteria. The authors noted that concentrations of resistant bacteria were very high in control soils (averaging $10^6$ to $10^7$ CFU $g^{-1}$ soil) (Brooks et al., 2007), indicating that indigenous organisms were producing natural antibiotics. It has recently been suggested that the antibiotic resistance gene pool in the environment is much larger than the gene pool observed among pathogens (Bondarczuk et al., 2016). If this is indeed the case, the release of resistant bacteria into natural environments and/or agricultural soils should be a cause for concern. Bondarczuk et al. (2016) suggest two reasons: first, pathogens residing in soils may acquire new antibiotic resistance genes from environmental strains. Second, in an opposite scenario, environmental microorganisms may capture clinically important genes evolved under a strong selective pressure from pathogens. Both scenarios pose a potential public health impact and could threaten the future of antibiotic therapy. However, studies indicate that certain activities associated with release of subtherapeutic antibiotic concentrations to soil not only fail to spread resistance to native bacteria but sometimes result in a notable reduction of resistant bacteria and resistance gene abundance (Negreanu et al., 2012; Walsh and Duffy, 2012). Cytryn (2013) suggests that this phenomenon may be associated with inverted selection, where certain bacterial resistance mechanisms actually lower fitness, and thus reduce overall bacterial survival, when exposed to particular antibiotic compounds (Bollenbach et al., 2009; Chait et al., 2007).

### Biosolids-Mediated Transmission of Antibiotic Residual to Soil and Water

Although studies have correlated an increased incidence of antibiotic resistance among culturable bacteria in surface water (Harwood et al., 2000; Reinthaler et al., 2003) and groundwater (McKeon et al., 1995) impacted by wastewater effluent discharge, no significant correlations between biosolids application and resistant bacteria have been reported. In 2003, Yang and Carlson identified biosolids as a potential source of antibiotics in runoff to surface waters, yet current work reveals limited mobility of residual antibiotics in biosolids following soil application. Wu et al. (2010) simulated a 200 mm rainfall event and reported that the majority of applied pharmaceuticals were retained in the biosolids mixed layer (0–5 cm). Working in the Chihuahuan Desert in Texas, Wester et al. (2011) showed that water leached through biosolids applied at rates up to 34 Mg $ha^{-1}$ retained water quality well within USEPA drinking water standards. Furthermore, biosolids reduced soil erosion and soil water runoff and increased soil water infiltration. Even the lowest application rate (7 Mg $ha^{-1}$) reduced erosion by about 40% compared to bare areas not treated with biosolids. Adverse quality effects were observed in water leached through soils at the highest biosolids application rate (90 Mg $ha^{-1}$), but the quality of surface runoff water was generally not adversely affected by surface-applied biosolids.

## Risk Assessment Data Needs: Biosolids Application, Antibiotic Resistance, and Human Health Risk

Although quantitative microbial risk assessment (QMRA) has evolved rapidly over the last decade, a definitive QMRA has not yet been performed for biosolids-borne pathogenic antibiotic-resistant bacteria with regard to human exposure. The most recent attempt to establish a QMRA framework was published by Ashbolt et al. (2013), who outlined the complex nature associated with this type of analysis. The greatest uncertainty in such a QMRA is related to the estimation of dose response resulting from human exposure (Haas et al., 1999). At the most basic level, it can be assumed that pathogenic antibiotic resistant bacteria would behave similarly to their nonresistant pathogenic counterparts regarding their ability to cause disease, and thus the dose response would be the same. However, considerations would have to be made regarding the human response to treatment. Furthermore, to properly define the risks posed by biosolids land application, a more complete understanding of pathogen abundance, infectivity, and diversity is required. Some data suggest that an antibiotic resistant bacterial pathogen may be more infectious than its counterpart nonantibiotic resistant pathogen (Ashbolt et al., 2013). In the case of pathogens that are not generally treated with antibiotics (e.g. *Salmonella* spp.), disease manifestation would most likely be the same whether antibiotic resistant or not. To date, no infectious dose-response assessment has been conducted for an antibiotic resistant pathogen, simply because the data do not exist or have not been collected. Without proper dose-response data for an antibiotic resistant pathogen, no true QMRA can be conducted.

In addition to the need for detailed dose-response data for antibiotic resistant pathogens, accurate QMRA requires standardized methods for the detection of pathogens, including methods for identification of newly recognized pathogens for which data are extremely limited (NRC, 2002). Development of databases must also consider demographics of target populations. For example, the presence of older and more immune-compromised individuals who have a greater risk of serious illness are increasing in number (Gerba and Smith, 2005). These population subsets could be more vulnerable to infections of antibiotic resistant pathogens.

Of course, an accurate risk assessment for biosolids-borne contaminants would consider not just antibiotic resistant microbes, but also would include risk posed by the antibiotics themselves. Data sets developed to assess such risk should include half-life data for antibiotics developed using field studies rather than from laboratory experiments (Walters et al., 2010). In addition, accurate environmental risk assessments should consider not only acute but also chronic toxic effect threshold values of chemicals on nontarget organisms due to chemical longevity in soil (Chalew and Halden, 2009). The potential for plants and crops to take up microcontaminants from biosolids-amended soils is an additional area requiring further research (Jones et al., 2004). It has been documented that certain antibiotics, specifically tetracyclines and fluoroquinolones, can be taken up by crop plants (Migliore et al., 2003; Kumar et al., 2005), and thus risk assessment must consider potential effects of plant uptake on human populations and grazing animals. However, such assessments must consider that doses calculated for multiple routes of human exposure to antimicrobials following biosolids application may be substantially less than the no observable adverse effect level. For example, Aryal and Reinhold (2011) calculated that human exposure to triclocarban from eating

pumpkin and zucchini grown in fields receiving biosolids was two orders of magnitude less than exposure from using products containing triclocarban.

As detailed in the preceding sections, the persistence of enteric bacterial pathogens after land application is believed to be limited. Land application at the practiced loading rate of one to three tons per acre results in about a 100- to 1,000-fold dilution of biosolids-borne bacteria when added to the soil, and Gerba et al. (2008) reported that *Salmonella* in biosolids did not regrow under these conditions in agricultural soil. Multiple studies indicate that, though short-term increases in resistance can be detected, long-term biosolids application does not increase the number of antibiotic resistant bacteria (Zerzghi et al., 2010; Brooks et al. 2012). It has been reported that when the EPA-mandated waiting period was implemented after biosolids application, no antibiotic resistant bacteria or genes were detected in the soil or on the crops above normal background levels (Rahube et al., 2014), suggesting that adherence to the aforementioned regulations for the growth of produce traditionally eaten raw in biosolids amended soils will minimize the contamination of said crops from enteric antibiotic resistant bacteria and genes. Since the 503 regulations have been in use there has not been a documented transmission of an enteric pathogen from land application of biosolids.

## Need for Further Research: Biosolids and Environmental Antibiotic Resistance

The extent of bacterial resistance associated with biosolids application in the environment has yet to be clearly defined, and as illustrated in the preceding sections, contributions from agriculture are debated and often contradictory. Assessment of risk is currently not possible, as the state of knowledge of antibiotic-resistant microbial distributions and diversity in soils with applied biosolids remains limited. We also highlight above the importance of obtaining science-based information on the survival and transport of resistant bacteria after field application in conditions representative of realistic agricultural practices. Further work focused on the ability of bacterial species to transfer resistance genes is also needed.

The most significant confounding factor preventing accurate correlations of biosolids use and antibiotic resistance is the presence of native soil antibiotic resistance, which must be considered in any scientific study. A myriad of published experimental data indicates that anthropogenic activities may considerably expand soil antibiotic resistance, but it is becoming increasingly clear that resistance in the soil microbiome exceeds that known in clinical settings. Because this complexity has only recently been recognized, it is only beginning to be addressed in scientific studies. Thus, it remains unclear whether biosolids application to soil has contributed resistant bacteria and/or resistance genes to the pathogen resistome.

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# 14

# High Throughput Method for Analyzing Antibiotic Resistance Genes in Wastewater Treatment Plants

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Traditionally, the research related to antibiotic resistance has focused mainly on pathogens and used cultivation-based methods such as antibiotic susceptibility testing of bacteria. While susceptibility testing gives important data on resistance patterns that are needed for designing treatments for patients, it gives little information on the resistome present in wastewater treatment plants (WWTPs) or the dynamics of the resistome since only a minority of the species present in the WWTPs can be cultivated with the current methodology. To find the best treatment processes for WWTPs, it is essential to find high-throughput methods to detect antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs), which may contribute to more far-reaching antibiotic resistance.

Quantitative PCR (qPCR) methods can determine the amount of the genes from WWTP DNA samples without the need of cultivation. They give results directly on the abundance of ARGs or other genes of interest and are widely used. Recently developed qPCR arrays give the opportunity to quantify hundreds of genes, making it possible to study the whole resistome instead of selected genes. Rigorous statistical analysis combined with DNA sequence analysis makes it possible to analyze co-occurrence patterns of ARGs and microbial populations. In this chapter we will introduce the qPCR array, explore the advantages and disadvantages of the method, and review the literature on the research on WWTPs and related environments utilizing qPCR arrays.

## Introduction

Urban WWTPs have been considered hot spots for horizontal gene transfer (HGT) and they are recognized as key environments where antibiotic resistance evolves (Baquero et al., 2008; Rizzo et al., 2013). WWTPs are one of the main point sources of antibiotic resistance to the environment, and the resistance has been found to be enriched by the treatment processes (Harris et al., 2012). In addition, the use of reclaimed water is increasingly common due to the emerging threat of water scarcity. Reclaimed water

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is commonly used in irrigation of parks in arid areas and as household water in many countries. In the future there will be more pressure for the efficient utilization of the solid by-products from wastewater treatment process as the result of expected phosphorus depletion. Therefore, these by-products should be considered as possible disseminators of ARGs.

The increased use of recycled water and other by-products of WWTPs may contribute to the spread of antibiotic resistance. The large and growing number of antibiotic resistance genes calls for high-throughput methods for surveillance of ARGs in anthropogenic environments. There is a need for efficient methods for analyzing the dynamics of the resistome in WWTPs and their abundance in the final effluents that are released to the environment.

More data are also needed to understand the effects of different methods used for water purification in WWTPs. For example, ozonation has been considered as a resolving treatment, since it would also remove some of the organic contaminants, including traces of antibiotics. However, ozone treatment of conditioned wastewater was recently shown to select antibiotic resistance genes and opportunistic bacteria (Alexander et al., 2016). This is another example to support the need for high-throughput methods to find the best alternatives for technologies to be used in WWTPs to decrease the risks caused by dissemination of ARGs.

Since most environmental bacteria are not culture-grown, and cultivation-based methods give very little information on the dynamics of the resistome in WWTPs, culture independent methods are needed for studying the resistance potential and dynamics in WWTPs. Quantitative PCR (qPCR) is a suitable method for quantifying the copy numbers or analyzing the abundances of ARGs or other genes of interest. The limitation of qPCR is that the sequences of the studied genes must be known in order to design primers; however, a wide range of primers targeting ARGs is available in the literature. Several studies have focused on analyzing a few relevant genes from WWTPs with qPCR, but the studies determining a large number of genes are rare. One possible reason for this is that qPCR studies can become costly and time consuming since studying several genes requires multiple qPCR runs and also trained laboratory professionals.

Recently developed qPCR arrays can address the throughput limitations associated with traditional qPCR (Stedtfeld et al., 2008; Looft et al., 2012; Zhu et al., 2013). With the qPCR array, the simultaneous quantification of hundreds of ARGs and other genes of interest is possible as parallel assays in just one run. This creates an opportunity for quantification of a large number of relevant ARGs, sequences related to mobile genetic elements, and genes specific to certain bacterial species in WWTPs or related environments. The literature uses two different terms for these arrays, high-throughput qPCR (HT-qPCR) and qPCR array. In this chapter the term qPCR array will be used for both.

There are currently at least three qPCR array systems commercially available for quantification of ARGs. The Fluidigm Access Array provides quantitative information on the abundance of the ARGs and allows collection of sequencing-ready barcoded amplicons postrun. Reactions are run on a chip that has the capacity for 48 assays and 48 samples (2304 reactions). Users can select the assays and primers according to their preferences. The system is yet to be used to analyze wastewaters, but it has been used to study the enrichment and clustering of ARGs in swine agriculture, and it should be well suited for analysis of waters as well (Johnson et al., 2016). Qiagen has developed an Antibiotic Resistance Genes Microbial DNA qPCR Array, which includes primers for

quantification of ARGs and virulence factors and is available in either 96- or 384-well formats. In the Qiagen system, it is not possible to customize the assays and primers. The third qPCR array system, Wafergen Bio-systems SmartChip Real-Time PCR, allows qPCR arrays with selected primers. Both of these latter technologies have been used in studying the dynamics of ARGs from various samples including agricultural environments, WWTPs, reclaimed water, downstream environments, and irrigated samples, and this literature is reviewed in the following discussion.

## The Basic Principle of qPCR Arrays

The qPCR array chips or plates can hold numerous qPCR assays targeting up to hundreds of different genes, which are analyzed in a single run. The reaction mixes are loaded on an array chip (Wafergen) or a plate (Qiagen) with preloaded primer sets. This way it is possible to combine the screening capacity of microarrays and the sensitivity of qPCR. The Wafergen Bio-systems SmartChip Real-Time PCR array chip has 5,184 nanowells. With one chip, it is possible to run up to 384 reactions with different primer pairs and 12 samples simultaneously. The sample capacity can be further increased up to 384, samples narrowing the number of targeted genes to 12 assays. Qiagen has several microbial DNA qPCR arrays, which are designed to target virulence factor genes or ARGs. These qPCR arrays can be ordered with 96- or 384-well plates with integrated controls in addition to preloaded primer sets. The Qiagen Microbial DNA qPCR Array plates can be run on standard real-time PCR instruments. The other two platforms need specialized instruments.

In qPCR array systems, the assays are generally analyzed without standard curves because it is not practical to include standards for hundreds of genes into the chip. The quantification is therefore relative to a control gene or a housekeeping gene, which in the case of bacteria and ARGs is commonly the 16S rRNA gene, but other control genes are also possible. Absolute gene copy numbers can be calculated according to Equation 14.1, assuming 100% PCR efficiency (Looft et al., 2012):

$$\text{Gene Copy Number} = 10^{\left((31-\text{CT})/(10/3)\right)}, \tag{14.1}$$

where 31 is the threshold cycle (CT) used as a detection limit in this example and CT is the threshold cycle of the ARG. Another term in the exponent, 10/3, is derived from the qPCR slope, where the 10-fold difference in gene copy numbers is 3 cycles, if the efficiency is close to 100%. The results have usually been calculated according to comparative CT method, which is also known as the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Equations 14.2 and 14.3 are used in calculations in the comparative CT method, according to Schmittgen and Livak (2008):

$$\Delta CT = CT_{ARG} - CT_{16S} \tag{14.2}$$

$$\Delta\Delta CT = \Delta CT_{\text{Treatment}} - \Delta CT_{\text{Reference}}, \tag{14.3}$$

where CT is the threshold cycle, ARG refers to one antibiotic resistance gene assay, 16S is the 16S rRNA gene assay, "Treatment" is the experimental sample and "Reference" is the reference sample. Relative abundances, that is, ARG copy numbers normalized to

16S rRNA gene copy number, have also been used in comparing different treatments. Relative ARG abundances (R) can be obtained with Equation 14.4 and fold changes (FC) according to Equation 14.5:

$$R = 2^{-\Delta CT} \tag{14.4}$$

$$FC = 2^{-\Delta\Delta CT} \tag{14.5}$$

In the first studies, which utilized qPCR array, the results were reported as fold changes and copy numbers of ARG were relative to 16S rRNA gene copy number (Karkman et al., 2016; Wang et al., 2014; Zhu et al., 2013). Results were calculated according to the equations presented above. An alternative approach is to transform the relative copy numbers of ARGs to absolute copy numbers. This requires quantification of the absolute 16S rRNA gene copy number in different samples with traditional qPCR separately from qPCR array and then normalizing the ARG copy numbers from the array, obtained with Equation 14.1, to the absolute copy numbers of 16S rRNA gene. Several studies have used this approach (Ouyang et al., 2015; Xu et al., 2016; Su et al., 2015). However, the relative quantification might be sufficient for answering the research question if, for example, two treatments or samples are compared.

## Advantages and Disadvantages of the qPCR Array

One disadvantage of the qPCR array is that it is not possible to optimize the conditions for each individual qPCR reaction since the run is performed on an array chip or a plate. The limit of detection in qPCR arrays has not been as low as in the optimized traditional qPCR assays. However, the limit of detection in qPCR arrays is likely to be reduced when the assays become further optimized such that reliable comparisons can be made.

Despite the drawbacks described above, qPCR array is extremely useful for comparing effects of treatments in different samples, since it is possible to analyze several genes from numerous samples in high throughput and absolute quantification is not needed. On the other hand, by using a large set of genes in one array, it is possible to narrow down the candidates for marker or indicator genes to be used in broader studies or for monitoring. Research projects of this kind are currently ongoing with the StARE (Stopping Antibiotic Resistance Evolution) consortium, as an example (https://stareeurope.wordpress.com/).

The use of qPCR array generates an immense amount of data, which gives plenty of opportunities for various statistical analyses and visualization of the results. Very often the ARGs are divided into categories according to the antibiotics for which they confer resistance and their resistance mechanisms. As in the studies conducted with microarrays, a common way to present qPCR array data are the heat map figures. Values presented in heat maps are usually fold changes, copy numbers, or relative abundances. Ordination plots are also common, which show the similarities or dissimilarities between different samples or treatments. Furthermore, correlations between ARGs, MGEs, antibiotics, and environmental conditions have been applied, as has network analysis between ARGs, MGEs, and bacterial communities, when qPCR data can be linked to sequencing data.

## Use of qPCR Arrays in Studying the ARG Dynamics in Wastewater Environments

The qPCR array was first applied for studying antibiotic resistance in agroecosystems (Zhu et al., 2013). Following that, the method has also been used in studying the resistome in WWTPs and related environments. Typically, the research studies utilizing qPCR array have compared different sample types or treatments. The results are obtained with the equations explained above. In many presented studies, the results produced by qPCR array have been linked to sequencing data, environmental factors, antibiotic concentrations, or cultivation methods.

## Park Soils and Effects of Irrigation with Reclaimed Wastewater

Wang et al. (2014) used the Wafergen qPCR array to quantify and determine the enrichment of 244 ARGs and 9 transposase genes in park soil samples irrigated with reclaimed water. Gene abundances in these reclaimed water irrigation (RWI) soil samples were compared with park soil samples that were not irrigated with reclaimed water. Samples were collected from seven Chinese cities and analyzed for traces of antibiotics as well. Han et al. (2016) also studied the effects of RWI on park soils in Victoria, Australia. The researchers analyzed abundances of 84 ARGs from park soil samples and compared RWI park soils with soil samples collected from natural parks and urban parks that were not irrigated with reclaimed water (Han et al., 2016). Han et al. used the qPCR arrays offered by Qiagen and in addition manually quantified copy numbers of one transposase and one integrase gene.

With the use of qPCR arrays, the influence of various treatments on the park soil resistome profiles could be monitored. The researchers in China detected a total of 147 ARGs among all of the studied samples, and the enrichment was significant in 105 unique ARGs in the RWI samples (Wang et al., 2014). The researchers found that ARGs were very common in RWI soils; however, an average of 81 ARGs were detected even in the control samples. Han and colleagues (2016) found that the copy numbers and fold changes of the detected ARGs were significantly higher in parks with RWI than those without RWI, suggesting that RWI could disseminate and enrich the ARGs in park soils (Han et al., 2016).

Wang et al. (2014) discovered that different ARGs existed in large extent in the RWI samples, though the distribution of enriched ARGs varied in different areas. In Australia, the effects of RWI on the ARG profile seemed to be soil dependent, and in addition, soil pH correlated significantly with the enrichment of total ARGs (Han et al. 2016). The researchers also reported an enrichment of ARGs in urban parks without RWI compared to the remote national parks. The authors suggested that, in addition to RWI, other anthropogenic activities might contribute to selection pressure in the ARGs in the soil microbiome (Han et al., 2016). This is a noteworthy observation and shows that in the studies concentrating on the effects of anthropogenic activities on the resistome, it is important to include pristine sites as well.

Wang et al. (2014) reported a significant correlation between transposases and resistance genes against tetracycline and sulfa drugs. It was pointed out that RWI can

introduce transposons or other MGEs harboring ARGs to the soil microbiome, and it was concluded that these elements may contribute to ARG dissemination among soil bacteria (Wang et al., 2014). Two beta-lactam resistance genes demonstrated significant positive correlations with the integrase gene in the study by Han et al. (2016). However, according to the authors, the RWI did not significantly enrich the *intI1* and *tnpA* genes, which suggested that RWI did not increase the HGT in the investigated urban parks (Han et al. 2016). In addition to qPCR array, Han et al. analyzed the bacterial communities in the parks with T-RFLP and linked those results to qPCR array results. The results supported each other such that the RWI parks were clearly separated from those parks without RWI. However, the bacterial communities between the remote national parks and the urban parks without RWI were not clearly different (Han et al., 2016).

## Water Environments and Treatment Plants

The Wafergen qPCR array technique has been used to examine various compartments of the urban water cycle, including urban WWTP, drinking water treatment plants, river water, and drinking water. The method has included 295 primer sets, of which 285 targeted for ARGs, 8 for tranposase genes, and 1 for integron genes, as in the study by Wang et al. (2014). One set of primers was used in quantification of the 16S rRNA gene to allow normalization.

Ouyang et al. (2015) compared urban sites of the Jiulongjiang River in China to a pristine site. In this study the relative ARG copy numbers obtained with qPCR array were calculated to absolute ARG copy numbers by multiplying them with the absolute 16S rRNA gene copy numbers quantified separately from the array.

It was discovered that the total abundance of ARGs in urban stream samples was over two orders of magnitude higher than in a pristine source, and furthermore those ARGs had significant correlation with MGEs (Ouyang et al., 2015). The authors stated that this indicates higher potential for HGT of ARGs in urban samples. In addition, Ouyang et al. (2015) evaluated the relative abundance of ARGs in the total bacterial community by normalizing the copy numbers of ARGs to an estimate of bacterial cell numbers, by using the approximation that bacteria have four copies of 16S rRNA genes (Klappenbach et al., 2001). It was found that each bacterial cell could harbor at least 2.4 MGEs and ARGs in urban samples, whereas in the pristine source each bacterial cell harbors only 0.01 and 0.26 MGEs and ARGs, respectively (Ouyang et al., 2015). The researchers also discovered that the abundance of multiresistant bacteria was elevated in the urban stream using traditional culture dependent methods. The study conducted by Ouyang et al. (2015) is an example of how qPCR array can be utilized: the qPCR array was used for screening and profiling ARGs, and according to those results the researchers studied bacteria carrying ARGs in more detail by traditional microbiological methods.

Ouyang et al. (2015) concluded that anthropogenic activities change the bacterial composition of the river water. Using qPCR array together with cultivation methods, it was possible to show that anthropogenic activities could cause the enrichment of ARGs, the bacteria carrying ARGs, and possibly induce HGT in the river environment.

Xu et al. (2016) investigated two different drinking water treatment plants (DWTPs), drinking water distribution systems, and tap water samples in Hangzhou City, China.

The researchers compared advanced DWTP and conventional DWTP, evaluating the effects of different water purification methods; absolute quantification of ARGs and MGEs was also used in their study. Both of the studied DWTPs were used to purify the raw surface river water as drinking water, which was distributed to the local population through water supply systems. The publication compared the effects of different treatment steps on the abundances of ARGs and MGEs (Xu et al., 2016).

Up to 122 ARGs were detected from tap water in the supply area of the conventional DWTP in the study by Xu and colleagues (2016). The tap water samples had clearly higher abundances of MGEs than the raw water, and most of these genes also showed significant correlation with the ARGs found in tap water. The abundance of ARGs was clearly elevated in tap water samples compared to the finished water samples from the DWTP. The authors suspected regrowth of resistant bacteria in the water distribution system and that gene transfer occurred during the water distribution processes (Xu et al., 2016).

Karkman et al. (2016) studied an urban wastewater treatment plant in Helsinki, Finland, over four seasons. The study focused on the resistome of raw inflow, dried sludge, and final effluents and also assessed the long-term effects of purified wastewater release to Baltic Sea sediments. Altogether, the researchers detected 175 ARGS and 9 transposase genes from the WWTP. The results were presented as relative abundances of ARGs to 16S rRNA gene.

