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Biosensors for the Environmental Monitoring of Aquatic Systems

**Bioanalytical and Chemical
Methods for Endocrine Disruptors**

**The Handbook of
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Bioanalytical and Chemical Methods
for Endocrine Disruptors

Volume Editors: Damià Barceló · Peter-Diedrich Hansen

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The Handbook of Environmental Chemistry, Subseries 5 ISSN 1433-6863

ISBN 978-3-540-00278-9

e-ISBN 978-3-540-36253-1

DOI 10.1007/978-3-540-36253-1

Springer Dordrecht Heidelberg London New York

Library of Congress Control Number: 2009926177

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Cover design: SPi Publisher Services

Printed on acid-free paper

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Preface

This book on “Biosensors for the Environmental Monitoring of Aquatic Systems” is based on the scientific developments and results achieved within a group of European Union (EU) funded projects that will be briefly discussed below. Indeed, everything started with the development of biosensors for environmental measurements in 1991 as part of the EC Environment Programme (1991–1994) under Area II: Technologies and engineering for the environment, section 1: Assessment of environmental quality and monitoring. In the context of this Programme, a biosensor was defined as a compact analytical device containing a biological or biologically derived sensing element (e.g., enzymes antibodies, microorganisms, or DNA) either integrated with or in intimate contact with a physicochemical transducer (e.g., electrochemical, optical, thermometric, or piezoelectric). The first European Workshop on Biosensors for Environmental Monitoring took place at the Technische Universität Berlin (BIT - Berlin Institute of Technology) in February 1993, organized by Prof. P.D. Hansen and Dr. J. Büsing. This was the first focused workshop of a series of workshops in London (1994) organized by P. Bennetto and J. Büsing, Florence (1995), Barcelona (1996), Freising (1997), Kinsale (1998), Paris (1999), Cascais (2000), and Alcala de Henares (2001). All these workshops covered a large variety of technical and scientific topics related to biosensors for environmental monitoring with additional presentations of the progress reports of the specific EU projects. The workshop in Florence had already presented ten biosensor projects supported under the EC Environment Programme, dealing with pesticide detection, microbiological contamination, and xenobiotics in water and air. This workshop involved some 100 scientists from 15 European countries and from Japan. The objective of the workshop in Florence (1995) was to present the state of European research activities supported under the EC Environment and Climate Programme (1995–1998) in the field of biosensor development and to identify priorities for future Research & Development.

During that period the European Commission’s Environment and Climate Research Programme supported a three-year co-ordination project to guide technological developments in Biosensors for Environmental Technology (BIOSET). The aim of BIOSET was to enhance the development of biosensors for practical applications in monitoring pollutants in the environment. BIOSET maintained close contact with European industry and explored possibilities for collaboration with the commercial

sector. This ensured eventual transfer of the technology demonstrated at the workshops and Technical Meetings to marketable devices. BIOSET provided assistance for researchers from European laboratories to meet to exchange ideas, use equipment, and establish a basis for new joint projects. The secretariat of the Concerted Action BIOSET supported the Technical Meetings.

There were three Technical Meetings held, two in Berlin in 1997 and 1998, and the third in Barcelona, in April 2000. The goal of these technical meetings was to join different research and industrial teams to evaluate the performance of their biosensor technology in field conditions with common and standardized surface and waste waters.

As a result of these field experiments, the additional information that biosensors can offer to environmental monitoring was also evaluated. Thus, these three Technical Meetings were useful accompanying measures and practical additions to the currently organized yearly workshops. The concerted action BIOSET was followed by the SENSPOL network. The 1st SENSPOL Workshop was held on the 9–11 May 2001 on Sensing Technologies for Contaminated Sites and Groundwater at the University of Alcala. There was one special Workshop on “Genotoxicity Biosensing (TECHNOTOX)” supported by the European Commission DG XII D-1 and BIOSET in the year 2000. The TECHNOTOX meeting at the Flemish Institute for Technological Research (VITO) in Mol was organized by Phillippe Corbisier (VITO), Peter-D. Hansen (TU Berlin) and Damia Barcelo (CSIC Barcelona). Thirty scientists participated in this meeting and 14 genotoxicity tests were performed on-site simultaneously on a maximum of 11 samples (chemical compounds and environmental samples). The AMES Test (ISO 16240) and the well-accepted umuC Test (ISO 13829) were performed as reference genotoxicity tests. The panel of genotoxicity tests included six prokaryotic tests, one eukaryotic test, four mammalian tests, one DNA test, and one bacterial test. Four assays were commercially available. It was demonstrated that no single test is capable of detecting all relevant genotoxins. Therefore, a Test Battery for genotoxicity is recommended (see the chapter by T. Grummt et al., this volume). The concept was to transform the standardized and harmonized microplate assay into an automated on-line assay and/or finally into a biosensor. The EuCyanobacteria Electrode was one of the first biosensors developed under the EC Environment Programme (1991–1994) initiated by Jürgen Büsing. The EuCyanobacteria Electrode was developed and optimized as an on-stream biosensor for pesticide detection (EV5V-CT92-0104). The EuCyanobacteria Electrode, in principle a so-called Rawson sensor, was transformed into a single rod electrode. The EuCyanobacteria Electrode with detection limits for atrazine <10 microgram per liter and diuron <2.5 microgram per liter was implemented in the monitoring programme on the river Rhine after the SANDOZ disaster in 1986 and optimized in several research projects in the following years. Special catch system devices were coupled with secondary electrochemical methods of signal transduction based on enzyme or whole-cell reactions. With the aid of mass spectrometry it was possible to evaluate the bio-recognition and detection components.

The Technical Meetings showed that biosensors are useful tools for controlling the degree of pollution of surface waters and of WWTP (wastewater treatment plants).

All biosensors evaluated showed much higher “alarm” values for the influent waste waters as compared to the final treated WWTP effluent. This is already remarkable, since the main advantage of the different biosensors evaluated, like BOD, ToxAlert, or phenols is that the measurements are being carried out very rapidly—a maximum of 20 minutes—so the information is available immediately. This is certainly an advantage over conventional methods that require several days, either for the BOD-5 or for conventional chromatographic/mass spectrometric methods.

The contributions in this book intend to summarize some of the practical achievements in the field of biosensors for environmental monitoring in Europe focusing, mainly, on examples of chemical groups of analytes (or effects) in different water matrices and more specific aspects of biosensor technology. The target objective of this book is to provide an overview of biosensors as a practical alternative and complementary or additional measurement methodology to traditional chromatographic techniques. Emphasis will also be given to the validation of the applied technology and its application to real-world environmental samples. The various chapters cover examples in different areas that are listed below:

General parameters of water quality. Biochemical Oxygen Demand (BOD) which is usually determined by measuring the oxygen consumption of a mixed culture over 5 days (BOD5). In the Third Technical Meeting held in Barcelona, the commercial firm Biosensores presented an on-line biosensor technique for the determination of BOD. This on-line biosensor device was installed in the la Llagosta wastewater treatment plant and after the Technical meeting, was installed in two more WWTP for a period of one month. This is an example of the implementation of biosensors into real-world situations, since the authorities are already using two of their devices for routine control.

Trace organics in surface water. The River ANALyzer (RIANA), an optical immunosensor coupled to a FIA system based on a competitive solid-phase fluorescence immunoassay with immobilized analyte derivative and free fluorescence-labeled anti-atrazine or anti-simazine antibodies has been developed and applied for the measurement of chlorotriazine pesticides in river water samples. A pilot monitoring survey in the Ebre area (Tarragona, Spain) was carried out over a three-month period. Simazine and atrazine were detected at levels ranging from 0.5 to 1 microgram per liter and in soil samples from 19 to 40 g kg⁻¹. Phenylurea herbicides and paraquat were also determined by this immunosensor approach. The validation of the RIANA biosensor for the determination of phenylurea herbicides was carried out by LC-MS whereas in the case of paraquat capillary electrophoresis(CE)-UV detection was used. Enzymatic biosensors based on catalytic inhibition have been widely used and applied for the analysis of organophosphorus and carbamate pesticides in water samples. A cholinesterase biosensor using acetylthiocholine chloride as substrate was able to distinguish between different batches of water containing 3–10 ppb levels of pesticides.

Toxic and endocrine-disrupting effects. There are different methods of measuring toxicity. Two of the most commonly used methods are Microtox® and the recently developed ToxAlert® system. Although they are not strictly biosensors, they are a

kind of bioprobe used in a standardized way to measure toxicity of the effluents. They are widely accepted and internationally recognized. During the Third Field Experiment held in Barcelona, Spain more data was generated and a variety of cytotoxic compounds were identified in the wastewater treatment plant during this field experiment. The efficiency of the WWTP was evaluated by collecting and analyzing samples at various stages of the water treatment process. The chemical compounds identified could explain the toxicity observed at the different steps of the WWTP. Endocrine-disrupting effects are today widely studied. One of the most current measurements is the so-called vitellogenin (VTG) level in male fish. If VTG levels are high as compared to control values, exposure to endocrine-disrupting chemicals can be concluded. This approach has been used for the development of a biosensor called VITELLO within the EU projects PRENDISENSOR and SANDRINE. Within the EU project SANDRINE different biosensors, bioassays, and receptor assays are being developed for detecting endocrine-disrupting compounds in WWTP. In this respect, MCF-7 and vitellogenin by the ELRA and Vitello sensor of the SANDRINE project are a few of the different assays in use. A practical example was the determination of the presence and the effects of endocrine-disrupting compounds in two tributaries of the Llobregat river (NE Spain). Water samples and carp, *Cyprinus carpio*, were collected from selected sites along a transect, for chemical and biological determination, respectively. Western blot analysis of male carp plasma using a polyclonal antibody raised in the cyprinid Koi carp, detected this protein in all samples, the VTG increase being more evident at the vicinity of the treatment plants. A certain correlation was also found between NP in water and VTG induction in fish ($r = 0.75$). Within the EU project ALGAETOX a bio-analytical system was developed with the immunotoxic relevant endpoint. Phagocytosis was measured by oxidative burst (ROS) and luminescence. There is now a recent application of this system in the COLUMBUS/BIOLAB programme. The bio-analytical system contributes to risk assessment concerning immunotoxicity under space-flight conditions. A schematic work flow demonstrates the close connection between environmental chemistry and the development and validation of a biosensor.

All these promising results demonstrate some of the European efforts to implement biosensor technology and other assays, including toxicological relevant assays and ELISA, in environmental analysis. The developments achieved during the last years by the different EU Environment Programs have permitted us to show some key examples of the application of biosensors for environmental monitoring, demonstrating not only cost-effective alternatives for screening but also effect-related readouts where new and unpredictable approaches are expected. Biosensor technology has reached maturity for BOD, pesticides, and phenols incorporating whole cells, affinity or catalytic events with electrochemical or optical transduction. Complex and difficult analytes (e.g., surfactants) are progressively being approached by biosensors. The large variety of analytes, the complexity of environmental matrices, the low limits of detection, and the legislative compliance should not shadow the progress achieved in this area. On the contrary, biosensor research is

offering new strategies for the evaluation of biological effects where cocktails of compounds may show synergistic, additive, or antagonistic effects that will remain unsolved by chromatographic or spectroscopic techniques. The challenge for environmental biosensors remains open and further research in this area will only prove that new parameters are needed for safer and more complete environmental risk assessment. It is expected that collaborative work, focused research, and interdisciplinary approaches may lead to biosensor indexes that will certainly contribute to a better knowledge and real-time characterization of environmental samples. The book is organized to provide information on relevant principles, novel biosensor techniques and their applications. It is hoped that it will stimulate much discussion and bring new ideas to one of the most exciting and environmentally beneficial areas of environmental chemistry and eco-toxicology. It is hoped that the book and its methods contribute to the broader goal of conserving and protecting our aquatic ecosystems and their biota for future generations.

The book will be of interest to a broad audience of analytical chemists, biologists, environmental chemists, water management operators, and technologists working in the field of wastewater treatment, or newcomers who want to learn more about the topic using new measurement devices such as biosensors. Finally, we would like to thank all the contributing authors of this book for their time and effort in preparing this comprehensive compilation of research papers. Special thanks to Dr. Juergen Büsing for “pushing” this topic since 1991 at the EU level thus allowing excellent scientific and technological developments. The book that you have in your hands is part of such achievements.

February 2009

Damià Barcelò
Peter-Diedrich Hansen

Contents

Biosensors for Environmental Monitoring at Global Scale and the EU Level	1
Marinella Farré, Sara Rodriguez-Mozaz, Miren López de Alda, Damià Barceló, and Peter-Diedrich Hansen	
Achievements of the RIANA and AWACSS EU Projects: Immunosensors for the Determination of Pesticides, Endocrine Disrupting Chemicals and Pharmaceuticals.....	33
Sara Rodriguez-Mozaz, Maria J. López de Alda, and Damià Barceló	
Biosensors for Pharmaceuticals and Emerging Contaminants Based on Novel Micro and Nanotechnology Approaches.....	47
Javier Adrián, Fátima Fernández, Alejandro Muriano, Raquel Obregon, Javier Ramón-Azcon, Nuria Tort, and M.-Pilar Marco	
Recombinant Yeast Assays and Gene Expression Assays for the Analysis of Endocrine Disruption	69
Benjamin Piña, Susanna Boronat, Marta Casado, and Alba Olivares	
Biosensors for Aquatic Toxicology Evaluation	115
Marinella Farré and Damià Barceló	
Genetically Engineered Bacteria for Genotoxicity Assessment	161
Alva Biran, Rami Pedahzur, Sebastian Buchinger, Georg Reifferscheid, and Shimshon Belkin	

Adverse Effects in Aquatic Ecosystems: Genotoxicity as a Priority Measurement	187
Tamara Grummt, Petra Rettberg, Petra Waldmann, Jürgen Zipperle, and Peter-Diedrich Hansen	
Genotoxicity in the Environment (Eco-Genotoxicity)	203
P. D. Hansen, E. Wittekindt, J. Sherry, E. Unruh, H. Dizer, H. Tüg, H. Rosenthal, V. Dethlefsen, and H. von Westernhagen	
Liquid Chromatography–Mass Spectrometry Methods for Analysis of Endocrine-Disrupting Chemicals in Wastewaters.....	227
Cristina Postigo, Marina Kuster, Marta Villagrasa, Sara Rodríguez-Mozaz, Rikke Brix, Marinel la Farré, Miren López de Alda, and Damià Barceló	
Index.....	273

Biosensors for Environmental Monitoring at Global Scale and the EU Level

Marinella Farré, Sara Rodriguez-Mozaz, Miren López de Alda, Damià Barceló, and Peter-Diedrich Hansen

Abstract Biosensor technology is based on a specific biological recognition element in combination with a transducer for signal processing. Since their inception, biosensors have been expected to play a significant analytical role in environmental monitoring. The use of biological data to complement chemical analysis, as well as the development of biosensors and other biological approaches, has grown steadily in recent years. However, the commercialization of biosensor technology has significantly lagged behind their search output as reflected by a plethora of publications and patenting activities. The rationale behind the slow and limited technology transfer could be attributed to cost considerations and some key technical barriers.

During the last 15 years a relevant support has been provided by public institutions for biosensor research in the USA, Japan and, especially, in Europe. In addition, biomonitoring is an essential tool for rapid and cost-effective environmental monitoring. For this reason, biosensor technology has been considered as a key tool for the complete implementation of the new European Union (EU) directives, such as the Water Framework Directive (WFD), and other related directives such as the recent Marine Strategy Framework Directive.

Analytical chemistry has changed considerably, driven by automation, miniaturization and system integration with high throughput for multiple tasks. Such requirements pose a great challenge in biosensor technology where the biosensor is

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often designed to detect a single or a few target analytes. Successful biosensors must be versatile to support interchangeable biorecognition elements, and, in addition, miniaturization must be feasible to allow automation for parallel sensing with ease of operation at a competitive cost. A significant up front investment in research and development is a prerequisite in the commercialization of biosensors. The progress in such endeavours is incremented with limited success, thus, the market entry for a new venture is very difficult unless a niche product can be developed with a considerable market volume.

Recent technological developments in the miniaturization of electronics and wireless communication technology have led to the emergence of Environmental Sensor Networks (ESN). These will greatly enhance monitoring of the natural environment and in some cases open up new techniques for taking measurements or allow previously impossible deployments of sensors.

Herein we present the principles, advantages and limitations of biosensor technology for environmental diagnosis, with special emphasis on those based on nanomaterials and technologies for remote biosensing developed under the support provided by public institutions for research in USA, Japan and Europe.

Keywords Biosensor, Environmental monitoring, Network, Sensor, Water Framework Directive

Contents

1	Introduction.....	2
2	Biosensor Research Funding Schemes	4
3	Biosensors.....	7
3.1	Classes and Fundamentals	7
3.2	Biosensors for Environmental Monitoring	11
3.3	Autonomous Biosensor Wireless Networks.....	22
4	Future Trends and Conclusions.....	25
	References.....	26

1 Introduction

Because of the difficulty of predicting the collective effects of the increasing number of pollutants in receiving ecosystems, there is a need for screening methods in environmental monitoring.

The implementation of safety programs calls for environmental analysis comprising two parts: screening methods based on high-throughput analysis and capable of continuous monitoring on-line at site with low costs, and analysis of the positive samples with confirmatory analytical techniques.

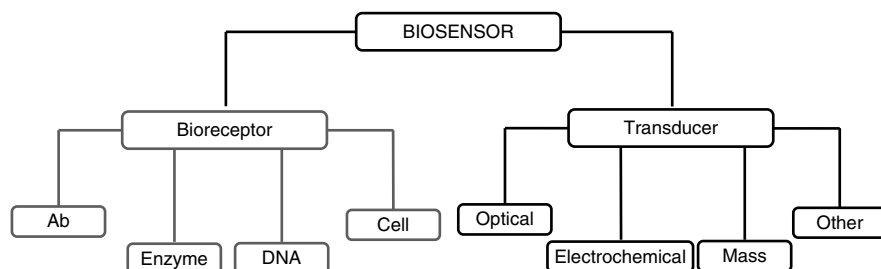


Fig. 1 General scheme of biosensors

Bioresponse-linked instrumental analysis combines two processes, biological recognition initiating a biological effect, and chemical analysis. The biomolecular components are used as targets for active substances. Although it is possible to apply, for example, binding assays that provide effects-linked equivalents, information on the responsible contaminants is only accessible by chemical analysis. Therefore a subsequent step is provided by the chemical analysis of the substances that are bound by the biorecognition components and that are therefore bio-effective.

Biosensors are usually classified either according to the transduction element (e.g. electrochemical, optical, piezoelectrical, or thermal), or according to the biorecognition principle (enzymatic, immunoaffinity recognition, whole-cell sensors, or DNA) (see Fig. 1).

The main advantages of biosensors are short times of analysis, low cost of assays, and possibility of portability, real-time measurements and remote control. These new technologies have been applied equally in the quantitative analysis of target analytes and in ecotoxicological measurements.

At present, biomonitoring is an essential tool for the complete implementation of the EU directives, such as the Water Framework Directive (WFD), and the Marine Strategy Framework directive. Nevertheless, many of these new technologies are still being developed and rely on combined efforts from diverse scientific fields. In Europe, the European Commission (EC) has been the flagship for the development of these new technologies in environmental analysis. Under the 3rd, 4th, 5th and 6th Framework Programs (FP) a great number of funded research projects were dedicated to the development of new analytical tools, and more than 300 projects on sensors, biosensors and biological techniques have been carried out [1].

Several reviews on biosensors, optical sensors for different analytical fields, or biological techniques for environmental analysis have been published during the last years: see Rodríguez-Mozaz [1], González-Martínez [2], Kurosawa [3], Farré [4].

Herein we aim to present the principles, advantages and limitations of biosensor technology for environmental monitoring, and summarize the advances obtained in this field under the support provided by public institutions.

2 Biosensor Research Funding Schemes

Europe, USA and Japan have been trend leaders in biosensor research and manufacture, especially for health, food and environmental analysis.

The European research funding in the area of biosensors for environmental monitoring as a specific topic started in 1992 and the first workshop was held in Berlin in 1993. A selection of projects developed in the past years under different framework programs is listed in Table 1.

During the first EU Framework Programmes, FP4 and FP5, funding was devoted to supporting research projects related to the determination of pesticides in water, in support to the European Union Drinking Water Directive (98/83/EC).