Karkman et al. (2016) found a high diversity and abundance of ARGs and MGEs in the raw sewage; however, they also observed a reduction in richness and relative abundance from raw inflow to final effluent. Nevertheless, some ARGs were enriched in the effluent community, which indicates that different genes may have different behavior during the process. Xu et al. (2016) noticed that there was an increase in the diversity and relative abundance but a decrease in the absolute abundance of ARGs and MGEs in the advanced DWTP after the biological activated carbon and chlorination treatments. Based on qPCR array results, Xu et al. (2016) could conclude that the advanced drinking water treatment did not add expected benefit in eliminating ARGs compared to conventional methods.

Both studies by Xu et al. (2016) and Karkman et al. (2016) show the advantages of qPCR arrays over traditional qPCR. The number of assays in one array made it possible to quantify several genes at the same time and to compare resistance profiles over single genes in different stages of the processes. The differences between the various genes showed that looking only at a few genes would have lost the overall picture.

Karkman et al. (2016) discovered that the few genes that were enriched in the sediments near the release site were the same genes that were enriched in the RWI park soil samples in the study by Wang et al. (2014). These genes were also more abundant in the urban stream samples in the study by Ouyang et al. (2015) than in the pristine site. Since these genes can also be found in two different *E. coli* plasmids, it is possible that these genes are selected together. This is an example of the advances of qPCR array. It is possible to identify possible coselection patterns without sequencing or laboratory experiments.

Karkman et al. (2016) also studied the sludge of settled biosolids that is applied to land after composting. The sludge had a few more enriched genes than the effluent water. The authors stated that the sludge may play an important part in environmental ARG contamination, and therefore the use of sludge in land application should also be

studied. Despite the enrichment of a few genes in the sludge, it was concluded that the studied urban WWTP provided efficient removal of ARG contamination from the sewage (Karkman et al., 2016).

## Sewage Sludge and its Effects on Soil Resistome

Su et al. (2015) conducted a lab-scale experiment composting urban sewage sludge from municipal sewage. The compost was used as a soil fertilizer. The researchers compared the sewage sludge before composting with samples collected at different phases during the composting process, with the endpoint being the final compost. Chen et al. (2016) studied the effects of long-term field application of sewage sludge on the soil resistome. The samples compared in the study by Chen et al. (2016) were soil samples treated with sewage sludge and chicken manure against soil samples treated with chemical fertilizers. The methods used by both research groups were a combination of qPCR arrays including 295 primer sets for ARGs and MGEs and Illumina sequencing of bacterial 16S rRNA genes (Chen et al., 2016; Su et al., 2015). With these methods the research groups were able to link the changes in the bacterial community compositions with changes in the resistome of the samples.

Composting of the municipal sewage was found to significantly increase the diversity of ARGs and MGEs (Su et al., 2015). The absolute copy numbers of ARGs also increased; however, elevated copy numbers of MGEs were not detected. Chen et al. (2016) made an observation that the effect of sewage sludge on the abundance of ARGs depends on the dose and that the lower dose caused no significant increase in the abundance of ARGs. Therefore, the researchers suggested that 4.5 t $hm^{-2}$ would be a suitable safe limit for the use of sewage sludge as soil fertilizer.

In the composting experiment it was discovered that environmental factors, such as temperature, pH, total nitrogen concentration, and dissolved organic carbon together with bacterial compositions explained 39.9% of the variation of redundancy analysis, while the MGEs contributed to 2.6% variation individually (Su et al., 2015). Chen et al. (2016) found that MGEs had significant positive correlation with genes, conferring resistance to some antibiotics but not to all. It was discussed that the distribution of ARGs and MGEs would be dependent on the host range (Chen et al., 2016). The researchers found that MGEs explained only 4.1% variation of ARGs, and thus the authors suggested that the major driver of antibiotic resistome alternation was bacterial community shift instead of HGT (Chen et al., 2016). Su et al. (2015) had a similar postulate and stated that since the ARG profiles were positively correlated with the relative abundance of thermophilic and multiresistant *Firmicutes* and *Actinobacteria*, the elevated abundance and enrichment of ARGs during composting could happen as a consequence of selecting a bacteria population, which is best adapted to the environment rather than HGT.

Su et al. (2015) concluded that direct application of sewage sludge compost on a field could lead to further dissemination of ARGs. By using network analysis for determining possible co-occurrence patterns between ARGs and microbial taxa, Chen et al. (2016) found that four bacterial families might be possible ARG hosts. The researchers identified bacterial pathogens using a Blast tool based on the 16S rRNA gene sequence from each sample against the bacterial pathogen 16S rRNA database. Two human pathogens,

*Mycobacterium tuberculosis* and *Pseudomonas aeruginosa*, were identified. However, Chen et al. (2016) stated that this result might be inaccurate and suggested the metagenomic approach for the future studies.

## Conclusions

Many studies have investigated the role of WWTPs and related environments in the dissemination and enrichment of ARGs using PCR and qPCR approaches. Since the diversity of ARGs is high in natural environments, qPCR arrays offer a unique opportunity for broad-spectrum screening of ARGs. This method thus enables researchers to compare the profiles of ARGs in samples exposed to antibiotics or resistant bacteria loads or different treatments. The quantitative information of large numbers of genes can also be used for the selection of indicator genes that should be analyzed for future monitoring purposes. On the other hand, qPCR arrays also allow high-throughput analysis of large numbers of samples. The method is very useful in assessing the efficiency or effects of different processes carried out in WWTPs. Often, absolute copy numbers of ARGs or MGEs are not needed and the relevant information is achieved by comparing the ARG profiles in samples before and after the treatments. In addition, if regulations require that concentrations be reported as copy numbers per volume, the relative copy numbers can be transformed to absolute copy numbers by quantifying the copy number of the gene used in normalization separately from the qPCR array.

Many researchers conclude that further studies are needed to investigate the resistomes at different stages of WWTPs and especially in the influent and effluent water. This can be achieved in high throughput by using the qPCR array method. Moreover, it is important to categorize which specific ARGs could be the genes of most interest or which genes could be used as indicators to monitor the quality of reclaimed water in order to avoid health risks.

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# 15

# Antibiotic Resistance and Wastewater Treatment Process

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## Introduction

There is an urgent need to specifically monitor and address the problem of antibiotic and antibiotic resistance (AR) spread into the environment, namely into water, by introducing regulations intended to minimize this risk to water quality and human health. This aim can be pursued by introducing actions to reduce pollution sources. Only specific regulations can steer possible stakeholders (e.g., national/local authorities, farmers, pharmaceutical industries, hospitals infrastructures, water/wastewater treatment managers, pharmaceuticals end users, and others) toward adopting the necessary actions to minimize the risk of antibiotic, pathogenic, and AR water contamination. As AR is of global clinical concern, and is regarded as an emerging contaminant in water, it is important to address this health risk to ensure continuous provision of safe water. However, we must also include the standard indicators of pollution, *Escherichia coli* and intestinal enterococci, in order to leverage current standard operating procedures utilized globally for comparability of the novel monitoring technologies developed. Current practices for testing water quality in terms of pathogenic pollution involve monitoring indicator organisms according to the criteria defined when using culture-based techniques. There are a myriad of tools currently used to detect and measure AR in water and wastewater treatment plants (WWTP).

Over the last decade, studies on the distribution and evolution of AR bacteria and genes in the environment, in particular in wastewater, have gained considerable attention (Berendonk et al., 2015). However, the knowledge regarding antibiotic contamination, AR bacteria, and antibiotic resistance genes in water ecosystems is still fragmentary, mainly due to methodological bias. The methods used to detect pathogenic bacteria involve culturing and require laboratory facilities. Portable monitoring technologies are needed that do not require the use of laboratory facilities to enable

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the generation of datasets required to analyze risk. Once the risk from emerging contaminants is measured and tracked to its source, then policies can be developed to reduce or minimize the risk to water quality.

## Techniques Used to Measure Antibiotic Resistance Genes and Antibiotic Resistant Bacteria

There are multiple techniques available to measure antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in wastewater treatment plants. They range from culture-based techniques and antibiotic susceptibility tests used to identify ARB, to culture-independent molecular methods to target ARGs.

### Culture-Dependent Methods

In order to identify phenotypes of specific bacterial taxa and establish resistance levels, it is necessary to isolate the bacteria found in WWTPs. The most commonly monitored microbiological indicators of fecal pollution in water are coliforms and enterococci. The membrane filtration method, in conjunction with selective culture media supplemented with antibiotics, is used to isolate bacteria and to determine antibiotic resistance patterns. The isolated bacteria can then undergo further antibiotic susceptibility testing and, through molecular methods, resistance genetic determinants can be detected (Ferreira da Silva et al., 2006; Rizzo et al., 2013b; Schwartz et al., 2003). The minimal inhibitory concentration (MIC) is established by determining the lowest concentration of antibiotic that inhibited growth (Jorgensen and Ferraro, 2009; Wiegand et al., 2008). By monitoring the number of bacteria growing in the presence or absence of the antibiotic, it is possible to determine the percentage of resistance (Figueira et al., 2011b; Novo and Manaia, 2010; Watkinson et al., 2007). This information can be used to calculate numbers of resistance rates. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) guidelines for clinical and veterinary microbiology describe various antibiotic susceptibility tests to differentiate between resistant and susceptible organisms (CLSI, 2015; EUCAST, 2016; Rizzo et al., 2013b).

### Macrobroth and Microbroth Dilution Tests

Macrobroth and microbroth dilution tests are commonly used antibiotic susceptibility tests for clinical pathogenic bacteria. For macrobroth dilution tests, two-fold dilutions of antibiotics (e.g., 1, 2, 4, 8, and 16 mg/L) are prepared in a liquid broth medium in test tubes. The tubes containing antibiotics are inoculated with a standardized bacterial suspension, McFarland 0.5 standard, of $1–5 \times 10^5$ CFU/mL. Following overnight incubation at 35°C, the tubes are examined for bacterial growth in the form of turbidity in order to calculate the MIC (Jorgensen and Ferraro, 2009; Wiegand et al., 2008). Advantages of this technique include the reduced cost and the establishment of MIC values; however, there are also a few disadvantages to the macrobroth dilution method. A considerable number of reagents are needed for each test, a large laboratory space is required to conduct the tests, and the preparation of the antibiotic solutions for each

test can be time consuming, laborious, and subject to human error (Jorgensen and Ferraro, 2009).

Microbroth dilution tests are similar to macrobroth dilution testing with the exception that they are conducted on a microscale on a 96-well plate. The 96-well plates contain 0.1 mL of broth in each well. As the plate contains 96 wells, this allows various antibiotics to be tested in a range of two-fold dilutions. It is possible to prepare a large number of identical plates from a single stock of dilutions in a relatively short time period. Each well is inoculated with 0.01–0.05 mL of a standardized bacterial suspension. MICs are determined after incubation by looking manually or using an automated device to check for bacterial growth. Each inoculation concentration analysis is performed in triplicate via parallel wells (Jorgensen and Ferraro, 2009; Wiegand et al., 2008). Some of the advantages of this method include the establishment of MIC values, the more economic use of reagents and space (as the test has been miniaturized), and the convenience of using preprepared plates, which also results in good reproducibility. On the other hand, using preprepared plates can be a disadvantage as there is not a wide selection of antibiotics in standard commercial panels (Jorgensen and Ferraro, 2009). Huang et al. (2012) isolated multiple-antibiotic-resistant bacteria from the secondary effluent of a WWTP and used the microbroth dilution tests to determine the MICs of penicillin G, ampicillin, cephalothin, chloramphenicol, tetracycline, and rifampicin to these bacteria. Their results proved that multiple-antibiotic-resistant bacteria are present in the effluent of WWTPs (Huang et al., 2012).

### Agar Dilution

Agar dilution is another widely used antibiotic susceptibility test for antibiotic resistance. It is similar to the broth dilution methods. Agar plates are prepared with different antibiotic concentrations and standardized suspensions of bacterial inoculum are spotted onto each plate. The plates are incubated and then MIC values can be established by determining the presence or absence of bacterial growth on the plates (Wiegand et al., 2008).

### Disk Diffusion

Disk diffusion is an easy and practical method for testing antibiotic susceptibility. A standardized suspension of bacterial cells of approximately $1–2 \times 10^8$ CFU/mL is spread on an agar plate. Paper disks containing fixed concentrations of antibiotics that have been commercially prepared are placed on the inoculated agar surface. The plates are then incubated for a time period at a temperature specific to the bacterial species. If the bacteria are susceptible to the antibiotic, an area of no growth, or zone of inhibition, will be visible around each of the antibiotic disks. The diameter of the zone is measured to the nearest millimeter. The size of the zone of inhibition will depend on the susceptibility of the bacteria to the antibiotic and the diffusion rate of the antibiotic through the agar medium. The CLSI guidelines have measurements for each of the zone diameters for each antibiotic in order to establish antibiotic resistance. There are several advantages to the disk method: the test is easy to use, there is a large selection of antibiotics for testing, it is the cheapest susceptibility method, no specialized equipment is needed, and clinicians can understand the results without

difficulty. There are some disadvantages to this method: it is not possible to accurately test all slow-growing or fastidious bacteria by this method, and there is no automation of the test (Jorgensen and Ferraro, 2009). Several studies have used disk diffusion effectively to test antibiotic susceptibility from wastewater effluent (Koczura et al., 2012; Sidrach-Cardona et al., 2014).

### Antimicrobial Concentration Gradient

The commercially available *E*-test method (BioMerieux AB BIODISK) establishes an antimicrobial concentration gradient in an agar medium to determine antibiotic susceptibility. A dried antibiotic concentration gradient is on the underside of thin plastic test strips with the upper surface marked with a concentration scale. An agar plate is inoculated with a standardized bacterial suspension, and several strips can be placed in a radial fashion on the surface of the plate (Jorgensen and Ferraro, 2009). Advantages of this test include its ease of use and that the results generally correlate well with MIC values from broth or agar dilution methods. There are some disadvantages: the method can be quite expensive if more than one or two antibiotics need to be tested, and when testing certain organism-antimicrobial agent combinations it can be difficult to avoid some systematic biases toward higher or lower MICs. Several studies have used the *E*-test method to determine the MICs of coliforms, such as *E. coli*, and enterococci from wastewater effluent to ampicillin, amoxicillin, ciprofloxacin, ofloxacin, tetracycline, trimethoprim, sulfamethoxazole, gentamicin, and vancomycin (Akiyama and Savin, 2010; Rizzo et al., 2013a; Talebi et al., 2007).

### Automated Systems

There are a variety of automated systems available that use specific sets of tests or inbuilt algorithms for identification and antibiotic susceptibility testing. Some of the common ones are the VITEK 2 system (bioMerieux S.A.) (Barry et al., 2003; Livermore et al., 2002), the Phoenix system (Becton Dickinson Biosciences, Sparks, Md) (Leverstein-van Hall et al., 2002), the Sensititre ARIS 2X (Trek Diagnostic Systems), and the MicroScan WalkAway instrument (Siemens Healthcare Diagnostics) (Jorgensen and Ferraro, 2009). These automated systems analyze subtle changes in the bacterial growth and use antibiotic reagent cards to evaluate the MICs. An advantage of the automated systems is that they allow for rapid standardized interpretive reading of the MICs, but they can be very expensive systems (Jorgensen and Ferraro, 2009; Livermore et al., 2002). Reinthaler et al. (2003) used the VITEK 2 system to determine the antibiotic resistance profile of *E. coli* in WWTP effluent.

## Molecular-Based Methods

### Polymerase Chain Reaction (PCR)

PCR is the most commonly used molecular technique to detect ARG (Bauer et al., 2014; Fluit et al., 2001; Sundsfjord et al., 2004; Rizzo et al., 2013b). Environmental target DNA or RNA found at low concentrations in WWTPs can be amplified and detected

using PCR. Multiple PCR assays have been designed to target specific antibiotic resistance genes encoding for resistance to aminoglycoside (Mohapatra et al., 2008; Taviani et al., 2008), chloramphenicol (Dang et al., 2008), β-lactam (Taviani et al., 2008), macrolide (Chen et al., 2007; Jensen et al., 2002; Patterson et al., 2007), penicillin (Srinivasan et al., 2005), sulphonamide (Agersø and Petersen, 2007), tetracycline (Ardic et al., 2005; Jacobs and Chenia, 2007), trimethoprim (Moura et al., 2007; Ramachandran et al., 2007), sulfamethoxazole (Ramachandran et al., 2007), and vancomycin (Bell et al., 1998; Caplin et al., 2008).

### Multiplex PCR

Multiplex PCR methods use several primer sets in the same PCR reaction system, allowing the amplification of various ARG fragments simultaneously. This is advantageous as different target regions of diverse environmental ARGs can be detected rapidly and conveniently at the same time, which saves time and money (Gilbride et al., 2006). A disadvantage is that care must be taken when designing multiplex assays as it is possible that a few of the DNA amplifications could be inhibited due to all of the reactions taking place under the same parameters, which could result in false-negatives. There can also be a decrease in assay sensitivity, which can be problematic (Fluit et al., 2001; Sundsfjord et al., 2004; Zhang et al., 2009b).

### Quantitative PCR (qPCR)

qPCR is a molecular method that can be used to target ARGs. It is based on the idea of using a fluorescent reporter to measure the changes in concentration of PCR products after each DNA amplification cycle in order to determine absolute or relative amounts of target DNA in a sample (Zhang and Fang, 2006). There are two common types of fluorescent reporter that are used for qPCR: Taqman and SYBR Green. Taqman probes, or hydrolysis probes, are hairpin-shaped oligonucleotide probes with a fluorophore covalently attached to the 5' end of the sequence and a quencher attached to the 3' end. The quencher suppresses the fluorophore until the probe hybridizes with the target, and the fluorophore is released. This allows measurement of the fluorescence, which is directly proportional to the concentration of DNA present (Fluit et al., 2001; Zhang and Fang, 2006). SYBR green is a cyanine dye that intercalates between base pairs to specifically bind double-stranded DNA and then fluoresces. The fluorescent signal is measured at the end of either the annealing or the extension cycle (Sharkey et al., 2004; Zhang and Fang 2006). Both methods have their advantages and disadvantages. TaqMan probes have been designed to target *vanA*, *mecA*, *tet*(O), *tet*(W), *tet*(Q), and *ampC* genes found in wastewater (Smith et al., 2004; Volkmann et al., 2004). SYBR Green assays are available to target *tet*(G), *tet*(Q), *mef*, and *erm* genes found in activated sludge and wastewater (Auerbach et al., 2007; Fluit et al., 2001; Morsczeck et al., 2004; Reinert et al., 2004; Zhang et al., 2009a). qPCR is a rapid and efficient technique with high levels of sensitivity; however, inhibitors in the wastewater can interfere with its performance, although it can be overcome by using environmental mastermixes, spiking methods, or sample dilution (Cao et al., 2012; Green and Field, 2012; Shanks et al., 2009).

### DNA Microarrays

In the DNA microarray method, gene-specific oligonucleotides or probes are transferred to a solid surface, after which the target-labeled DNA is hybridized to the array. A reporter system then detects the probe-target hybridization and measures the concentration of the DNA. This is a very rapid technique as results can be obtained in a few hours, and it has high throughput as a large number of resistance genes can be targeted on a single array. On the other hand, few studies have used this technique to detect the ARGs in environmental samples. This could be due to its low detection limit, which could be overcome by using PCR in conjunction with the microarray (Gilbride et al., 2006; Zhang et al., 2009b). Another factor is that there are inhibitors present in environmental samples that can interfere with DNA extraction and/or target gene amplification (Fluit et al., 2001; Sundsfjord et al., 2004; Zhang et al., 2009b).

### DNA Sequencing

DNA sequencing can be used alongside PCR to determine the presence of ARG in wastewater samples. In this method, multiple nucleotide sequences from the target of interest are compared to nucleotide sequences available in online public databases to establish the ARGs present. These sequences can then be used to design primers and probes for qPCR or microarray. In recent years, DNA sequencing has become faster and more cost effective (Fluit et al., 2001; Sundsfjord et al., 2004). Novel molecular high throughput technologies such as next generation sequencing and metagenomics are becoming important methods for analyzing microbial communities for functional gene dynamics and diversity, and for establishing a deeper understanding of vertical and horizontal gene transfer events in WWTP. This can be important for determining ARGs in wastewater (Fitzpatrick and Walsh, 2016; Fouhy et al., 2015; Schluter et al., 2008; Szczepanowski et al., 2009). Szczepanowski et al. (2009) used metagenomic analysis to detect 140 clinically relevant ARGs in a WWTP, showing that WWTPs are a hot spot for ARG transfer.

While molecular methods have many advantages due to their ability to give accurate, rapid, and nonbiased results, it is important to note that they do not allow the identification of ARB, nor do they distinguish between live and dead organisms (Rizzo et al., 2013b).

## Which Resistance Genes and Bacteria Have Been Tested and Identified Worldwide?

### Antibiotic Resistance in WWTP

WWTPs are one of the main sources of ARB and ARGs released into the environment. WWTPs are connected with households and hospitals releasing antibiotic and bacteria of human origin. Therefore, in WWTPs, bacteria from different environments can interact and exchange genetic materials by horizontal gene transfer. Once ARB successfully enters WWTPs, they can spread their resistance determinants among bacteria of the indigenous microbial community. Moreover, the presence of antibiotics may cause selective advantages for resistance bacteria. Some studies showed that antibiotic

resistant bacteria have been found in WWTPs and in their effluent (Bergeron et al., 2015; Łuczkiewicz et al., 2010a; Rizzo et al., 2013a; Ferreira da Silva et al., 2006). Resistance to beta-lactams, quinolones, tetracycline, and sulfamethoxazole/trimethoprim was detected in WWTPs all over the world by using classical microbiology methods such as cultivation and antibiotic-susceptible testing as well as culture-independent methods (Galvin et al., 2010; Karkman et al., 2016; Łuczkiewicz et al., 2010b; Novo and Manaia, 2010; Szczepanowski et al., 2009; Tao et al., 2014; Watkinson et al., 2007).

It is usually considered that hospitals are an important source of antibiotics and ARB that can be released into WWTPs; thus, the role of hospital wastewater in the development and the spread of antibiotic resistance is of interest. Multidrug resistant (MDR) bacteria and ARGs have been identified in hospital effluents (Picão et al., 2013). However, in another study, no significant difference was detected between the amount of ARB found in the effluent of a hospital intensive care unit and those found in the influent of municipal sewage treatment plants. In this study, the detectable resistance bacteria in WWTPs with or without hospital effluents were almost the same. The hospital effluents actually contribute less than 1% of the total amount of municipal sewage; thus, the hospital wastewater was diluted extensively in WWTPs, suggesting that municipal wastewater also contains high concentrations of ARB (Kümmerer, 2004). The number of MDR bacteria found in WWTPs was found to be highly variable between different studies of WWTPs receiving hospital effluents. Therefore, the separate treatment of hospital wastewater before discharge to WWTPs is an issue that needs to be addressed and may depend on local circumstances.

In the following section we will discuss the ARB and ARGs that have been tested and identified in/from WWTPs around the world.

### Antibiotic Resistant Bacteria in WWTPs

Antibiotic resistant bacteria that have been studied in WWTPs mainly belong to the common indicators of fecal contamination, namely, coliforms and enterococci (Araújo et al., 2010; Boczek et al., 2007; Figueira et al., 2011a; Martins da Costa et al., 2006; Reinthaler et al., 2003; Sabaté et al., 2008), because these bacteria are closely associated with humans and easy to identify. In order to establish the relationship between the environment and clinical settings, research on the last generation antibiotic resistant bacteria has also been conducted in WWTPs. Methicillin resistant *Staphylococcus aureu*s, vancomycin resistant *Enterococcus* spp., and Gram-negative bacteria (enterobacteria, pseudomonads, acinetobacter) that are resistant to fluoroquinolones, carbapenems, and producers of extended-spectrum beta-lactamases have all been reported in WWTPs (Araújo et al., 2010; Boczek et al., 2007; Figueira et al., 2011a; Martins da Costa et al., 2006; Reinthaler et al., 2003; Sabaté et al., 2008).

### Cultivable Heterotrophic Bacteria

Heterotrophic bacteria in the activated sludge process have an important role in WWTPs. It has been reported that the number of bacteria including the total number of resistant bacteria significantly decreases downstream of the wastewater treatment process (Guardabassi et al., 2002; Huang et al., 2013). Some studies reported a decrease in the concentration of ARB after treatment, and an increase of the proportion of ARB

was also observed. Guo et al. (2013) found the reduction of the proportion of heterotrophic bacteria resistant to erythromycin, cephalexin, gentamicin, and ciprofloxacin, while the proportion of bacteria resistant to sulfadiazine, vancomycin, rifampicin, and tetracycline increased after UV treatment in WWTPs. The operating conditions of the treatment system in WWTPs have different effects on the fates of ARB. For instance, in the study of Munir et al. (2011), the concentrations of resistant bacteria to tetracycline and sulfonamide decreased several orders of magnitude in the treated water in comparison with the raw water, but the concentration of ARB almost remained the same in pre-and postdisinfected effluents. However, the conditions in WWTPs may be favorable for the development of ARB, which can in turn transfer the resistant determinants to susceptible bacteria (Baquero et al., 2008; Goñi-Urriza et al., 2000; Iwane et al., 2001; Schwartz et al., 2003).

## Indicators of Fecal Contamination

### *Escherichia coli* (Coliforms)

Coliforms are intestinal flora of human and warm-blooded animals. The fecal coliforms can be discharged into the environment through inadequate treatment of wastewater in WWTPs. Human and animal health may be impacted by exposure to contaminated water. The monitoring of the fecal contamination in water may provide valuable insights into water reuse and the routes of fecal contamination (Whitlock et al., 2002; Young and Thackston, 1999). Coliforms including *E. coli* are used as indicator organisms to monitor microbial quality of water. Even though most strains of *E. coli* are nonpathogenic, some of them may still cause diseases. In WWTPs, the high resistance rates of *E. coli* were detected for aminopenicillins, sulfonamides, and tetracycline, while the lower rates were observed for quinolones and gentamycin (Literak et al., 2011; Manaia et al., 2011; Ferreira da Silva et al., 2006). However, the wastewater treatment processes cannot decrease the antibiotic resistance rates in fecal coliforms. The slight increase of resistance to antibiotics from different classes (penicillin, fluoroquinolones, trimethoprim/sulfamethoxazole and tetracycline) was reported (Ferreira da Silva et al., 2007). The *E. coli* resistant to ciprofloxacin and cephalothin was found at higher proportions in treated water than in the raw inflow of an urban wastewater treatment plant (UWWTP) in Portugal (Łuczkiewicz et al., 2010b; Ferreira da Silva et al., 2007). The mechanism behind this observation is not well understood; however, there is a hypothesis that the increase of antibiotic resistance may be associated with the selection of ARB or that the susceptible bacteria were eliminated more intensively by the treatment processes (Figueira et al., 2011a; Rizzo et al., 2013a).

In the WWTP receiving the hospital's wastewater, there was a high rate of antibiotic resistant strains of *E. coli* detected (Reinthaler et al., 2003). Indeed, the most prevalent resistance phenotype in the penicillin group was found for ampicillin (18%) and piperacillin (12%); in the cephalosporin group, cefalothin (35%) and cefuroxime-axetil (11%); in quinolone class, nalidixic acid (15%); for tetracycline the rate was 57%; and for trimethoprim/sulfamethoxazole, 13%. Infections caused by carbapenem resistant *E. coli* are very difficult to treat and may be lethal for upwards of 50% of patients who become infected.

### Extended-Spectrum Beta-lactamase (ESBL)–Producing *E. coli*

WWTPs can be a source of antibiotic resistant and virulent *E. coli*, especially ESBL genes such as $bla_{CTX-M}$, $bla_{CMY-2}$, and *qnrS* (Čornejová et al., 2015). Bréchet et al. (2014) reported that WWTPs released a large amount of extended-spectrum beta-lactamase–producing *E. coli* into the environment. It was estimated that about $6 \times 10^{11}$ ESBL *E. coli* are discharged daily into the receiving river in France, and the sludge used as fertilizer contains $2.6 \times 10^5$ ESBL *E. coli* per gram. The ESBL *E. coli* were detected in almost all environmental samples and at the rate of 0.3% in the untreated water upstream of the WWTP. The concentration of *E. coli* was observed to be similar between hospital and urban wastewater, but some studies indicated there are a higher proportion of ESBL *E. coli* in hospital than in urban wastewater (Blaak et al., 2015; Bréchet et al., 2014). This may relate to the high concentrations of antibiotics in hospital wastewater and the high frequency and density of ESBL *E. coli* carriers among patients compared to the community (Bréchet et al., 2014; Hocquet et al., 2016). However, the volume of urban wastewater is much higher in WWTPs compared with hospital wastewater; thus, the hospital wastewater is not considered as the main source releasing ESBL *E. coli* into the water environment. Wastewater treatment greatly reduced the *E. coli* as well as ESBL *E. coli* load, but the proportion of ESBL *E. coli* among *E. coli* significantly increased after treatment. This may be due to the presence of antibiotics in WWTPs, which causes a selective pressure for ARB.

In another publication, Amos et al. (2014) also reported the detection of ESBL *E. coli* when analyzing water samples upstream and downstream of a WWTP effluent point in the United Kingdom. A significant increase in number of third-generation cephalosporin (3GC) resistant *E. coli* was observed in the river sediment downstream of the WWTP effluent discharge, compared with the upstream samples during all the sample dates. The WWTP effluent also had an effect of the prevalence of 3GC resistant *E. coli*, with 0.95% of *E. coli* resistant to 3GCs downstream compared with 0.13% of *E. coli* upstream. They also pointed out that the 3GC resistance prevalence is associated with the dissemination of $blac_{ctx-m-15}$, which is the most common ESBL in clinical pathogens *E. coli* and *Klebsiella* spp. In addition, an imipenem resistant *E. coli* was found in the river in the United Kingdom, which indicated the spread of carbapenem resistance in the water environment and is a cause of great concern.

### Resistant Enterococci

Enterococci are among the most studied bacteria in WWTPs. Many studies have reported that the resistance to erythromycin was common among enterococci; the high resistance rates of these bacteria were observed for ciprofloxacin and tetracycline, and lower resistance rates were observed for aminopenicillins and sulfonamides (Celik et al., 2014; Łuczkiewicz et al., 2010b; Manaia et al., 2011; Martins da Costa et al., 2006; Ferreira da Silva et al., 2006). The treatment process in WWTP considerably reduced the number of bacteria, but it seemed to have no effect on the decrease in resistance rates. The positive selection of ARB was observed during treatment of wastewater (Ferreira da Silva et al., 2006). Like *E. coli*, compared with raw inflow, the proportion of resistant enterococci to ciprofloxacin is higher in treated water (Martins da Costa et al., 2006; Ferreira da Silva et al., 2007). The high prevalence of resistant enterococci in the WWTP effluent has been reported. The proportion of *E. faecalis* and *E. faecium*

resistant to fluoroquinolones increased after wastewater treatment in Poland (Łuczkiewicz et al., 2010b). With similar results, Martins da Costa et al. (2006) reported that almost 50% of all isolates identified from the WWTPs in Portugal showed resistance to multiple antibiotics.

### Vancomycin Resistant Enterococci

The occurrence of vancomycin resistant enterococci (VRE) has been demonstrated in WWTPs. VRE are resistant to vancomycin, an important antibiotic to treat serious enterococci infections. VRE were detected in wastewater samples in Europe ranging from 2% to 52%. Indeed, VRE were found in 2%–3% of samples from European secondary WWTPs (Łuczkiewicz et al., 2010a; Morris et al., 2012) and in 52% of wastewater samples from a tertiary WWTP that uses chlorination as a final disinfection process (Kotzamanidis et al., 2009). Recently, VRE were detected in unchlorinated effluent from U.S. WWTPs associated with reuse sites (Rosenberg Goldstein et al., 2014). In this study, VRE were identified in influent and biologically treated samples but not in the chlorinated effluent. This result indicated that the chlorination treatment is effective in reducing VRE to an undetectable level. The concentration of enterococci in urban and hospital wastewater was found to be more or less the same, but the concentration of VRE was higher in hospital effluent than in urban effluent. CC17 *E. faecium* associated with hospital VRE outbreak worldwide is over-represented in hospital wastewater (Leclercq et al., 2013; Sadowy and Luczkiewicz, 2014).

## Other Bacteria

### *Acinetobacter* Spp.

The study of the water samples from a UWWTP showed the significant increase of the proportion of antibiotic resistant *Acinetobacter* spp. because of the wastewater treatment (Zhang et al., 2009d). Indeed, the resistance to amoxicillin/clavulanic acid, chloramphenicol, rifampicin, and even the proportion of MDR bacteria increased in final effluent samples in comparison with the raw influent.

### *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* are ubiquitous in hospital wastewater discharges. In comparison with *E. coli* pathogens, *P. aeruginosa* is not a human commensal, and the rate of infection among patients is low. The infections caused by *P. aeruginosa* become more complicated over time due to the intrinsic resistance of these bacteria to many classes of antibiotics and their ability to acquire the resistance to effective antibiotics.

The concentration of *P. aeruginosa* was found to be higher in hospital wastewater than in urban wastewater, and the proportion of antibiotic resistant *P. aeruginosa* was also much greater in hospital effluent compared with urban wastewater (Fuentefria et al., 2011; Slekovec et al., 2012; Tuméo et al., 2008). The MDR *P. aeruginosa* developed through mutations or horizontal gene transfer. Most of MDR *P. aeruginosa* isolated

from hospital wastewater mainly belongs to the few high risk clones ST235, ST 111, and ST 175 (Schwartz et al., 2006). The treatment process in WWTPs may reduce the number of resistant *P. aeruginosa* at the rate of 97%; otherwise, the resistant, especially MDR, strains will be discharged into the water environment.

### Aeromonas

Aeromonads are ubiquitous and considered indigenous to the water environment (Janda and Abbott, 2010). It has been demonstrated that Aeromonads can potentially develop and spread antibiotic resistance in both clinical settings and in the environment (Arias et al., 2010; Blasco et al., 2008; Cattoir et al., 2008; Goñi-Urriza et al., 2000; Gordon et al., 2008; Huddleston et al., 2006; Walsh et al., 1997). The members of the genus *Aeromonas* may carry genes encoding resistance to beta-lactams as well as plasmid-mediated quinolone resistance, which can be transferred between bacteria (Cattoir et al., 2008; Chen et al., 2012; Moura et al., 2012; Varela et al., 2016). Figueira et al. (2011b) found the high-resistance prevalence to beta-lactam of *Aeromonas*. Indeed, amoxicillin resistance was found in almost all isolates in their work (except in an *Aeromonas enteropelogenes* strain). Similar results were reported by Igbinosa et al. (2012), in which they also found absolute resistance of *Aeromonas* to ampicillin and oxacillin. These findings indicated the intrinsic resistance of *Aeromonas* to beta-lactam antibiotics, and it could be associated with the presence of beta-lactamase genes detected in these bacteria (Chen et al., 2012; Igbinosa and Okoh, 2012). The quinolone resistance was found in two species, *A. media* and *A. punctata*, at a high frequency. The predominance of these bacteria in the wastewater environment may be considered as an explanation for the elevated rate of nalidixic acid resistance in wastewater. Similarly, sulfamethoxazole/trimethoprim resistance, found exclusively in those two species, was observed only in wastewater (Figueira et al., 2011b).

### Methicillin-Resistant *Staphylococcus aureus* (MRSA)

MRSA are resistant to all beta-lactam antibiotics used to treat bacterial infections. MRSA strains were found in the inflow and the activated sludge in a WWTP over time in Sweden (Börjesson et al., 2010, 2009). In this study, a high number of MRSA of genetically diverse species was observed in municipal wastewater. The treatment process decreased the number of MRSA, but it also stimulated the selection for bacterial strains that are more extensively resistant to clinical antibiotics and PVL + strains. The ratio of MDR MRSA was higher in the activated sludge than in influent samples. The MRSA isolated from wastewater carried only two types of SCC*mec*, I and IV, but not types III and V (Börjesson et al., 2009). It may be associated with the small size of SCC*mec* I (Deurenberg and Stobberingh, 2008), and carrying SCC*mec* IV may not impose a large fitness cost (Lee et al., 2007).

In another study, (Rosenberg Goldstein et al., 2012) researchers also detected MRSA and methicillin-susceptible *Staphylococus aureus* in four WWTPs in the United States. The number of MRSA isolates differed between WWTPs, sampling time, and location. MRSA was detected in 50% of all water samples. The wastewater treatment reduced the number of MRSA released into the effluent. Several isolates that could survive after treatment are likely MDR and are more virulent.

## Antibiotic Resistance Genes Identified in WWTP Influent and Effluent

The examples of ARGs detected in water samples from WWTPs are presented in Table 15.1.

It has been reported that the treatment process in WWTPs cannot remove ARGs, which are then discharged to the water environment (Munir et al., 2011). In general, ARGs conferring resistance to all classes of antibiotics were detected in WWTPs all over the world, and they can be also found in the WWTP effluents (Auerbach et al., 2007; Karkman et al., 2016; Laht et al., 2014; Munir et al., 2011; Rizzo et al., 2013b; Zhang et al., 2009d). Using metagenomic functional selection, Munck et al. (2015) identified a core WWTP resistome that contains mostly novel genes encoding resistance to all tested antibiotics. This evidence highlights WWTPs as a reservoir of ARGs that have not been characterized previously.

**Table 15.1** Antibiotic resistance genes detected in WWTP influent and effluent.

| Resistance Phenotype | ARGs in Influent and Effluent of WWTPs | Reference |
|---|---|---|
| Aminoglycoside | *aacA, aadA, strA, strB, aphA* | Moura et al., 2012; Silva et al., 2007 |
| Beta-lactams | Class A: $bla_{TEM}$, $bla_{CTX-M}$, $bla_{SHV}$, $bla_{VEB}$, $bla_{GES}$, $bla_{KPC}$, $bla_{NPS}$, $bla_{KPC,}$ $bla_{PER}$<br>Class B: $bla_{IMP}$, $bla_{VIM}$<br>Class C: *amp*C<br>Class D: $bla_{OXA}$<br>*mec*A, | Agga et al., 2015; Blaak et al., 2015; Börjesson et al., 2009; Čornejová et al., 2015; Drieux et al., 2016; Karkman et al., 2016; Laht et al., 2014; Szczepanowski et al., 2009 |
| Glycopeptides (vancomycin) | *van*A | Araújo et al., 2010; Morris et al., 2012; Rosenberg Goldstein et al., 2014 |
| Macrolides | *erm*A, B, F | Agga et al., 2015; Marti et al., 2013; Szczepanowski et al., 2009 |
| Quinolones | *qrn*A, B, S;<br>*accA6-ib-cr* | Agga et al., 2015; Figueira et al., 2011b; Szczepanowski et al., 2009 |
| Sulfonamides | *sul*1, *sul*2 | Burch et al., 2013; Laht et al., 2014; Marti et al., 2013; Szczepanowski et al., 2009 |
| Tetracycline | *tet* genes | Agga et al., 2015; Araújo et al., 2010; Auerbach et al., 2007; Mao et al., 2015; Schwartz et al., 2006; Zhang et al., 2009a |
| Trimethoprim | *dfr* genes | Moura et al., 2012; Schwartz et al., 2006; Silva et al., 2007 |
| Integrons | *Intl* 1, *Intl* 2 | Figueira et al., 2011b; Pellegrini et al., 2011 |

The prevalence of ARGs can be determined by either culture-dependent methods, where ARGs are detected from ARB, or culture-independent methods, whenre ARGs are identified directly from water samples. The abundance of 13 tetracycline-, sulphonamide-, streptomycin- and β-lactam-resistance genes in ARB was higher in influent than in effluent samples, except for *sulA* and CTX-M (Zhang et al., 2015). The culture-dependent methods for identification of ARGs give excellent results for known pathogens. In parallel, the culture-independent methods can measure high numbers of ARGs/associated resistance determinants. According to the number of metagenome reads, tetracycline, macrolide, and multidrug resistant genes are highly represented, while other ARGs such as aminoglycosides, sulfonamides, and bacitracin are less frequent. In a study of wastewater samples in Germany, 140 clinically relevant antibiotic resistance genes were detected, including aminoglycoside, beta-lactam, chloramphenicol, fluoroquinolone, macrolide, rifampicin, tetracycline, trimethoprim, and sulfonamide, as well as multidrug efflux resistance genes (Szczepanowski et al., 2009). They also found the ARGs in the WWTP's final effluent, which may be further disseminated in the bacterial community downstream of the WWTP discharge point.

Some other studies indicated that the WWTP effluent may spread and increase the prevalence of ARB and ARGs in the receiving rivers (Amos et al., 2014; Marti et al., 2013). The analysis of the ARG flow through each unit of the WWTPs in northern China showed the proliferation and the release of ARGs. In the final effluent there was a significant release of ARGs in combination with ARB, which was also resistant to chlorination (Mao et al., 2015). In this study, 30 ARGs encoding resistance to tetracycline, sulfonamides, quinolones, or macrolides were identified by qPCR from the activated sludge of two WWTPs. It was shown that there was the reduction of the abundance of ARGs from the raw influent to the effluent; however, 12 ARGs (*tet*(A), *tet*(B), *tet*(E), *tet*(G), *tet*(H), *tet*(S), *tet*(T), *tet*(X), *sul1*, *sul2*, *qnrB*, *ermC*) were discharged from WWTPs at higher rates than the influent.

The analysis of WWTP in Hong Kong by metagenomic sequencing showed the seasonal change of few ARG types and the decrease of genes in the WWTP effluent (Yang et al., 2014, 2013). Yang et al. (2014) indicated that most of the ARGs were removed from the UWWTP influent after the wastewater treatment. Indeed, by metagenomic sequencing they pointed out a more than 98% reduction of ARGs in the effluent in comparison with the raw influent. The reduction of ARGs after the treatment process was also reported by Laht et al. (2014). Other studies demonstrated that there is no change in the relative number of ARGs or that the number increases. In contrast, the enrichment of some ARGs was observed in the effluent community (Auerbach et al., 2007; Harris et al., 2012; Laht et al., 2014). This could be explained by the selective conditions in WWTPs that may favor ARGs, ARB harboring genes, or horizontal gene transfer (HGT) among the bacterial community. Karkman et al. (2016) showed that *ermF* from macrolide, lincosamide, and streptogramins B (MLSB) resistance genes, *tetPA* and *tetPB*, were the most enriched genes in the dried sludge. Similar results were found for genes encoding resistance to polymyxin, tetracycline, vancomycin, and MLSB class antibiotics in the UWWTP sludge (Yang et al., 2014). The UWWTP sludge was recognized as the main source of tetracycline and sulfonamide resistant bacteria and genes discharged into the water environment (Munir et al., 2011). These results show that UWWTP sludge can play important role in the selection and spread of ARGs.

## Antibiotic Resistance Gene Transfer

In WWTPs, wastewater often contains bacteria from different origins, including human and animal pathogens as well as antibiotic residues. The high density of a bacterial community may establish an ideal environment for HGT among environmental bacteria and human pathogens (Watkinson et al., 2007). The resistance mobile genetic elements usually are identified in genera such as *Enterococcus* and *Escherichia*, using culture-dependent methods. Based on metagenomic analysis, Proteobacteria of the classes Alpha-, Beta-, Gamma-, and *Bacillus*, *Mycobacterium*, or *Nocardiopsis* are the most abundant bacteria carrying antibiotic resistance plasmids (Zhang et al., 2011). The location of ARGs on mobile genetic elements such as plasmids, transposons, and integrons makes the transfer of resistance possible and easy to achieve among bacteria with the same or different origins (Allen et al., 2010).

The transfer of resistant plasmids of *Enterococcus faecalis* in the activated sludge of two WWTPs in Germany was examined (Marcinek et al., 1998). It was shown that the transfer rates between different strains of *E. faecalis* resistance plasmids that are broad host range for Gram-positive bacteria in the activated sludge conditions were at least 10 times lower than they were under laboratory conditions. MDR bacteria and ARGs were found in the WWTP effluent in Poland. The ARGs were located in the MDR plasmids, which could be transferred into an *E.coli* recipient strain, indicating a high possibility of HGT among bacteria in the water environment (Osińska et al., 2016).

Resistance integrons are also well investigated for their dissemination of ARGs into the water environment. In resistance integrons (RIs), ARGs can be clustered in the same unit with other elements that may have ecological advantages (Stokes and Hall, 1989). Such structures of the integron allow different ARGs to be selected and transferred together under the selection of one antibiotic. RIs were detected in the activated sludge process in WWTPs (Ma et al., 2011; Moura et al., 2007). It was found that 12% of resistant plasmids extracted from the WWTP sludge carried resistant class 1 integrons. More than half of these plasmids have broad host ranges that have high transfer rates (Moura et al., 2007; Tennstedt et al., 2003). Based on the q-PCR analysis, Zhang et al. (2009c) showed that gene copies of class 1 integrons and ARGs significantly differed between the activated sludge samples at different treatment steps in five WWTPs. Indeed, the activated sludge process successfully removed more than 90% of class 1 integrons and ARGs in two WWTPs, while the disinfection process in one WWTP eliminated 94% of integrons and 77% of ARGs. It was also found that thermophilic anaerobic digestion can significantly reduce the relative abundance of class 1 RIs (Ghosh et al., 2009).

In the study of RIs from the influent, the activated sludge, and the effluent of WWTPs in China, Ma et al. (2011) found the class 1 integrons in bacterial isolates from all water samples, and the occurrence frequency of the class 1 RIs was 20.4% for influent, 30.9% for activated sludge, and 38.9% for effluent water samples. This result was confirmed further by qPCR that showed the abundance of RIs was higher in the effluent than in the influent. This finding indicated the spread of RIs in WWTPs and suggested that activated sludge can be a potential hot spot for the transfer of RIs and selection of MDR bacteria. However, the differences in the observation of the abundance of ARGs and class 1 RIs were found between five WWTPs in China (Du et al., 2014). The concentration of ARGs decreased in the effluents of WWTPs treating domestic or/and industrial wastewater with anaerobic/aerobic or membrane bioreactor technologies. The decrease

of the ARG abundance was found in the treatment plant treating vitamin C production wastewater by anaerobic/aerobic technologies. Therefore, it is difficult to identify which treatment process had the most effective removal of ARGs in WWTPs. The implications of antibiotic resistance entering our environment after wastewater treatment are important in terms of the potential environmental consequences, particularly when water is reused as irrigation water that could potentially extend exposure pathways by entering the animal and human food chains.

ARB and ARGs should be considered biological contaminants of emerging concern (CECs). According to the U.S. Geological Survey (2015), CECs have been defined as "any synthetic or naturally occurring chemical, or any microorganism, that is not commonly monitored in the environment but has the potential to enter the environment and cause known or suspected adverse ecological and (or) human health effects."

Antibiotic resistance can develop through random mutations in the genes and/or by HGT. Mobile genetic elements carrying ARGs in gene-transfer units can auto-replicate and can be maintained in the bacterial population over time unless they have a fitness cost for the recipients. Reducing the antibiotic loads can decrease the amount of ARGs. Gonzalo et al. (1989) found that the dilution of WWTP effluent in rivers decreased R-plasmids in *E. coli*. However, the decline of resistance is slow and the ARB and ARGs remain in bacterial populations (Andersson, 2003).

The development and dissemination of antibiotic resistance including ARB and ARGs can be induced by human activities such as clinical use of antibiotics, wastewater treatment, and agriculture. Acquired resistance was found in pathogens in places with low antibiotic loads (Pallecchi et al., 2008). This could indicate that when ARB or ARGs are introduced into the environment, they may disseminate and persist even without constant exposure to the antibiotic pressure. Once the WWTP effluent is discharged into the aquatic environment, ARB and ARGs can be spread and transfer into the surrounding environments. In this context, water can be considered as an effective vector for carrying and transferring ARB and ARGs between ecological compartments (Baquero et al., 2008). The coselection of ARGs and other genes, such as heavy metal resistance genes, is one of the key factors in the dissemination and maintenance of ARGs in the water environment (Hellweger, 2013). The study of Hellweger et al. (2011) showed that antibiotic resistance in the Poudre River receiving WWTP effluent, as well as urban and agricultural runoff, developed based on the input of ARGs, antibiotic pressure, and the presence of heavy metals. The genes providing resistance to heavy metals as well as ARGs were located in the same gene cassettes; thus, they were cross-selected in this case. In the last few years the role of WWTPs as the reservoirs of ARB and ARGs have been given more attention, with many different approaches being used to investigate their occurrence and spread during the WWTP treatment process as well as in downstream environments.