Table 1 Research projects involving biosensors supported by European Union funding

Title, acronym	Contract no.	Duration
Optical biosensing techniques for monitoring organic pollutants in the aquatic environment, BIOPTICAS	EV5V-CT92-0067	1993–1996
Development of new multi-sensing biosensors for the detection of bioavailable heavy metals in solid matrices, BIOMETSENSOR	ENV4-CT95-0141	1996–1999
Integrated immuno extraction sampling and portable biosensor prototype for in field monitoring, INExSPORT	ENV4-CT97-0476	1997–2000
Prediction of the behaviour of potential endocrine disruptors in soils using vitellogenin ELISA as biosensors, PRENDISENSOR	ENV4-CT97-0473	1997–2000
The application of integrated biosensors with antibody and macrocyclic receptor libraries in the measurement of algal cells and toxins in water, ALGAETOX	ENV4-CT98-0784	1999–2002
Development of improved detection systems for monitoring of toxic heavy metals in contaminated groundwaters and soils, DIMDESMOTOM	EVK1-CT-1999-00002	2000–2003
Protection of groundwater resources at industrially contaminated sites, PURE	EVK1-CT-1999-00030	2000–2003
Automated water analyzer computer supported system, AWACSS	EVK1-CT-2000-00045	2001–2004
Artificial recharge demonstration project, ARTDEMO	EVK1-CT-2002-00114	2002–2005
Water catchment areas: tools for management and control of hazardous compounds, WATCH	EVK1-CT-2000-00059	2001–2004
A biological nanoactuator as a molecular switch for biosensing, BIONANO-SWITCH	Specific Targeted Research Project 43288	2006–2009
Tools and technologies for the analysis and synthesis of nanostructures TASNANO	NMP-2003-3.4.1.4-1	2005–2007
Diagnostic NANotech and MICrotech Sensors, DINAMICS	Integrated Project 26804	2007–2010

A good example of such projects was BIOPTICAS, which was followed up by RIANA, AWACSS and SANDRINE being the most successful projects at the EU level in that area. In the frame of these projects biomolecular receptors were obtained, and different biosensor configurations were developed and applied to real water samples. Under the RIANA and AWACSS projects optical immunosensors were developed and validated against several groups of organic pollutants, for example, for the simultaneous determination of pesticides and estrogens in natural water samples [5]. It is important to highlight BIOSET and SENSPOL concerted actions, which have been organizing various annual workshops and technical meetings on different areas of biosensors for environmental monitoring in order to stimulate research in that topic in Europe and to demonstrate the applicability of biosensors under real-world conditions.

At the same time, in the USA, “biodefence” and research with potential contribution to national security was an issue of particular concern, and biosensors for biohazards detection were developed [6, 7]. In addition, important initiatives were carried out under the auspices of the National Science Foundation (NSF), which funded biosensing network proposals, and “The National Centre For Environmental Research” (NCER), which funds research grants and graduate fellowships in numerous environmental science and engineering disciplines. The Office of Naval Research also promoted the science and technology programs through government laboratories, such as the “Naval Research Laboratory”, which is one of the leading groups in biosensors research in the USA. They have developed several single and multi-analyte biosensors for toxins, bacteria and virus detection in clinical and environmental samples. Another remarkable research group, the biosensor group at UC Davis undertakes research into the development of miniaturized, fast and sensitive biosensors for use in environmental research and monitoring.

Regarding biosensor research in Japan, it is important to mention that bio-computing and nanotechnology are areas of increasing interest. One of the main research groups devoted to biosensor development is Karube’s group in the “Research Centre for Advanced Science and Technology”, at the University of Tokyo. Japan Fund for Global Environment (JFGE) provides grants for environmental conservation activities. For example, support is provided for activities, such as conserving air and water quality, and organizing symposiums, seminars and workshops. For example, Biosensors 2002, the Seventh World Congress on Biosensors, was held in Kyoto, Japan.

More recently, in Europe under FP6 less funding was directly devoted to biosensors for environmental monitoring; however, other important fields related to the development of environmental biosensing were boosted, such as nanotechnology. Some examples of research funded projects in this period are BIONANO-SWITCH, BIOMED-nano, OPTANANOGEN, TASNANO, DINAMICS and ISAMCO, most of them related to nanotechnology and material science advancements. For example, BIONANO-SWITCH is still on-going, and the main aim is the development of a nanoactuator/biosensor system.

The BIOMED-nano STREP-project aimed at improving enzymatic electron transfer reactions for application towards integrated bio-powered biosensing systems for diagnosis and healthcare. Such improvements were based on novel enzymes; modification of enzymes; design of novel nano-structured scaffolds for enzyme immobilization, in order to obtain biosensing devices with improved stability and electron transfer efficiency. Another example was OPTONANOGEN: Integrated opto-nanomechanical biosensor for functional genomic analysis. The aim of this project was to develop a portable genotoxicity biosensor microsystem able to detect hybridization of nucleic acids with sensitivity to distinguish single base substitutions, deletions and insertions.

In addition to initiatives for biosensing development, an important restriction is the successful marketing of biosensors for environmental applications. In order to overcome implementation barriers and bridge the gap between innovative and accepted techniques for environmental monitoring in Europe an Environmental Technology Verification (ETV) system has been developed following the model of the US and Canada. Therefore, Europe is now heading towards an Environmental Technology Verification System. Under the 6th FP several projects have been devoted to the development of this verification system including PROMOTE-ETV, Eurodemo, Testnet, AirETV and Tritech-ETV. A proposal for an EU framework on ETV is planned to be published by the European Commission in autumn 2008.

Research based on nanotechnology has been extremely important worldwide, and has been a new motor for biosensors development. In addition to the use of nanomaterials to improve analytical properties of new biosensors, the development of nanobiosensing devices is one of the main promising perspectives for future developments.

In this sense, during the last years in the US the National Science Foundation (NSF), through the Directorate for Engineering and the Directorate for Computer and Information Science and Engineering, carried out a broad interdisciplinary program of research and education in the area of advanced sensor and biosensors development. An example of successful achievements has been the creation of the world's first all-integrated sensor circuit based on nanowire arrays, combining light sensors and electronics made of different crystalline materials, by Scientists at the U.S. Department of Energy's Lawrence Berkeley National Laboratory and the University of California at Berkeley [8, 9]. Several projects are under development such as MEMS-Based Magnetic Probe Microscopy Project, in which a scanning-probe magnetic microscope based on a high-resolution magnetic tunnel junction (MTJ) sensor is under construction. The completed microscope will be used to characterize the magnetic properties of ferromagnetic metal samples.

In the same way the Japan Science and Technological Agency has been promoting a number of initiatives focused on nanobiosensors.

In Europe the 7th FP presents a thematic area on Nanosciences, nanotechnologies, materials and new production technologies. Also under the 7th FP, the first call has included funding for projects to develop sensors and biosensors platforms for remote environmental monitoring. The last tendency in Europe is research in new autonomous platforms able to operate on-line in an autonomous manner.

3 Biosensors

Biosensors can be defined as an integrated receptor-transducer device, which is capable of providing selective quantitative or semi-quantitative analytical information using a biological recognition element. A biosensor converts a biological event into a detectable signal by the action of a transducer and a processor.

The automation and the miniaturization of biological analytical techniques, as well as the development of on-line and remote measurement equipment, can be achieved through biosensor technology. In recent years a growing number of biosensor applications have been developed for environmental analysis [10] for targeted analysis and for monitoring whole biological effects such as toxicity or estrogenicity [11].

3.1 *Classes and Fundamentals*

Biosensors can be classified according to the bioreceptor elements involved in the recognition and according to the physicochemical transduction elements.

The main classes of bio-receptors applied in environmental analysis of organic pollution are: enzymes, antibodies, DNA and whole cells.

On the other hand, there are four basic groups of transduction elements: electrochemical, optical, mass sensitive and thermal sensors, with most of the biosensors described in the literature being electrochemical biosensors. The following sections discuss the main classes of biosensors according to the transduction element.

3.1.1 **Electrochemical Transduction**

Electrochemical sensors measure the electrochemical changes that occur when chemicals interact with a sensing surface of the detecting electrode. The electrical changes can be based on a change in the measured voltage between the electrodes (potentiometric), a change in the measured current at a given applied voltage (amperometric), or a change in the ability of the sensing material to transport charge (conductometric). Such effects may be stimulated electrically or may result in a spontaneous interaction at the zero-current condition. Voltammetric, amperometric, potentiometric, potentiometric solid electrolyte gas sensor and chemically sensitized field effect transistor (CHEMFET) are subgroups that may be distinguished.

The principle operation of amperometric biosensors is defined by a constant potential applied between a working and a reference electrode. The imposed potential promotes a redox reaction, which produces a current. The magnitude of this current is proportional to the concentration of electro active species present in solution.

Oxidase enzymes have been the most frequently investigated and applied. A number of amperometric biosensors are based on the monitoring of oxygen consumption, or hydrogen peroxide generation. Both are electrochemically active; oxygen can be electrochemically reduced, and hydrogen peroxide can be oxidized. The current generated is proportional to the concentration of the enzyme substrate present in a sample.

The use of mediators should permit one to replace oxygen as an electron acceptor and to operate at a much lower potential, reducing the effects of other electrochemically active species found in complex matrices.

Potentiometric biosensors are based on the monitoring of the potential at a working electrode, with respect to a reference electrode.

During the last few years this type of transduction has achieved a great development. Recent research has led to powerful nanomaterial-based electrical biosensing devices and examines future prospects and challenges. New nanoparticle-based signal amplification and coding strategies for bioaffinity assays are in use, along with carbon-nanotube molecular wires for achieving efficient electrical communication with redox enzyme and nanowire-based label-free DNA sensors.

3.1.2 Optical Transducers

Optical transducers are based on various technologies of optical phenomena, which are the result of an interaction of an analyte with the receptor part. Optical transduction may employ linear optical phenomena, including adsorption, fluorescence, phosphorescence, polarization, rotation, interference, etc., or non-linear phenomena, such as second harmonic generation.

Reflectometric techniques are based on a white incident light passing the interface between different refractive indices, which will be reflected in part. These reflected beams superimpose and build a characteristic interference spectrum. The binding of a biological receptor such as an antibody to the surface changes the thickness of the toggling layer, which causes a change in the reflectance spectrum (Fig. 2).

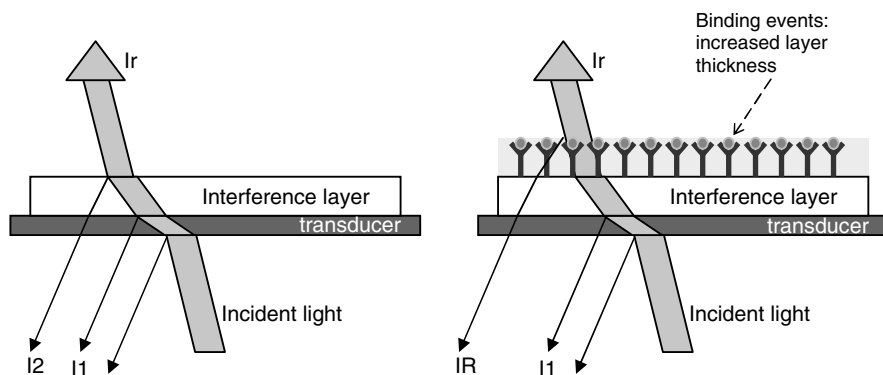


Fig. 2 Reflectometric based biosensors scheme

Thus, the interaction process between the bioreceptor and the analyte can be detected time-resolved [12].

Interferometry has also been exploited for biosensor development. When an immunoreaction takes place on the waveguide surface, it produces a change in the refractive-index profile within the evanescent field volume; correspondingly, the effective refractive index of the waveguide system is changed. In Mach-Zehnder interferometry (MZI), an optical waveguide is split into two arms and after a certain distance they are recombined again. The sensor arm will be exposed to a variation of the refractive index due to a biorecognition reaction such as an immunoreaction in the sensor channel. Over this distance, light travelling in the sensing arm will experience a phase shift in comparison with guided light in the reference arm [13].

Optical waveguide lightmode spectroscopy (OWLS) is a new sensing technique using an evanescent field for the in situ and label-free study of surface processes at molecular levels. It is based on the precise measurement of the resonance angle of linearly polarized laser light, diffracted by a grating and incoupled into a thin waveguide layer. The incoupling is a resonance phenomenon that occurs at a defined angle of incidence that depends on the refractive index of the medium covering the surface of the waveguide. In the waveguide layer, light is guided by total internal reflection to the edges where it is detected by photodiodes. By varying the angle of incidence of the light, the spectrum is obtained from which the effective refractive indexes are calculated for both electrical and magnetic field waves [14].

Total internal reflection fluorescence (TIRF) has been used with planar and fibre optic waveguides as signal transducers in a number of reported biosensors. In these transducers, light is propagated down a waveguide which generates an electromagnetic wave (evanescent wave) at the surface of the optically denser medium of the waveguide and the adjacent less optically dense medium. The amplitude of the standing wave decreases exponentially with distance into the lower refractive index material. The fluorescence of a fluorophore excited within the evanescent field can be collected.

At low angles, total internal reflection results when light propagating within a dense medium (e.g. quartz) reaches an interface with a less dense medium (e.g. aqueous solution). Although the light is fully reflected, an evanescent field is generated that extends beyond the interface and into the aqueous solution. Typically, the penetration depth (or the thickness of evanescent field) is in the range of half the wavelength of the light. The evanescent field provides the surface selectivity of TIRF. Only fluorophores adsorbed, adhered, or bound to the surface will be excited and therefore fluoresce. Conversely, fluorophores in bulk solution will not be excited. Therefore, if the surface is made biologically active so that one may "trap" fluorescently labelled compounds of interest, one can detect analytes within complex sample solutions. Because the excitation light is totally reflected away from the detection, one can easily discriminate the fluorescence signal from the excitation light and achieve high sensitivities and low detection limits. TIRF systems provide measurement of real time kinetics of a bioanalyte's binding to a surface immobilized sensor molecule. TIRF is a fast, non-destructive, sensitive and versatile technique that is well suited for monitoring biomolecular interactions. TIRF allows monitoring conformational

changes, orientation changes and lateral mobility of biomolecules. TIRF has been used in a high number of biosensors. Examples of application are RIANA and AWACSS devices [10, 15, 16, 17].

The measurement based on evanescent waves has been exploited for environmental biosensor development. Evanescent waves propagating from waveguides can be used to probe only the sensing film to decrease the optical background signal from the sample.

Another important type of transduction is *surface plasmon resonance* (SPR). A surface plasmon wave can be described as a light-induced collective oscillation in electron density at the interface between a metal and a dielectric. At SPR, most incident photons are either absorbed or scattered at the metal/dielectric interface and, consequently, reflected light is greatly attenuated. The resonance wavelength and angle of incidence depend upon the permittivity of the metal and dielectric. SPR biosensors are based on the special electromagnetic waves—surface plasmon polaritons—to probe interactions between an analyte in solution and a biomolecular recognition element immobilized on the SPR sensor surface. Under the frame of different EU projects different SPR biosensors have been developed for environmental applications. Under the MENDOS project one of the main objectives was the development of an SPR optical sensor system and application for its assessing and screening endocrine disrupting compounds (EDCs).

Fluorescence/luminescence occurs when a valence electron is excited from its ground state to an excited singlet state. The excitation is produced by the absorption of light of sufficient energy [18]. The common principle of luminescence immunosensors is that an indicator or chemical reagent placed inside or on an immunoreactor is used as a mediator to produce an observable optical signal. Typically, conventional techniques, such as spectrometers, are employed to measure changes in the optical signal. This approach has been reviewed by Schobel et al. [19]

The general advantages of the optical techniques are the speed and the reproducibility of the measurement, and the main drawback is the high cost of the apparatus. Several applications [20, 21, 22, 23] have been described during the last years for environmental analysis; some of them are revised in the next section.

Optical biosensors offer different advantages such as low limits of detection and robustness and, are, in general, suitable for screening a large number of samples simultaneously; however, as a main limitation they cannot be easily miniaturized.

3.1.3 Mass-Sensitive Sensors

Measurements of small changes in mass is a transduction form that has been used for biosensor development [24, 25]. Piezoelectric devices and surface acoustic wave devices can be grouped under this category.

The vibration of piezoelectric crystals produces an oscillating electric field in which the resonant frequency of the crystal depends on its chemical nature, size, shape and mass. These crystals can be made to vibrate at a specific frequency of oscillation which is dependant on the electric frequency. The frequency of oscillation is therefore

dependant on the electrical frequency applied to the crystal as well as the crystal's mass. When the mass increases due to the binding of analytes, the oscillation frequency of the crystal changes and the change can be measured. The general equation of crystal microbalances can be summarized as follows when the change in mass (m) is very small compared to the total mass of the crystal:

$$\Delta f = Cf^2 \Delta m/A,$$

where f is the vibration frequency of the crystal in the circuit, A is the area of the electrode and C is a constant determined in part by the crystal material and thickness. Piezoelectric crystals, sometimes referred to as quartz crystal microbalances (QCM), are typically made of quartz and operate at frequencies between 1 and 10MHz. These devices can operate in liquids with a frequency determination limit of 0.1Hz; the detection limit of mass bound to the electrode surface is about 10^{-10} to 10^{-11} g.

Acoustic wave devices, made of piezoelectric materials, are the most common sensors, which bend when a voltage is applied to the crystal. Acoustic wave sensors are operated by applying an oscillating voltage at the resonant frequency of the crystal, and measuring the change in resonant frequency when the target analyte interacts with the sensing surface.

Limitations for this transduction method involve format and calibration requirements, which are time consuming.

3.1.4 Thermometric Sensors

Thermometric sensors are based on the measurement of the heat effects of a specific chemical reaction or adsorption which involve the analyte. In this group, the heat effects may be measured in various ways, for example in the catalytic sensors the heat of a combustion reaction or an enzymatic reaction is measured by use of a thermistor. Calorimetric biosensors detect variations of heat during a biological reaction.

3.2 *Biosensors for Environmental Monitoring*

The following sections discuss the biosensors developed and applied in environmental monitoring, classified according to the biological recognition element.

3.2.1 Enzyme Biosensors

Enzyme electrodes have been widely used for monitoring a wide range of clinically or environmentally important substrates, especially using oxido-reductases (as tyrosinase, peroxidase and laccase) [26, 27], and hydrolases (choline estearases)[28].

Enzyme biosensors can be distinguished into those that measure the inhibition of a specific enzyme due to the presence of target analytes, and those that measure the catalytic transformation of target analytes by a specific enzyme.

Most of the transduction elements associated to enzyme-based biosensors are electrochemical: amperometric or potentiometric. Some examples of environmental applications of electrochemical biosensors are reported in Table 2.

Enzymes typically used in amperometric biosensors are oxidases. The main advantage of this class of transducer is the low cost; it is also highly reproducible and a wide variety of disposable electrodes are often available. The instrumentation is also very easy to obtain and can be inexpensive and compact; this allows for the possibility of on-site measurements. Limitations for amperometric measurements include potential interferences to the response if electroactive compounds are present in the sample, because they can generate false current values.

The development of electrochemical DNA and protein sensors on screen-printed electrodes (SPEs) based on the catalytic activity of hydrazine has been described by Shiddiky et al. [51] A high sensitive flow enzymatic biosensor for the detection of 2,4-D, atrazine and ziram has been reported by Kim et al. [52]. The same group [53] has also recently reported the preparation of an optimized coverage of gold nanoparticles at a tyrosinase electrode for the measurement of the pesticide in various water samples. Screen-printed electrodes containing immobilized acetylcholine esterase (AChE) were used for the electrochemical determination of organophosphorous and carbamate pesticides [54]. In this work, the extent of AChE deactivation by the pesticides was determined in the presence of acetylcholine (AChCl) substrate. In this approach AChE was attached to the SPE by in situ bulk polymerization of acrylamide to ensure efficient adherence within the membrane with minimal losses in enzyme activity. Responses were observed for the pesticides monocrotophos, malathion, metasytox and lannate over the concentration range 0–10 $\mu\text{g L}^{-1}$.

Most of the recent advances in electrochemical biosensors have been based on nanotechnology. The huge interest in nanomaterials is driven by their many desirable properties. In particular, the ability to tailor the size and structure and hence the properties of nanomaterials offers excellent prospects for designing novel sensing systems and enhancing the performance of the bioanalytical assay.

An extremely important challenge in amperometric enzyme electrodes is the establishment of satisfactory electrical communication between the active site of the enzyme and the electrode surface. The redox centre of most oxidoreductases is electrically insulated by a protein shell. Because of this shell, the enzyme cannot be oxidized or reduced at an electrode at any potential. In general, there is a need for co-substrates or mediators to achieve efficient transduction of the biorecognition event. Aligned carbon nanotubes (CNT), prepared by self assembly, can act as molecular wires to allow electrical communication between the underlying electrode and redox proteins (covalently attached to the ends of the single wall nanotubes (SWNT))[55, 56].

The catalytic properties of metal nanoparticles have also facilitated the electrical contact of redox centres of proteins with electrode surfaces. For example, gold nanoparticles were shown to be extremely useful as electron relays for the alignment of glucose oxidase on conducting supports and wiring its redox centre [57].