## Impact of Antibiotic Resistance from WWTP on Environment and Human Health

The incomplete removal of ARB and ARGs as well as antibiotics in WWTPs leads to the transport of these components from WWTPs to the receiving water environment and then into other terrestrial environments such as plants and soils. The transport of

antibiotic resistance can impact the ecosystem of receiving environments. There were significantly high levels of ARB and ARGs found in the raw influent, effluent, and biosolids samples of a WWTP using more advanced technologies such as membrane biological reactors (Munir et al., 2011). Other important ARGs (*ampC*, *vanA*, *mecA*, $bla_{\text{CTX-M-15}}$) as well as the resistant mobile genetic elements (such as plasmids and integrons) have been detected in wastewater samples from all over the world (LaPara et al., 2011; Mokracka et al., 2012; Pruden et al., 2006; Szczepanowski et al., 2009; Volkmann et al., 2004). These findings indicate the potential transfer of antibiotic resistance from WWTPs to the environment. Czekalski et al. (2012) found that the dissemination of antibiotic ARB and ARGs from WWTP effluents into Lake Geneva led to a significant proliferation of antibiotic resistance levels in both the water column and in sediments around the discharge point.

The selection and spread of ARB and ARGs from WWTPs, along with antibiotic pollutants, may play a role in shaping the bacterial community of the receiving water. The contamination of these elements may have an effect on the physiological behavior and dynamics of microbial populations (Martinez, 2009b). How do recipient bacteria change after taking in new ARGs? In fact, the structure of the Gram-positive bacterial cell wall is strongly modified due to the changes in the structure of peptidoglycan when bacteria become resistant to beta-lactams or glycopeptides (Mainardi et al., 2008). The study of the clinically relevant phenotype of small colony variants of *S. aureus* revealed an alteration in metabolism resulting in slow growth (Heinemann et al., 2005). These findings demonstrate that the acquisition of antibiotic resistance has unpredictable effects on bacterial metabolism and morphology; thus, it may cause changes in the evolution of environmental bacteria.

## Impact of Antibiotic Resistance from Wastewater on Human Health

In addition to the ecological factors, the potential impacts of ARB and ARGs from WWTPs on human health are of interest. The World Health Organization (2014) classified the development of antibiotic resistance as one of the major global threats to society and that intensive monitoring was recommended for the identification and control of hot spots such as WWTPs in order to decrease the propagation of antibiotic resistance.

Some studies have demonstrated that the application of manure and biosolids along with the release of WWTP effluents may lead to the expansion of environmental antibiotic resistance reservoirs (Binh et al., 2008; Davies and Davies, 2010; Knapp et al., 2010; Munir and Xagoraraki, 2011), which in turn can serve as hot spots for spreading ARB and ARGs between bacteria, including human pathogens (Martinez, 2009a; Xi et al., 2009). In fact, WWTPs provide the ideal conditions for the development and spread of ARB and ARGs. Contamination with ARB and ARGs may result in the high possibility of human pathogens acquiring antibiotic resistance. In India, antibiotic resistance was detected everywhere in water: ARB downstream of WWTPs, ARGs in drinking water, and MDR *Salmonella* in water sprayed on vegetables (Ansari et al., 2008; Chitnis et al., 2004; Parvathi et al., 2011). MDR *E. Coli* O104:H4 were identified from water sprayed on vegetables causing the European outbreak of bloody diarrhea

and hemolyticuremic syndrome in 2011 (Rubino et al., 2011). The outbreak began in Germany in early May and was still reported during the first 10 days of June. During that time, thousands of people were infected, resulting in 877 cases of hemolyticuremic syndrome with 32 deaths and 3043 cases of enterohemorrhagic *E.coli*. This outbreak spread mainly in Germany, but sporadic cases were identified in 15 other countries, including Denmark, France, Greece, United Kingdom, Holland, Norway, Austria, Spain, Czech Republic, Luxembourg, Poland, Sweden, Switzerland, Canada, and United States. Further research on the dissemination of antibiotic resistance in/from WWTPs needs to be conducted in order to minimize the risk to human health.

## Reusing Treated Wastewater (TWW) in Agriculture Irrigation

There is an increasing shortage of water in many areas in the world, especially in arid and semi-arid regions such as Africa, south Asia, southern Europe and the Middle East. The reuse of TWW is becoming a practical solution for mitigating the problem of water scarcity (Levine and Asano, 2004; Tal, 2006). Approximately 85% of TWW is reused in Israel, mainly for agricultural irrigation. TWW is also used for other applications, such as irrigation of public gardens (Levine and Asano, 2004) and stream flow augmentation (Arnon et al., 2015; Halaburka et al., 2013).

Reuse of treated wastewater for agricultural irrigation has implications for the spread of antibiotic resistance to the soil microbiome, and to human and animal pathogens. The wastewater treatment process in WWTPs does not completely remove ARB and ARGs; thus, these elements can be disseminated to the natural soil and then enter into the animal and human food chains. TWW can contain high levels of ARB. As an example, an estimate of the release of ciprofloxacin resistant coliforms suggested that per minute, a treatment plant discharges $10^9$ ARB (Vaz-Moreira et al., 2014). The rate of resistance to antibiotics (aminopenicillins, sulfonamides, tetracycline, and erythromycin) can be more than 50% of some bacterial populations released into the final effluent of WWTPs with conventional treatment (Manaia et al., 2011; Rizzo et al., 2013b).

Changes in the soil microbiome have been observed after soil was irrigated with TWW. The application of TWW irrigation induced the development of copiotrophic bacteria and fungi, and a significant increase of microbial biomass was detected in soils irrigated with TWW (Filip et al., 2000, 1999). In the study of Al-Jassim et al. (2015), the potential risk associated with reused TWW from ARB and ARGs was highlighted. The presence of MRD bacteria and ARGs in TWW could represent health hazards to the general public and to agricultural farmers. The fate of ARB and ARGs in soil after TWW irrigation is still poorly understood. When bacteria from wastewater enter the soil, it may lead to the following negative consequences: (i) some ARB can proliferate into plants or soil and (ii) some ARGs can be transferred from wastewater bacteria to soil/plant bacteria (Becerra-Castro et al., 2015). Several investigations of the fate of antibiotic resistance from the application of TWW irrigation have shown variable results. Some studies indicated that ARB from TWW are unable to compete or survive in soil after a long irrigation time, and these bacteria did not have high ARG contributions to soil bacterial populations (Gatica and Cytryn, 2013; Negreanu et al., 2012). The opposite results were reported in the work of Wang et al. (2014). They found a high density and abundance of ARGs and *Int* 1 gene in the soil that was continuously irrigated with

TWW. The presence of class 1 integrons indicated the high potential of HGT among bacteria in soil.

There is still a general lack of knowledge about the serious risks of contamination to soil, water environments, wildlife, and food chains from reusing TWW for irrigation (Nereus COST Action ES1403). There is also a methodological gap in environmental risk assessments of wastewater irrigation since there are only two possible routes for pollutants to enter the soil environment in the European Commission's (2003) risk assessment guidelines. The advanced treatment of wastewater may be required for safe reuse of TWW in irrigation.

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# 16

# Antibiotic Pollution and Occurrence of Bacterial Antibiotic Resistance Genes in Latin American Developing Countries

## Case Study of the Katari Watershed in the Bolivian Highlands

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## Latin American Developing Countries' Context in Relation to Antibiotic Pollution and Bacterial Resistance to Antibiotics

As social inequality indices and inadequate social services remain very high in the Latin American region, development challenges are still very important in this area. The growth rate of the Latin American region has remained above that of the world population since the eighteenth century (Guzmán et al., 2006). Urban growth in this region is principally driven by industrial development and economic opportunities (McMichael, 2000), with industrialization being typically faster than the creation of basic infrastructure, such as wastewater treatment facilities. The creation of sanitation, health, and environmental policies in developing countries is usually delayed and occurs much slower than the rapid economical and industrial development, as it is generally not considered a necessity (WHO, 2004). The lack of basic infrastructure for water supply, sewage systems, or wastewater treatment plants (WWTPs) induces wastewater discharges into the environment and inappropriate disposal of wastes, impacting environmental quality.

The expansion of cities in Latin America, just as in other parts of the world, generates a wide range of pollutants, leading to multicontaminated situations. These multicontaminations include nutrient and chemical compounds for which knowledge about their fate and impacts is available, but also other compounds for which we strongly lack such knowledge. These compounds are known as "emerging contaminants." Among them, pharmaceuticals, and especially antibiotics, are particularly worrying. The presence of antibiotics in the environment has raised particular attention because

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it can lead to the development of resistant bacterial strains, which can induce severe public health problems (Mojica and Aga, 2011). Bacterial resistance to antibiotics can occur not only in natural polluted areas but also inside human patients or treated animals, in relation with the overuse, abuse, and misuse of antibiotics. Pollution by urban wastewaters will modify the structure and possibly the function of native bacterial communities through the exposure of native bacteria to antibiotics that acquire resistance by successive mutations (Rysz and Alvarez, 2004; Schauss et al., 2009) or by horizontal transfer of resistance genes to native bacterial population (Rizzo et al., 2013; Martinez, 2009).

Misuse of antibiotics can be particularly worrying in Latin America countries as the lack of pharmaceutical use and control policies as well as social-economic factors favor self-medication and low-cost antibiotics use. This can lead to important dissemination of antibiotics in the environment and to the development of antibiotic resistant bacteria (Segura et al., 2015). These antibiotic-resistant bacteria from urban/anthropogenic origin reach natural environments by direct discharges of wastewater or sludge from WWTPs or through inappropriate livestock waste disposals.

While infections in patients from developed countries by bacterial resistant strains are quite well controlled, health problems relating to antibiotic resistant pathogens could be very important in Latin America due to lacks in sanitation culture (Muñoz et al., 2011) and to limited availability of resources and infrastructure for controlling such infections (Sosa et al., 2010). Factors contributing to the presence and persistence of antibiotic resistance genes in soil and water compartments in Latin America and especially in rapidly growing cities also include untrained health care professionals, deficient laboratory facilities, inadequate access to health care, lack of funds for appropriate antibiotic doses, or the distribution of substandard and counterfeit drugs (Bartoloni and Gotuzzo, 2009).

Although Latin America has a Network for Antimicrobial Resistance Vigilance (RELAVRA) (Ramón-Pardo et al., 2011), which is part of the WHO, the lack of infrastructures induces unequal vigilance coordination in the whole region.

## The Katari Watershed in the Bolivian Highlands

The Katari watershed has an area of approximately 2,022 $km^2$ and is located in the western part of Bolivia in the Bolivian Altiplano region (PNUMA, 2011). This region has an extreme climate due to high diurnal variations in solar radiation, temperature, and humidity (Bartoloni and Gotuzzo, 2009). Rainfall is low in the watershed with average annual precipitation of 490–620 mm $yr^{-1}$ (MMAyA, 2010) concentrated between December and March, while the southern winter (June to August) is very dry.

The Katari watershed has four main rivers: the Seke, Seco, Pallina, and Katari rivers. The Katari River is the main river with a length of 90 km; its outlet is Cohana Bay located in the Bolivian part of Lake Titicaca. The main tributary of the Katari River is the Pallina River, which crosses the city of Viacha. The Seke and Seco rivers run through the city of El Alto before joining the river Pallina, after crossing Viacha (Figure 16.1). The volume of water discharged from the Katari watershed to Lake Titicaca for the year 2008 was about 242.42 mcm (million cubic meters), representing an average flow of 7.7 $m^3 s^{-1}$ (MMAyA, 2010).

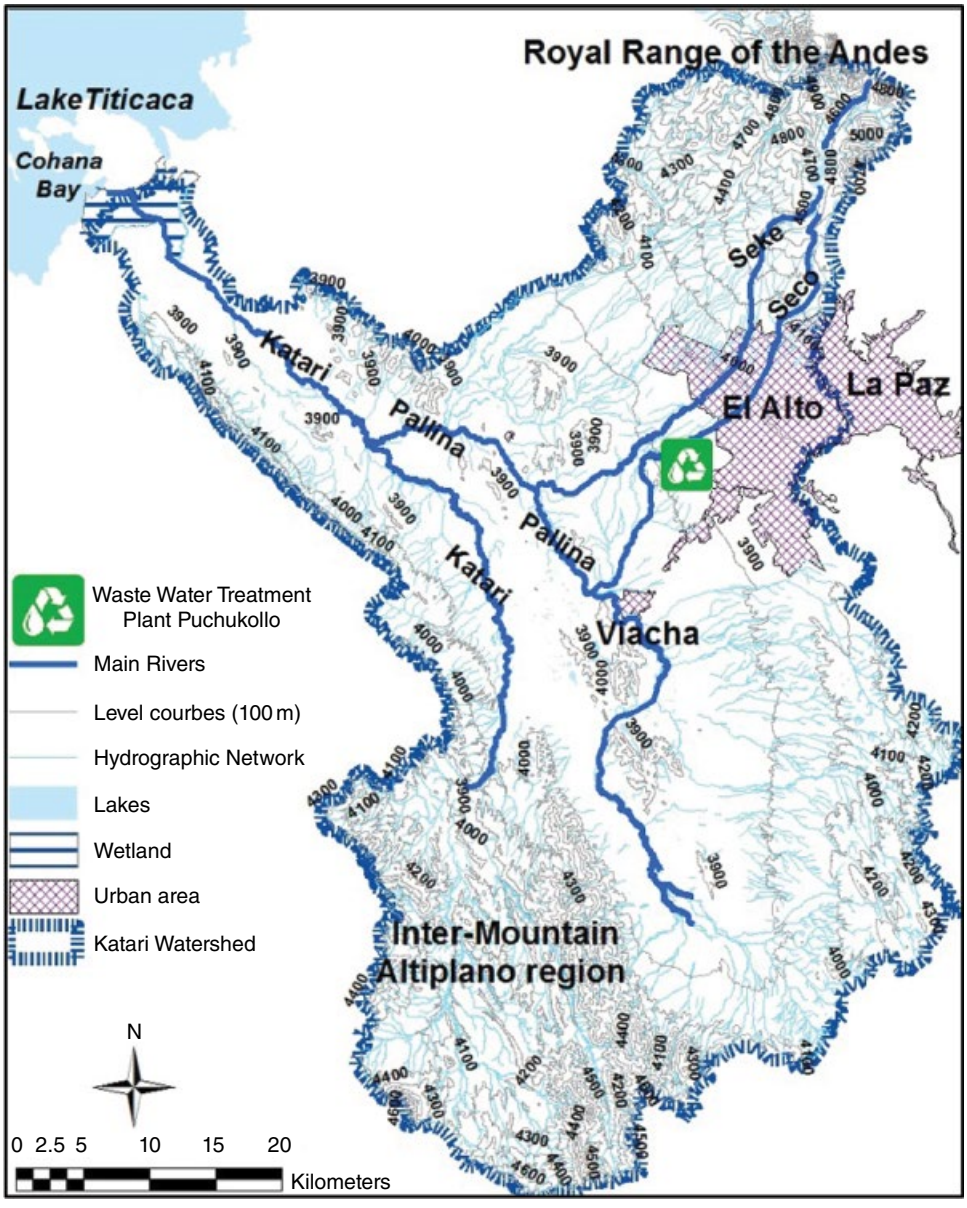


**Figure 16.1** The Katari watershed: hydrography, topography and urban area. (*See insert for color representation of the figure.*)

The Royal Range of the Andes (Cordillera Real) lies at the northeast of the watershed, with high peaks like the Huayna Potosi (6088 m); the altitude at this point of the basin is between 4200 m and 5720 m. It corresponds to the sources of the Seco and Seke rivers. The Intermountain Altiplano region, "Serranías Inter-altiplánicas," is situated in the Southwest of the watershed and delimits the North Altiplano from the Southern

Altiplano. It corresponds to the sources of the Katari and Pallina rivers. The central part of the watershed is characterized by altitudes below 4200 m, the minimal altitude being 3800 m in the Cohana Bay (Titicaca Lake).

## The Urban Area of the Katari Watershed and the Fast-Growing City of El Alto

The two main cities of the basin are Viacha City, which has a population of about 80,000 inhabitants, and El Alto City, which has the largest population density in the basin with a population of about 1.2 million of inhabitants (Figure 16.1). El Alto City is the second-largest city in Bolivia and the largest city in the Altiplano.

El Alto was one of the Latin America's fastest growing cities in the 1980s when it grew with an average of about 9% per year (Arbona and Khol, 2004). Its population has increased sharply in relation to two events. First, the occurrence of a drought event in the northern Bolivian Altiplano during the years 1982 and 1983, and second, the closure of the state mines in the Cordillera in 1985 (McFarren, 1992). Thus, El Alto's population grew from 95,000 inhabitants in 1976 to about 1.2 million according to the last census (Mazurek, 2012).

This rapid urban growth was accompanied by minimal land planning and lack of basic infrastructure and environmental policies (Hardy et al., 2015). For example, in the basin there is only one WWTP. The Puchukollo wastewater treatment plant is located below the city of El Alto and discharges its treated waters into the Seco River (Figure 16.1). The plant treats around 500 $Ls^{-1}$, officially providing a secondary type treatment (filtration through grills and percolating filters, and organic matter removal through anaerobic ponds). It was estimated that in 2001, around 70% of the El Alto population was connected to the Wastewater Collection and Treatment System (Instituto Nacional de Estadistica de Bolivia, 2001; MMAyA, 2013). However, as the city continued to grow, this percentage certainly decreased, probably below 50%. Untreated wastewaters go directly into soils through septic tanks or to rivers through pipes.

Nowadays, El Alto is a major industrial city with more than 5,000 industries, including textile, tannery, furniture, food, painting, metallurgic (PNUMA, 2008), and pharmaceutical industries. Most of the industries have no WWTP, increasing wastewater discharges, and the pollution of ecosystems in the basin. Due to its rapid and uncontrolled development, El Alto is now considered the main source of pollution in the Katari watershed, being seen as the main contributor to the eutrophic state of the Cohana bay in Lake Titicaca (Ribera Arismendi, 2010; Duwig et al., 2014; Fonturbel, 2005).

## Bolivian Public Health Policy and Antibiotic Use

One of the key objectives of the Bolivian government in recent years was to ensure the availability and the access of the population to effective and safe medications at a reasonable price. However, until today, about 50% of the population has no access to medications. This is due to the inaccessibility of certain remote geographic areas, the entrenched culture of indigenous traditional medicine, and the economic situation of

the majority of the Bolivian population, all of which inhibit access to medical insurance (Martin, 2013).

This public health situation is at the origin of the presence of a highly active black market for medications in the country. The pharmaceutical market in Bolivia is thus approximately distributed as follows: 33% from local production, 42% from import, and 25% from adulteration or counterfeiting (Martin, 2013; Montes, 2012). Furthermore, pharmaceuticals and antibiotics are available without medical prescription, which, together with illegal sales, results in high self-medication rates.

In the country, socioeconomic factors greatly influence the pharmaceutical consumption patterns, including antibiotics. For example, it has been reported that the quantities of antibiotics dispensed by pharmacies in Bolivia vary according to a patient's ability to pay (Bartoloni et al., 1998). Partially due to the socioeconomic situation of developing countries, patients often cannot afford the cost of a full treatment. Thus, in Bolivia as in other developing countries, pharmacies generally dispense an inappropriate and ineffective antibiotic regime (quantity and time of treatment), which could contribute to the selection of resistant microorganisms.

The type of consumed antibiotics is also highly influenced by the socioeconomic status of the population. For example, low-cost antibiotics are in general more often used in developing countries as they are more affordable (Cameron et al., 2011).There is evidence that lower cost antibiotics such as sulfonamides (especially sulfamethoxazole) are predominantly consumed in developing countries, compared to more expensive antibiotics such as macrolides (Segura et al., 2015).

## Wastewater Rejects as a Source of Antibiotic Pollution and Resistant Bacteria in the Katari Watershed

### Sampling and Analytical Procedures Used

#### Determination of Antibiotic Concentrations

A first sampling campaign was carried out in June 2012, during the dry season, for the determination of antibiotic concentrations in surface waters of the Katari watershed. For that, 1 L samples were collected in 17 amber glass vials of 60 mL at five strategic points of the studied watershed (Figure 16.2, sampling points: 4, 6, 8, 9, and 10). Sampling point 6 corresponds to treated wastewater discharges from the WWTP. Collected samples were frozen at −20°C until analysis in France. Once in France, antibiotic concentrations were determined at the Institute of Analytical Science (Lyon, France). The determined antibiotic families were tetracyclines, sulfonamides, phenicols, penicillins, and trimethoprim, which are often detected in surface waters all around the world. Samples were first filtered and concentrated with an automatic Solid Phase Extractor (AutoTrace SPS Workstation, Caliper) with the following conditions: cartridge (Oasis HLB, 3 mL, 60 mg), conditioning (methanol followed by pure water), loading of sample (200 mL of extract volume), drying of cartridge, and elution (ultrapure grade methanol). Eluent was then evaporated until dryness under a gentle flow of nitrogen and concentrated by the addition of 200 μL of a solution of water/methanol (V/V: 95/5). Sulfamethoxazol-d4 was added to each sample before extraction as internal standard. Analyses were carried out by LC-MS (Argilent/1100 and ABSciex/3200 QTRAP) with the following conditions:

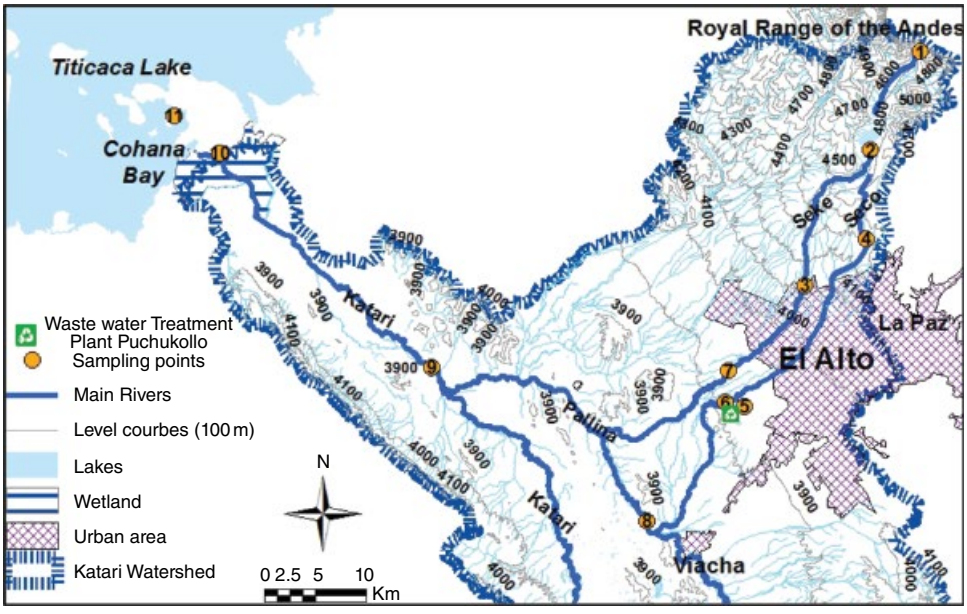


**Figure 16.2** Surface waters sampling points for the determination of antibiotic concentrations and bacterial resistance to antibiotics. Sampling point 5 corresponds to wastewaters entering the WWTP. Sampling point 6 corresponds to treated wastewater discharge from the WWTP. (*See insert for color representation of the figure.*)

column (Zorbax Eclipse Plus C18 1.8 μm (50×2.1 mm)), injection volume (25 μL), temperature of column (50°C), flow (0.3 mL $min^{-1}$), and ionization source (electrospray in negative and positive mode). Mobile phase consisted of (i) pure water with the addition of formic acid (100 μL $L^{-1}$) and (ii) methanol (ultrapure grade). Data acquisition was conducted with the Analyst 1.5 software.

#### Assessing the Presence of Bacterial Resistance to Sulfonamides

A second sampling campaign was conducted in February 2013, during the wet season, to evaluate the presence of bacterial resistance to sulfonamides in surface waters (Figure 16.2, sampling points 1–12). Sampling point 5 corresponds to wastewaters entering the WWTP. About 500 mL of water were sampled in sterile bottles (first rinsed with water from the site). They were then cooled to 4°C and transported to the laboratory (in Bolivia) within 4 hrs after sampling.

Once in the laboratory, 100 mL (in triplicate) of samples were filtered (within 48 hrs after sampling) through sterile cellulose nitrate filters (SARTORIUS, 0.45 μm porosity). Filters were then placed in sterile containers and frozen at −20°C until analysis. DNA extraction from filters was performed using the FastDNA® SPIN Kit for Soil (MP Biomedicals). Slight modifications were made to the manufacturer's protocol; thus, the filters were folded and placed into a lysing matrix tube. The filter was immersed in liquid nitrogen for freeze-drying and further crushed with a sterile micro spatula. The quality of DNA was checked by agarose gel electrophoresis (1%) in 1XTris-Borate-EDTA buffer and UV visualization of ethidium bromide stained gels with a Molecular imager Gel DocTM XR (Biorad) device. DNA concentrations were determined on a Qubit fluorometer TM using the QuBit dsDNA BR Assay kit (Invitrogen).