A wide range of enzyme electrodes based on dehydrogenase or oxidase enzymes rely on amperometric monitoring of the liberated NADH or hydrogen peroxide products. The anodic detection of these species at ordinary electrodes is often hampered by the large over voltage encountered for their oxidation. The greatly enhanced redox

Table 2 Examples of electrochemical biosensors reported for environmental analysis

Analyte/effect	Biosensor: Type AND Basis	Matrix	Limit of detection	Reference
Carbamates	AChE, BChE: Potentiometric detection	Synthetic samples	1.5×10^{-5} – 2.5×10^{-3} M	[29]
Dimethyl- and diethyl-dithiocarbamates	Tyrosinase amperometric detection	Aqueous solution	0.2 mM	[30]
2,4,6-Trichloroanisole	Amperometric immunosensor	Water	1 ng mL ⁻¹	[31]
2,4-Dichlorophenoxyacetic acid	Amperometric immunosensor	Water	0.1 µg L ⁻¹	[32]
Bisphenol A	Tyrosinase immobilized on different carbon carriers with amperometric detection	Drinking and surface water	0.02 µM	[33]
Bisphenol A	Potentiometric immunosensor	Water	0.6 ng mL ⁻¹	[34]
Bisphenol A, 17-β-estradiol	Tyrosinase-modified boron-doped diamond electrodes with amperometric detection	Water	10^{-6} M for bisphenol A	[35]
Catechol, <i>p</i> -cresol, phenol, <i>p</i> -chlorophenol, and <i>p</i> -methylcatechol	Tyrosinase immobilized in titania sol-gel with amperometric detection	Spring and surface water	9×10^{-8} M	[36]
Dichlorvos	AChE inhibition amperometric detection	River water	70 nM	[37]
Fenitrothion and ethyl <i>p</i> -nitrophenol thiobenzenephosphonate	Amperometric whole-cell biosensor	Water	1.4 ppb of fenitrothion and 1.6 ppb of EPN	[38]
Genotoxicity	DNA amperometric detection	Wastewater		[39]
Genotoxicity	Voltammetric DNA			[40]
Genotoxicity	DNA electrochemical biosensors using four types of DNA (calf thymus ssDNA, calf thymus dsDNA, salmon testis ssDNA and salmon testis dsDNA) were constructed using graphite screen printed electrodes	Water		[41]
Heavy metals	Amperometric DNA	Water	4.0×10^{-11} , 1.0×10^{-10} , 1.0×10^{-9} , and 5.0×10^{-9} M for Cu(II), Pb(II), Cd(II), and Fe(III), respectively	[42]
Organophosphates	Single-walled carbon nanotube (SWNT) amperometric whole-cell biosensor	Water	Phosphate levels in the marine system were 0.04 mg L ⁻¹	[43]

(continued)

Table 2 (continued)

Analyte/effect	Biosensor: Type AND Basis	Matrix	Limit of detection	Reference
Parathion	Parathion hidrolase enzyme action with amperometric detection	River water	10ngmL ⁻¹	[44]
PAT gene	Nano-SiO2/p-aminothiophenol self-assembled films, DNA Impedance measurement	Water	1.0×10 ⁻¹¹ ML ⁻¹	[45]
Phenol, <i>o</i> -cresol, <i>p</i> -cresol, <i>m</i> -cresol, catechol, dopamine, and ephinephrine	Multi-walled carbon nanotube-Nafion-Tyrosinase nanobiocomposites with amperometric detection	Aqueous solution	303µAmM ⁻¹	[46]
Polycyclic aromatic hydrocarbons	Amperometric immunosensor based on screen-printed electrodes	Water	Phenanthrene LOD of 2ngmL ⁻¹	[47]
Progesterone	Colloidal gold-graphite-Teflon-Tyrosinase composite with amperometric detection	Milk	0.43ngmL ⁻¹	[48]
Surfactants	Amperometric bacterial biosensor	Water	0.25mgL ⁻¹	[49]
Toxicity	Amperometric whole-cell biosensor	Water and wastewater		[50]
Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE)				
Phosphinothricin acetyltransferase (PAT) gene				

activity of hydrogen peroxide [58] and NADH [59] at CNT-modified electrodes addresses these over voltage limitations. The ability of CNT to promote electron transfer reactions is attributed to the presence of edge plane defects at their end caps.

The deposition of platinum nanoparticles onto CNT has led to further improvements in the detection of the enzymatically liberated peroxide species [60]. In addition to CNT films, it is possible to use CNT-based inks [61]. The excellent electrocatalytic properties of metal nanoparticles (compared to bulk metal electrodes) can also benefit amperometric enzyme electrodes. For example, You et al. [62] dispersed iridium nanoparticles in graphite-like carbon for improved amperometric biosensing of glutamate.

Another promising and controllable route for preparing conducting-polymer nanowire enzyme sensors involves electro-deposition within the channel between electrodes [63].

Recently, a lactate electrochemical biosensor with a titanate nanotube as a direct electron transfer promoter has been reported by Yang et al. [64]. In this work hydrogen titanate ($\text{H}_2\text{Ti}_3\text{O}_7$) nanotubes were synthesized by a one-step hydrothermal processing. Lactate oxidase (LOx) enzyme was immobilized on the three-dimensional porous nanotube network to make an electrochemical biosensor for lactate detection. Cyclic voltammetry and amperometry tests revealed that the LOx enzymes, which were supported on tunnelling nanotubes (TNTs), maintain their substrate-specific catalytic activity. The nanotubes offer the pathway for direct electron transfer between the electrode surface and the active redox centres of LOx, which enables the biosensor to operate at a low working potential and to avoid the influence of the presence of O_2 on the amperometric current response.

In another recent study Kochana et al. [36] reported the development of a titania sol-gel-derived tyrosinase-based amperometric biosensor for determination of phenolic compounds in water samples.

3.2.2 Immunosensors

The recognition elements are immunochemical interactions between antibody and antigen. This type of device combines the principles of solid-phase immunoassay with physicochemical transduction elements (electrochemical, optical, piezoelectric, evanescent wave and surface plasmon resonance).

Because most small molecular weight organic pollutants in the environment have few distinguishing optical or electrochemical characteristics, the detection of stoichiometric binding of these compounds to antibodies is typically accomplished using competitive binding-assay formats. The main limitation of these techniques is the electrochemical detection of the immunoreaction, because it is necessary to use enzymes that will generate electrochemically active compounds.

Electrochemical immunosensors have been widely used for environmental analysis in amperometric, potentiometric and conductimetric configurations. Amperometric immunosensors measure the current generated by oxidation or reduction of redox substances at the electrode surface, which is held at an appropriate electrical potential. A high number of successful applications have been reported during the last decade. Among them Wilmer et al. determined 2,4-dichlorophenoxyacetic (2,4-D) acid in

water by an amperometric immunosensor with a limit of detection of $0.1\mu\text{gL}^{-1}$ [32], and the same group developed also a flow injection system [65]. Screen-printed electrodes also have been extensively used. An example is the system developed by Fahnrich et al. [47] for the detection of polycyclic aromatic hydrocarbons (PAHs). Moore et al. [31] described the development of a biosensor for the quantitative detection of 2,4,6-trichloroanisole.

Another configuration used in electrochemical immunosensors for environmental analysis has been based on the use of recombinant single-chain antibody (scAb) fragments, this approach was used for atrazine determination [66]. The label-free detection of bisphenol A using a potentiometric immunosensor was reported by Piao et al. [34] with a detection limit of 0.6 ngmL^{-1} .

The use of nanomaterials in order to improve electrochemical properties of immunosensors has also been studied. A nanostructured progesterone immunosensor using a tyrosinase-colloidal gold-graphite-Teflon biosensor as the amperometric transducer has been developed by the Pingarron group [48].

Acoustic transducers have also been applied in immunosensors for water analysis [67, 68]. The resonant frequency of an oscillating piezoelectric crystal can be affected by a change in mass at the crystal surface. Piezoelectric immunosensors are able to measure a small change in mass. Last publications have been based on immunosensors using a quartz crystal microbalance for the detection of trace amounts of chemical compounds, such as dioxins [3].

In general, a vast number of optical transduction techniques can be used for biosensor development. These may employ linear optical phenomena (e.g. adsorption, fluorescence, phosphorescence and polarization) or non-linear phenomena (e.g. second harmonic generation). The choice of a particular optical method depends on the analyte and the sensitivity needed.

Total internal reflection fluorescence has been used with planar and fibre-optic wave-guides as signal transducers in a number of biosensors. In these devices, light is propagated down a wave-guide, which generates an electromagnetic wave (evanescent wave) at the surface of the optically denser medium of the wave-guide and the adjacent less optically dense medium. The amplitude of the standing wave decreases exponentially with distance into the lower refractive index material. The fluorescence of a fluorophore excited within the evanescent field can be collected either outside the wave-guide, or by coupling the emission frequencies back into the wave-guide. The biological sensing element is immobilized on the side rather than at the end of the wave-guide. On the basis of the evanescent wave transducing principle, atrazine was detected at concentrations around $0.1\mu\text{gL}^{-1}$ [69] and cyclodiene insecticides in the μgL^{-1} range [70].

Binding of the bioreceptor to the transducer is an important aspect of immunosensor fabrication. A method for the immobilization of hytidine-target antibodies onto a gold surface for surface plasmon resonance measurements has been reported by different authors [71].

A significant high number of SPR immunoassays have been recently used for rapid analysis of pollutants in different water matrices. In these assays the incident light is reflected from the internal face of a prism in which the external face has

been coated with a thin metal film. At a critical angle, the intensity of the reflected light is lost to a resonant oscillation in the electrons created at the surface of the metal film. This method has been used to measure the binding of antibodies to antigens immobilized at the sensor surface because the critical angle depends on the refractive index of the materials of the metal surface. An assay approach was exploited by Nabok et al. [72] for in situ registration of low molecular weight toxins with specific antibodies immobilized onto the gold surface via a (poly)allylamine hydrochloride layer using an electrostatic self-assembly (ESA) technique. A simple and rapid detection method using surface plasmon resonance for dioxins, polychlorinated biphenyls and atrazine was described by Shimomura et al. [73, 74], with limits of detection ranging between 0.1 and 5 ng mL⁻¹, requiring 15 min for a single sample measurement. Mauriz et al. [75] presented an immunoassay based on a binding inhibition test using a portable surface plasmon resonance immunosensor for the continuous monitoring of the organophosphate pesticide chlorpyrifos in real water samples with limits of detection from 45 to 64 ng L⁻¹. Sensor reusability was assured through the formation of alkanethiol self-assembled monolayers (SAMs). Using the same principle Mauriz et al. [76] developed single and multi-analyte surface plasmon resonance assays for simultaneous detection of cholinesterase inhibiting pesticides.

An immunosensor chip utilizing surface plasmon resonance was fabricated for detecting carcinoembryonic antigen based on protein A-conjugated [77].

In another example, a SPR immunosensor was developed for the detection of 2,4-dinitrophenol at ultra-low concentrations (immunoassay range between 1 ppt and 1 ppb). The sensor strategy was based on a competitive immunoreaction between 2,4-dinitrophenol and a 2,4-dinitrophenol-protein conjugate, namely DNP-bovine serum albumin conjugate (DNP-BSA). Anti-2,4-dinitrophenol monoclonal antibody was immobilized on a gold thin-film coated SPR-sensor chip by means of a chemical coupling process [78].

Farré et al. [22] described the development of a part-per-trillion immunoassay also based on SPR for atrazine determination in natural waters.

Among this variety of different immunosensor systems two optical immunosensor devices for environmental applications must be mentioned: RIANA and AWACSS. These immunosensors are based on solid-phase fluoro-immunoassays combined with an optical transducer chip chemically modified with an analyte derivative. The RIANA immunosensor was successfully applied in different environmental applications [5], and, along the same lines, a new analytical system, AWACSS, was developed and evaluated to measure several organic pollutants at low nanogram per litre level in a few minutes of analysis without pre-treatment steps [10, 15, 16].

A summary of recent applications of optical biosensors for environmental analysis is presented in Table 3.

3.2.3 DNA Biosensors

DNA's structure is very sensitive to the influence of environmental pollutants, such as heavy metals [89, 90], polychlorinated biphenyls (PCBs) [91, 92] or polyaromatic

Table 3 Examples of optical biosensors for environmental monitoring

Analyte	Type and basis	Sensitivity	Reference
Pesticides and herbicides			
Atrazine	Inhibition assay optical detection	1–10 $\mu\text{g L}^{-1}$	[79]
Isoproturon	Indirect assay and TIR-fluorescence detection	0.01–0.14 $\mu\text{g L}^{-1}$	[17]
Triazines	Inhibition assay SPR detection	0.16 $\mu\text{g L}^{-1}$	[80]
Triazines	Inhibition assay surface refractive index change detection	15 $\mu\text{g mL}^{-1}$	[81]
Pesticides	Competitive immunoassays fluorescence detection		[10]
Carbaril	Competitive immunoassay SPR detection	54–64 ng L^{-1} 1.38 $\mu\text{g L}^{-1}$	[75]
DDT and related compounds	Competitive immunoassay SPR detection	15 ng L^{-1}	[82]
Phenols	Indirect assay and TIR-fluorescence detection	0.9, 1.0, 1.0, 3.0 μM and 0.038, 0.037, 0.056, 0.201 mM for 4-chlorophenol, phenol, <i>m</i> -cresol and <i>p</i> -cresol, respectively	[83]
Phenols			
Phenols, organic acids	Whole cell optical biosensor with immobilized bioreporter <i>Pseudomonas fluorescens</i> HK44	1.2 and 0.5 mg L^{-1} for naphthalene and salicylate	[84]
EDCs			
Hormones	Optical immunosensor based on fluorescence	Sub ng L^{-1}	[85]
Bisphenol A	Optical immunosensor based on fluorescence		[11]
Bisphenol A	SPR immunosensor		[86]
Surfactants			
Alkylphenols and their ethoxylates	Immunosensor using electrochemical transduction	$\mu\text{g L}^{-1}$ range	[87]
Nonylphenol	Immunosensor using electrochemical transduction	10 $\mu\text{g L}^{-1}$	[88]

compounds (PAHs) [93]. These substances are characterized by a great affinity to DNA, causing mutagenesis and carcinogenesis. So, it is very attractive to use DNA-containing systems, for example DNA-based biosensors [94, 95] to perform genotoxic assays, or for rapid testing of pollutants for mutagenic and carcinogenic activity. Lazarides et al. [96] presented gold colloidal-nanoparticle aggregates that were linked by short pieces of DNA [96] and that exhibited a colour change due to electromagnetic coupling between the gold non-spherical nanoparticles after DNA hybridization.

Electrochemical biosensors based on immobilized DNA and its parts integrate detection sensitivity with a high specificity of biomolecules. This reduces the

consumption of DNA and gives rise to the development of modern methods of analysis of DNA effectors, including toxic ones, in environmental and biological effects [97, 39, 98, 42, 40].

The development of electrical DNA hybridization biosensors has attracted considerable research efforts [99]. Such DNA sensing applications require high sensitivity through amplified transduction of the oligonucleotide interaction.

Nanoparticle-based amplification schemes have led to improved sensitivity of bioelectronic assays by several orders of magnitude. The groups of Wang [100] and Limoges [101], both in 2001, reported the use of colloidal gold tags for electronic detection of DNA hybridization. This protocol relies on capturing the nanoparticles to the hybridized target, followed by highly sensitive anodic stripping electrochemical measurement of the metal tracer. Electronic DNA hybridization assays have been extended to other metal tracers, including silver [102] and iron [103]. Commonly the coupling of the biorecognition element to surfaces of magnetic beads offers an effective minimization of non-specific binding.

The hybridization of probe-coated magnetic beads with the metal-tagged targets results in three-dimensional network structures of magnetic beads, cross-linked through the DNA and gold nanoparticles. The placing of such a magnetic-bead/DNA/metal-label assembly onto the electrode leads to direct contact of the metal label and the surface and enables chronopotentiometric measurements without dissolving the metal tag [104].

Several amplification processes can be used to dramatically enhance the sensitivity of particle-based bioelectronic assays.

Treatment of the gold-linked DNA-hybrid assembly with silver ions in the presence of hydroquinone results in catalytic deposition of silver on the gold tracer (acting as a catalyst), leading to a dramatic signal amplification [105].

Wang et al. [106] described a triple amplification bioassay, coupling the carrier-sphere amplifying units (loaded with numerous gold nanoparticle tags) with the “built-in” pre-concentration of the electrochemical stripping detection and a catalytic enlargement of the multiple gold-particle tags. The success of these and other nanoparticle-based amplification strategies depends on maintaining a low background response.

Other strategies are based on the use of inorganic nanocrystals offering an electro diverse population of electrical tags as needed for designing electronic coding. Inorganic-nanocrystal tracers have been used for a multi-target electronic detection of DNA [103] or proteins [107]. Three encoding nanoparticles (zinc sulphide, cadmium sulphide and lead sulphide) have thus been used to differentiate the signals of three protein targets in connection with a sandwich immunoassay and stripping voltammetry of the corresponding metals. Each binding yields a distinct voltammetric peak corresponding to a different antigen. This concept can be scaled up and multiplexed.

Nanoparticle-induced changes in the conductivity across a microelectrode gap can also be exploited for highly sensitive and selective electronic detection of DNA hybridization [108]. One-dimensional nanowires can also be used for bridging two closely spaced electrodes for label-free DNA detection. For example, a p-type silicon

nanowire functionalized with PNA probes has been shown to be extremely useful for real-time label-free conductometric monitoring of the hybridization event [109]. This relies on the binding of the negatively charged DNA target that leads to an increase in conductance, reflecting the increased surface charge.

Similar improvements have been reported in connection with nanowires and carbon nanotubes functionalized with other receptor molecules.

Ramanathan et al. [63] demonstrated the real-time monitoring of nanomolar concentrations of biotin at an avidin-embedded polypyrrole nanowire.

Carbon nanotubes can also lead to ultrasensitive bioelectronic detection of DNA hybridization [110, 111]. For example, carbon nanotubes can be used as carriers for several thousands of enzyme tags and for accumulating the *a*-naphthol product of the enzymatic reaction. Such a carbon nanotube-derived double-step amplification pathway (of both the recognition and transduction events) allows the detection of DNA down to the 1.3 zmol level.

Several works developed different electrochemical DNA systems for environmental analysis. Disposable electrochemical DNA-based biosensors have been used for the determination of low molecular weight compounds with affinity for nucleic acids and for the detection of the hybridization reaction. The rapid screening of genotoxic compounds using the molecular interaction between surface-linked DNA and the target pollutants or drugs has been applied in different configurations.

Genotoxic compounds were measured by their effect on the oxidation signal of the guanine peak of calf thymus DNA immobilized on the electrode surface and investigated by chronopotentiometric analysis by Mascini et al. [111]. This type of DNA biosensor is able to detect known intercalating compounds, such as daunomycin, polychlorinated biphenyls, aflatoxin B1 and aromatic amines. Applicability to river and waste water samples is also demonstrated [112]. Disposable electrochemical sensors for the detection of a specific sequence of DNA were realized by immobilizing synthetic single-stranded oligonucleotides onto a graphite screen-printed electrode. The probes became hybridized with different concentrations of complementary sequences present in the sample. The hybrids formed on the electrode surface were evaluated by chronopotentiometric analysis using daunomycin as the indicator of the hybridization reaction.

Recently, Feng et al. [113] constructed gold nanoparticle/polyaniline nanotube membranes on a glassy carbon electrode (Au/nanoPAN/GCE) for sensing of DNA hybridization. The properties of the Au/nanoPAN/GCE and the characteristics of the immobilization and hybridization of DNA were studied by cyclic voltammetry, differential pulse voltammetry and electrochemical impedance spectroscopy. The synergistic effect of the two kinds of nanomaterials, nanogold and nanoPAN, could enhance dramatically the sensitivity of the DNA hybridization recognition.

A simple and sensitive electrochemical DNA biosensor based on in situ DNA amplification with nanosilver as the label and horseradish peroxidase (HRP) as the enhancer has been designed by Fu [114].

The good performance of DNA electrochemical biosensors has been proved in several works. For example, the electrochemical detection of harmful algae and

other microbial contaminants in coastal waters using hand-held biosensors has been reported by LaGier et al. [115].

3.2.4 Whole-Cell Biosensors

A microbial biosensor is an analytical device that couples microorganisms with a transducer to enable rapid, accurate and sensitive detection of target analytes in fields as diverse as medicine, environmental monitoring, defence, food processing and safety. Earlier microbial biosensors used the respiratory and metabolic functions of the microorganisms to detect a substance that was either a substrate or an inhibitor of these processes.

Recently, genetically engineered microorganisms based on fusing of the *lux*, *gfp* or *lacZ* gene reporters to an inducible gene promoter have been widely applied to assay toxicity and bioavailability.

Whole-cell biosensors in environmental analysis have been applied for the assessment of biological effects such as biological oxygen demand (BOD), toxicity, genotoxicity or estrogenicity, and also for the measurement of target pollutants or groups of pollutants. However, most applications have focussed on whole biological effects. Ecotoxicological assessment using whole-cell biosensors is reviewed in depth in the chapter by J. Adrián et al. (this volume).

Most reported transduction elements in bacterial biosensors are electrochemical and optical. One of the most relevant applications has been the determination of the biochemical oxygen demand [116].

Most BOD sensors rely on the measurement of the bacterial respiration rate in close proximity to a transducer, commonly the Clark type (an amperometric sensor developed by Clark in 1956 for measuring dissolved oxygen [117]). Some BOD sensors have been developed and marketed by various manufacturers in both biofilm and bioreactor-type configurations.

Instrument information about many BOD commercial biosensors was provided by Liu and Mattiason [117]. BOD biosensor systems still present a series of limitations that restrict their applications: the lack of standardization and legislation in most countries, complicated maintenance requirements, and insufficient resistance to various toxic compounds. It is possible to eliminate the toxic effects of heavy-metal ions by using a chelating agent that complexes the ions, for example ethylene diamine tetra-acetate (EDTA) or sodium diethyl dithiocarbamate (DDTC) [118]. Prevention of contamination by other microbes is also important.