Presence of bacterial resistance to sulfonamides (genes *sul*I and *sul*II) was examined by PCR assays. For that, three genes coding for sulfonamide resistance were amplified from extracted crude DNA: *sul*I, *sul*II and *sul*III. The primers used for their amplification were the following: for *sul*I: *sul*I-F5'-TCACCGAGGACTCCTTCTTC-3' and *sul*I-R5'CAGTCCGCCTCAGCAATATC-3', for *sul*II: *sul*II-F5'-CCTGTTTCG TCCGACACAGA-3' and *sul*II-R5'-GAAGCGCAGCCGCAATTCAT-3', for *sul*III: *sul*III-F5'GAGCAAGATTTTTGGAATCG-3' and *sul*III-R5'-CATCTGCAGCTAACC TAGGGCTTTGGA-3'.

The reaction medium (25 µL) was composed of 12.5 µL of water, 5 µL of Green GoTaq® Flexi (Promega) buffer (5X), 2 µL of MgCl2 (25 mM), 2 µL of dNTP (2.5 mM), 0.5 µL of each primer (10 µM), 0.625 µL of BSA (10 mg $mL^{-1}$), 0.125 µL of DMSO, and 0.125 µL of GoTaq® Flexi DNA Polymerase at 5U $\mu L^{-1}$. This reaction medium, supplemented with one microliter of metagenomic DNA crude extract ($10^{-1}$ ng µL), was placed in a thermocycler (Biorad) and subjected to the following program for genes *sul*I and *sul*II: 95°C for 10 min, followed by 30 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min. A final extension was performed at 72°C for 7 min. *Sul*III gene thermal cycler conditions were 95°C for 10 min, followed by 30 cycles of 95°C for 30 s, 51°C for 1 min, and 72°C for 1 min. A final extension was performed at 72°C for 7 min. Amplification was verified by agarose gel electrophoresis (1%) as for DNA extractions.

Real time PCR (qPCR) was used to determine the number of gene copies of *sul*I and *sul*II genes. qPCR reactional medium volume was of 20 µL: 10 µL of SsoADV Univer SYBR Green Supermix 200 (Bio-Rad), 0.5 µL of each primer, 1 µL of template, and 8 µL of $H_2O$. The thermocycler was programmed for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 seconds at appropriate annealing temperatures (60°C for *sul*I and 53°C for *sul*II). Number of copies of *sul*I and *sul*II resistance genes was expressed as the ratio of gene copies of *sul*I or *sul*II genes and the copies of 16S rRNA gene. For that, a PCR and a qPCR of the 16S rRNA gene were performed. The primers used for their amplification were Eub338F 5'-ACTCCTACGGGAGGCAGCAG-3' and Eub518R 5'-ATTACCGCGGCTGCTGG-3'. qPCR conditions were the same as for *sul* resistance genes, and annealing temperature was 53°C. The product specificity was confirmed by melting curve analysis (5 sec at 95°C and 1 min. 65°C–97°C). Calibration curves were generated with plasmids carrying target genes: *sul*I, *sul*II, and 16SrDNA. Plasmids carrying target genes were generated using the PROMEGA pGEM®-T easy vector systems method (ref.A1360) with a laboratory strain of *Escherichia coli* (commercial competent cells pGEM®-T) harboring the recombinant plasmids.

## Antibiotic Pollution in Surface and Treated Waters of the Katari Watershed

Determined antibiotics and their observed concentrations (ng $L^{-1}$) are shown in Table 16.1. Generally, observed concentrations were lower in sampling points 4 and 11, which are located upstream of El Alto and on Cohana Bay in Lakae Titicaca, respectively. Greater concentrations were observed for sampling points 6, 8, and 9. Thus, antibiotic pollution appears to be related to proximity to the urban area of the watershed. We can also notice that most of the observed antibiotics belong to the sulfonamide family (Table 16.1). In regard to observed sulfonamides, the sulfadimethoxine, sulfadimerazine, and sulfamethoxidiazine antibiotics concentrations were higher in rural areas (sampling points 4 and 11), which probably relates to an agricultural use of these

**Table 16.1** Analyzed antibiotic concentrations (ng $L^{-1}$) in surface waters of the Katari watershed. DL = detection limits; DQ = quantification limits. DL and DQ were the same for samples 4 and 11, while DL and DQ were the same for samples 6, 8, and 9.

| Antibiotic | DL | DQ | 4 | 11 | DL | DQ | 6 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| **Tetracycline** | 1 | 5 | <DL | <DL | 4 | 20 | <DL | <DL | <DL |
| **Oxytetracycline** | 1 | 5 | <DL | <DL | 4 | 20 | <DL | <DL | <DL |
| **Chlortetracycline** | 1 | 5 | <DL | <DL | 4 | 20 | <DL | <DL | <DL |
| **Sulfathiazole** | 0.2 | 1.5 | 1.5 | <DQ | 0.8 | 6 | 114 | 137 | 59 |
| **Sulfadimethoxine** | 0.2 | 1.5 | 3.4 | 20 | 0.8 | 6 | <DQ | <DQ | <DL |
| **Sulfadimerazine** | 1 | 5 | <DL | 6.2 | 4 | 20 | <DL | <DL | <DL |
| **Sulfanilamide** | 5 | 50 | <DL | <DL | 20 | 200 | <DL | <DL | <DL |
| **Sulfadiazine** | 1 | 5 | <DL | <DL | 4 | 20 | <DL | <DL | <DL |
| **Sulfabenzamide** | 0.2 | 0.5 | <DL | <DL | 0.8 | 2 | <DL | <DL | <DL |
| **Sulfamethoxidiazine** | 0.2 | 0.5 | 1 | 6.5 | 0.8 | 2 | <DL | <DL | <DL |
| **Sulfamethoxazole** | 0.2 | 0.5 | 63 | 86 | 0.8 | 2 | 8832 | 14624 | 8893 |
| **Penicillin G** | 5 | 50 | <DL | <DL | 20 | 200 | <DL | <DL | <DL |
| **Trimethoprim** | 1 | 5 | 31 | 26 | 20 | 2632 | 2632 | 4474 | 28 |
| **Chloramphenicol** | 1 | 5 | 23 | <DL | 20 | 20 | 20 | 28 | <DL |

antibiotics as they are frequently used in veterinary medicine. Sulfathiazole and sulfamethoxazole antibiotic concentrations were greater in the urban area and in its vicinities, indicating an anthropogenic origin for these antibiotics, probably linked with wastewater discharges. The sulfanilamide, sulfadiazine, and sulfabenzamide antibiotics remained under detection limits in all studied sampling points. The studied tetracyclines as well as the penicillin G remained below detection limits in all sampling points.

Sulfamethoxazole (SMX) and trimethoprim (TMP) antibiotics showed the higher concentrations. SMX and TMP levels also seem to be linked with urban wastewater discharges. These antibiotics are commonly employed together for bacterial disease treatment in human and animal medicine. For SMX, the higher concentrations were recorded downstream of both urban areas of the watershed, El Alto and Viacha cities (sampling points 6 and 8) and in the Katari River (sampling point 9) (Table 16.1 and Figure 16.2). SMX and TMP were still detected downstream of the Katari River (sampling point 9) and in Lake Titicaca (sampling point 11) (Table 16.1), which could be related to the persistence of the compounds as well as to local contamination in rural areas.

Wastewaters seem to be the principal source of antibiotic pollution in the watershed. A clear relation between wastewater discharges and antibiotic pollution has already been observed in other parts of the world. For example, Hoa et al. (2011) studied antibiotic contamination in aquatic environments in Northern Vietnam and observed greater concentrations in urban environments. In this study, sulfonamides were detected in all collected samples and SMX was the main antibiotic in the studied urban water bodies (city canals). Observed SMX concentrations in this study ranged from 612 to 4330 ng $L^{-1}$.

**Table 16.2** Hydrological and population information about the study site and other antibiotic-polluted rivers.

| Study site | Population | Mean Flow ($m^3\ s^{-1}$) | Author |
|---|---|---|---|
| **Katari watershed** | 1,280,000 (in El Alto and Viacha) | 0.98 (mean of the urban and the lower part of the basin) | This study |
| **Seine River** | 12,341,418 (in Paris) | 310[a] | Tamtam et al., 2008 |
| **Mekong River** | 1,237,000 (in Can Tho, connected to the urban drainage) | 13700[b] | Shimizu et al., 2013 |

[a] Interannual mean flow at Paris (http://sigessn.brgm.fr/spip.php?article175).
[b] Smajgl and Ward, 2013.

In the Seine River, SMX was also the most frequently detected antibiotic (Tamtam et al., 2008). Nevertheless, our observed concentrations are higher than maximum detected concentrations in the Seine River (500 ng $L^{-1}$). This attests to a greater consumption of SMX in the studied watershed and less dilution as the population in the vicinity of the Seine River is much greater than the population of the Katari watershed (Table 16.2).

The SMX concentrations measured in the present study were more comparable with those observed on the Mekong River in Vietnam, which were of about 1720 ng $L^{-1}$ (Shimizu et al., 2013). The Katari population is much lower than the population of the Mekong River, and water fluxes are much lower in the Katari watershed, thus generating an antibiotic concentration effect (Table 16.2), which could be partially responsible for the observed similarities. Nevertheless, the presence of a high SMX consumption in the study site cannot be excluded.

Antibiotic loads (in g $d^{-1}$) at different points of the Katari watershed are shown in Figure 16.3. We can see the contributions of the two cities, El Alto (sampling point 6) and Viacha (sampling point 8), in the watershed to dissolved antibiotics concentrations. It can be noted that SMX and TMP loads almost doubled between the exit of the WWTP (sampling point 6) and the Pallina River (sampling point 8), which receives

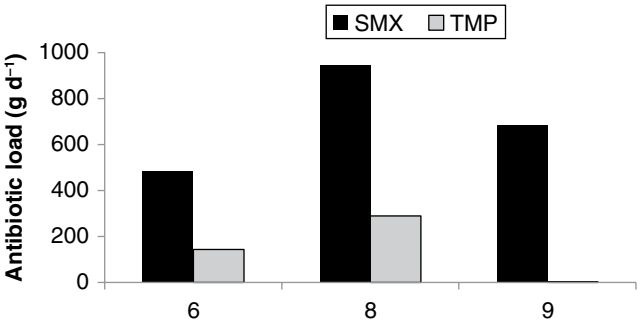


**Figure 16.3** Antibiotic loads (g $d^{-1}$) of sulfamethoxazole (SMX) and trimethoprim (TMP) at different points of the Katari watershed. 6 = exit of WWTP; 8 = Pallina River after El Alto and Viacha cities; 9 = downstream Katari river.

treated and untreated waters from El Alto as well as wastewaters from Viacha City. Important SMX concentrations were still detected at sampling point 9, showing that SMX is highly mobile and persistent. Nevertheless, its higher presence could also relate to local emissions. Even if observed concentrations are lower than therapeutic doses, they could be sufficient to induce bacterial resistance, which is known to develop at subtherapeutic doses.

## Occurrence and Prevalence of Antibiotic Bacterial Resistance to Sulfonamides in the Katari Watershed

As most of the antibiotics detected in the surface waters belong to the sulfonamide antibiotic family, we looked for the presence of sulfonamide resistance genes in bacteria. Table 16.3 shows the PCR results indicating the presence of sulfonamide resistance genes, *sul*I, *sul*II, and *sul*III. As can be seen, the majority of the collected waters had SMX resistance genes present (*sul*I, *sul*II, or *sul*III), with exception of sampling points 2 and 4. At sampling point 2, the highly acidic pH (pH 3) can explain the absence of bacterial resistance genes as it can affect the development of bacterial communities. For sampling point 4 the absence could be explained by its location, as it is located above the urban area and probably receives a much lower or a negligible quantity of wastewater.

The most frequently observed sulfonamide resistance genes were *sul*I and *sul*II, which were present in samples collected from points 1, 3, 5, 6, 7, 8, 9, 10 and 11. Accordingly, Hsu et al. (2014) observed that the *sul*I gene was dominant in the surrounding river of a swine farm in northern Taiwan. The *sul*III gene was the less frequently detected sulfonamide resistance gene (sampling points 1, 5, 6, 7, 8, and 9).

The consistent detection of antibiotic resistance genes in the sampling points located in and after the urban area suggests that their presence relates mainly to discharges of treated and untreated wastewaters. On the other hand, resistance genes were observed at sampling point 1, the highest sampling point in the watershed, where no human activity is expected. This could be linked to the presence of farm animals such as llamas potentially treated with antibiotics (and already carrying resistant bacteria), to sporadic human presence (for illegal mining activity), or to naturally occurring resistance genes.

Mean relative abundances of the *sul*I and the *sul*II are expressed as the ratio of the 16s gene to the number of *sul*I or *sul*II genes and are shown in Figure 16.4. In the case of the *sul*I genes, higher relative abundance was observed at the outlet of the WWTP

**Table 16.3** Presence of bacterial resistance genes to sulfonamides (*sul*I, *sul*II and *sul*III) in surface waters of the Katari watershed. + refers to the presence, – refers to the absence.

| | Sampling Point | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Gene** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** |
| ***sul*I** | + | – | + | – | + | + | + | + | + | + | + |
| ***sul*II** | + | – | + | – | + | + | + | + | + | + | + |
| ***sul*III** | + | – | – | – | + | + | + | + | + | – | – |

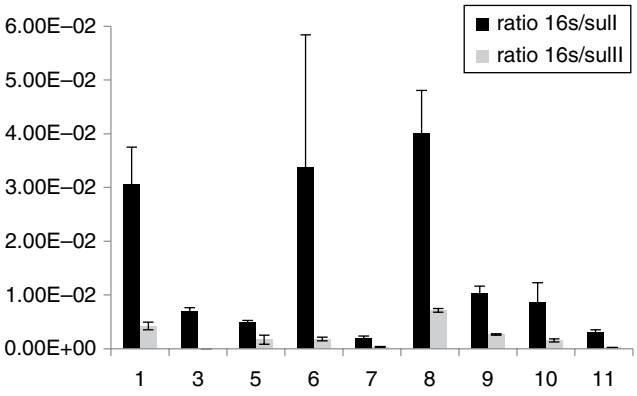


**Figure 16.4** Mean relative abundances of *sul*I (black) and *sul*II (grey) genes. Relative abundances are expressed as the ratio of the 16s gene and the *sul*I or the *sul*II genes. Error bars represent standard deviations calculated with triplicates.

(sampling point 6), which tends to decrease toward Lake Titicaca. The fact that *sul*I abundance was higher at the outlet of the WWTP (sampling point 6) than at the inlet (sampling point 5) shows that the wastewater treatment favors the concentration or development of antibiotic resistance genes and that treated wastewater discharges contribute to the spreading of antibiotic resistance genes in the environment. This is supported by the tendency toward a decrease in the number of *sul*I genes downstream in the watershed (samples 8 to 11). WWTPs are nowadays considered one of the main hot spots for spreading and transferring antibiotic resistance (Rizzo et al., 2013; Michael et al., 2013). The abundance of *sul*II genes was greater at sampling point 8, located after the two cities of the watershed (El Alto and Viacha). The abundance of *sul*II genes also tended to decrease gradually as the river approached Lake Titicaca, which suggests that its presence and abundance is also principally linked to urban discharges.

The abundance of *sul*I gene at sampling point 1 was comparable with its abundance observed at the outlet of the WWTP (sampling point 6). This observation was unexpected as no human activity was expected in this area, suggesting that resistance genes' persistence at this point of the watershed relate to other mechanisms (such as the coexistence of resistance genes to metals and antibiotics), as high metal concentrations of geogenic origin were detected at sampling point 1 (Archundia et al., 2017b). Wright et al. (2006) found that bacteria sampled along a gradient of metal contamination were more tolerant to antibiotics and metals compared to bacteria from a reference site free of metal pollution.

Another explanation could be related to the fitness of resistance genes: there is evidence that the fitness cost of some resistance genes has a negligible impact on their prevalence (Gullberg et al., 2011). Moreover, some authors have observed that the cost of some antibiotic resistance mechanisms is negligible (Zhang et al., 2006; Luo et al., 2005).

As we can see, bacterial resistance to sulfonamides is present in the study site, even in remote areas free of anthropogenic activity and antibiotic pollution. Disease-causing bacteria that have become resistant to antibiotic drugs are an increasing public health problem all over the world and especially in developing countries. Thereby, human

populations of the Katari watershed who are in contact with surface waters containing resistant bacteria could be in danger through their enhanced exposure to antibiotic resistant microorganisms. Further information about sulfonamide resistance genes in the Katari watershed can be found in Archundia et al. (2017a).

## Conclusion

Bolivia is an example of Latin American developing countries, where rapid demographic growth and industrial development has led to the rapid expansion of cities (such as El Alto). This rapid growth has been accompanied by a lack of basic infrastructure (principally sewage systems and WWTPs), as well as the indiscriminate use of pharmaceutical products in the case study region. The consequence is the presence of antibiotic pollution in surface waters of the Katari watershed. Sulfonamide antibiotics were the most observed antibiotic family. Sulfamethoxazole and trimethoprim antibiotics were present at high concentrations in relation with wastewater discharges in the urban area (El Alto and Viacha). SMX was still detected downstream of the Katari River and in Cohana Bay (Lake Titicaca), likely related to its high mobility and poor degradation or to local contamination from rural areas. Other sulfonamide antibiotics that are more frequently used in veterinary medicine (sulfadimethoxine, sulfadimerazine, and sulfamethoxidiazine) were more specifically detected in the rural areas.

The other consequence of wastewater discharges in the river network is the presence of sulfonamide resistance genes in surface and wastewaters of the watershed, the most frequently observed resistance gene being *sul*I. The abundance of resistance genes increased from the inlet to the outlet of the WWTP but decreased again downstream from the urban area, supporting the urban origin of these genes to Cohana Bay. This study provides evidence to support the importance of surveying and establishing control measurements to prevent antibiotic pollution and the development of bacterial resistance to antibiotics in the Latin America region.

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# 17

# Antimicrobial Resistance in Hospital Wastewaters

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Evidence suggests that frequent use of broad-spectrum antibiotics leads to higher concentrations of active or residue compounds, higher numbers of resistant bacteria, and higher proportions of resistance genes in hospital compared to other wastewaters. The unique characteristic of spatially concentrated and seriously ill patients, many who receive antimicrobial therapy for life-threatening infections, makes hospital wastewater point source discharges a public health risk. Worldwide, very few hospitals have wastewater treatment processes in place prior to discharge into sewers. Hospital wastewater therefore represents an important source of antimicrobial resistance–related contaminants that are taken into wastewater treatment facilities and are eventually distributed in various ecosystems, including water networks, food systems, and the natural environment. When hospital waste joins other effluent in community wastewater treatment plants (WWTP), it may also contribute to a complex milieu that results in further antibiotic resistance through gene transfer. This chapter highlights hospital wastewaters as potential reservoirs for development of antimicrobial resistance in bacteria and suggests some strategies for approaching this global public health challenge.

## Introduction

Hospitals discharge unique microbiological effluents into wastewater systems. They are centers that continue to evolve but that have not always used optimal infection prevention practices. From a historical infection control perspective, Ignaz Philipp Semmelweis and his efforts to protect women from fatal streptococcal postpartum infections acquired in health care settings shine a still poignant light on patient risks from infections. Hospitals and linked facilities currently use a variety of programs and tools to protect patients, but much less has been done to protect the environment from the contamination and the public health threat of antibiotic resistance.

As knowledge of infectious diseases grew, so did antibiotic treatment options. As antibiotic usage grew, so did selective pressures producing antibiotic resistance.

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Hospital acute care sites developed an ecological niche that included highly resistant bacterial strains, and nosocomial spread became high risk. Infection prevention and control programs were created to prevent cross-infections and to decrease specific hospital communicable disease risk through better staff education and hospital infection surveillance. Other multidisciplinary hospital responses included quality improvement work. These policy, practice, and procedure improvements followed quality management system standards that began to be applied to health care with accrediting bodies holding hospitals, and later hospital networks or groups, accountable for infection rates. Improved hand hygiene, a basic hospital infection control practice, has been recently highlighted at national levels as a top health care priority with increasing government level accountability (Semmelweis, be pleased). As new antibiotic resistance patterns emerged, hospital laboratories improved monitoring bacterial susceptibility patterns (to guide treatment) and resistance (to monitor infection control). Faster, automated microbiology methods enabled better data analysis for rapidly identifying new trends and resistance patterns. As patients moved back and forth between acute and community care, alerts for infection control practitioners helped prevent antibiotic resistant bacteria spreading from one hospital facility to another, in the same way as human pathogens are controlled. Patient-based information and trends are shared outside the hospital setting, including with public health agencies and sometimes governments. Within hospitals and their networks, drug formulary and antimicrobial stewardship committees manage the use of potentially last-line-of-defense antibiotics in these complex, highly acute settings. The focus has been to decrease patient risk within hospitals. There has been much less focus on the impact of hospitals on the environment and on population level risks.

In many parts of the world, health care systems have evolved with single independent hospitals being merged into large medical networks. Within these networks, as acute care hospitals took up increasingly sophisticated, expensive technologies and cost containment pressures grew, patients began to be more rapidly decanted for care in the community. Community care facilities including residential care sites have become major settings of broad-spectrum antibiotic usage. While acute care hospital resistance selection pressure is intense, community care antibiotic usage also adds to selective pressure, less from antibiotics such as vancomycin but more from more sustained use of broad-spectrum antibiotics. And as acute care infection control programs decreased acute care patient risk, community antibiotic stewardship programs also emerged. Thus, community care, including residential care for chronically ill patients, is part of the human health setting that contributes to antibiotic resistance. Although hospitals are the focus of this brief overview, all health care system waste-producing facilities should be included in further studies.

Another recent phenomenon related to health care systems and the dynamics of antibiotic resistance is medical tourism. Patients infected in a hospital on another continent may suddenly present in regional hospitals with concerning and complex antibiotic resistance susceptibility patterns. Infection control practitioners and public health, for example, were highly concerned when Gram-negative bacteria identified from such imported infections showed previously unreported genetic elements resulting in resistance to the most powerful group of antibiotics, the carbapenems (Chan et al., 2011).

As hospitals in health care systems evolve, what is the role of waste from these settings, and do acute care, residential care, and community care have unique effects on

ecological factors impacting the natural environment? In particular, what is known about antibiotic compounds after their use in patients as they leave the hospital and residential care settings? What is the fate of bacterial resistant bacteria in urine and feces of patients colonized in health care settings, and do bacterial mechanisms of genetic exchange pose a threat? And what is the role of wastewater treatment related to these settings?

## Antibiotics Persisting in Hospital Wastewater

The type and intensity of antibiotic usage varies by patient populations in different health care settings. Although the pharmacology of the wide array of antibiotics also varies, they can be grouped into classes based on mechanisms of action and host metabolism; from a waste stream perspective, most antibiotic classes are excreted in feces or urine. Once entering waste streams, some persist in nonmetabolized or altered but active states. Studies of waste downstream of hospitals show that some classes are more persistent than others (Brown et al., 2006; Huang et al., 2001; Rodriguez-Mozaz et al., 2015). Sulfonamide and fluoroquinolone groups appear to persist in the environment while the beta-lactams, which hydrolyze easily, do not (Baquero et al., 2008). Persisting antibiotics, however, have been detected at concentrations less than their lethal or inhibitory levels required for therapy, an important factor contributing to selective pressures (Kohanski et al., 2010).

In a recent study (Oberle et al., 2012), antibiotics excreted from patients in a hospital and retirement facility were measured in samples of the waste stream from medical site effluents to a wastewater treatment plant and then to discharge into a river. Contamination by antibiotics (and antibiotic resistant *E. coli*) decreased along the continuum, with only some antibiotic groups persisting in river water. Some differences in persistence were noted between the hospital and retirement center settings. Of 34 target antibiotics, only 15 were detected; concentrations decreased along the waste stream with 8 detected in the river. Only ofloxacin, sulfamethoxazole, and trimethoprim persisted in all samples, including discharge samples after wastewater treatment (primary and secondary treatment processes). They were also detected in a separate sampling site upstream of the WWTP discharge, consistent with the observation that there are broad ranges of possible contamination sources. Although last-line-of-defense antibiotics are excreted into hospital waste streams, beta-lactams including carbapenems or vancomycin do not appear to persist into the environment. On the other hand, antibiotics commonly used in settings such as long-term and community care, where higher loads of antibiotics such as amoxicillin and various quinolones are excreted from chronically ill patients, appeared to persist less frequently and at low levels at least into WWTP. Once persistent antibiotics enter the WWTP or directly into the environment and its ecosystems, they can impact bacterial communities and their evolution (Bouki et al., 2013).