However, the conventional standard method for the determination of BOD measures the microorganisms oxygen consumption/respiration over a period of 5 days [116] and is reported as BOD₅. While BOD₅ is a good indicator of the concentration of organic pollutants in water, it is extremely slow and hence not suitable for process control [119]. To address this limitation, several BOD biosensors based on amperometric oxygen electrode transducers modified with microorganisms degrading/metabolizing organic pollutants have been reported. The microbial strains used as the biological sensing

element include among others *Torulopsis candida* [120], *Trichosporon cutaneum* [121], *Pseudomonas putida* [122], *Klebsiella oxytoca* AS1 [123], *Bacillus subtilis* [118], *Arxula adenivorans* LS3 [124] and *Serratia marcescens* LSY4 [125].

However, bacterial biosensors using amperometry as the transduction method have also been relevant in other fields such as in environmental toxicity assessment. Neurotoxic organophosphate (OP) compounds have found wide application as pesticides and insecticides in agriculture and as chemical warfare agents for the military [119]. Amperometric biosensors based on genetically engineered *Moraxella* sp. and *P. putida* with surface-expressed organophosphorus hydrolase (OPH) have been developed by Lei et al. [119] for the detection of organophosphorus pesticides. The biosensor consisted of recombinant PNP-degrading/oxidizing bacteria *P. putida* JS444, and under the optimum operating conditions, the biosensor was able to measure as low as 55 ppb of paraxon and 53 ppb of met.

Recently, a novel single-walled carbon nanotube-based biosensor for real-time detection of organophosphate has been developed by Liu [43]. Horizontally aligned SWNTs were assembled to desirable electrodes using the AC dielectrophoresis technique. Organophosphorus hydrolase immobilized on the SWNTs by non-specific binding triggers enzymatic hydrolysis of organophosphates, such as paraoxon, consequently causing a detectable change in the conductance of the SWNTs. The conductance change is correlated to the concentration of organophosphate.

3.3 Autonomous Biosensor Wireless Networks

A wireless sensor network is a wireless network consisting of spatially distributed autonomous devices using sensors and/or biosensors to cooperatively monitor physical or environmental conditions, such as temperature, pressure, salinity, motion or pollutants, at different locations (Fig. 3). This approach could be in the near future one of the main advances for continuous environmental monitoring, and for the implementation of new EU directives, especially in environments difficult to monitor, such as coastal areas and the open sea.

Pioneers of internet-scale control were the TJ Watson IBM researchers Alex Morrow and Ron Ambrosio. They postulated that the future world would operate on the basis of complex interlocking control loops that range from localized sensor-actuator systems to platforms that aggregate information from multiple heterogeneous sources.

Today, molecular and biological events can be monitored and translated to digital signals by means of biosensors and this fact opens up a new window in analytical devices. This new approach could be a revolution in the future of environmental monitoring. However, the cost of reliable autonomous biosensing is still far too great for massively scaled-up deployments. Some of the drawbacks that should be first overcome are: the complexity of biosensing processes, the need for regular calibrations, which should be minimized, and the need for more stable biological reagents; energetic costs and residue production should also be minimized.

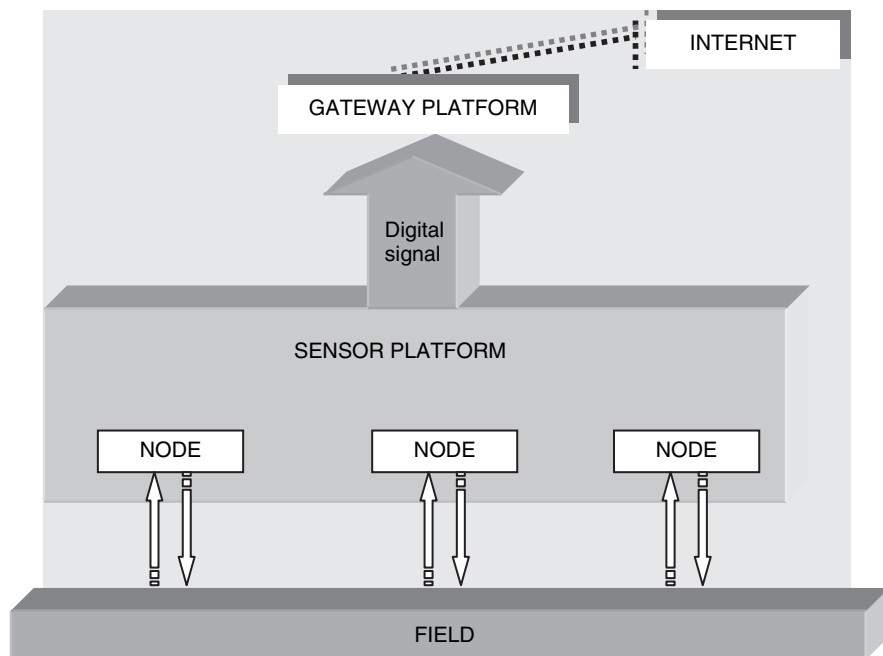


Fig. 3 Wireless sensors network diagram

Considerable advances have been made in recent years in hardware and software for building wireless biosensor networks. However, to ensure effective data gathering by biosensor networks for monitoring remote outdoor environments, the following problems remain:

- *Reactivity*: the ability of the network to react to its environment and provide only relevant data to users;
- *Robustness*: the ability of network nodes to function correctly in harsh outdoor environments;
- *Network lifetime*: maximizing the length of time the network is able to deliver data before nodes' batteries are exhausted.

The first step in wireless sensor networks is the development of autonomous blocks of sensing platforms. A sensor *node* is the smallest component of a sensor network that has integrated sensing and communication capabilities. It contains basic networking capabilities through wireless communications with other nodes as well as some data storage capacity and a microcontroller that performs basic processing operations. They usually come with a sensor board that usually slots onto the controller board, which allows the user to interface with other sensors, provided the signal is presented in the appropriate form for the controller. They also include a power supply, usually provided by an on-board battery.

In recent years, there has been a focus on power consumption as the small lithium button batteries commonly employed have limited lifetime and regular manual replacement is unrealistic. The sensor nodes within a wireless sensor network are also commonly referred to as “motes”. During the last years, the most widely used motes have been those provided by Crossbow Technologies Inc., based in San Jose, CA, which is a spin off from Berkeley.

The hardware requirements for wireless sensors include robust radio technology, a low-cost and energy-efficient processor, flexible signal inputs/outputs for linking a variety of sensors, a long-lifetime energy source, and a flexible, open source development platform. They also outline a number of software requirements for a wireless sensor node which include a small footprint capable of running on low-power processors, small memory requirement, and high modularity to aid software rescue. Thus, the basic components of a sensor node are a microcontroller, radio transceiver, set of transducers, and power source, and the software that runs on these nodes must be small and allow for efficient energy use.

In the simplest case, motes are programmed before deployment to perform measurements at a particular sampling rate and return the captured data in a prearranged format. In more sophisticated deployments, the motes are programmed to facilitate sampling rates that adapt to external events and function cooperatively in terms of finding the optimum route for returning data to remote base stations. However, new transducer interfaces are presently under development.

Because of the limited computing power of sensor motes, they often employ an operating system called TinyOS, although more recently, products that are fully C compliant have become available, and these are generally preferred by experienced programmers. TinyOS is an operating system written in the nesC programming language, which is a dialect of C specifically designed for restricted operating environments as exists on sensor motes where there is limited memory and processor power available [126].

On the basis of those computing, communication, and robotic schemes researchers have investigated biosensors and ancillary technologies for environmental applications, especially those that require continuous monitoring. Biosensors are currently available for monitoring biochemical oxygen demand [127] and bacteria [128]. The Oak Ridge National laboratory USA has developed a 1.5-cm² infrared microspectrometer which uses a light source to excite certain types of compounds in gases, liquids and solids which emit infrared light of various wavelengths [129].

However, sensor and biosensor networks are in their infancy, an example is the wireless, remote query ammonia sensor developed by Cai et al. [130]. Recently, the same group has described a wireless magnetoelastic biosensor for the detection of acid phosphatase [131].

Larger projects include an investigation of air quality on the MIR space station [132], and air pollution monitoring in Ferrera [133, 134, 135].

Bendikov et al. [136] performed research on developing sensors on the millimetre scale as well as on sampling. Under the EU FP programmes, the Automated Water Analyser Computer Supported System (AWACSS) project developed an optical sensor to detect river pollution [16]. These projects address the problems of mobility, scale and sensing in water as well as the development of new sensors.

4 Future Trends and Conclusions

Biosensor technology constitutes a rapidly expanding field of research that has been transformed over the past two decades through new discoveries related to novel material technologies, novel means of signal transduction, and powerful computer software to control devices.

However, in order to obtain improved and more reliable devices, future trends in biosensor research activities will be focused on the achievement of continuous monitoring, multi-analyte determinations, using robust transduction elements, and miniaturized devices.

Some systems have been developed for continuous monitoring and can provide easy, rapid and on-site measurements [137, 138]. They may also be useful for mapping of contamination when it is important to obtain rapid results in the field, such as after accidental spills or pollution events [139].

In addition, autonomous sensor platforms and wireless network technologies are very promising for the development of real-time warning systems, revealing dynamic changes in monitored variables in the environment. But to date there are significant problems to be overcome.

As regards advances in sensing technologies, the advancements in optical transduction, which offers robustness, versatility, and facility of miniaturization, must be emphasized. For example, SPR devices appear as some of the most promising technologies to be implemented in environmental warning programmes. Several examples of SPR applications have been reported in recent years. Another important objective is the development of sensors capable of determining several analytes simultaneously. Large-scale biosensor arrays, composed of highly miniaturized signal transducer elements, enable the real-time parallel monitoring of multiple species and are an important driving force in biosensor research [140].

Also relevant advances in electrochemical devices have been achieved recently thanks to the introduction of new nanotechnologic materials, such as nanoparticles and carbon nanotubes, which have been the basis of new systems with improved responses.

On the other hand, the advances in microelectronics and microfluidics have permitted the miniaturization of analytical systems, allowing reduction of energetic requirements, handling of low volumes of samples and therefore a reduction in reagent consumption and waste generation, as well as increasing sample throughput.

New sensing elements that can improve the affinity, specificity and mass production of the molecular recognition components may ultimately dictate the success or failure of detection technologies. In this sense, it should be emphasized that the contribution of gene engineering focuses on two main fields in the area of biosensors: genetically transformed cells and genetically engineered receptor molecules.

Finally, the last limitation to be overcome by biosensors for environmental monitoring is validation. This is an important issue for their integration into pollution control programs.

Acknowledgements The work described in this article was supported by the Spanish Ministerio de Ciencia e Innovación Project CEMAGUA. This work reflects only the author's views and the European Community is not liable for any use that may be made of the information contained therein.

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Achievements of the RIANA and AWACSS EU Projects: Immunosensors for the Determination of Pesticides, Endocrine Disrupting Chemicals and Pharmaceuticals

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Abstract In the last 15 years the research projects supported by the European Commission River Analyser (RIANA) and Automated Water Analyser Computer Supported System (AWACSS) have developed quick, intelligent and cost-effective biosensors that allow the monitoring of different organic pollutants, ranging from regulated ones such as pesticides to emerging contaminants such as pharmaceuticals and endocrine disrupting compounds. Herein we give an overview of the aims, scope and main achievements of the RIANA and AWACSS projects as well as details about basic technology, immunoassays, software and networking developed within the research project. We also report on the systems performance, real water sample measurements and validation of the biosensors with conventional analytical methods. The biosensors developed were able to measure several organic pollutants at low nanogram per litre level in an analysis taking only a few minutes without any prior sample pre-concentration or pre-treatment steps. This work is a proof-of-concept that biosensors are a practical alternative or complementary methodology to traditional chromatographic techniques.

Keywords Automated Water Analyser Computer Supported System, Environmental monitoring, Emerging pollutants, Immunoassay, Network system, Optical immunosensor, River Analyser, Validation

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Contents

1	Introduction.....	34
2	Achievements of the RIANA and AWACSS Projects.....	35
2.1	RIANA System: Key Features.....	35
2.2	AWACSS System: Advanced Features	36
2.3	Limit of Detection.....	38
2.4	Multi-Analyte Determination.....	38
2.5	Automatisation	39
2.6	Network System.....	39
2.7	AWACSS-Marketing Aspects: Water Quality Survey	40
3	Environmental Applications.....	41
3.1	Target Pollutants.....	41
3.2	Real-World Water Analysis.....	41
3.3	Validation	43
3.4	Water Monitoring	44
4	Other Applications	44
5	Conclusions.....	45
	References.....	45

1 Introduction

Environmental and water pollution is a global problem which has led to increasing water quality demands and to more stringent legislation measures in the European Union (EU). Standardised methods for water monitoring need to be developed and implemented throughout Europe to ensure effective enforcement of EU water quality directives. Until now, environmental monitoring has been accomplished by the use of classical analytical tools such as liquid and gas chromatography, which are often lengthy and limit the number of analytes that can be determined in a single run to specific chemical families [1]. Therefore, new tools are demanded for the simultaneous monitoring of organic micro-pollutants in water in real-time and at reasonable cost. In the last 15 years two research projects supported by the European Commission – River Analyser (RIANA) and Automated Water Analyser Computer Supported System (AWACSS) – have developed quick, intelligent and cost-effective instruments that allow the simultaneous detection of different organic pollutants, ranging from regulated ones such as pesticides to emerging contaminants such as pharmaceuticals and endocrine disrupting compounds (EDCs). Herein we intend to provide an overview of the main practical achievements of these research projects. Two different biosensor prototypes were developed for a different set of trace organic contaminants and applied in different water matrices during the projects course. The biosensor RIANA (1995–1999; ENV4-CT95-0066) was developed within the project with the same name, funded by the European Commission under the Environment and Climate Program (Fourth Framework Program). Three RIANA biosensor prototypes were constructed by Perkin Elmer (Ueberlingen, Germany). The system was optimised toward ultra-sensitive detection of pesticides and EDCs in water samples. A new more advanced prototype was constructed by

Central Research Laboratories (CRL, Middlesex, UK) within the AWACSS project (2001–2004) (EVK1-CT-2000-00045) under the Fifth Framework Programme. The AWACSS consortium included nine European partners from four different European countries. Four partners out of the nine were also part of the former RIANA consortium. This new and versatile, multi-sensor system was designed based on reliable and low-cost instrumentation for a quick multi-analyte analysis. Another objective of the AWACSS project was the design of an intelligent warning system with a number of remote measurement stations linked by a communications network.

2 Achievements of the RIANA and AWACSS Projects

The main achievements attained during the RIANA and AWACSS projects are summarised in the following sections. Key features of the two biosensors developed in the corresponding projects are also reviewed.

2.1 RIANA System: Key Features

The RIANA immunosensor was described in detail by Klotz et al. [2]. Basically, the system consists of a flow injection system, a transducer mounted in a flow cell and an optical excitation and detection system. The flow injection system, FIAS 3000, from Perkin Elmer (Ueberlingen, Germany), consists of a six-way distribution valve equipped with a 1-mL syringe pump and connected through a Teflon tubing to the flow cell (dimensions of $40 \times 1.7 \times 0.06$ mm). The transducer is mounted in this flow cell, which includes a flow channel of 1.7 mm width and 0.1 mm depth, and is sealed by an O-ring. Light from a collimated and modulated He–Ne laser source (633 nm, 7 mW) is directly coupled into a glass-slide via a bevelled end-face and guided through the length of the transducer by total internal reflection. An evanescent field is produced at each reflection spot and penetrates a few hundreds of nanometres into the external medium. This configuration avoids interferences from bulk fluorescence and permits the excitation of fluorescently labelled antibodies that are locally and specifically bound to the analyte derivatives. Fluorescence light is subsequently collected through optic fibres, filtered, and detected by photodiodes using lock-in detection. An autosampler AS90/91 from Perkin Elmer is used to deliver the samples to the FIA system. Fluid handling and data acquisition is fully automated and computer controlled.

Immunochemical techniques can be particularly suited for the measurement of organic pollutants because of their specificity, high sensitivity, adaptability for field use and ability to recognise a wide range of substances [1]. Both in the RIANA and AWACSS systems, a solid phase fluoroimmunoassay was selected as an appropriate option to meet the requirements of a useful monitoring immunoprobe. In this

immunoassay the immobilisation of an analyte derivate was preferred to the immobilisation of labile antibodies in order to increase the stability and shelf life of the transducer. The configuration also enables its regeneration for re-use without loss of activity, thus allowing semi-continuous water monitoring.

Polyclonal antibodies were prepared against target analytes by immunising sheep with the corresponding hapten–protein conjugates. After about 8 months pure antibodies were isolated from the polyclonal antisera of the sheep by affinity chromatography. After purification, the antibodies were labelled with a fluorescent marker. The immunochemistry utilised in the project takes advantage of a binding inhibition test that requires that either the antibodies directed against specific analytes or analyte derivatives can be covalently bound to a transducer surface. The immobilisation of the immunoassay component in the surface of the transducer was achieved by binding aminodextran derivatives of the target analytes on separated areas on the transducer glass slide following the procedure described by Barzen et al. [3]. The objective of achieving spatially resolved excitation and collection of fluorescence from fluorescently labelled antibodies locally bound at a planar interface can be met by the evanescent field excitation of the fluorophore. Excitation light is guided by total internal reflection within the transducer structure resulting in an evanescent wave which allows the excitation of the fluorophores bound to the transducer surface (Fig. 1). The total internal reflection fluorescence (TIRF) principle has important advantages compared to direct illumination of the active area of the transducer: it allows selective detection of surface bound fluorophores and, therefore, on-line monitoring of binding events [1].

2.2 AWACSS System: Advanced Features

On the basis of the same immunoassay and optical detection principle of the biosensor RIANA, the new device AWACSS was designed to overcome RIANA drawbacks, with major improvements in three critical areas: (1) novel design approaches to the optical detection and fluidics including miniaturised integrated optics and micro-fluidics; (2) expanded multi-analyte analysis capability allowing for simultaneous measurements of up to 32 analytes; and (3) intelligent remote surveillance and control for unattended continuous monitoring [5].

With the RIANA design, up to six different analytes can be determined simultaneously, whereas the AWACSS instrument allows the performance of up to 32 different immunoassays simultaneously. The AWACSS biosensor is based on the same fluorescence detection principle but the fibre-pigtailed chip, driven by a semiconductor laser, consists of a waveguide circuit that distributes excitation light to 32 separate sensing patches on the chip surface. A fibre-coupled detection array is used to monitor the 32 separate fluorescence signals. The optics with 32 channels and 32 photodiodes was a major part of the AWACSS instrument design [5]. In addition, both the optical bench and the photodiode block were fitted with a semiconductor temperature detector. Algorithms to correct for temperature variation

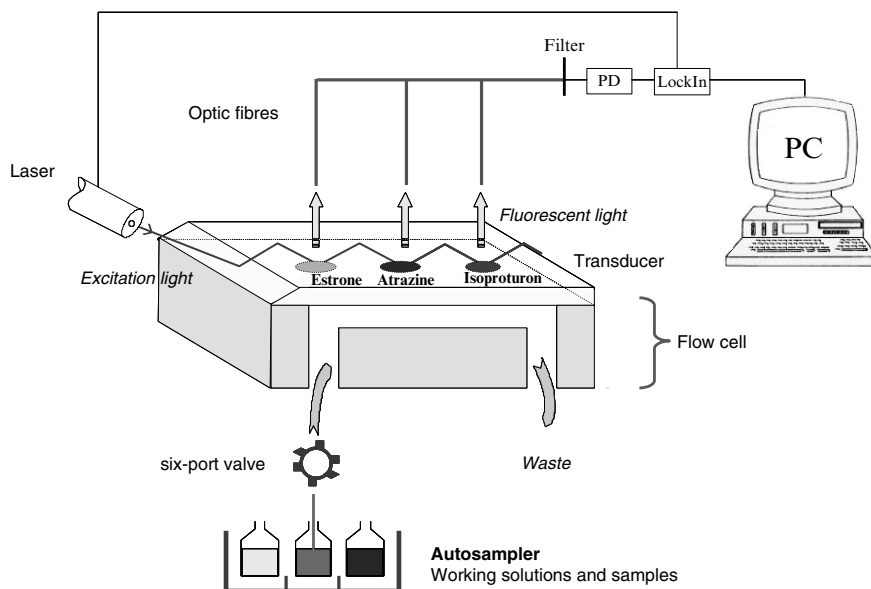


Fig. 1 Scheme of the immunosensor RIANA for a particular application, the simultaneous analysis of atrazine, isoproturon and estrone. The FIA system, equipped with a six-port valve and a 1-mL syringe pump, delivers the buffer solution, the sample or the regeneration solutions to the flow-cell. The transducer – a 1.5-mm thick surface polished sheet glass – is mounted on the flow cell and is sealed with an O-ring. The excitation light (He-Ne laser) is totally internally reflected at the transducer surface. The collected fluorescent light is filtered and detected by photodiodes. Reprinted from [4] with permission of Elsevier

(positive or negative trends) can therefore be programmed into the measurement software. The electronics with low noise, linear amplifier and filter behaviour, external data conversion, and RS232 communications with an external data acquisition unit was the second major part. The electronics design required the assembly of 32 independent linear amplifier circuits. The fluidics box with fully automated sample handling, micro-fluidic flow channel, and interfacing to autosampler was the third major part of the instrument design. The enclosure part with high-quality RF shielding and three separate sections for optics/electronics/fluidics brought the three major parts together. The casing for the instrument was designed as three compartments, separating the main electrical components from the optical bench and amplifier circuits. In this way it was possible to screen the outputs from the 32 channels from digital electronic interference. The optical bench and electronic amplifier compartments were also made light tight to prevent stray light from impinging on the sensor chip or photodiodes. The inside surfaces of the light tight compartments were also blackened to eliminate the possibility of reflected light interfering with the signal [5].