## Antibiotic Resistant Bacteria in Hospital Wastewater

New antibiotic resistance patterns of importance to human (and animal) health appeared soon after each new pharmaceutical discovery. Gram-positive bacteria quickly became resistant to key antibiotics such as penicillin and then methicillin. When

resistance was acquired by significant human pathogens such as *Stapylococcus aureus* (methicillin resistant *Staphylococcus aureus*, MRSA), the impact on hospitals was significant because nosocomial spread became high risk. MRSA continues to be an infection risk in health care facilities and more recently has been spread in the community setting (David and Daum, 2010). Vancomycin resistant enterococci (VRE), another Gram-positive resistant bacteria but residing in the gastrointestinal tract, has also been identified as a potential high risk for hospital spread and become a focus for infection control (Goossens et al., 2003). MRSA is less frequently found in sewage compared to gastrointestinal bacteria VRE, extended-spectrum beta-lactamase-producing (ESBL) *Escherichia coli,* or the environmentally adapted and intrinsically highly resistant Gram-negative, *Pseudomonas aeruginosa* (Wheater et al., 1980; Rosenberg Goldstein et al., 2012). All of these organisms, however, have been detected in hospital wastewater (Fuentefria et al., 2011; Thompson et al., 2012; Korzeniewska and Harnisz, 2013; Varela et al., 2013; Santoro et al., 2015; Varela et al., 2016; Drieux et al., 2016). Recently carbapenamase-producing organisms (CPOs) harboring a variety of genes conferring resistance in Gram-negative bacteria have been identified in infected patients from non–North American hospitals (Nordmann et al., 2011). CPOs have been shown to be transmitted within hospitals and are a cause for concern with resistance to even the latest broad-spectrum penicillins (Peleg and Hooper, 2010; Snitkin et al., 2012).

Hospital bacterial ecological niches intersect with those in the environment through discharge of wastewater effluents. MRSA from patients colonized on their skin (or mucosa) are discharged into waste but more significantly to this intersect, are bacteria excreted in patient feces, including ESBL *E. coli* and the CPOs. As a result of selective pressure from persistent antibiotics and disinfectants as well as higher loads from frequent patient carriage of ESBLs (Hocquet et al., 2016), it has been noted that the proportion of ESBL *E. coli* excreted from hospital waste is consistently higher than that found in community effluents. It was also noted that due to the high volumes arising from community settings, two-thirds of ESBL loads came from community settings; one-third came from from hospitals (Hocquet et al., 2016). This recent review also described the diversity of resistant *E. coli* genotypes, with some strains seemingly less adapted to survival in the environment, hence appearing less frequently downstream of hospital waste effluents. Other studies have reported that contamination by antibiotic resistant *E. coli* decreases as waste moves from hospital discharge to WWTP to surface water (Galvin et al., 2010; Berglund, 2015). It has been described (Hocquet et al., 2016; Bouki et al., 2013) that WWTP decreased bacterial loads from hospital effluent discharge along with the concentration of ESBLs and other resistant bacteria. The proportion of resistant *E. coli*, however, was not decreased, and treated effluents have higher proportions of antibiotic resistant bacterial populations in relation to those in surface water (Goñi-Urriza et al., 2000; Huang et al., 2001; Korzeniewska et al., 2013). Reduction in bacterial load, including antibiotic resistant bacterial concentrations, was noted when untreated hospital waste streams joined the sewer system (Katouli et al., 2012). Other studies have shown stepwise decreased numbers of resistant *E. coli* isolates in samples downstream of hospital effluents, through waste water treatment and then into a river system (Oberle et al., 2012). Chitnis et al. (2004) showed reduction in bacterial concentrations, including antibiotic resistant bacteria in effluents, and suggested that this reduction was probably due to WWTP flocculation processes.

Wastewater treatment plants, however, were designed primarily to protect the public from human pathogens and their spread through the environment; their technologies were not put into place to specifically remove bacteria with antibiotic resistance. This environmental contaminant has yet to benefit from significant research on water treatment methods that remove it, although considerable contributions to the scientific literature have been made in recent years (Karaolia et al. 2018 (this volume); Beier et al., 2012; Kovalova et al., 2013). Evidence has been collected to suggest that resistant bacteria isolates of hospital-treated wastewater could be passed to the microbial communities in the urban wastewater system via horizontal gene transfer (Kotzamanidis et al., 2009). Work so far (Dodd, 2012; Chen and Zhang, 2013; Lüddeke et al., 2015; Moreira et al., 2016) does show that the variety of wastewater treatment operating processes can impact antibiotic resistant bacteria differently. Although wastewater treatment of influents containing hospital waste have been shown to significantly reduce resistant *E. coli* through the process stream, the measured abundance in the effluent suggests resistant *E. coli* could be disseminated in the receiving environment (Reinthaler et al., 2003).

Some studies have characterized resistant human pathogens in which adaptations gained from hospital sources are likely to influence their survival in receiving environments (Sadowy and Luczkiewicz, 2014). Ongoing waste discharge from the hospital ecological niche implies a continuous input of various resistant microorganisms (Pauwels and Verstraete, 2006; Nunez and Moretton, 2007; Zhang et al., 2013; Narciso-da-Rocha et al., 2014). Other studies have concluded that the mechanisms of developing antimicrobial resistance in hospital wastewater isolates were different from those derived from human clinical strains (Tuméo et al., 2008). A recent study demonstrates that not all carbapenemase-producing *Enterobacteriaceae* in hospital wastewater originated from excreta from infected human patients, but rather other sources (including hospital pipework) may contribute to the concentration determined in the waste stream (White et al., 2016). At the same time there do not appear to be clear-cut relationships between the antibiotics used and those detected in the wastewater stream or bacterial resistant isolates. Clearly, many biological, ecological, and chemical factors are at play in this dynamic system.

Few countries require hospitals to treat their effluent discharges, which contain high percentages and heavy loads of antibiotic resistant bacteria, particularly those excreted in feces. There are no environmental infection control practices for these heavily contaminated effluents leaving hospitals (or, after wastewater treatment, discharging into rivers), similar to infection control practices used within hospitals to reduce risks to patients. In the developing counties where WWTPs are few or nonexistent, hospitals often discharge their contaminated waste directly into rivers and water networks.

## Antimicrobial Resistance Genes in Wastewater

Antibiotics or their residues as well as resistant bacteria are now regularly detected in hospital wastewater (Duong et al., 2008; Picão et al., 2013; Chonova et al., 2016). A third element, bacterial resistance genes, often found in groups or cassettes, has also been shown to be present in waste from hospitals and other health care settings (Chagas et al., 2011; Oberle et al., 2012). Hundreds of bacterial genes have been identified in a complex system of the resistance development (Bouki et al., 2013). Horizontal gene

transfer of resistance traits through vehicles such as plasmids, transposons, and integrons by cell-to-cell conjugation, transduction (through bacterial viruses), or transformation (resistant plasmid DNA transferred into bacteria) have been well described (Ochman et al., 2000; Berger-Bachi and Rohrer, 2002; Juhas et al., 2008; Barlow, 2009; Warnes et al., 2012; Stalder et al., 2014; von Wintersdorff et al., 2016). The environment itself has been recognized to have its own resistome (Cantas et al., 2013) and its own ecological niches, which include antibiotic resistant bacteria.

Like antibiotic groups and the concentration (not proportion) of resistant bacterial isolates, there is an observed decrease in some bacterial resistance genes downstream from health care settings and as well after wastewater treatment (Diwan et al., 2010; LaPara et al., 2011). Disturbingly, however, some genes/genetic elements have been observed to accumulate in the environment (Pei et al., 2006). Expanding pools of resistance genes and their transfer could result in hitherto unknown multiresistant bacterial strains. Risk of gene transfer could be increased with the accumulation of genes in the environment as well as the WWTP processes, with hospitals and related health care network waste discharges contributing to what was intended to decrease risks from public health pathogens. It has been suggested (Hocquet et al., 2016) that "nutrient-rich" high cell density environments may make wastewater treatment plants "hotspots" for the "transfer of antibiotic resistant genes" (Rizzo et al., 2013). Significant reduction of bacteria has been reported when an additional process of wastewater disinfection was used for wastewater treatment (Korseniewska et al., 2013), but it has also been suggested that this chemical stress may promote transfer of genes (Bouki et al., 2013). Public health emergencies have been caused by emerging highly virulent and resistant enteric pathogens such as the 2011 enterohemorrhagic *E. coli* outbreak in Germany spread by contaminated food (and water) that resulted in many deaths (53) and more than 800 cases of renal failure (Buchholz et al., 2011). Thus, bacterial genetic exchange can surface suddenly, causing significant health impacts. With concerns that waste treatment may become part of the problem and not part of the solution, the need to address the lack of fundamental information is high priority.

## Conclusions and Recommendations

Hospitals discharge unique waste into treatment plants and the environment. They add challenges to the ongoing antibiotic resistance issue in at least three different areas noted above, each of which is complex. Affecting these point source discharges through inadequate WWTP processes may actually increase risks of antibiotic resistance by enhancing gene transfer through its high cell density and high nutrient microenvironments. Interrupting these discharges through coordinated, multidisciplinary strategies is also basic to preventing further genetic materials conferring bacterial antibiotic resistance and accumulating in various ecosystem niches in the environment. Hospitals continue to evolve and now are sources of networks of discharges, each with varying antibiotic usages and resistomes. Hospitals are not the only source of this contamination, and it is still not clear if they contribute overall to the global epidemiology of resistance. Antibiotic use is not restricted to human health. Further research and effective solutions will require a suite of coordinated solutions developed and applied by multidisciplinary groups. In developing countries WWTP are used less frequently, and contamination of the

environment is direct. As well, in regions where even basic drinking water disinfection is not routinely available and food system health controls are not in place, spread of bacterial colonizers will depend on their adaptability under environmental conditions. While more multidisciplinary and coordinated research is needed to define the role of human health care settings, to develop effective removal by WWTP, and to inform hospitals and their facility networks as well as public health, agricultural, and veterinary practice, there is also a role for international standards based on new public polices to be created and adopted worldwide. Waste issues are outside hospital walls, and cost pressures and lack of risk incentives will probably require that public policies with regulations are enacted to ensure best practices are used. Approaches for targeting and providing research priorities could focus on hospital discharges along with improved WWTP practices that could be applied to defined point waste sites. International and multidisciplinary antibiotic stewardship groups could work together to reduce antibiotic usage, not just manage drugs in acute care settings. Wastewater antimicrobial monitoring could be combined with hospital infection prevention and control programs, with public health accountability for surveillance of global resistance/susceptibility patterns. This complex issue, while not ignoring the unique settings of hospitals and their networks of health care facilities, requires system priorities and strategies.

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# 18

# Curbing the Resistance Movement

## Examining Public Perception of the Spread of Antibiotic Resistant Organisms

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Over the past few years, there has been a distinct shift of the confidence in health outcomes at hospitals in many nations related to escalating fear of exposure to antimicrobial resistant bacteria. Public awareness of health risks linked to exposure to methicillin-resistant *Staphylococcus aureus* (MRSA) or "superbugs" has dramatically increased due to knowledge gained from direct or indirect personal experience, as well as information accessed through various media campaigns. This chapter will look through a sociological lens at the shift in the public's confidence that use of antibiotics will consistently successfully treat bacterial infections. It argues that public perception of the risk related to spread of antibiotic resistance has eroded and that fear plays a role in citizens' health decisions when hospital bacterial populations cannot be seen, known, or cultured. Greater public awareness and public perception of health risks can have important influence in contemporary regulation of antibiotics and policies to control the spread of antibiotic resistance on a broader scale. Thus, there is a two-fold resistance movement: the actual spread of antibiotic resistant organisms and the wave of influence that spreads as a result of the public managing their information (lay or science-based) related to the perception of health risk.

## Introduction

Our friend contracted an infection in his foot. He is a generally a healthy man with an above-average commitment to fitness and exercise. He loves the outdoors. During a family vacation on the East Coast, he spent lots of time swimming in the river and walking around barefoot near the riverfront. However, within a couple of days of returning home he came down with a high fever and a painful red rash on his right foot. The fever persisted and he sought medical care. The first cultures were done immediately and cephalexin was prescribed. The symptoms subsided for a while, but they soon returned. Another sample for culture was taken and cephalexin was prescribed together with

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another antibiotic. The symptoms subsided again and came back. The cycle repeated for several months. Eventually, he was referred to a specialist. Samples were cultured again and this time ciprofloxacin was prescribed. The symptoms disappeared and then recurred. Our best guess is that some form of antimicrobial resistance is likely at work.

* * * * *

Recent decades have seen a noticeable shift in public awareness of the health risks related to development of antimicrobial resistance (AR) in pathogens. Most of this awareness focused on methicillin-resistant *Staphylococcus aureus* (MRSA) or the "superbugs" that, until the late 1980s, the general public had thought were confined to hospital settings. In 1959, methicillin was first introduced for the treatment of infections caused by *Staphylococcus aureus* that were resistant to penicillin, but by 1961, the first reports of MRSA were already published (Jevons, 1961). Reports of community-acquired MRSA began appearing in the 1990s (Hussain et al., 2000). Public awareness of the lethal consequences of some infections by MRSA followed an exponential growth curve that mirrored the public's growing reliance on the internet to provide information about critical health risks. In 2003, the tragic death of a college football player, Rick Lannetti, became a trigger event for the awareness of increasing incidence of MRSA infections, particularly in team sports, including American NFL football (Drew, 2007). Now the ever-growing body of scientific literature has provided sufficient evidence such that an increasing fraction of the public recognizes that exposure to antimicrobial resistant pathogens is not limited to MRSA and that hospitals represent only one setting where exposures to such infections can occur.

Public perception of the risk of pathogenic infection is influenced by an element of fear. The notion of some sort of submicroscopic bacterial anarchy combined with an incomplete understanding of the epidemiology of infectious disease has contributed to the public perception of risk related to transmission of AR. The dramatic increase in use of hand sanitizers in public places attests to the consumer demand for reassurance that interventions to mitigate the spread of infectious disease are always accessible. The spotlight on hand hygiene has intensified over time as more and more people believe that unclean hands "are one of the main culprits in the spread of infections contracted in hospitals" (Owen, 2013). Purell is a hand sanitizing product originally developed by Gojo Industries in the late 1980s. The now ubiquitous Purell dispensers in many public spaces serve as practical and symbolic reminders of the importance of hand hygiene as a measure to interrupt the spread of infectious diseases, including the subset of these that may be due to antimicrobial resistant pathogens.

## Public Awareness of the Antimicrobial Resistance Health Risk

There have been relatively few scientific qualitative studies to investigate knowledge, belief, and perception of risk related to the development and spread of AR. McCullough et al. (2015) conducted a systematic review of knowledge about antibiotic resistance and found that more than 90% of clinicians believed that AR was a problem but was caused by other pathogens. The following year the McCullough team (2016) reported on another systematic review of the public's knowledge and beliefs about antibiotic

resistance in which they concluded that the public had "an incomplete understanding of antibiotic resistance and misperceptions about it and its causes and do not believe they contribute to its development." Results of the meta-analysis presented in the systematic review by Gualano et al. (2015) indicated that about 59% of respondents knew about AR, but of these about 27% failed to identify misuse of antibiotics as having something to do with the problem. Some surveys of attitudes and beliefs among the public have been previously reported from Europe (Emslie and Bond, 2003; André et al., 2010) and several other nations, including Malaysia (Ling et al., 2011), South Korea (Kim et al., 2011), Hong Kong (Wun et al., 2013), and Trinidad and Tobago (Parimi et al., 2002). In each of these studies, there were consistent observations that the public's knowledge of the relationship between use, misuse, and overuse of antibiotics and the development of AR appears to be incomplete.

> *"Antimicrobial resistance poses a catastrophic threat. If we don't act now, any one of us could go into hospital in 20 years for minor surgery and die because of an ordinary infection that can't be treated by antibiotics. And routine operations like hip replacements or organ transplants could be deadly because of the risk of infection."*
>
> Dame Sally Davies, Chief Medical Officer England
> March 2013

Considerable improvements have been made over recent years to advance public awareness of the health issues related to AR in human, animal, and environmental health. Many nations now devote a week in November to a campaign for Antibiotic Awareness Week (World Health Organization, 2016). Relatedly, beginning with a small pilot program in Grand Prairie Alberta in 1997, the Do Bugs Need Drugs? educational program grew to become a province-wide initiative in Alberta and British Columbia Canada in 2005 that uses televised infomercials, print media, and posters on public buses and bus shelters to improve public perceptions of the health risk (Do Bugs Need Drugs?, 2015). In 2014, filmmakers Michael Graziano and Ernie Park produced a documentary film titled, *Resistance—The Film*. In the film, several scientific and medical experts describe why overuse, misuse, and abuse of antibiotics leads to development of AR and how this, in turn, can render a given antibiotic ineffective against the infections it was originally intended to treat. A meteoric rise in public awareness of AR followed the release of England's Chief Medical Officer's second annual report on AR in March 2013, after which CMO Dame Sally Davies (quoted also above) said the infectious threat of antibiotic resistance was "as great a global threat as terrorism" (CMO England, 2013). The report included 17 recommendations for measures to combat the health risks of AR, and the wave of media attention that followed release of the report noticeably increased the public's interest in the issue.

## McLibel and the Rise in Public Awareness of Antibiotic Use in Meat Production for Fast Food Chains

As one of the longest running lawsuits in British history, McDonald's Corporation versus Steel and Morris, also called "the McLibel Case," became an unexpected and effective trigger event in the rise of public awareness of AR in food animals. Helen Steel and David Morris, together with three other members of London Greenpeace,

distributed a leaflet in the city titled "What's Wrong with McDonald's" that made several accusations that the company was involved in corrupt practices including cruelty to animals, selling unhealthy food products, exploiting their staff, and destroying rainforests (Vidal, 1997). In 1990, McDonald's Corporation brought libel charges against all five of the London Greenpeace activists, of whom only Steel and Morris chose to fight the case. Under English defamation law, the burden of proving the truth of allegedly defamatory statements is placed on the defendant, rather than the plaintiff. Thus, Steel and Morris embarked on a multiyear mission to systematically verify each of the allegations against McDonald's as true.

In the years that followed, Steel and Morris gathered research to support claims that McDonald's was aware that the company or its suppliers were involved in practices that could be interpreted as cruelty to animals or manufacturing food products to which a health risk could be linked (Vidal, 1997). As more and more information was collected, knowledge of high-intensity food animal production facilities, food animal feeding practices, and food production business strategies directed attention toward a number of potential human health risks linked to transfer of biological and chemical contaminants via the food chain. Over the course of the McLibel trial, mainstream media began publicizing many important issues related to food production, including practices such as the use of antibiotics in cattle, chickens, and pigs for growth promotion and the associated risk of development of antibiotic resistant infection in human patients. In June 2003, the McDonald's Corporation was the first fast food chain to announce its policy to require global meat suppliers to phase out the use of animal growth-promoting antimicrobials that are also used in human medicine in a direct attempt to address the health risks linked to antimicrobial resistant infections in people (AVMA, 2003). Although the English court found some of the statements to be libelous and some of the accusations to be true (Vidal, 1997), the long court case that Helen Steel and David Morris endured had lasting effects in raising the awareness of many important issues, including the use of antibiotics in meat production. In fact, A&W Food Services Canada Inc. "started to notice interest among Canadians about the food they eat and what's in it," and as a result, current advertising campaigns on media such as television, digital advertising, and social media include their "Chicken guarantee—Raised without the use of antibiotics" (Marketing PR, 2014).

## "Look at My Dog—I Know He Needs Antibiotics"

Nested within the contentious debate about the use of antibiotics in animals is an underappreciated connection that can be directly influenced by the public perception of AR as a health risk. Companion animals and pets represent a significant fraction of veterinary patients for which protecting the efficacy of antibiotics to treat pathogenic infections is critical. In 2012, it was estimated that about 40% of households in the US owned at least one dog (for a total of about 70 million dogs), and about 30% of households had at least one cat (totaling about 74 million cats) (AMVA, 2012). Pet demographic trends in Canada are very similar (CAHI, 2015). It is observed with increasing frequency that modern families include at least one pet but often no children. As well, there appears to be a growing trend of households composed of one individual and one

companion animal, usually a dog or cat. Pets and companion animals also include fish, turtles, birds, rabbits, ferrets, hamsters, gerbils, rats, guinea pigs, snakes, and other reptiles, and in many cases the emotional bond between owner and pet is very strong. In many cases, pet owners expect the same level of medical care as for a human patient, with the exception that owners make all health care decisions on behalf of their nonhuman patient.

In some cases, the discourse concerning banning the use of certain antibiotics in animals fails to highlight the important distinction between the use of antimicrobials in livestock and the use of antimicrobials in pets. Several of the same classes of antibiotics are used to treat pathogenic infections in food animals, companion animals, and human patients. However, for some infectious diseases in pets or companion animals (including horses), severe cases require individualized treatment using the same antibiotic that could be used for human patients. The One Health approach stresses the importance of prudent use of antibiotics in food animals and includes the recognition that prevention of the spread of AR in small or large animal clinics is as important as controlling the potential spread of AR in human hospital settings. Despite the widespread acceptance of the scientific medical community of One Health as a more holistic approach to considering the environment, humans, and animals together in addressing important health issues, the general public does not seem aware of the One Health approach, although to this author's knowledge, there have been no systematic studies to investigate this.

Pets have been recognized as reservoirs for AR (Guardabassi et al., 2004), and prudent use of antimicrobials in small animal veterinary clinics is a required best practice for responsible antimicrobial stewardship. However, the unique relationship between owners and their pets can influence veterinary prescription of antibiotics. Access to information via the internet, the consistent growth of the pet insurance industry, and extreme concern for pet health and well-being can lead to situations where owners request veterinary prescription of antibiotics when they may or may not be needed. Veterinary clinics usually operate with a for-profit business model, whereby failure to meet pet-owner demands can quickly erode a client base. This, among many other factors, has led to the development of educational campaigns by the veterinary associations, particularly in the United States, Canada, and Europe, to inform the pet-owning public of the importance of adopting appropriate strategies to limit the spread of antimicrobial resistant pathogens.

## Public Perception of AR Risk in the Context of Wastewater Treatment: Rio Olympics—The Facts or Fiction?

For most societies, the role of nature and the environment in human health and well-being is embedded in contemporary culture. Recognition of the impacts of human activities on ecosystems related to issues such as climate change, finite limits to resources, and sustainability are now common themes throughout many layers of society. However, despite warnings from the scientific community for decades, it is only recently that the general public seems to understand the importance of wastewater treatment plants as potential sources that could discharge contaminants into the environment.

In the time leading up to the 2016 opening of the Games of the XXXI Olympiad held in Rio de Janeiro, Brazil, media reports began appearing that criticized the water quality of Guanabara Bay since it was one of the locations used for some water sports. Preparation for the 2016 Rio Olympic Games included the construction of a new sewage treatment plant in Deodoro using state-of-the-art Nereda technology from Royal Haskoning DHV in the Netherlands (Royal Haskoning DHV, 2016). A number of reports began to appear in popular press that described a confusing array of information that related to public health risks of exposure to "superbugs" and "super bacteria" (and also "superbug bacteria") allegedly derived from wastewater and raw sewage discharged directly into the bay (Ecowatch, 2016). News articles referenced research of Professor Renata Picao and colleagues from the Federal University of Rio de Janeiro that served to amplify public and Olympic athletes' concern for exposure to antibiotic resistant microbes at Rio beaches and competition location waters (Ecowatch, 2016). Many of the reports discussing environmental issues connected with the Rio Olympics chose to highlight the public health risk posed by an army of antibiotic resistant microbes originating from sewage.

Although some media pointed out that research of Picao and others had been done several years prior to the summer of 2016, some of the global public developed the notion that evidence for the presence of antimicrobial organisms in coastal environments was new and noteworthy. Coutinho et al. (2013) presented a case study in which they determined that antibiotic resistant *Pseudomonas aeruginosa* could be found in discharge from a wastewater treatment plant that received hospital waste and released effluent to the Jacarepaguá lagoon system located within the metropolitan area of Rio de Janeiro. Extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumonia* was found to be present in the effluents and sludge of a sewage treatment plant located in the metropolitan area of the city of Rio de Janeiro that received hospital wastewater (Prado et al., 2008). A study of Chagas et al. (2011) demonstrated that the removal efficiency of a wastewater treatment system also located in the metropolitan region of Rio de Janeiro was insufficient to eliminate all antibiotic resistant microorganisms present in hospital wastewaters before discharge to the environment. Despite criticism and controversy linked to water quality and potential public health risk of exposure to antibiotic resistant microbes specifically associated with the 2016 Rio Olympic Games, the spike in media coverage has raised the overall public awareness of the relationship between wastewater treatment and the potential for transmission of infectious disease in the environment. From hand sanitizers to the Olympic Games, the past 50 years eclipsed a surging perception in the public regarding the spread of antibiotic resistant organisms. From a sociological point of view, this perception can be understood as a means of socializing both the public and the pathogens.