2.3 *Limit of Detection*

Immunoanalytical methods with very low limits of detection (LOD) and low limits of quantification (LOQ) are becoming increasingly important for environmental analysis and, especially, for monitoring drinking-water quality. Tschmelak et al. devoted some efforts to improve the limit of detection of the biosensor assay. The basis of a sensitive assay is an antibody with a high affinity constant toward the analyte [6, 7]. Reducing the amount of antibody per sample results in better validation parameters and lower limits of detection [7]. During the optimisation process, Tschmelak et al. compared different surface modifications (four different immobilised derivatives) and reduced the amount of antibody per sample. The optimisation of the immunoassay resulted in very low limits of detection in water: 0.6, 0.37 and 2 ng L⁻¹ for propanil, [8] progesterone [6] and estrone [7], respectively, without any sample pre-treatment or pre-concentration. These low LOD achieved, in the nanogram per litre range, show that biosensors can compete with common analytical methods in the field of water analysis [6].

2.4 *Multi-Analyte Determination*

Sensors capable of determining several analytes simultaneously allow shortening of the time of analysis and the volume of sample and other reagents, and thus constitute a valuable tool for environmental monitoring. The development of multi-analyte immunoassays was one of the aims of the RIANA project. The spatially resolved immobilisation of the biomolecular recognition elements in transducer surfaces enabled the real-time parallel monitoring of multiple organic contaminants by means of separate immunoreactions. Aminodextran derivatives of the target analytes were covalently bound on separated areas on the transducer glass slide following the procedure described by Barzen et al. [3]. Briefly, a glass interference layer of 58 × 10 × 1.5 mm with a 45° bevel on the short side was cleaned by immersion in a freshly prepared hot mixture of concentrated H₂SO₄/H₂O₂ 2:1 for 30 min, rinsed with water afterwards and dried at room temperature. Silanisation of the surface was achieved by treatment with 3-glycidoxypentyltrimethoxysilane (GOPTS) for not more than 1 h. Thereafter, it was rinsed with acetone and dried in a nitrogen stream. Conjugates of the analyte derivatives with aminodextran were then placed in separated areas of the activated transducer surface allowing the reaction to take place. Such a multi-analyte transducer with predefined detection spots permits the performance of simultaneous multi-analyte measurements.

The first attempts at multi-analyte determination were carried out during the RIANA project [3]. Feasibility studies on multi-analyte measurements were performed with a mixture of atrazine, isoproturon and estrone [9]. The method developed was used to investigate the occurrence and removal of these three compounds throughout the treatment process (sand filtration, ozonation, activated carbon filtration and chlorination) in a waterworks [4]. In the frame of the AWACSS project further efforts were devoted to this multi-analyte aspect; different studies and assays were developed

for the determination of pharmaceuticals, antibiotics, hormones, endocrine disrupting chemicals and pesticides. In the final stage of the project, the multi-analyte determination capability of the AWACSS was successfully tested with a mixture of six analytes. The AWACSS chip was modified with derivatives of atrazine, bisphenol A, estrone, isoproturon, sulphamethizole and propanil according to the previously described immobilisation protocol. Subsequently, a simultaneous calibration in Milli-Q water with all six analytes mixed with an antibody stock solution containing the six corresponding polyclonal antibodies (anti-atrazine, anti-bisphenol A, anti-estrone, anti-isoproturon, anti-mixed sulphonamides and anti-propanil) was performed. For all compounds, the calculated LOD was below $0.020 \mu\text{g L}^{-1}$ [10]. This multi-analyte calibration demonstrated the possibility to quantify pesticides from three different classes (triazines, phenylureas and anilides), EDCs (bisphenol A), steroid hormones (estrone), and pharmaceuticals (sulphamethizole) within one single measurement cycle, which only takes approximately 18 min. No cross-reactivity effect was observed for any of the tested analytes.

2.5 *Automatisation*

Automation and minimal or no sample manipulation are among the most important features in any analytical technique to enhance the precision and accuracy of the measurements and to help diminish potential contamination problems in the analysis process. An autosampler AS90/91 from Perkin Elmer was used in the RIANA project, and initially during the AWACSS project, to deliver the samples to the FIA system of the biosensor. Fluid handling and data acquisition was fully automated and computer controlled. Later, this autosampler was replaced with an autosampler from CTC Analytics, the HTC PAS, which was coupled both to the RIANA and to the new AWACSS device. With this new system there was no need to prepare and incubate samples and standards before analysis since the new autosampler was able to select, mix and inject the samples following a programmed sequence. The new autosampler also permitted improvement in reproducibility, by lowering the standard deviations (RSD), and as a consequence, the LODs.

2.6 *Network System*

Contaminant concentrations in water courses are dynamic, changing both as a result of receiving inputs and changes in water flow. With monthly sampling and analysis it is extremely unlikely that mapping of contamination profiles or obtaining rapid results will occur when it is important, such as after accidental spills or pollution events [11]. The establishment of network analytical systems generates huge interest. These network systems consist of multiple autonomous analytical stations that can control extensively the sites of interest in rivers, lakes, wells or even water treatment plants. In this respect, biosensors can be used as automated continuous

monitoring systems that can provide easy, rapid and on-site measurement. One of the main achievements of the AWACSS project was the establishment of an early warning system by means of a network of measurement and control stations [5].

Figure 2 illustrates the capabilities of the AWACSS monitoring network. The network concept is based on remote measurement stations and an internet-accessible database on the server station. The measurement station uploads the measurement data to the server and downloads new parameters for the next measurement. For each analyte, the incoming data on the server side is checked regarding the limit value concentration. Different limit value concentrations are held in the database depending on analyte type, sampling site location and additional factors. A comparison of these values with the incoming analyte concentration values originating from the different sampling stations is performed by a watch dog module. Automatic alert messages and e-mails are generated following the early-warning concept defined in the database and watch dog module [5].

2.7 AWACSS–Marketing Aspects: Water Quality Survey

A market survey covering 12 European countries and the USA was performed to check the feasibility of the AWACSS system in the water quality market [5]. A standardised questionnaire was completed by institutions dealing with water quality matters in the selected countries to help identify end-user requirements. The data from this survey represented the situation in analytical practice in the different

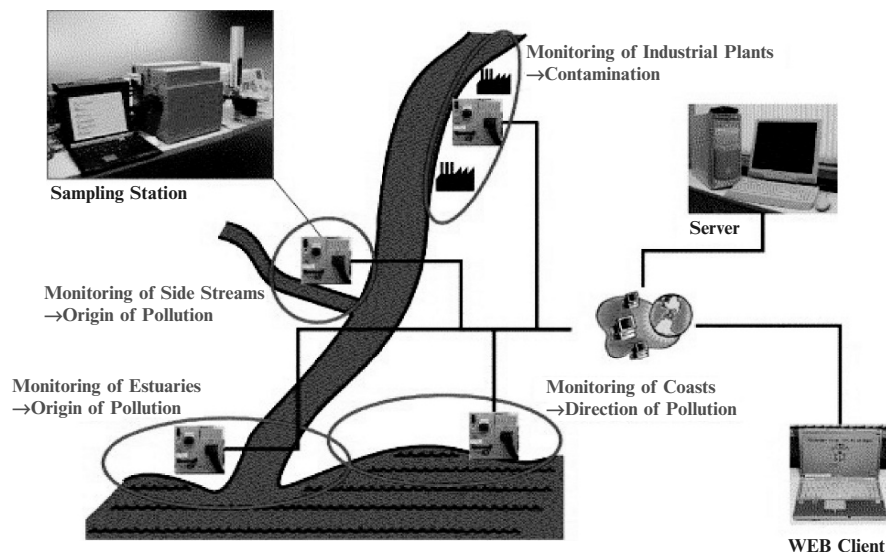


Fig. 2 AWACSS monitoring network. Reprinted from [5] with permission of Elsevier

countries based on the present legislative needs and available laboratory equipment and assays. The collected data covered lists of substances, number of sampling sites, sampling frequencies, number of samples, detection limits, methods applied, number of positive findings, determinations below $0.1 \mu\text{g L}^{-1}$, and analytical costs. Real-time sensing, improved regulatory and public acceptance and less expensive monitoring were found to be prime end-user requirements. The AWACSS system not only meets the market demand but exceeds the current competitive detection methods in terms of meeting end-user requirements for current and future expenditures and expectations: A system like AWACSS, equipped to measure up to 32 top compounds, could be well placed to the actual market with increasing acceptance in the future due to the flexibility of adaptation of new compounds as well as the capability of online, unattended and centrally controlled monitoring and surveillance.

3 Environmental Applications

In the previous section, biosensor features were described in terms of analytical achievements and contribution to biosensor field development. Practical aspects related to biosensor performance in the analysis of environmental samples as well as different aspects and achievements of the RIANA and AWACSS projects are reviewed in this section.

3.1 Target Pollutants

A set of immunochemical reagents was developed and generated for selected organic micro-pollutants of interest to be analysed in environmental water samples. Major criteria for selection of target analytes were their presence in the environment, the existing environmental legislation, the increasing interest in certain emerging contaminants and the technical possibilities to prepare sufficiently selective polyclonal antibodies and their corresponding analyte. Analytical methods for the analysis of pesticides, EDCs, WFD priority substances, industrial pollutants and pharmaceuticals have been developed using the biosensor devices from the RIANA and AWACSS projects. Table 1 gathers together the organic pollutants and the water matrices studied and the limits of detection achieved.

3.2 Real-World Water Analysis

Different assays using the RIANA and AWACSS biosensors have been developed in Milli-Q water in the process of developing and optimising the appropriate analytical method for the target compound analysis. This step is necessary before application

Table 1 List of target organic pollutants analysed by RIANA or AWACSS using either single or multi-analyte configurations

Analytes	LOD [ng L ⁻¹]	Environmental matrix	References
Paraquat	10	Milli-Q and river water	[12]
Isoproturon	10	Milli-Q and river water	[13]
Triazines	60–200	River water	[14]
Propanil	0.6	Milli-Q water	[6]
Bisphenol A	14	Waterworks	[15]
Progesterone	0.37	Spiked Milli-Q, drinking and river water	[8]
Testosterone	0.2	Surface water	[16]
Estrone	0.2	Milli-Q water	[7]
	1		[17]
Sulphonamides	2.7–6.6	Drinking, ground, and surface water	[18]
Multi-analyte determination			
Atrazine	30		
Simazine	30		
Isoproturon	110	Milli-Q Water	[3]
Alachlor	70		
2,4-D	70		
PCP	4,230		
Isoproturon	50	Natural water samples	[4, 9]
Atrazine	160		
Estrone	80		
Bisphenol A	5		
Atrazine	2	Milli-Q, surface water	
Estrone	19	Groundwater	[19]
Isoproturon	16		

2,4-D: 2,4-dichlorophenoxy acetic acid, PCP: Pentachlorophenol

to real-world analysis regardless of whether single or multi-analyte measurements are to be performed. The application of new biodevices to real-world environmental samples has to be accomplished in the final steps of development. However, despite the large number of newly developed biosensors, most of the biosensors described in the literature overlook this step and only report applications in distilled water or buffer solutions [20]. One reason is the matrix effect in the measurements accuracy. Sample matrix can affect dramatically the application of biosensors to real samples. RIANA and AWACSS are based on competitive interactions between antibodies, analytes and analyte derivatives. These interactions can be affected by the pH or the ionic strength of natural water matrices. It has also been reported that the presence of other natural substances, such as dissolved organic carbon that interact weakly with the antibody, can induce an overestimation of the immunoassay response [21]. The influence of ground and river water matrices on the immunosensor response was normally evaluated for the different assays developed for RIANA and AWACSS by spiking these matrices with analyte standard solutions and observing the displacement of the calibration curves.

On the other hand, several strategies have been proposed and tested to prevent matrix interferences of the water samples, such as diluting and buffering the sample, adjusting the sample pH and conductivity, sample clean-up, enrichment of the sample by solid phase extraction and subsequent reconstitution of the extract, and adding a background protein. The addition of detergents or immobilization proteins reduces the probability of the antibody to adhere to passive particles in water and has become a current routine to overcome matrix interferences in immunoassays [4, 22, 23]. This is the strategy adopted in the case of the RIANA and AWACSS assays where the background protein ovalbumine is added to a real sample as an immobilization protein.

As part of the AWACSS project, Tschmelak et al. proposed and demonstrated the efficacy of a new method to overcome the partly occurring matrix problems in real-world immunosensor monitoring [24]. They developed an easy matrix referencing method using a synthetic organic carbon standard that could be adapted to other applications depending on a similar test-format. The organic carbon level of monitored waters should be determined in parallel or in frequent intervals. With this referencing method, very good recovery rates for the ultra-sensitive estrone assay were possible.

3.3 Validation

Despite the practical advantages of biosensors, they need to be comparable to conventional analytical systems in terms of reliability, sensitivity, selectivity, specificity and robustness. Biosensor measurements need therefore to be verified and validated before being accepted. The RIANA and AWACSS biosensors were validated by comparing the biosensor performance with that of conventional methods. The overall performance of the new system in comparison to the conventional analytical and immunosensor techniques was tested in an inter-laboratory collaborative trial among the AWACSS partners at the late stages of the project. Different analytical set-ups were used in six laboratories; among them four automated online and offline solid-phase extraction (SPE) systems coupled to liquid chromatography and either mass spectrometry or diode array detection (SPE-LC-MS, SPE-LC-DAD), a large volume injection-gas chromatography-mass spectrometry system (LVI-GC-MS), two RIANA biosensors, one AWACSS device and one ELISA assay. The tested matrices were Milli-Q water and freeze-dried river sediments from the Nitra River in the Slovak Republic. Each of them was spiked with the analytes atrazine, bisphenol A and estrone at 0.1 and 1.0 $\mu\text{g L}^{-1}$ in water matrices and 50 and 500 ng g^{-1} in sediments. All samples, including blanks, were prepared in triplicate and distributed among the partners. For more details on the procedures, the reader is addressed to reference [10]. The results showed that, in terms of accuracy, the AWACSS performance in Milli-Q water and sediment samples is fully comparable to conventional chromatography-based techniques. In general, the AWACSS results were less biased towards higher values than the ELISA and RIANA immunosensor techniques.

3.4 Water Monitoring

The performance of both, the RIANA and the AWACSS biosensors has been tested for a variety of organic pollutants and water matrices. However, only two works have reported their use in environmental monitoring studies. The immunosensor RIANA was evaluated to monitor total chlorotriazine pesticides in river water samples in the Ebre area (Tarragona, Spain) from April to June 1998 [14]. Simazine and atrazine were detected (levels ranging from 0.18 to 1 $\mu\text{g L}^{-1}$) in only eight samples out of 18 measurements carried out. The results obtained with the immunosensor were validated by online solid-phase extraction followed by liquid chromatography-atmospheric pressure chemical ionisation-mass spectrometry (LC-APCI-MS). Although some overestimation was observed with the biosensor no false-positive was detected. Thus, the method developed with the biosensor allows the unequivocal determination of chlorotriazines in natural waters and can be applied to comply both, with the EPA legislation for drinking waters, which requires the monitoring of atrazine at the 2 $\mu\text{g L}^{-1}$ level and also with the European Community (EEC) Drinking Water Directive (DWD), which establishes limit values for individual and total pesticide concentrations at 0.1 and 0.5 $\mu\text{g L}^{-1}$, respectively.

The RIANA system was also applied to determine the occurrence of the organic pollutants bisphenol A, atrazine, isoproturon and estrone in the waterworks Sant Joan Despí (Barcelona, Spain) between May and August 2002 [4, 15]. The removal efficiency of the target pollutants throughout the different purification stages at the waterworks was assessed using two different biosensor configurations: single analyte mode for bisphenol analysis [15] and multi-analyte configuration for the determination of atrazine, isoproturon and estrone [4]. Chromatographic methods were also applied in parallel for the analysis of the samples. On the basis of the comparative performance of the biosensor and liquid chromatography based methods, the biosensor emerged as a suitable tool for fast, simple and automated screening of water pollutants without sample pre-treatment and was proved to be a useful tool for water resources protection; in most water production companies the quality of the raw water and the removal efficiency of target contaminants during drinking water production needs to be assessed.

4 Other Applications

The significant progress made with RIANA in environmental monitoring and water analysis was successfully adapted to milk analysis for use in the field of reproduction management, in particular for the sensitive determination of progesterone in cow milk, as an indicator of ovulation [25]. The sensitivity and robustness of the existing progesterone assay for water analysis was improved, resulting in a LOD of only 0.2 ng L^{-1} . Progesterone was then analysed in three different types of milk (UHT milk, fresh milk and raw milk) (levels typically in the range 5–15 $\mu\text{g L}^{-1}$). LOD achieved for added progesterone (i.e. spiked samples) were between 45.5 and 56.1 ng L^{-1} depending on milk type. In this work, a commercially available antibody

was incorporated for the first time into the immunosensor. The biosensor operation was fully automated and the LOD achieved for progesterone in bovine milk was below $1 \mu\text{g L}^{-1}$. The assay was further optimised by reducing the time per measurement to about 5 min and tested by measuring the progesterone level in daily milk samples for 25 days, covering a whole oestrus cycle [26].

5 Conclusions

Herein we have reported the main achievements attained during the EU research projects RIANA and AWACSS, and the successful application of biosensors for environmental monitoring. Two different biosensor prototypes have been designed and constructed during the projects and different software modules for data storage, communication, data treatment, networking and data evaluation have been successfully developed and combined into a software package. Feasibility studies have been conducted on multi-analyte analysis and on matrix effects. Detection limits of most developed immunoassays have been in the few nanogram or even sub-nanogram per litre range.

The biosensors capability for analysing a variety of environmental organic micro-pollutants, such as pesticides, EDCs and pharmaceuticals in surface, ground, drinking and waste water has been compared with conventional analytical methods for validation purposes. The biosensors described in this report have been demonstrated not only to be cost-effective alternatives to chromatographic techniques for environmental screening, but also very convenient techniques when the information is needed immediately (measurements are performed very rapidly; maximum 20 min).

Most of the compounds analysed with the RIANA and AWACSS biosensors meet the end-users demands, their monitoring is required by the present EU environmental legislation (98/83/EC, 1998; 2000/60/EC, 2000) and all of them are frequently detected in real water samples all over Europe. On the basis of the features reported, good perspectives are given to the biosensor system to be placed among the current state-of-the-art analytical instruments for water monitoring.

Acknowledgements This work was funded by the “Automated Water Analyser Computer Supported System” (AWACSS) (EVK1-CT-2000-00045) research project supported by the European Commission under the Fifth Framework Programme and contributing to the implementation of the Key Action “Sustainable Management and Quality of Water” within Energy, Environment and Sustainable Development. This work reflects only the authors’ views and the EU is not liable for any use that may be made of the information contained therein.

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Biosensors for Pharmaceuticals and Emerging Contaminants Based on Novel Micro and Nanotechnology Approaches

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Abstract The investigation of new sensing principles and technologies for the detection of molecular binding events has created great expectations on numerous major industrial sectors, such as healthcare, food, water and agriculture. Combining many of these advances with the potential of the immunochemical systems has allowed developing novel biosensors that provide interesting advantages against the traditional strategies for analysis, such as the possibility of multianalysis, development of field analytical methods and fabrication of easy end-user devices. Specifically, many efforts have been lately invested to control residues of pharmaceuticals in food and environmental samples, as an indication of the impact of the human activity in the media. Human and veterinary drugs, such as antibiotics, hormones, analgesics, cytostatics or β -blockers, show a high potential risk of negative effects in the environment and public health. Thus, there is a great need for low-cost and highly efficient tools for quick, reliable, and accurate detection of these contaminating bioactive agents. In particular, the scope of the present chapter is addressed to provide an overview of the potential of novel micro(nano)technology approaches to develop biosensors useful for the analysis of emerging pollutants.

Keywords Amperometric biosensor, Antibody, Antibiotics, Biosensor, Evanescent wave, Hormones, Impedimetric biosensor, Microcantilevers, Nanobiotechnology, Pesticides, Piezoelectric crystal, Plasmon resonance

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Contents

1	Introduction.....	48
2	Electrochemical Biosensors.....	50
2.1	Amperometric Biosensors.....	51
2.2	Impedimetric Biosensors.....	54
3	Optical Biosensors.....	56
3.1	Biosensors Based on the Evanescent Wave Optical Principle.....	57
3.2	Plasmon Resonance Biosensors.....	59
3.3	Nanomechanical Biosensors.....	62
4	Piezoelectric Immunosensors.....	63
5	Conclusions.....	64
	References.....	64

1 Introduction

Biomolecular analytical devices have evolved rapidly over the last years. Novel molecular diagnostic approaches to refine and extend the limits of detection have emerged as a consequence of the combination of micro(nano)technological and biotechnological advances. Biosensors are among the potential applications of new materials and devices and of novel nanobiotechnological approaches. Thus, subtle changes in properties such as the dielectric field or the refractive index produced after biomolecular recognition events can be detected if they are taking place at the surface of appropriately developed transducers. New knowledge of the unique properties of nanostructured materials has opened up the possibility to investigate the influence that biorecognition phenomena have on the new optical and/or electrical properties of these systems, possibly leading to the development of more sensitive and flexible biosensing systems.