* * * * *

Overall, our friend is doing alright; however, the rash on his foot is still very much present, although not as severely as before. At the time we are writing this chapter, his next specialist appointment is coming up. The cycle of infection has not yet disappeared since the very first occurrence, now nearly one and a half years later. We acknowledge his contribution to reviewing this text—he can now spot the error when some of the multidrug resistant bacteria species are spelled incorrectly.

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# 19

# Public Health Consequences of Antimicrobial Resistance in the Wastewater Treatment Process

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The twenty-first century trend of concentrating populations in cities has fueled a consistently growing demand on utilities to provide clean, safe drinking water and to ensure safe disposal of wastewater. Protection of public health is the number one concern in the management of water resources in large urban centers and smaller rural communities. Within this context, it is now well recognized that the development and spread of antimicrobial resistance represents an extremely important public health risk. Wastewater treatment plants, usually operated by public utilities, are known to act as point sources for the introduction of antibiotic compounds, antimicrobial resistant organisms, and antimicrobial resistance genes into the receiving environment (Rizzo et al., 2013). The potential for dissemination of antimicrobial resistance as a result of the collection, treatment, and disposal of wastewater requires careful consideration for protecting human and ecosystem health. Wastewater treatment plants serve as an intersection where the engineering related to operation of the process overlaps with the requirements for protection of public health from the spread of infectious disease. This chapter will summarize some of the current discourse related to the role of wastewater treatment processes in the transmission of antimicrobial resistance in the environment, with particular emphasis on the exposure pathways that potentially impact public health.

## Introduction

The relationship between the epidemiology of human disease and the engineering of water distribution systems was crystallized forever the moment John Snow removed the pump handle from the Broad Street Soho district well of London to arrest the cholera outbreak in 1854 (Snow, 1855). This pivotal event in history inspired fundamental changes in water management, sanitation, and engineering of water distribution systems to protect the health of citizens from infectious disease. Most of the water used

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on a daily basis in urban centers eventually finds its way to wastewater treatment facilities of some sort. As a consequence, wastewater treatment plants collect a plethora of contaminants that require application of removal technologies before the quality of discharged effluent can be considered safe for discharge to the receiving environment. The diverse range of contaminants found in urban wastewater include chemical substances (e.g., pharmaceuticals, personal care products, trace metals, soaps), biological organisms or biologically active agents (e.g., bacteria, viruses, endocrine-disrupting compounds) and insoluble physical entities (e.g., grit, sand, microplastics). Essentially all of the wastewater constituents and the processes involved in treating sewage affect the effluent quality that must meet criteria for discharge to ensure the protection of public health and the preservation of healthy ecosystems. As more and more evidence is gathered to suggest that the wastewater treatment process plays a particularly important role in the development and dissemination of antimicrobial resistance, understanding the impacts of antibiotics, antimicrobial resistance genes, and antimicrobial resistant organisms is essential to solve elements of the risk assessment puzzle for the protection of public health.

## Urban Water Cycle

The continuous transport of water throughout ecosystems is governed by the hydrologic cycle. Water that evaporates from ocean surfaces rises and condenses to form clouds. These clouds are transported in the atmosphere until the water is returned to the earth's surface as precipitation. The water travels on and below the land surface until it is returned to the atmosphere through evaporation or by transpiration from plant leaves. All human activities including patterns of settlement that concentrate large populations in cities have profound influence on the hydrologic cycle such that a subsystem, referred to as the urban water cycle, now dominates the circulation of water in many parts of the world.

In the urban water cycle (Figure 19.1), the transport of water begins at an environmental source. This water usually represents the highest quality that is available before being treated and disinfected to ensure that it is safe for drinking in the local communities. The urban water cycle involves the collection and storage of freshwater from the environmental source, treatment, and disinfection for provision of potable water, distribution of it through pipe networks to end users, consumer use of the water, collection of sewage after use, and treatment of wastewater such that it can be returned to the environment without compromising public health or ecosystem health. Reuse of water following wastewater treatment is now playing an increasing role in the urban water cycle for many global cities where limited water availability is shifting demands on reclaimed water to serve ever-growing needs.

Bacteria are present in most phases of the urban water cycle, and the tangential effects of human exposure to antimicrobial resistant organisms and antimicrobial resistance genes are important to consider throughout the urban water cycle (Varela and Manaia, 2013; Vaz-Moreira et al., 2014). Climate change can alter the urban water cycle in many ways, including unpredictable responses to drought or flooding conditions which, in turn, affect the exposure pathways of antimicrobial resistant bacteria and genetic determinants of resistance. Seasonal variability in the occurrence and persistence of some

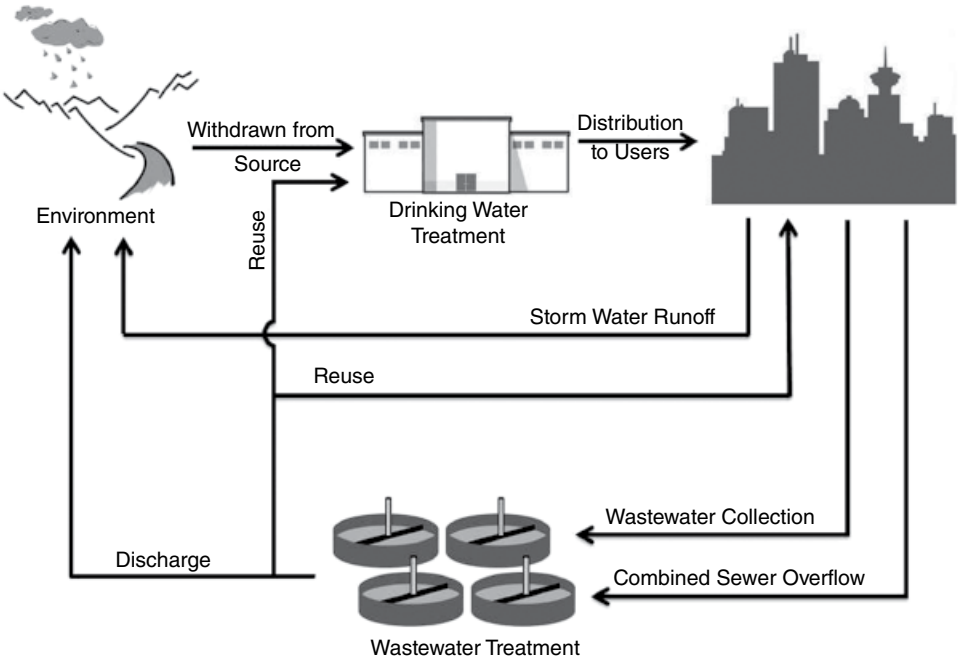


**Figure 19.1** The urban water cycle.

opportunistic pathogens derived from harvested rainwater has been observed (Hamilton et al., 2017), which has important implications for the potential for persistence of the antimicrobial resistant subset of such strains. Antimicrobial resistance genes and antimicrobial resistant organisms have been detected in drinking water treatment systems (Xi et al., 2009; Jia et al., 2015) and in the biofilms associated with drinking water distribution networks (Schwartz et al., 2003; Khan et al., 2016). A recent study has demonstrated that drinking water disinfectants (free chlorine, chloramine, and hydrogen peroxide) can affect horizontal gene transfer of antibiotic resistance genes (Zhang et al., 2017). In addition, several studies have reported that bottled water could serve as a possible source of antimicrobial resistance in human drinking water (Duquino and Rosenberg, 1987: Messi et al., 2005; Falcone-Dias et al., 2012; Ranjbar et al., 2016). Thus, the preconsumption and use phases of the urban water cycle have been demonstrated to offer multiple opportunities for the potential spread of antimicrobial resistance eventually to the human microbiome.

Moreover, as presented in previous chapters, antimicrobial resistance (AR) does not limit itself to resistance to antibiotics only, but to all classes of antimicrobials, including physical, chemical, and biological agents. Presence of such contaminants in the urban water cycle is of concern. For example, bacteria have shown an ability to acquire resistance to ultraviolet radiation (Arrage et al., 1993; Goldman and Travisano, 2011; Alcántara-Díaz et al., 2004), silver (Gupta et al., 1999; Percival et al., 2005), copper (Bondarczuk and Piotrowska-Seget, 2013; Dupont et al., 2011), nickel (Hu et al., 2017), and even phages (Seed, 2015; Samson et al., 2013; Labrie et al., 2010). Because bacteria are ever-evolving organisms, static tools as strategies to control them have limitations.

Bacterial infection control with a natural predator such as bacteriophages has been comprehensively reviewed (Sulakvelidze et al., 2001), and phage therapy is an avenue that several medical centers in Georgia and Poland have adopted and described as holding promise for the future (Reardon S., 2014; Abedon et al., 2011). Even though bacteria can adapt to phages, they share a predator-prey relationship, and phages will also adapt. The main downside (which is also an upside for some) is that phages need to be selected on-site and sometimes will be unique to a single patient or single ecosystem niche. Exploiting the natural predator-prey interaction between bacteria and bacteriophages in wastewater treatment has been investigated (Withey et al., 2005; Periasamy and Sundaram, 2013; Khairnar et al., 2014; Lui et al., 2015; Jassim et al., 2016), although successful application of this strategy requires a good understanding of the ecological structure and dynamics of the microbial communities in the subsystems of the process.

Wastewater treatment plays a central role in the urban water cycle that potentially influences development and circulation of AR through various environmental compartments. Because wastewater treatment plants eventually receive excreted gut bacteria and a continuous flow of rich organics nutrients, they provide "perfect mixing pots for bacteria carrying genes that confer antibiotic resistance" (Davies, 2012). As discussed throughout all of the chapters in this book, there is sufficient scientific evidence demonstrating that contaminants present in the wastewater treatment processes can alter bacteria at the genetic level and impact bacterial community structures and that some of these AR-related contaminants can be found in biosolids or effluents discharged to the receiving environment after treatment (Xu et al., 2015; Watkinson et al., 2007; Ouyang et al., 2015; Odjadjare and Olaniran, 2015; Xie et al., 2016). Additionally, with more and more emphasis placed on the need for reclaimed water that meets water quality criteria for the protection of human health, studies have examined antimicrobial resistance in recycled water (Fahrenfeld et al., 2013) and combined sewer overflows (Scheurer et al., 2015). Interconnected sectors of the urban water cycle can therefore permit exchange of antibiotic resistant bacteria and mobile genetic elements of resistance between unclean and clean water within a community.

Antimicrobial resistance has become an increasingly serious public health concern worldwide. Two specific actions are essential for controlling the spread of antimicrobial resistance in human populations: preventing infections and surveillance to detect emerging patterns of resistance. The same two specific actions are critical for controlling antimicrobial resistance in animal populations. Human and animal populations represent only part of the species composition of ecosystems and, as discussed in chapter 3, the One Health approach incorporates the role of the environment into assessing the risk of development and spread of antimicrobial resistance. Although still not universally accepted, there is a much greater appreciation that a One Health approach to antimicrobial resistance in human pathogens considers overall ecosystem health, animal health, and human health combined. Addressing this human health risk is a shared responsibility that requires interdisciplinary, coordinated, and sustained efforts, particularly on the part of health professionals, scientific researchers, policy makers, and citizens at large.

In our ever-changing globalized world, prevention of infection in the first place and the control of the spread of infectious diseases are of paramount concern in the protection of public health. The chain of infection is influenced by an extremely complex array of factors that govern the emergence of disease. When the etiologic agent is

bacteria, development of antimicrobial resistance in pathogenic species assumes a worrisome role in the control of infectious disease. Reservoirs of antimicrobial resistant bacteria are found in humans, animals, and the environment. Human and animal reservoirs can be subdivided into acute clinical cases (often concentrated in a health care facility) and carriers (who do not necessarily present with symptoms of illness but represent a greater transmission risk since their activities are not restricted by their illness). Environmental reservoirs include all major biophysical compartments of natural ecosystems, such as soil, sediment, plants, and water. Strains of bacteria resistant to common antibiotics in clinical use today and antimicrobial resistance genes have been detected in environmental reservoirs, some of which date back millions of years (Mindlin et al., 2008; D'Costa et al., 2011). Direct and indirect modes of transmission connect the portal of exit from a reservoir into the portal of entry of a susceptible host. Given that food products, consumer goods, animals, and humans traverse the globe every minute of every day, preventing the spread of infectious disease and in tandem, the spread of antimicrobial resistance means interrupting the chain of infection at any opportune link. Host susceptibility may also be potentially enhanced in the absence of a healthy array of resident flora.

Surveillance is an integral element in meeting the global challenge of preventing the spread of infectious disease, preventing the spread of antibiotic resistant bacterial infections and protecting public health. Measuring, monitoring, and tracking the epidemiology of infectious diseases and AR are imperative. Surveillance programs usually include information on bacterial resistance patterns in health care facilities, community isolates, veterinary practice, on-farm, and at various points in the food chain. Complete surveillance should also include efforts to track the pressure for natural selection by recording rates of antimicrobial utilization. Most first-world and several developing countries have operated national surveillance programs collecting data on antibiotic resistant infections in human patients and on the utilization of antibiotic drugs for several years. Increasingly (and with increasing adoption of a One Health approach to managing the spread of antimicrobial resistance), national surveillance programs gather data on patterns of resistance detected in infections in animals (primarily food animals but now sometimes pets) and on the use of antibiotics in veterinary medicine. The Sixty-eighth World Health Assembly adopted the Global Action Plan on Antimicrobial Resistance held in May 2015, which identified the strengthening of global surveillance and research as one of its priority strategic objectives in combatting the public health threat of antimicrobial resistance (WHO, 2015). Success in meeting the public health challenge of antimicrobial resistance in human pathogens is enhanced by organized, collaborative, integrated, and sustained surveillance of antimicrobial resistance and antibiotic use. This integrated approach to surveillance could, in the future, include environmental monitoring at points of effluent discharge from wastewater treatment plants.

## Antimicrobial Stewardship Programs

Antimicrobial stewardship programs are now recognized as a valuable component of health care to provide coordinated interventions intended to improve therapeutic outcomes of antibiotic use and to limit the spread of antimicrobial resistance.

Although these programs have previously been primarily associated with hospitals, the growing awareness of antimicrobial resistance in community settings and in environmental reservoirs is leading to the extension of antimicrobial stewardship outside of health care facilities. Some core elements of good antimicrobial stewardship programs (CDC, 2014) are now becoming more widely accepted. A commitment to leadership that provides adequate human, financial, and information technology resources is essential. Program accountability is best served by appointment of a single leader who is responsible for program outcomes. Improvements in antibiotic use can benefit from the expertise provided by pharmacists. Monitoring antibiotic prescription and reporting patterns of resistance in pathogens is a required element of good antimicrobial stewardship programs (CDC, 2014). Imbued throughout all of the working parts of a good antimicrobial stewardship program is continuous education—coordinated, multidisciplinary, and sustained interventions to reduce the public health risk of antimicrobial resistance depend on education available to all strata of society. Finally, there is mounting evidence that processes that provide feedback to individual prescribers or exert a degree of administrative control over the use of some agents can improve the performance of stewardship efforts.

Gradually antimicrobial stewardship programs are establishing a greater presence in the veterinary medical community as well. There are promising trends of voluntary data collection for antibiotic use and patterns of antimicrobial resistance on the part of small animal clinics and food animal production facilities in many areas of the world. Greater efforts are being made to harmonize measurements and adopt a more standardized approach to the recording of data in the human medical community and in animal health care. To date, however, there are no reports of surveillance programs or antimicrobial stewardship programs that record information concerning presence or concentrations of antibiotics, antibiotic resistant organisms, or antibiotic resistance genes as environmental contaminants.

## Antimicrobial Resistance Stewardship and Engineering

Within the urban cycle, civil engineers are involved in all key technical activities related to collection, purification, transmission, and distribution of potable water and the treatment of wastewater to kill pathogens and remove contaminants. Contaminants that influence the development of antimicrobial resistance in bacteria can be distributed via this transmission network, ultimately being released into the receiving environment. Engineered systems throughout the urban water cycle are points where the presence of such contaminants could potentially be monitored and where mitigation strategies could potentially be deployed. As more and more civil engineers build an understanding of risk factors that influence development and spread of antimicrobial resistance, engineered processes can strengthen the link between stewardship practices and protection of public health.

In Canada and probably elsewhere, there are very few examples where engineers serve on hospital antimicrobial resistance stewardship committees. In one Canadian hospital, a civil engineer responsible for overall building operation regularly attends AR stewardship meetings, participates in AR stewardship educational activities, and is fully aware of hospital antibiograms, hospital-specific AR stewardship practices, hospital

cleaning procedures, and all site operations related to water and air circulation in the hospital. This situation is cited because it represents an example of an important opportunity for regular dialogue between health care professionals in the hospital and the engineering team responsible for its day-to-day operations. Such communication is particularly valuable for educating building operations staff about hospital-specific issues related to antimicrobial resistance (including risk communication regarding occupational exposure and workplace safety) and for educating medical personnel about building and infrastructure-related issues that could affect exposure pathways of contaminants related to AR. A hospital can be thought of as a smaller scaled ecosystem containing part of a condensed urban water cycle. With few exceptions (including small rural hospitals), most hospitals do not treat wastewater on site but discharge effluent to sewers. As discussed in chapter 17, evidence strongly suggests that hospital wastewater is an important reservoir for conditions that influence development of antimicrobial resistance. There may be opportunities within the hospital microcosm where engineered systems can play a greater role for monitoring AR-related contaminants and mitigating impacts of these contaminants before hospital effluent is discharged to regional sewer systems and the environment.

The "smart city" model is becoming infused throughout many layers of society. The quantum leap in technological capabilities in only a few decades has enabled application of "multi-scalar smartness" to everyday life. Coupled with this are vast improvements in our ability to collect and manage extremely large quantities of data. Integrated smart technologies can now enable the modern homeowner to monitor the lights and temperature in any room of their house at any time using their cellphone. At the opposite end of the scalar spectrum, air quality and pollutant dispersion can be measured in real time using sensors located on buses in an urban transit system utilizing a smart framework of data collection (Wen and Li, 2014). At this intersection between applied information technology and the protection of public health is a particularly valuable role for engineers at the building and community-wide scales.

First, engineers at all hospitals should be invited to join antimicrobial resistance stewardship committees. Engineers at all hospitals should know where the hospital wastewater is discharged to sewer. Ideally, smart systems could be installed for gathering basic water quality data within the hospital water system and for regularly monitoring basic air quality parameters in hospital buildings. Information could be easily accessed and seasonal trends established. Hospital antibiograms and hospital pharmacy formularies can provide important information that, in combination with basic water quality data and effluent flow rates, could indicate whether hospital wastewater offers a low, medium, or high risk of transmitting AR-related contaminants into the regional urban water cycle. This practice could be extended to the urban water cycle through the collaborative efforts of engineers and the stewardship committee members of primary and secondary health care professionals networked throughout any community. In view of the growing body of scientific evidence related to antimicrobial resistance in wastewater treatment processes, large urban sewage treatment centers offer the unique opportunity to sample the resistance patterns from the microbial flora of an entire human population and represent ideal surveillance spots in their own right.

As mankind looks toward the sky with projects such as the Chinese plan to walk on the moon within 10 to 15 years or projects seeking to establish colonies on Mars, the reality of antimicrobial resistance needs to be taken into account. In fact, in space travel,

environmental compartments are completely interconnected and virtually perfectly closed-loop. Given the current knowledge of antimicrobial resistant bacteria and resistance gene removal, careful attention should be given to the AR aspect of engineering systems in space crafts to treat not only water/wastewater but air and physical surfaces as well (NASA, 2016).

Education plays a massive role in building the foundations for good practices in antimicrobial resistance stewardship—for informing policy makers, health professionals, citizens at large, and indeed, all of society. This book is intended to serve as an educational platform for strengthening conversations between the dimensions of microbiology, health, and engineering such that antimicrobial resistance as a public health threat can diminish to a more manageable condition. Hopefully the next generation of innovative solutions in wastewater treatment and application of responsible practices in antimicrobial stewardship will ensure that the lifetime of the antibiotic era can be prolonged well into the future.

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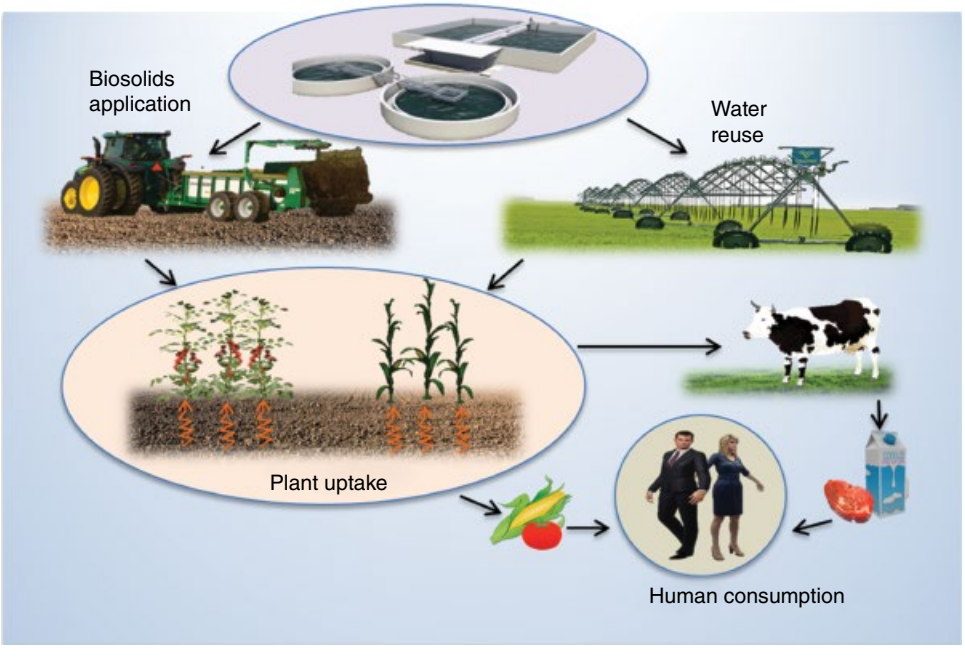


**Figure 5.1** Schematic diagram illustrating how antimicrobials can be introduced into the human food systems. Water reuse, which is a common practice in arid and semiarid regions, uses treated wastewater to irrigate food crops; biosolids are typically land-applied as fertilizer. Both practices can introduce antimicrobial residues into the human food cycle.