Among biosensors, immunosensors using antibodies as biorecognition elements have fascinating features such as the possibility to respond selectively to biological or bioactive substances and the capability to respond in a physiological manner. The unique properties of certain nanomaterials combined with the excellent features of the antibodies allow envisaging novel exquisitely sensitive chemical and biological sensors. Thus, antibodies are natural molecules with inherent capabilities to specifically react with their counter antigen. Antibodies can be produced, in principle, against all kinds of substances and their features can be tailored according to the requirements of each application. Their homogeneity regarding chemical structure and properties allow standardization of several procedures. Moreover, antibodies show greater improved stability, when compared to other biomolecules.

A very important aspect in biosensor development is the construction of biofunctional surfaces. Sensor solid surfaces are in general solid inorganic matter and per se not suitable for immobilizing biomolecules. Hence, further modification is required to adapt them for the immobilization of biomolecules. In addition, functional sensor surfaces place several demands such as biocompatibility, homogeneity, stability, specificity, and functionality. Thus, a challenge in biosensor development

is to construct adequate surfaces as well as to design molecules suitable for site-directed immobilization. Surface architecture depends on the nature of the transducer and on the features of the biomolecule, as well as the type of measurements needed [1–4]. The surface has to be activated appropriately for further tethering of the proteins with a particular immobilization method. Subsequent layers can be generated in place, and textured following specific demands.

Environmental monitoring of chemical and biological hazard is a potential application of biosensors, and therefore several developments can be found in the literature trying to provide better analytical tools to assess any risk derived from contamination. This interest in developing more efficient bioanalytical devices responds to the increasing production of these chemicals, including so-called emerging contaminants such as pharmaceutical active ingredients and personal care products (PPCPs) [5] and the potential toxicological risks derived from them. Pharmaceuticals and biogenic hormones are normally discharged to the environment through wastewater treatment plants (WWTP), which are often not designed to remove them from the effluent. From there on, transport, fate, or possible adverse consequences of these pollutants on human health and on the ecosystem, are frequently unknown or not clearly understood [6]. Potential concerns include reproductive impairment [7–9], increased incidence of cancer [10], development of antibiotic-resistant bacteria [11], or the potential increased toxicity of chemical mixtures due to synergistic effects [12].

In Europe, regulations and regulatory methods to assess and control the impact of these substances in the aquatic environment, aim to protect the ecosystem and public health while monitoring contamination levels and any potential negative effects. In addition to the REACH (Registration, Evaluation, Authorization, and Restriction of Chemicals) law (EC 1907/2006), regarding chemicals and their safe use, which entered into force on 1st June 2007, there are specific regulations addressed to protect health and ensure quality of all water resources such as the Drinking Water Directive (DWD, Council Directive 98/83/EC), the Bathing Water Directive (2006/7/EC), and the Urban Waste Water Directives (91/271/EEC and 98/15/EC). Moreover, the Water Framework Directive (WFD, 2000/60/EC) intends to provide an overall framework for a cleaner and safer water ecosystem, particularly regarding surface freshwater and ground water bodies (i.e., lakes, streams, rivers, estuaries, coastal waters etc.). Thus, consistent with this directive, the Marine Strategy Framework Directive (2008/56/EC) aims to achieve good environmental status of the EU's marine waters by 2021. Similarly, a River Basin Management Plan (RBMP) is being developed to be implemented for each river basin district which must include knowledge of the particular pressures and impacts of human activity, protection programs, controls, and remediation measures. The first RBPM is scheduled to be published at the end of 2009 and according to it the new Groundwater Directive (2006/118/EC) establishes a regime which sets underground water quality standards and introduces measures to prevent or limit input of pollutants into the groundwater.

On the other hand, pharmaceutical residues can also contaminate food through animal treatment with veterinary drugs. During their lifetime animals may have to be treated with medicines for prevention or cure of diseases. In food-producing

animals such as cattle, pigs, poultry, and fish this may lead to residues of the substances used for the treatment in the food products derived from these animals (e.g., meat, milk, eggs). The residues should, however, not be harmful to the consumer, for which reason EC legislation requires that the toxicity of potential residues is evaluated before use of a medicinal substance in food-producing animals is authorized. If considered necessary, maximum residue limits (MRLs) are established and in some cases the use of the relevant substance is prohibited. Thus, Directive 96/23/EC establishes the frequencies and level of sampling and the groups of substances to be controlled for each food commodity. Moreover, Commission Decision 2002/657/EC (and the following amending directives) establishes criteria and procedures for the validation of analytical methods to ensure the quality and comparability of analytical results generated by official laboratories. Additionally, the Decision introduces a procedure to progressively establish minimum required performance limits (MRPL) for analytical methods employed to detect substances for which no permitted limit (maximum limit) has been established, such as hormones.

Pharmaceuticals are by definition very active substances and therefore there is an increased demand for new and more efficient analytical methods in order to protect ecosystems and public health. Herein we aim to illustrate this with examples of recent developments of new biosensor devices reported to detect certain emerging pollutants, antibiotics, and androgenic anabolic steroids. The physical principles and micro/nanotechnological approaches behind the examples presented herein should be contemplated as universal and with general applicability to the analysis of other chemical or biological hazards as soon as a bioreceptor is available to specifically detect these substances.

2 Electrochemical Biosensors

Because of its simplicity electrochemical transduction has given rise to a great variety of low-cost devices based on different formats and configurations (for recent reviews see [13–16, 17]). From all possible electrochemical transduction systems those based on recording the amperometric signal generated after an enzymatic reaction have seen the greatest development [16]. However, new approaches have been described aimed at directly detecting biomolecular recognition reactions without using enzyme labels, such as the impedimetric devices, or incorporating nanoparticles to generate or increase the electrochemical signal. As an example, gold nanoparticles that provide a suitable microenvironment for biomolecule immobilization whilst retaining biological activity, and to facilitate electron transfer between the immobilized proteins and electrode surfaces, have led to an intensive use of this nanomaterial for the construction of electrochemical biosensors with enhanced analytical performance with respect to other biosensor designs [13, 15]. Similarly, the particular structure of carbon nanotubes and their unique properties make them a very attractive material for the design of electrochemical biosensors. Among the potential advantages are the promotion of different electron transfer reactions, especially those related to

biomolecules and the increase of the active surfaces which has led to different strategies for constructing carbon nanotube-based electrochemical sensors [14].

2.1 Amperometric Biosensors

Amperometric biosensors are based on the measurement of the current generated by oxidation/reduction of redox species at the electrode surface, which is maintained at an appropriate electric potential. The current observed has a linear relationship with the concentration of the electroactive species. The electrode is usually constructed of platinum, gold, or carbon. Adjacent to the electrode, entrapped by a membrane or directly immobilized, the bioreceptor is placed. Label enzymes used in electrochemical immunoassays are usually oxidoreductases such as horseradish peroxidase (HRP), or hydrolytic enzymes, such as alkaline phosphatase (AP), which yield electroactive species as a product of the enzymatic reaction (see Fig. 2). Other enzymes used as sensing elements for environmental monitoring are tyrosinase, laccase, aldehyde dehydrogenase, etc. Sometimes the substrate or the product of the enzymatic reaction can be monitored amperometrically, without the need for a mediator. These electrodes are called *unmediated amperometric enzyme biosensors*. However, a number of factors must be taken into account when assessing the suitability of an enzyme substrate to be used in an electrochemical detection system: the electrochemistry of the substrate, the electrochemistry of the product of the enzymatic reaction, the medium in which the measurements will be performed, and the electrochemistry of endogenous materials in the test sample. A problem often encountered with unmediated sensors is that other species present in the samples being analyzed are also electroactive at the potential applied. For example ascorbic acid and uric acid, present in many biological samples, are oxidized at an anodic potential of +0.35 V. AP combined with *p*-aminophenyl phosphate (PAPP) as a substrate has been shown to be a good alternative when measuring with such a system. Although *p*-aminophenyl phosphate has an irreversible wave in cyclic voltammetry at around 0.45 V vs. Ag/AgCl, its hydrolysis product *p*-aminophenol (PAP) shows a reversible electrochemistry with a half wave potential of -0.065 V vs. Ag/AgCl. Consequently, measurements on biological matrices can take place at lower potentials avoiding interference of endogenous compounds. Choosing an alternative electron transfer acceptor can also circumvent these problems. Usually the *mediator* is a species of low molecular weight (MW) that shuttles electrons between the redox center of the enzyme and the working electrode. These sensors are called *mediated amperometric enzyme biosensors*. A mediator should react rapidly with the enzyme, exhibit reversible heterogeneous kinetics, possess a low overpotential for regeneration, and be stable at a certain range of pH, temperature, redox state, and dioxygen. Some mediators frequently used are I^- , $[Fe(CN)_6]^{4-}$, *o*-phenylenediamine, diaminobenzidine, hydroquinone, and 5-aminosalicylic acid.

A recent example has been reported to detect xenoestrogens using an amperometric immunosensor based on a quinoprotein glucose dehydrogenase (GDH)-modified

thick-film sensor. The immunosensor is used to detect the tracer used in a capillary immunoassay (CIA) [18]. With this configuration, detection of the alkylphenols and their ethoxylates in $\mu\text{g L}^{-1}$ range is possible using a β -galactosidase tracer and anti alkylphenol ethoxylate antibodies. The assay is performed off-line in small disposable PVC capillaries coated with immobilized antibodies, which allows the combination of the assay with a small portable device potentially useful for on-site environmental monitoring. The bioelectrocatalytic properties of this biosensor offer an additional amplification and allow a very sensitive quantification of the 4-aminophenol, generated by the β -galactosidase. However, in the last few years, the advances and possibilities presented by easily fabricated screen-printed electrodes (SPEs) has given rise to a significant number of examples demonstrating the potential and the flexibility of this technology to develop low-cost biosensor devices for a variety of targets including microorganisms, pesticides, and also pharmaceuticals. As an example, disposable carbon SPE immunosensors have been described for the detection of anabolic androgenic steroids (AAS) using horseradish peroxidase as the enzyme label and tetramethylbenzidine/hydrogen peroxide (TMB/ H_2O_2) as the substrate for chronoamperometric detection at +100 mV [19–21]. The immunosensors were fabricated by immobilizing hapten-bovine serum albumin conjugates on the surface of the SPEs and followed by the competition between the free analyte and coating conjugate with corresponding antibodies. The signal is recorded after an anti IgG-HRP conjugate determining the degree of competition. By using different antibodies with different affinities and cross-reactivity patterns the immunosensor detects different AAS with limits of detection (LOD) below 200 pg mL^{-1} . Thus, LODs of $30.9 \pm 4.3 \text{ pg mL}^{-1}$ for boldenone and $120.2 \pm 8.2 \text{ pg mL}^{-1}$ for methylboldenone, respectively, have been reported. Testosterone can be detected in buffer with a LOD of 26 and 1.8 pg mL^{-1} in a complex matrix such as bovine urine. Similarly, nortestosterone can be detected in the same matrix with a LOD of 15 pg mL^{-1} .

The possibility to analyze progesterone using amperometric immunosensors has been recently reported using a colloidal gold-graphite-Teflon-tyrosinase composite biosensor as the amperometric transducer [22]. A sequential competitive configuration between the analyte and progesterone labeled with alkaline phosphatase was selected using in this case phenyl phosphate as the AP-substrate and the enzyme reaction product, phenol, was oxidized by tyrosinase to o-quinone, which was subsequently reduced at -0.1 V at the biocomposite electrode. A LOD of 0.43 ng mL^{-1} was accomplished while the time needed to reach the steady-state current from the addition of phenyl phosphate was 30–40 s. A lifetime of 14 days with no need to apply any regeneration procedure was reported. The immunosensor was applied to the detection of progesterone in milk spiked after just sample dilution.

Recently, several papers have been published combining magnetic particles with electrochemical amperometric detection. Magnetic beads are known to be a powerful and versatile tool in a variety of analytical and biotechnology applications. The use of magnetic beads greatly improves the performance of the immunological reaction, minimizing the matrix effect due to improved washing and separation steps. Additionally, the magnetic beads can be easily magnetically manipulated by using permanent magnets or electromagnets. Therefore, the analysis of samples performed

on magnetic beads can be easily achieved without any pre-enrichment, purification, or pretreatment steps, which are normally necessary for standard methods. In this context, an electrochemical immunosensing strategy based on magnetic sensors is presented as a sensitive, simple, cheap, and user-friendly analytical alternative to classical methods for the detection of residues. The strategy combines the advantages taken from immunological assays, magnetic bead separation, and electrochemical transduction and is based on the immunochemical competitive immunoreactions between the contaminant and the enzyme tracer with the specific antibody immobilized on magnetic beads followed by its separation from the biological matrix by the use of a magnetic field. Thus, after the immunological reaction, the magnetic beads can be easily captured by a novel electrochemical and magnetic transducer, based on a magneto graphite-epoxy composite [12, 13] (m-GEC), by merely dipping the magneto electrode in the solution where the beads are suspended (Fig. 1). This strategy has been used for the detection of sulfonamide antibiotics in milk samples [23]. The antibodies raised to provide an appropriate recognition pattern of this antibiotic family [24] are immobilized on the surface of magnetic beads through covalent attachment using tosyl-activated commercially available magnetic beads. Other immobilization strategies tested before also gave suitable results, but this strategy was demonstrated to be the one providing the best performance [25]. The immunological reaction for the detection of sulfonamide antibiotics is based on a direct competitive assay using a peroxidase (HRP) tracer as the enzymatic label. Excellent results were accomplished detecting sulfamethazine directly in a diluted raw full cream sample with detectability values far below the MRLs established by the EC in those types of samples (IC₅₀ of 9.8 $\mu\text{g L}^{-1}$ and LOD of 0.36 $\mu\text{g L}^{-1}$). The same results were obtained with UHT (ultra high temperature) milk, such as full cream (about 3.25% fat), semi-skimmed (about 1.5–1.8% fat), and

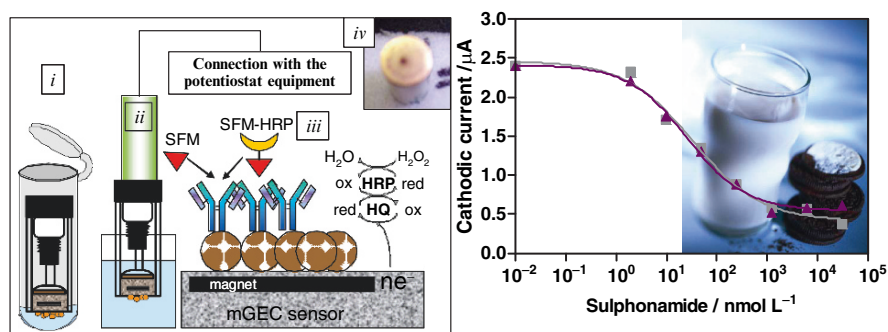


Fig. 1 Left: Schematic representation of the electrochemical magnetosensing strategy for the detection of sulfonamides. After the immunoreaction, the antibody-modified magnetic beads were captured for the m-GEC electrode (i). Chemical reactions occurring at the m-GEC surface polarized at -0.150 V (vs. Ag/AgCl) upon the addition of H_2O_2 in the presence of mediator (HQ) (ii and iii). Appearance of the m-GEC electrode covered with the magnetic beads (iv). Right: Calibration curves for sulfapyridine run in PBS and in 1:5 whole milk. Parts of this figure are reprinted from Zacco et al. [23] with permission from Elsevier

skimmed (0.1% fat). This strategy has been proven to be useful for the analysis of other residues in complex matrices such as atrazine in orange juice [25] or fluoroquinolone antibiotics in milk (unpublished results).

The same strategy has also been applied to the detection of polychlorinated biphenyls (PCBs) though in this case using SPEs [26, 27]. The sensor was based on a direct competitive immunoassay scheme in which antibody-coated magnetic beads were used on SPEs to detect the product of the enzymatic reaction of alkaline phosphatase used as the tracer. α -Naphthyl phosphate was used as the substrate and the α -naphthol produced during the enzymatic reaction was detected using differential pulse voltammetry (DPV). Aroclor 1248 PCB mixture could be measured with a detection limit of 0.4 ng mL^{-1} . The performance of the sensor was demonstrated analyzing PCBs on marine sediment extracts and spiked soil samples.

2.2 Impedimetric Biosensors

Different sensor approaches have been accomplished to avoid the use of labels when the target analyte is a biomacromolecule, bacteria, or virus, but only on a few occasions has the performance of these transduction principles been demonstrated to detect small organic molecules at low concentration levels. In this context, *electrochemical impedance spectroscopy* (EIS) is being rapidly developed because of the possibility to record directly information on biorecognition events occurring at the electrode surfaces and inducing capacitance and resistance changes [28, 29]. Thus, when a biological receptor binds to its counterpart, there is a change in the impedance of the system that enables direct measurement of an electrical signal, allowing the development of label-free biosensing devices. A major drawback of early sensors was that impedance changes due to biorecognition are very small. However, new electrode configurations have significantly increased the detectability of these devices. Thus, impedimetric label-free devices have been described to detect DNA hybridization [30] or biomolecules such as interferon-gamma [31], but direct detection of contaminants and residues in complex matrices is still a challenge. Label-free detection of bisphenol A (BPA) based on the impedance measurement has also been achieved with an impedimetric immunosensor [87]. The immunosensor was fabricated by the covalent immobilization of a polyclonal antibody on carboxylic acid group-functionalized nano-particle comprised conducting polymer and bisphenol A haptene, (4,4-bis(4-hydroxyphenyl) valeric acid, BHPVA) BSA conjugate. The immunosensor showed specific recognition of BPA with a LOD of $0.3 \pm 0.07 \text{ ng mL}^{-1}$. The proposed immunosensor was applied to the analysis of BPA in human serum samples.

In this context, interdigitated microelectrodes (ID μ E) have recently drawn great attention since their sensitivity is higher than conventional electrodes [32–35]. Using thin Au/Cr ($\sim 200 \text{ nm}$ thickness) ID μ E's ($3.85 \text{ }\mu\text{m}$ thick and with an electrode gap of $6.8 \text{ }\mu\text{m}$) on a Pyrex 7740 glass substrate, our group has recently reported detection of atrazine without the use of any label with a limit of detection of $0.04 \text{ }\mu\text{g L}^{-1}$ [36]. The sensor has been evaluated to assess its potential to analyze pesticide residues in a complex sample matrix, such as red wine. An atrazine hapten-BSA

conjugate was covalently immobilized within the μ -electrodes on the glass substrate. An appropriate functionalization of the transducer surfaces was crucial in this case. A key problem is the nonspecific binding of molecules present in the sample matrix to the surface of the sensor. This concerns any surface from a lower to higher extent, but particularly, gold is very suitable to capture nonspecifically organic molecules and components from the media. For this reason, a two-step activation procedure based on the distinct properties of the gold (electrodes) and the silicon oxide surfaces (between the electrodes) was used. The whole activation procedure was monitored by impedance spectroscopy using ID μ E as is shown in Fig. 2 In a first step, modification of the gold surfaces was accomplished making use of thiol chemistry using N-acetylcysteamine to cover the gold electrodes and to protect the sensor from undesired nonspecific absorptions (Step I). Following this, immobilization of the glass material between the electrodes was activated making use of silane chemistry, using (3-glycidioxypropyl)trimethoxysilane (GOPS) (Step II). The epoxy group provided the necessary reactivity for further attachment of the immunoreagents through a nucleophilic attack of the amino groups of the lysine

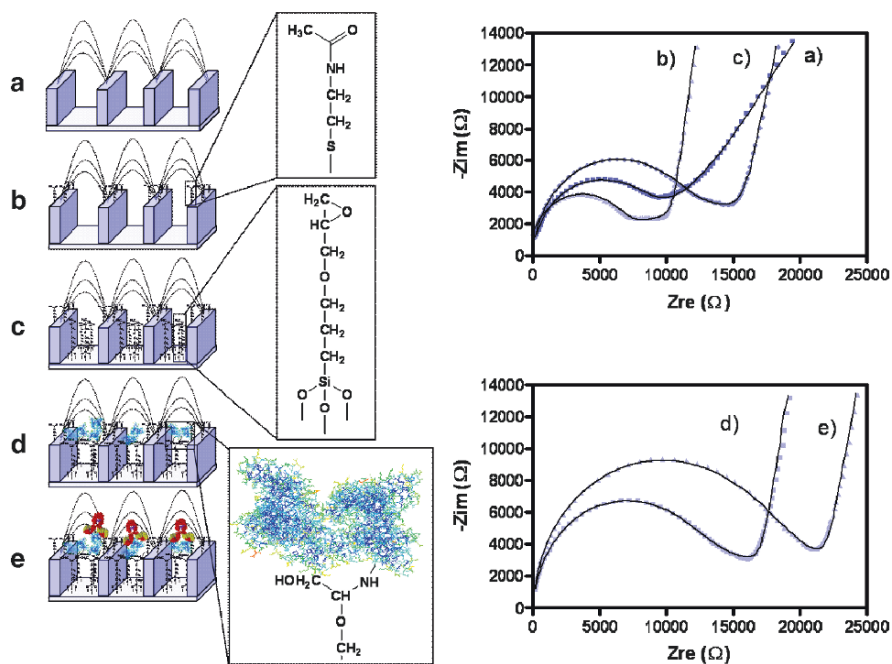


Fig. 2 *Left:* Scheme showing steps used to prepare the immunosensor surfaces and antibody binding. *Right:* Nyquist plots of impedance spectra corresponding to: **(a)** IDμE, **(b)** Step I: N-acetylcysteamine, gold protection, **(c)** Step II: functionalization of Pyrex substrate with (3-glycidoxypropyl)trimethoxysilane, **(d)** Step III: coating antigen 2d-BSA, covalent immobilization ($1 \mu\text{g mL}^{-1}$), and **(e)** Step IV: Antibody Ab11, incubation step ($1 \mu\text{g mL}^{-1}$). *Symbols* represent the experimental data. *Solid curves* represent the computer fitting data with the parameters calculated by the commercially available software Zplot/Zview (Scibner Associates). Parts of this figure are reprinted from [36] with permission from Elsevier

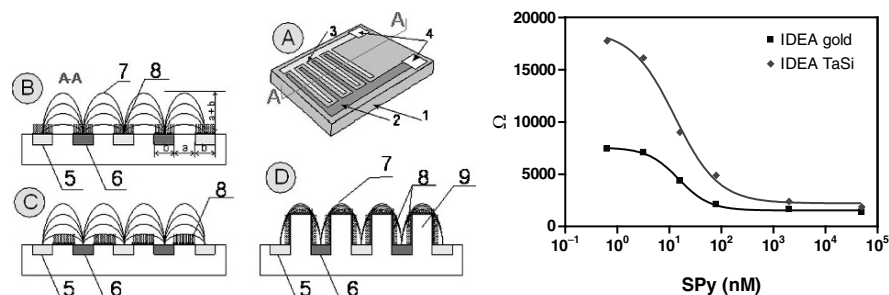


Fig. 3 *Left*: Schemes of the planar and barrier electrodes: (A) Planar IDEA device; (B), (C) cross-section A-A of the planar IDEA device; (D) IDEA device with insulating barriers between electrode digits. The numbers represent: 1-Insulating substrate; 2, 3-electrode collector bars; 4-contact pads; 5, 6-electrode “digits”; 7-electric field lines; 8-immobilized biomolecules; 9-insulating barrier. *Right*: Calibration curves obtained for sulfapyridine using either the planar electrodes or the electrodes with barriers. Parts of this figure are reprinted from Bratov et al. [37] with permission from Elsevier

residues of the antigen. As can be observed, the covalent immobilization of the atrazine antigen 2d-BSA (Step III) produced a significant change in the Nyquist plot. Finally, exposure of the immunosensor to a solution of the specific antibody (Step IV) produced a shift in the impedance spectra that is dependent on the concentration of atrazine present in the sample.

Also based on ID μ E an improved immunosensor for sulfonamide antibiotic residue analysis has been reported [37, 38]. This new transducer used a three-dimensional interdigitated electrode in which the different electrode digits are separated by insulating barriers. Using this strategy it was possible to use most of the electric field instead of using just a small percentage (Fig. 3, left). Moreover, there is a significant increase of the available area to immobilize the immunoreagents. On the other hand, the electrodes in this case are made of TaSi instead of gold, diminishing the nonspecific adsorptions on the surface. The immunosensor allows direct detection of the antibiotic sulfapyridine (SPY) and shows an IC_{50} value of $5.3 \mu\text{g L}^{-1}$ in buffer. As can be observed in Fig. 3 (right), the three-dimensional sensor shows considerable improvement compared with a standard planar InterDigitated Electrode Array (IDEA) design.

3 Optical Biosensors

Optical biosensors show great promise for residue analysis. Although first developments took advantage of the flexibility and low cost of the optical fibers measuring the absorption or emission of light of one of the components of the bioreaction [39], later developments were mainly based on micro/nanotechnology and on the possibility

to fabricate complex micro/nanostructures to guide light which has opened up the possibility to investigate novel optical phenomena and the optical properties of materials at the nanometric level (for reviews on new optical biosensing systems see [40–44]). Some of these developments have found application in the detection of emerging pollutants and also pharmaceuticals (i.e., [45–47]) and herein we will describe some of these examples.

3.1 Biosensors Based on the Evanescent Wave Optical Principle

An evanescent wave (EW) is produced in the external media (refractive index, n_2) of a waveguide (n_1) by the electromagnetic field associated to the light guided by total internal reflection (TIR). The electromagnetic field does not abruptly switch to zero at the interface between the two media ($n_1 > n_2$), but decays exponentially with distance from the interface. The penetration depth of the evanescent field is defined as the distance where its strength is reduced to $1/e$ of its value at the interface and generally has a value around a hundred nanometers. The penetration depth is dependent on the incidence angle at the interface and is proportional to the wavelength of the excitation light. When molecules with an absorption spectrum including the excitation wavelength are located in the evanescent field, they absorb energy leading to attenuation (attenuated total reflection, ATN) in the reflected light of the waveguide. One of the advantages of the biosensors based on this principle is that possible interferences from the bulk media are avoided since only directly absorbed substances interfere with the electromagnetic field.

With the aim to improve detectability, many reported immunosensors combine this principle with the use of labeled molecules that are able to re-emit the absorbed evanescent photons at a longer wavelength as fluorescence. Part of this emission is coupled back to the waveguide and in this way is transmitted to the receptor. This phenomenon is known as total internal reflection fluorescence (TIRF). As an example a fully automated TIRF-based biosensor study for progesterone in bovine milk has recently been reported measuring progesterone levels in daily milk samples for 25 days, covering the whole estrus cycle [48]. The immunochemical assay was designed as a binding-inhibition test with a progesterone derivative covalently immobilized on the sensor surface and a monoclonal antiprogesterone antibody as the biological recognition element. Progesterone levels can be measured in about 5 min with a LOD of 0.04 ng mL^{-1} . The biosensor can be used in-line in the milking parlor and thus could be an important tool for reproductive management of dairy cattle detecting heat and predicting pregnancy, which are critical parameters in milk production. Similarly, the River Analyzer (RIANA) is also a highly sensitive fully automated biosensor able to detect multiple organic targets rapidly and simultaneously [49–51]. Thus, the system has been used to analyze a variety of analytes in environmental samples with very good limits of detection. Hormones, pesticides, antibiotics, and endocrine-disrupting chemicals are among the organic pollutants that have been detected with this biosensor system [47, 49, 52, 53].

More recently, other EW immunosensor approaches such as *Grating Couplers* (i.e., [54–56]) or *Mach-Zehnder Interferometers* (MZI) [57–60] have been investigated in order to make possible direct measurements of small analytes without the use of fluorescent labels. Thus, planar optical waveguides with an input grating coupler, or input/output gratings, have been reported with the aim to be used as label-free biodetection devices. Nanoimprint lithography is used to integrate the grating patterns with thin-film waveguides [55]. An optical waveguide lightmode spectroscopy (OWLS)-based biosensor is a recently developed device in the field of integrated optics, and exploits the science of light guided in structures smaller than the wavelength of the light. The system OWLS has been commercialized by MicroVacuum Ltd (Budapest, Hungary). The immobilization of a bioligand to an OWLS-based biosensor occurs by the silanization of the waveguide layer and the coupling of it to the silanized sensor surface. The structure of the chip consists of an input grating and a waveguide layer. The light of a He-Ne laser ($\lambda = 632.8$ nm) is diffracted by the grating at the interface and starts propagating via TIR inside the waveguide. At a well-defined incident angle (α), the phase shift during one internal reflection equals zero (constructive interference) and a guided mode is excited, which generates an evanescent field penetrating into the covering medium. A change in refractive index at the surface as a consequence of a biorecognition reaction is monitored on-line by precise measurement of incoupling angle as a function of time with the operating software of the immunosensor system. This principle has been used to analyze sulfamethazine, by immobilizing the antibody on the surface of the chip and reaches a LOD of 10^{-8} M.

A similar system has been developed by the Centre Suisse d'Electronique et de Microtechnique (CSEM, Neuchâtel, Switzerland) [61–63]. The wavelength interrogated optical immunosensor (WIOS) also measures changes in the refractive index of the evanescent wave. In this case, the chip has an input and an output grating (Fig. 4). Light from a VCSEL laser emitting at around 763 nm is incident on a first grating and when coupling takes place, the waveguide mode is excited and propagates into the waveguide layer until the second grating where the light couples out

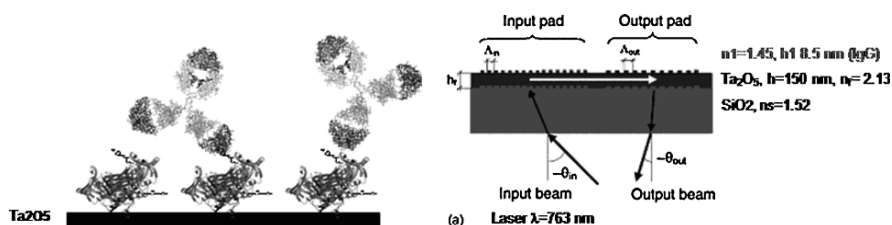


Fig. 4 Scheme of the WIOS system. The chip is a customized platform that contains 24 sensing sites. Each site is waveguide grating. Light from a VCSEL laser emitting at around 763 nm is incident on a first grating. The waveguide mode is excited and propagates into the waveguiding layer. The second grating couples out the guided light (at a different angle), which is collected with large plastic optical fibers. Electronics controls the laser wavelength modulation and amplifies the detected signals. A computer does the data acquisition and processing

of the waveguide (at a different angle) and is collected with large plastic optical fibers. Since the resonant coupling occurring at the first grating is also dependent on the wavelength (λ_i) of the incident light, an optical configuration has been envisaged that allows working at a fixed incidence angle (θ) while the wavelength at which coupling takes place is monitored through the output grating. This allows recording of effective index variations of the waveguide by continuously monitoring this parameter. On the basis of this principle, an immunosensor to detect sulfonamide antibiotic residues in milk samples has been developed. Immunoreagents appropriately produced to detect a wide range of sulfonamide antibiotic congeners [64] have been incorporated on the surface of the chip making use of a photopolymerizable dextran layer. The WIOS immunosensor is able to reach the necessary detectability to determine the most frequently used sulfonamide antibiotics used in the veterinary field, in compliance with the EC regulations. Thus, sulfapyridine can be detected in milk with a LOD value of 0.50 ± 0.12 and $0.24 \pm 0.06 \mu\text{g L}^{-1}$ and buffer samples, respectively. Other sulfonamides tested showed LODs below the MRLs values [65].

On a *Mach-Zehnder interferometer* the propagating light is split into two arms, one with the appropriate sensing layer and the other acting as a reference. The evanescent field of the measuring arm collects information regarding the bioreaction via the change produced in the refractive index. Consequently, the velocity of the wave in this arm varies. At the end recombination of the waves from both arms allows observation of a constructive or destructive interference, which is related to the extent of the bioreaction that has occurred on the sensing arm. Although there is intensive research in this field to our knowledge no applications to the development of biosensors for pharmaceuticals or emerging pollutants have been reported [57–60].

3.2 Plasmon Resonance Biosensors

The plasmon resonance is an evanescent electromagnetic field generated at the surface of a metal conductor (usually Ag or Au) when excited by the impact of light of an appropriate wavelength at a particular angle (θ_p). Surface plasmons are generated by electrons at the metal surfaces that behave differently from those in the bulk of the metal. These electrons are excited by the incident light, producing an oscillation (resonance) at different frequency from that in the bulk of the metal film. The absorption of light energy by the surface plasmons during resonance is observed as a sharp minimum in light reflectance when the varying angle of incidence reaches the critical value. The critical angle depends on the wavelength and polarization state of the incident light, but also on the dielectric properties of the medium adjacent to the metal surface and therefore is affected by analytes binding to that surface. This principle has been extensively investigated for monitoring of biological interactions in different types of configuration. We will provide herein some examples of late developments regarding detection of pharmaceuticals using this principle, however for additional information the reader is directed to some recent reviews (i.e., [39, 43, 66]).

Since distinct surface plasmon resonance (SPR) prototypes (Biacore, IASys, etc.) have appeared in the market, a significant number of applications of this principle have been reported during the last year. For example, in a recent paper surface plasmon resonance has been used to analyze 17 β -estradiol (E2) in sewage in the coastal marine environment [67]. Using the commercial Biacore system, the small molecule E2 is immobilized on a CM5 sensor chip for an indirect competitive immunoassay. The LOD achieved was 0.445 $\mu\text{g L}^{-1}$ although to analyze environmental samples, the SPR was coupled to a solid-phase extraction system [67]. Similarly, several examples of the use of SPR commercialized by Sensia to analyze environmental organic pollutants (DDT, atrazine, chlorpyrifos, and carbaryl) in natural water samples have been reported [46, 68–70]. BPA has also been analyzed using a SPR biosensor immobilizing a BPA-ovalbumin (BPA-OVA) conjugate on the Au thin film of the SPR sensor chip by just physical adsorption [71]. The lowest detection limit for BPA by SPR was around 1 ppb, almost the same as the LOD reached by ELISA (enzyme-linked immunosorbent assay) using the same immunoreagents.

An interesting example also reports the development of a novel surface plasmon resonance-based biosensor assay for the bioeffect-related screening of chemicals with thyroid-disrupting activity [72]. For this purpose two thyroid transport proteins (TPs), thyroxine-binding globulin (TBG) and recombinant transthyretin (rTTR), were applied in an inhibition assay format in a Biacore 3000 using CM5 biosensor chips coated with L-thyroxine (T4), the main hormone of the thyroid system. Known thyroid disruptors and structurally related compounds such as halogenated phenols, halogenated bisphenols, bisphenol A, 3,5-dichlorobiphenyl, and its hydroxylated metabolite 4-hydroxy-3,5-dichlorobiphenyl (4-OH PCB 14) were tested in this system to their relative potency (RP) with T4. The TBG-based assay was highly specific for T4, and the rTTR-based assay was sensitive toward several compounds, the highest sensitivity (RP = 4.4) being obtained with 4-OH PCB 14, followed by tetrabromobisphenol A (RP = 1.5) and tetrachlorobisphenol A (RP = 0.75).

Regarding antibiotic residues, a rapid and sensitive screening qualitative method using a surface plasmon resonance biosensor has been reported that detects all fenicol antibiotic residues in shrimps from a single sample extract [88]. This method requires ethyl acetate extraction followed by a single wash with isooctane/chloroform. Each sample extract is injected over the surfaces of two biosensor chip flow cells, one surface having the capability to detect florefenicol amine (FF amine), florefenicol (FF), and thiamphenicol (TAP) and the second surface for chloramphenicol (CAP) detection. The estimated detection capabilities (CC β) were 0.1, 0.2, 250, and 0.5 ppb for CAP, FF, FF amine, and TAP, respectively. This quick, simple test allowed the detection of CAP residues in shrimps at the minimum required performance limit of 0.1 $\mu\text{g kg}^{-1}$ for this compound and of FF, FF amine, and TAP below their maximum residue limits. Previously, detection of chloramphenicol and the mammalian metabolite chloramphenicol glucuronide (CAP-Glu) with the Biacore Q biosensor has also been reported. Honey, porcine kidney, and prawn extracts were analyzed after solvent extraction and solid-phase clean-up. Similarly, the determination of β -lactams using

Biacore has been reported, by immobilizing either an enzyme receptor [73] or a specific antibody [74]. In the first case, the specific recognition is based on the enzymatic activity of a carboxypeptidase converting a 3-peptide into a 2-peptide, a reaction that is inhibited in the presence of β -lactams. Antibodies were used to measure either the amount of formed enzymatic product or the amount of remaining enzymatic substrate. Different β -lactams were detected at or below European maximum residue limits. Thus, the LOD for penicillin G was 1.2 and 1.5 g kg⁻¹, depending on whether the 2- or 3-peptide was detected, respectively. The Biacore biosensor was also employed to detect β -lactams using a commercial antibody against ampicillin which had much higher affinity for the open β -lactam ring than for the closed ring. Two different pretreatments were tested prior to the biosensor assay to open the ring in order to increase the assay sensitivity. The LODs achieved for ampicillin in milk were 33 and 12.5 μ g L⁻¹ after enzymatic and chemical pretreatment, respectively. Conversely, penicillins G and M could be detected at or below their respective MRLs (4 and 30 μ g L⁻¹ in milk, respectively) and ampicillin and amoxicillin could be detected only at about the MRLs after chemical pretreatment.

A step forward in the use of plasmon resonant (PR) properties of materials is the use of particles for biological detection. Thus, colloidal gold or silver particles, typically 10–120 nm in diameter, are plasmon resonant. The particle geometric confinement of the surface plasmon gives rise to a spectral resonance in the light scattering that does not occur in thin films such as those used in the Biacore. The light scattered by nanometer-sized colloidal metal particles is dominated by the collective oscillation of the conduction electrons induced by the incident electric field (light), as mentioned above for the plasmon resonance of flat surfaces. However, in this case, the frequency band (specific color) of the scattered light is a function of the size, shape, and material properties of the particle. Thus, silver or gold nanoparticles, with diameters of 30–120 nm, efficiently scatter light in the visible spectrum. Variations in the refractive index around the nanoparticle surface also affect the resonance peak (frequency band of the scattered light), for which reason biomolecular interactions can be detected using nanoparticles (Fig. 5). This physical phenomenon is known as localized surface plasmon resonance (LSPR). An example of the application of this physical transducer principle to the analysis of an anabolic androgenic steroid such as stanozolol, has been recently reported [75, 76]. The chip could be fabricated in house by first activating the glass substrate with a silanizing agent possessing either a NH₂ or a SH group to bind the gold nanoparticles on the top of a flat SiO₂ (glass) surface without the observation of clusters. Following this, a stanozolol hapten-BSA conjugate was used to coat the gold nanoparticles and the detection of the hormone was made under competitive conditions by just mixing the sample with the antibody and adding a drop on top of the LSPR chip. The extinction spectra of the gold colloids were measured by conventional dark-field spectroscopy, illuminating the sample with a halogen lamp (100 W). The light scattered by the particles was analyzed with a microspectrometer and a CCD camera. Using this simple set-up stanozolol could be measured to a level below the minimum required performance level.

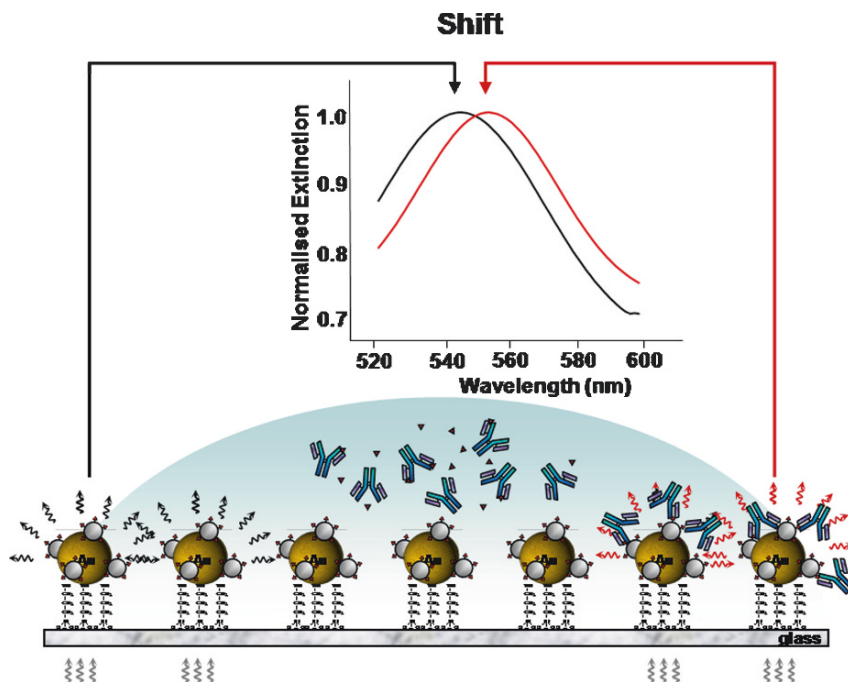


Fig. 5 Schematic of the chip using Au nanoparticles on top of a glass substrate used to detect stanozolol, and androgenic steroid used illegally to increase meat production or athletic efficiency in sport competitions. Specific binding of the antibody to stanozolol hapten-BSA coated on top of the nanoparticles, produces a shift in the resonance peak of the nanoparticles that has been used to detect this anabolic androgenic steroid. Reproduced from [75]

3.3 Nanomechanical Biosensors

Finally, biosensors based on microcantilevers (MCLs) have become a promising tool for directly detecting biomolecular interactions. Microcantilevers translate molecular recognition of biomolecules into nanomechanical motion that is commonly coupled to an optical or piezo-resistive read-out detector system. Biosensors based on cantilevers are a good example of how nanotechnology and biotechnology can go together. Microcantilever sensors rely on their deflection to indicate sensing. Thus, molecular adsorption onto the sensing element shifts the resonance frequency and changes its surface forces (surface stress). Surface stress due to conformation change of proteins and other polymers has been a recent focus of MCL research. Since conformational changes in proteins can be produced through binding of analytes at specific receptor sites, MCLs that respond to conformational change-induced surface stress are promising as transducers of chemical information and are ideal for developing microcantilever-based biosensors. On the other hand, high-throughput platforms using arrays of cantilevers have been developed for simultaneous measurement and read-out of hundreds of samples. Although many interesting applications in the environmental and clinical diagnostic field have been reported

(for recent reviews see [42, 77–80]), examples of their use to detect pharmaceuticals are still scarce.