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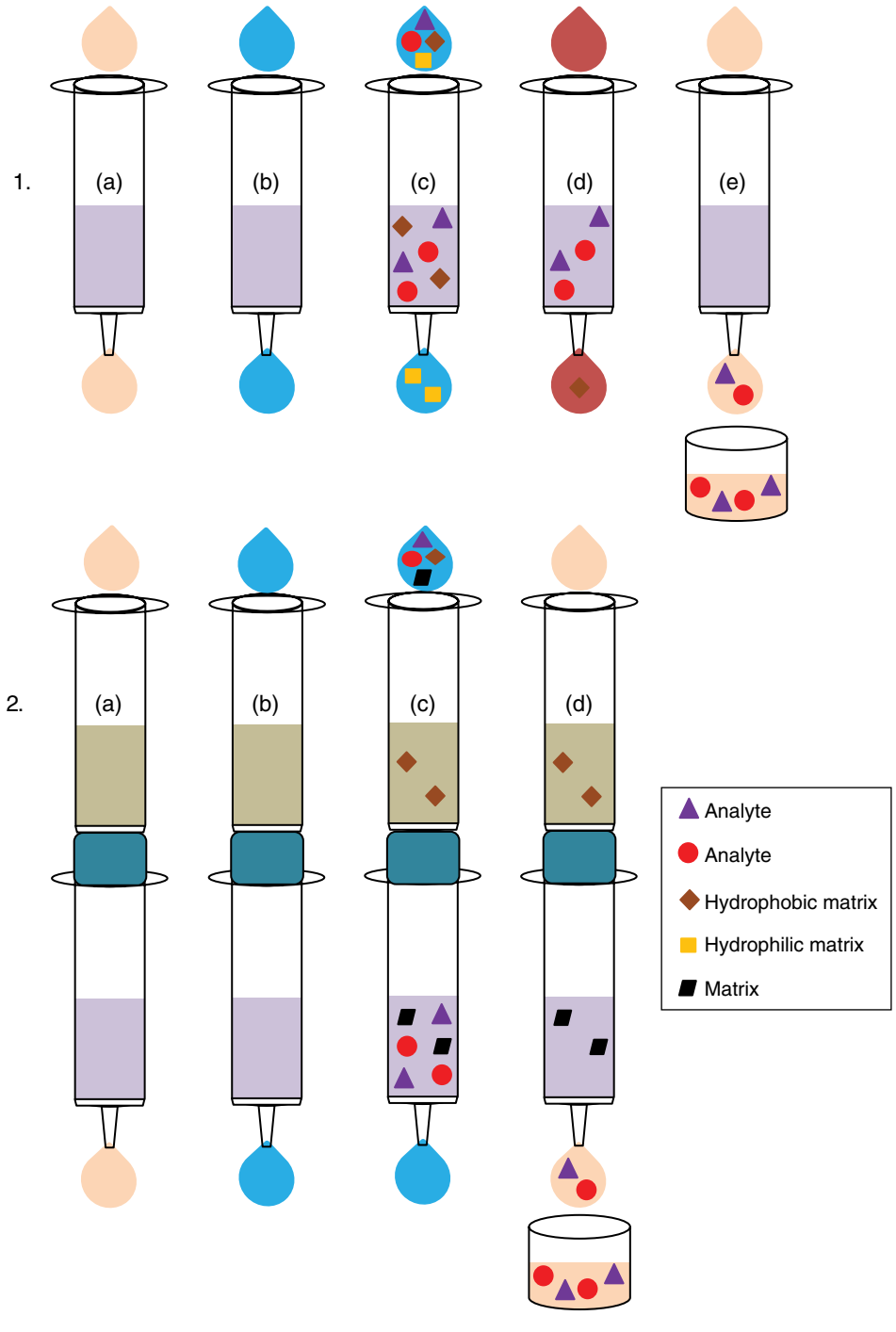



**Figure 5.2** Two different solid phase extraction strategies for the analysis of antimicrobials in aqueous samples.

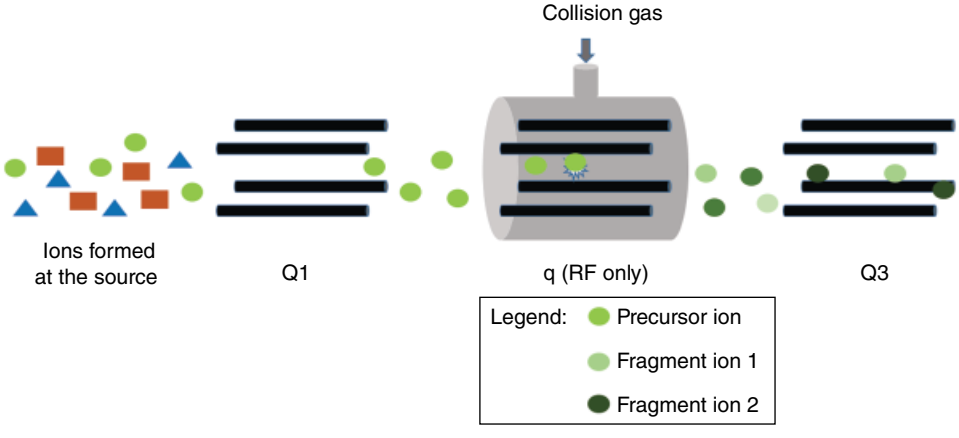



**Figure 5.3** Schematic diagram of how ions are fragmented inside a QqQ. After ions are formed at the source, they are directed to Q1, where the quadrupole filters the masses and sends the precursor ion of interest to q, where collision-induced fragmentation is accomplished by bombarding the precursor ion with a specific collision gas. The fragment ions are sent to Q3 for mass filtering then subsequently sent to the detector.

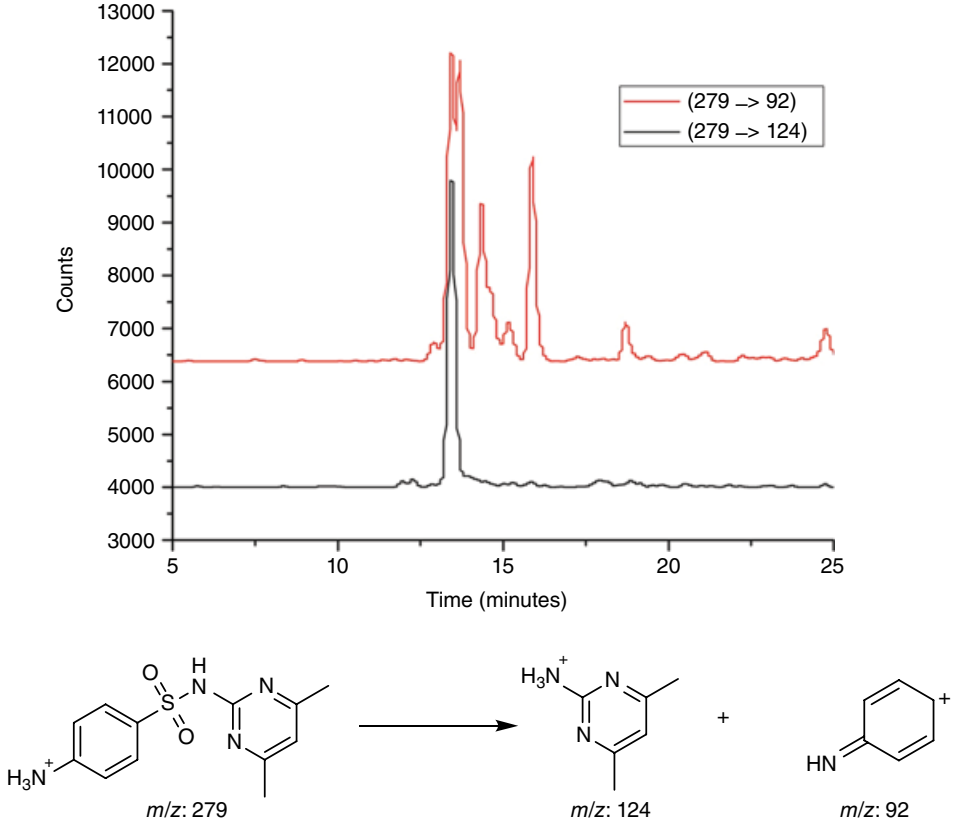



**Figure 5.4** By monitoring two fragments per antimicrobial compound, sulfamethazine in this example, one is able to discriminate between the real peak (overlapping red (top) and black (bottom) trace) and interfering peaks in wastewater matrix. The structure of sulfamethazine and its fragment ions are also shown.

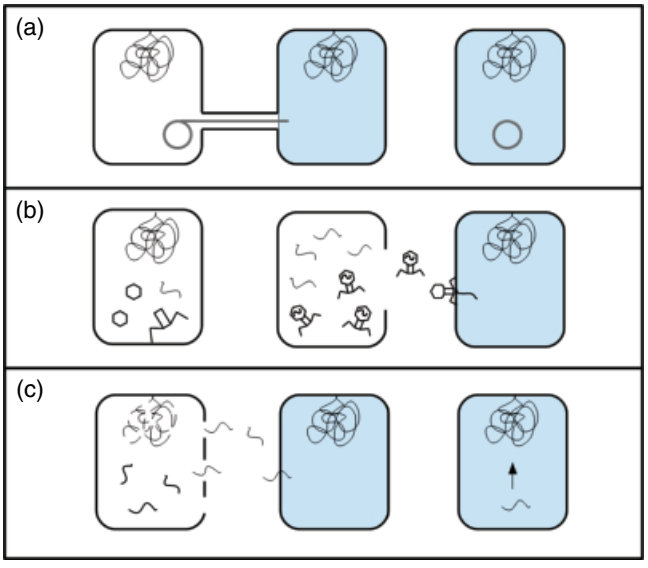



**Figure 8.1** Primary mechanisms of HGT in bacteria. Donor cell: white; recipient cell: gray. (a) Conjugation: transfer of conjugative plasmids or conjugative transposons through direct cell-to-cell link established by the donor cell. (b) Transduction: host genetic material may sometimes be included in the capsule of the forming phages and subsequently be transferred to a new host cell by infection. (c) Transformation: naked DNA is taken up by the cell from its surrounding environment, integrated into the host genome and expressed.

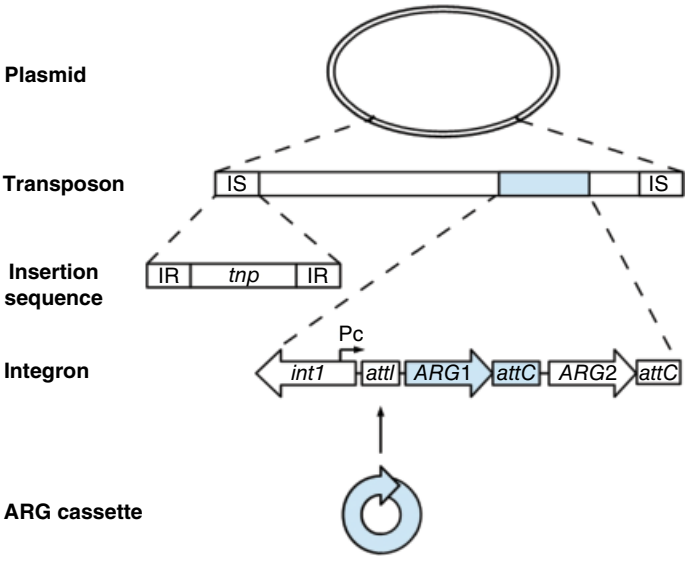



**Figure 8.2** Schematic representation of MGEs in hypothetical hierarchical configuration. In transposons, the insertion sequence (IS) consists of two inverted repeats (IRs) flanking a transposase (*tnp*) gene; these sequences guide mobilization and integration of the whole transposon. In Class 1 integrons, GCs (in this case an ARG-harbouring cassette) consist of a functional genetic element (e.g., ARG1 or ARG2) and an *attC* site. Integrase1, encoded by *int1*, promotes integration of GCs by facilitating recombination between *attC* and *attI* sites. The *Pc* promoter, located in the *int1* sequences, promotes the expression of the acquired GCs.

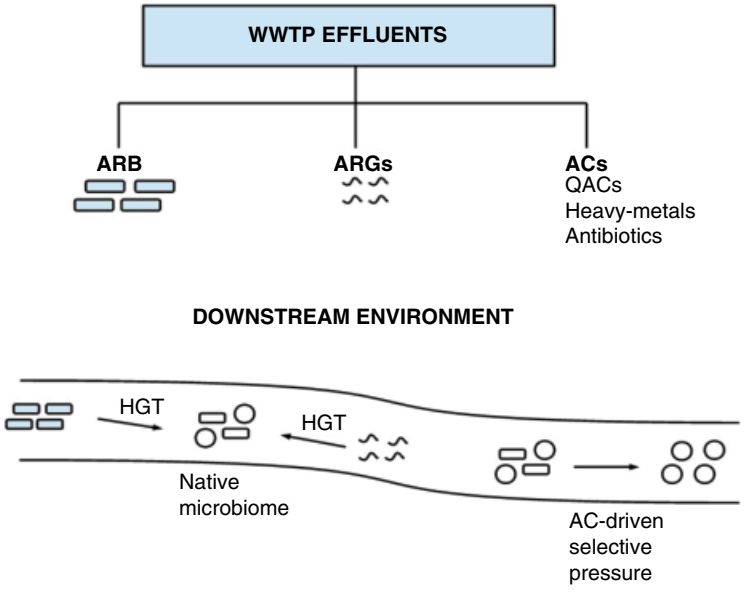



**Figure 8.3** Hypothetical scenario depicting the impact of WWTP effluents on downstream environments. Bacteria from native aquatic and terrestrial microbiomes can acquire ARGs by HGT of effluent-derived ARB or ARGs, by conjugation or transformation, respectively. Additionally, ACs in effluents can exhert selective pressure on the native microbiome, enhancing soil- or water-born ARG abundance.

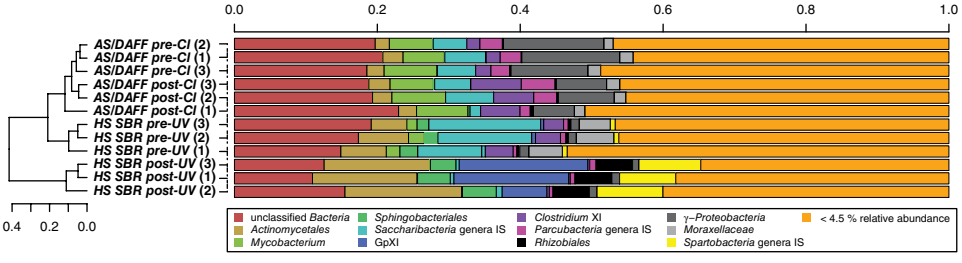



**Figure 9.1** Taxonomic annotation-based hierarchical clustering of dominant taxa (>4.5% of the total community) in pre- and postdisinfection effluent samples from two colocated wastewater treatment plants in Australia. AS/DAFF indicates activated sludge (AS) treatment followed by dissolved air flotation and filtration (DAFF). HS/SBR is a sequencing batch reactor (SBR) treating high salinity (HS) influent. Results are based on total DNA extraction from 1L water samples (0.22 µm filtered) and Illumina MiSeq partial 16S rRNA gene sequencing. This figure shows triplicate samples from a single sampling date for each site, including pre- and post- chlorination at the AS/DAFF plant and pre- and post-UV disinfection at the HS/SBR plant.

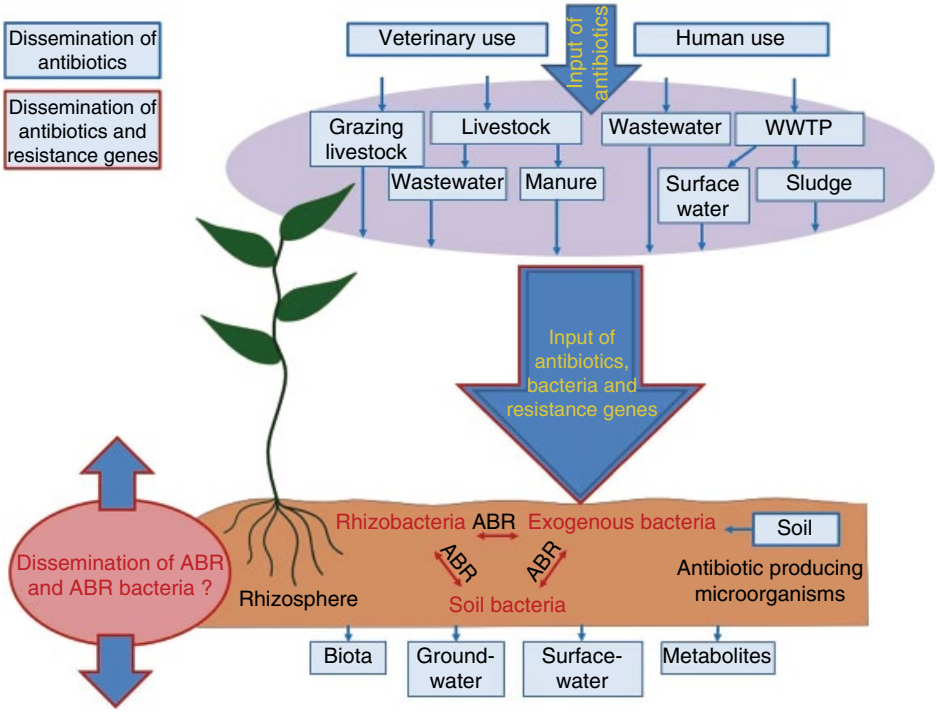
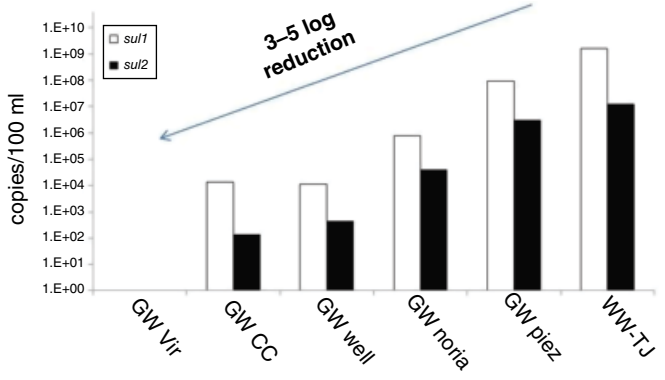



**Figure 11.1** Spread of antibiotics, bacteria, and resistance genes in the environment. By anthropogenic activities (e.g., manure application and wastewater irrigation), antibiotics (and other selectors for resistance genes), bacteria and antibiotic resistance genes are released into the environment. This might promote their accumulation and dissemination in the soil (transfer of antibiotic resistance genes from exogenous to soil bacteria and rhizobacteria) and might lead to the distribution of resistant bacteria via biological and physical forces (e.g., by wildlife, wind, water, or by consumption of near-ground plants). ABR = antibiotic resistance/antibiotic resistant; WWTP = wastewater treatment plant.

(a)




(b)

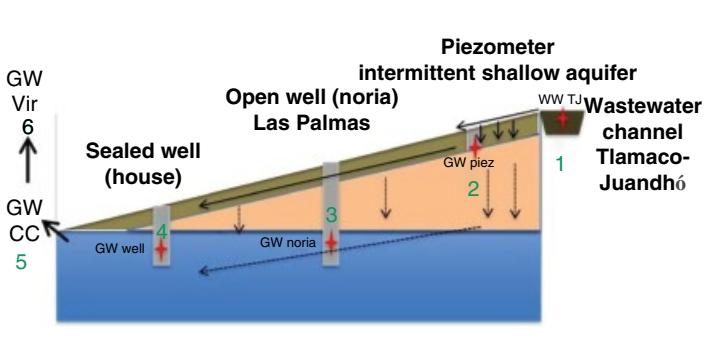



**Figure 11.2** Reduction of concentrations of antibiotic resistance genes by SAT (modified from Broszat, 2013). (a) Reduction of *sul* genes during SAT. GW Vir = spring water after chlorination; GW CC = spring recharged by SAT-treated WW; GW well = sealed well; GW noria = open well; GW piez = piezometer installed in 3 m depth; WW-TJ = WW channel. (b) Sampling sites 1: WW channel, 2: piezometer in 3 m depth, 3: open well, 4: sealed well, 5: spring, 6: chlorinated spring water. WW = wastewater; GW = groundwater.

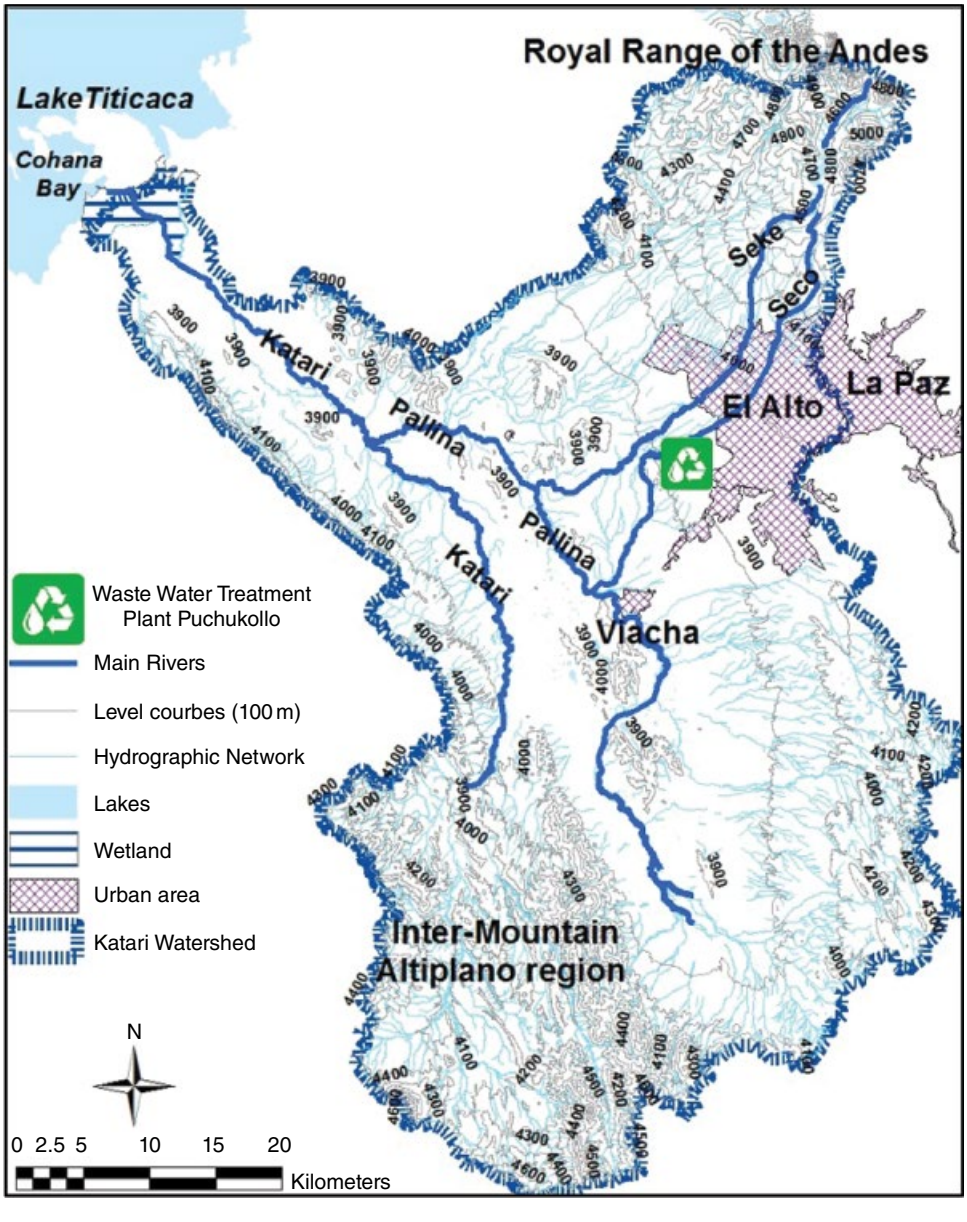



**Figure 16.1** The Katari watershed: hydrography, topography and urban area.

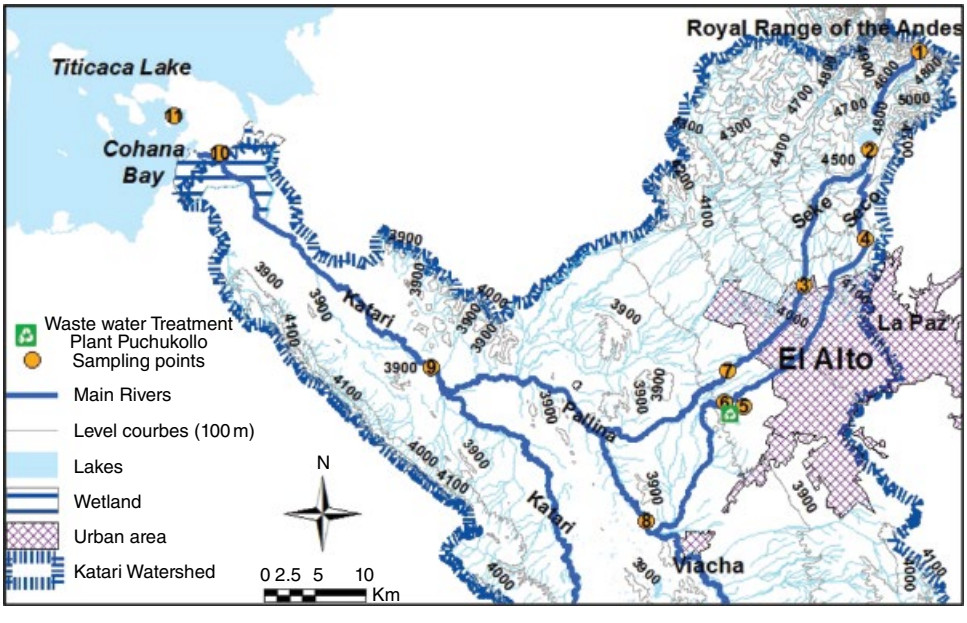



**Figure 16.2** Surface waters sampling points for the determination of antibiotic concentrations and bacterial resistance to antibiotics. Sampling point 5 corresponds to wastewaters entering the WWTP. Sampling point 6 corresponds to treated wastewater discharge from the WWTP.

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