4 Piezoelectric Immunosensors

Piezoelectrics are materials that may be brought into resonance by the application of an external alternating electric field. The frequency of the resulting oscillation is determined by the mass of the crystal. By coating a piezoelectric with an appropriate biomolecule such as an antibody, these kinds of immunosensors can, in principle, directly detect the binding of the corresponding analyte (for reviews see [81, 82]). Piezoelectric immunosensors may adopt two modes: (1) *Bulk acoustic (BA) devices* where adsorption of the analytes occurs on the coated surface of a piezoelectric crystal connected to an oscillator circuit. Resonance occurs on the entire mass of the crystal. If for example an antibody-coated crystal is placed in an atmosphere containing the selected analyte the immunoreaction will produce an increase in the mass of the crystal. The resonant frequency will therefore decrease according to the Sauerbrey equation: $\Delta f = -2.3 \times 10^6 f^2 (\Delta m/A)$, where f is the oscillation frequency in Hz, Δm is the adsorbed mass in g, and A is the sensing area in cm^2 . (2) *Surface acoustic wave (SAW) devices* where an acoustic wave moves just at the surface of the crystal. Mass loading on the acoustic path between two sets of electrodes will alter the phase wave velocity and cause a shift in the frequency. This technology can, in principle, detect binding events produced at the surface of the piezoelectric material.

Several examples can be found in the literature regarding the use of this type of sensor for environmental, food safety, and clinical applications. Liu et al. reported the detection of various polycyclic aromatic hydrocarbons (PAHs) by immobilizing the antigen (benzo[a]pyrene-BSA conjugate, BaP-BSA) on gold-coated quartz crystals, with a basic resonant frequency of 10 MHz, through thioctic acid [83]. The monoclonal antibody 10c10 (mAb10c10) allowed one to determine benzo[a]pyrene (BaP) in the flow injection system by using a competitive pattern, down to the nanomolar level. Similarly, a piezoelectric immunosensor system was developed for the rapid detection of polychlorinated dibenzo-p-dioxins (PCDDs) using a direct enzyme immunoassay format [84]. The purpose of using the enzyme was just to increase the mass of the binding molecule. The mouse monoclonal antibody against 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) was deposited on a 10-MHz AT-cut quartz crystal resonator modified with a self-assembly monolayer of dithiobis-N-succinimidyl propionate. Competition took place between the analyte and a conjugate of a dioxin-like competitor coupled to the enzyme horseradish peroxidase. Measurement of the frequency responses allowed detection of 2,3,7,8-TCDD in the concentration range of 0.01–1.3 ng mL^{-1} . In another example BPA was detected down to 10 ng mL^{-1} using antibodies coupled to nanoparticles, approximately 200 nm in diameter, with the aim to increase the mass change on the surface of the immunosensor and thereby increase the frequency shift detected [85]. PCBs have also been detected using a piezoelectric immunosensor, by immobilizing a polyclonal antibody on a gold electrode via protein A. Detection in the range of 1–50 ppm was achieved using either

enzyme or polystyrene particles to increase the response of the sensor [86]. As a representative example of the use of this principle to analyze pharmaceutical residues, Park et al. reported the direct detection of chloramphenicol by covalently immobilizing specific antibodies onto the gold electrode surface of piezoelectric crystals using a self-assembled monolayer, obtaining a significant shift of the resonance frequency in about 10 min [85]. However, the detectability achieved was much worse than in those formats where a competitive configuration was used.

5 Conclusions

Future advances in biosensor development will need scientists from different disciplines combining their research efforts. First, improvement of transducer technology is necessary in order to allow direct detection on environmental samples and to confer the necessary flexibility to develop field analytical methods. Frequent new micro/nanotechnological advances are opening up new and exciting possibilities that should be investigated in order to find novel ways to convert biomolecular recognition events into electrical or optical signals. Second, increasing the number of analytes that can be measured using the biosensor technology is necessary. Especially interesting are those compounds that are difficult to measure using currently accepted methods. Antibody-based biosensors continue to show great potential due to the excellent features of this type of biomolecule (stability, chemical homogeneity etc.) and the capability to produce them against almost any type of analyte, which may broaden the number of compounds that can be analyzed. This versatility of the immune system combined with the progress that recombinant DNA technology is making in this field may increase the potential of these bioreceptors. A third trend should be developing integrated units able to perform multianalyte measurements. And finally the extensive research that is taking place in this area will not give the expected fruit without performing rigorous validation studies with environmental samples. All these facts will contribute to improving investment by private companies to finally deliver reliable devices into the market.

Acknowledgments This work was supported by the Ministry of Science and Technology (Contract numbers AGL2005-07700-C06-01, NAN2004-09415-C05-02, NAN2004-09195-C04-04, and DEP2007-73224-C03-01) and by the European Community (Confidence, Proposal No.: 211,326 - CP Collaborative Project). The AMR group is a Group de Recerca de la Generalitat de Catalunya and has support from the Departament d'Universitats, Recerca i Societat de la Informació la Generalitat de Catalunya (expedient 2005SGR 00207). We would also like to give our thanks for the contribution from research groups from the CNM-CSIC, CSEM, Unisensors, and UAB to some of the results presented herein.

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Recombinant Yeast Assays and Gene Expression Assays for the Analysis of Endocrine Disruption

Benjamin Piña, Susanna Boronat, Marta Casado, and Alba Olivares

Abstract Endocrine disruption is a particularly pervasive form of pollution, as it affects the reproductive and metabolic systems at very low concentrations. In its most usual form, endocrine disruption is mediated by the binding of exogenous substances to certain natural hormone receptors that regulate the endocrine system. Here we describe two tools to study potential or actual impacts of endocrine disruptors in wild-life and humans. The Recombinant Yeast Assay, RYA, is an in vitro method to evaluate the ability of a given compound or a mixture of substances to bind a specific receptor and to elicit, at last partially, the physiological responses associated to this binding in vivo. The second tool, the analysis the expression of hormone-responsive genes by quantitative real-time PCR, measures directly endocrine disruption effects in exposed organisms in vivo, either in the field or in controlled experiments. Both strategies are based on the concept that hormone receptors are ligand-dependent transcription factors and that the principal primary responses to the endocrine regulation (and also to endocrine disruption) are changes on the expression of hormone-responsive genes. These methods are aimed to the rapid screening for potential ligands for hormone receptors and to the identification of endocrine disruption signals at lower concentrations and shorter times of exposure than those required to develop the associated pathologies.

Keywords Biomarkers, Bioassays, Endocrine disruption, Gene expression, Hormone receptors, Recombinant Yeast Assay, Quantitative Real-Time PCR

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Contents

1	The Recombinant Yeast Assay.....	74
1.1	The Genetic Setup.....	77
1.2	Mathematical Modelling.....	81
1.3	Practical Examples.....	86
2	Gene Expression Biomarkers in the Study of Endocrine Disruption	88
2.1	Methodology of the Analysis of Gene Expression	91
2.2	Practical Uses of Gene Expression Biomarkers: What are they Useful for?.....	97
3	Conclusions.....	103
	References.....	103

Till, piercing each the other's flesh, they run/Together, and incorporate in one:[...] Shoot up the same, and wear a common rind:/Both bodies in a single body mix./A single body with a double sex.

The story of Salamacis and Hermaphroditus, *Metamorphoses*/Book IV, Ovid

Separation of sexual roles has been a constant for most human cultures for as long as we have historical records. Deviations from the standard male/female dichotomy have been historically regarded as a perversion of the natural order or considered a sophisticated way of divine punishment, like in the Hermaphroditus myth. This cultural background fuelled the almost universal upheaval provoked by revelations in the early 1960s indicating that chemical pollutants may perturb reproductive functions of wildlife and humans, including sterility and sex reversal. This was the origin of the concept of endocrine disruption [1, 2]

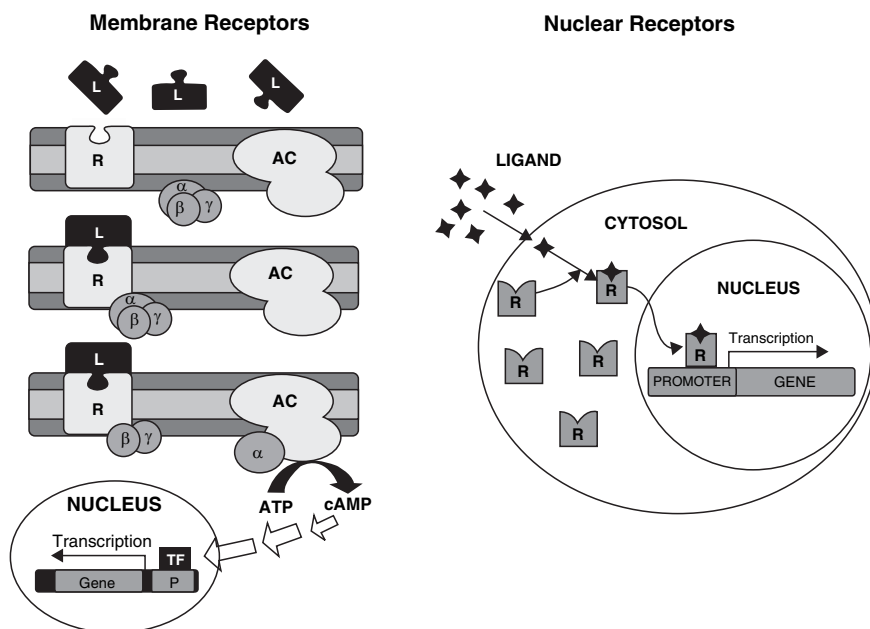
The international definition for endocrine disruptors, agreed in the 1996 Weybridge workshop, defines them as “exogenous substances which cause adverse effects in an organism, or its progeny, subsequent to changes in the endocrine system” [3]. Endocrine disrupting compounds include a variety of chemical classes, including hormones, plant metabolites, pesticides, compounds used in the plastics industry and in consumer products, and other industrial by-products and pollutants. Some of them are persistent organic pollutants (POP's) that can be transported long distances across national boundaries and have been found in virtually all regions of the world. Others are rapidly degraded either by physical agents or microbiological metabolism in the environment, remaining active for only short periods of time. Finally, many of these compounds may be metabolised in the exposed individuals, with subsequent changes in their chemical and biological properties.

Since the seminal publication of Rachel Carson's *Silent Spring*, endocrine disruption effects have been recorded in different wildlife populations: adverse effects include abnormal thyroid function and development in fish and birds; decreased fertility in shellfish, fish, birds and mammals; decreased hatching success in fish, birds and reptiles; demasculinisation and feminisation of fish, birds, reptiles and mammals; defeminisation and masculinisation of gastropods, fish and birds; decreased offspring survival; and alteration of immune and behavioural function in birds and mammals [4, 5].

Epidemiological effects of endocrine disruption in humans are (luckily) much scarcer, although this has not mitigated the general concern about them, under the general understanding that human health is inextricably linked to the health of the

environment. Many reports of endocrine disruption in humans came not from environmental risks, but rather from side effects of hormonal and obstetric medication. A paradigmatic case was the anti-abortion drug diethylstilbestrol (DES), which was discovered to affect the development of the reproductive system and cause vaginal cancer in the offspring of treated mothers [4]. Nevertheless, many health effects have been attributed to endocrine disrupting compounds, including a range of reproductive problems: reduced fertility, male and female reproductive tract abnormalities, skewed male/female sex ratios, loss of foetus, menstrual problems, changes in hormone levels; early puberty; brain and behaviour problems; impaired immune functions; and different cancers [6].

Different toxicity mechanisms may result in endocrine disruption, and their effects can be as complicated as the endocrine system is. Endocrine systems display a delicate conjunction of production of small molecules that are released into the bloodstream (the ligands) by specific secretory glands, and the presence of equally specific proteins in the target tissues, the hormone receptors, which recognise the ligands and that have the capacity to modify the cell metabolism according to their presence or absence. Generally speaking, there are two versions of this mechanism, depending on the nature of both the receptors and the ligands (Fig. 1). Membrane receptors are anchored in the cell membrane and are structured in two well-defined functional domains. The outer domain interacts with the cell media and recognises the ligand, whereas the inner domain originates a secondary signal as a response to this interaction. This secondary signal is transmitted from the inner plasma membrane into the



L: Ligand; R: Receptor; AC: Adenylate Cyclase; P: Promoter; TF: Transcription Factor

Fig. 1 Model of mechanisms of action of membrane-bound (*left*) and nuclear hormone receptors (*right*)

cell nucleus through a cascade of protein kinases, which result in a modification of the functionality of transcription factors and a subsequent change in the expression pattern of the target cells. In contrast, nuclear receptors are soluble proteins present in the cytoplasm or in the nucleus of target cells waiting for their ligands to cross the plasma membrane. In the presence of the ligand, the resulting receptor–ligand complex acts as a transcription factor, binding to DNA and modifying the expression of hormone-responsive genes. The physiological ligands for membrane receptors are typically small peptides, like insulin or neuropeptides, whereas nuclear receptors bind to small organic molecules, in many cases related to the steroid pathway. In consequence, nuclear receptors are sometimes dubbed as “steroid hormone receptors”, although in some cases their ligands are definitively not steroids; the best known example is the thyroid hormone receptor.

Cell receptors vary in their ability to bind exogenous substances. Because of the proteinaceous nature of their ligand, membrane receptors are in general less prone to be fooled by non-endogenous substances, and therefore, they are more resilient to endocrine disruption. There are notorious exceptions to this rule, opioids being an excellent example. Nevertheless, most current research on endocrine disruptors focuses on nuclear receptors, for anthropogenic compounds may easily share critical structural features with their natural ligands.

The so-called nuclear hormone receptor superfamily includes receptors for thyroid and steroid hormones (estrogens, progestins, androgens, mineralocorticoids and glucocorticoids), retinoids and vitamin D, ecdysone in invertebrates, as well as different orphan receptors of unknown ligands [7]. New ligands for some of these receptors have been identified, such as fatty acids [8], prostaglandin [9], or cholesterol derivatives [10]. All these receptors share a common ancestor; representatives of the superfamily are found in all metazoans but, as far as we know, they are absent in fungi, plants and other eukaryotes [11]. Up to date, a single representative of this family has been reported in Porifera [12]. The different receptor families and their ligands are shown in Table 1 (modified from [7]). Many potential exogenous ligands have been reported for several of these receptors, particularly for hormone receptors related to sex steroids (ER, AR and PR, Table 1, [4]), and many of them may cause endocrine disruption in exposed fauna and/or human populations. A deeper knowledge of the mechanisms of control of the endocrine system resulted in a notable increase in the number of receptors for which putative exogenous ligands have been reported, including TR, RAR, PPAR, PXR, CAR and RXR [13–17] (Table 1). The possible effects of these exogenous ligands in exposed populations are largely unknown, but it has been suggested that molecules interacting with the PPAR/RXR complex may promote obesity in humans [16]; in any case, it is clear that these ligands may affect sexual development and other physiological processes in molluscs [18, 19].

An unexpected addition to the list of endocrine disruptors occurred with the discovery that toxic effects of dioxines and benzopyrenes were mediated through their interaction with the Aryl hydrocarbon receptor (AhR); mice devoid of this receptor become refractory to the effects of TCDD [20] and of B[a]P [21]. The AhR belongs to a completely different family of transcriptional regulators, the bHLH-PAS family [22], with representatives in both Prokaryotes and Eukaryotes, although their ability to interact with ligands originated apparently with Chordates [22]. The

Table 1 Subfamilies of mammalian nuclear receptors

	Receptor	Denomination	Ligand	Binding Pattern ^a	Reported disrupters ^b
Class I	TR	Thyroid hormone receptor	Thyroid hormone (T3)	H	X
	RAR	Retinoic acid receptor	Retinoic acid	H	X
	VDR	Vitamin D receptor	1-25(OH) ₂ vitamin D3	H	
	PPAR	Peroxisome proliferator activated receptor	Benzotriene B4; Wy 14,643 Eicosanoids; thiazolidinediones (TZDS); 15-deoxy-12,41 - polyunsaturated fatty acids	H	X
	PXR	Pregnane X receptor	Pregnanes; C21 steroids	H	X
	CAR/MB67	Constitutive androstane Receptor	Androstanes; 1,4-bis[2-(3,5- dichloropyridyloxy)]benzene	H	X
	LXR	Liver X receptor	Oxysterols	H	
	FXR	Farnesoid X receptor	Bile acids	H	
	RevErb	Reverse ErbA	Unknown	M, D	
	RZR/ROR	Retinoid Z receptor/ retinoic acid-related orphan receptor	Unknown	M	
	UR	Ubiquitous receptor	Unknown	H	
Class II	RXR	Retinoid X receptor	9-Cis-retinoic acid	D	X
	COUP-TF	Chicken ovalbumin upstream promoter transcription factor	Unknown	D, H	
	HNF-4	Hepatocyte nuclear factor 4	Fatty acyl-CoA thioesters	D	
	TLX	Tailles-related receptor	Unknown	M, D	
	PNR	Photoreceptor-specific nuclear receptor	Unknown	M, D	
	TR2	Testis receptor	Unknown	D, H	
Class III	GR	Glucocorticoid receptor	Glucocorticoids	D	X
	AR	Androgen receptor	Androgens	D	X
	PR	Progesterone receptor	Progestins	D	X
	ER	Estrogen receptor	Estradiol	D	X
	MR	Mineralocorticoid receptor	Mineralocorticoids	M, D	X
	ERR	Estrogen-related receptor	Unknown		

^aM, monomer; D, homodimer; H, heterodimer.^b“X” indicates receptors for which that putative or bona fide exogenous ligands have been reported (see text)

physiological role of AhR, as well as the precise nature of its presumptive physiological ligands has not been completely elucidated yet, but it is known to become activated by a numerous family of aromatic compounds and to regulate specific phase I and II metabolic enzymes, among others [23, 24]. Exogenous ligands of AhR are known to elicit a broad range of physiological effects, including immune dysfunction, endocrine disruption, reproductive toxicity, developmental defects, and cancers in vertebrates [25–27].

There are essentially two ways to study potential or actual impacts of endocrine disruptors in wildlife and humans. First, there is an array of *in vitro* methodologies to evaluate the endocrine-disruption potential of a given compound or of an environmental sample [28–33], methodologies that can be coupled to surveillance protocols or to quantitative structure-activity relationship (QSAR) analysis [34]. A more direct approach is to analyse endocrine disruption effects in exposed organisms, either in the field or in controlled experiments. Different end-points for endocrine disruption have been described for both human populations and wildlife (reviewed in [6]). Here we describe two tools covering both strategies, both aimed at the rapid screening for potential ligands for hormone receptors and the identification of endocrine disruption signals at lower concentrations and shorter times of exposure than those required to develop the associated pathologies: yeast-based receptor binding assays and the quantification of gene expression biomarkers.

1 The Recombinant Yeast Assay

Genetically modified yeast strains have become common tools to detect exogenous ligands for different receptors. These tools have been applied to detect the presence of receptor-binding activity in the environment and to screen for new putative receptor binding substances among the ever-increasing number of new chemical compounds appearing in the market every year [29, 35–37] (Table 2). Yeast bioassays have been used to correlate the presence of suspected or bona-fide endocrine disruptors and estrogenic activity in environmental samples [38–40], and to establish relationships between chemical structures and affinity for vertebrate hormone receptors [37, 41–44]. These systems, also known as recombinant yeast assays (RYA) do not provide a precise chemical characterisation of the hormone-receptor ligands, but their low cost and easiness of handling makes them a first choice when testing large numbers of ill-characterised samples or compounds. A very detailed protocol to perform RYA has been recently published [33].

Yeast-based bioassays consist of an engineered yeast strain harbouring at least two foreign genetic elements, one of them allowing the expression of a given vertebrate nuclear receptor in the yeast cell and the second one devised to allow monitoring of the activity of the expressed receptor. In this second genetic element, a convenient gene whose expression is easy to quantify (the reporter gene) is under transcriptional control of the expressed nuclear receptor. The setup ensures that the genetic product of the reporter gene (an enzyme or a fluorescent protein, for example) will only be expressed in the presence of the receptor–ligand complex.

Table 2 Examples of use of RYA to monitor exogenous ligands for vertebrate hormone receptors

Receptor	Species	Receptor structure	Receptor expression/ Promoter	Reporter	Applications	References
PR	chicken	Ub-PR	Inducible/CUP1	PRE-LacZ	Test strain	[130]
	human	Full length	Inducible/CUP1	PRE-LacZ	Test ligands	[41]
	human	GAL4DBD-hPRDN23	Constitutive/ADH1	GAL1-LacZ	Test ligands	[131]
	human	Full length	n.a.	PRE-LacZ	Environment	[132]
	human	Full length	n.a.	PRE-LacZ	Environment	[133]
	human	Full length	n.a.	PRE-yEGFP	Test ligands	[134]
	chicken	Ub-PR	Inducible/2 × ERE- CYC1-ub-cPRA	PRE-LacZ	Test strain	[135]
	human	lexADBDBD-hPRLBD	Constitutive/ADH1	lexA-LacZ	Test strain	[60]
	rat	Full length	Constitutive/ADH1	PRE-LacZ	Environment	[29]
	human	Full length	Inducible/CUP1	ARE-LacZ	Test ligands	[41]
AR	human	Full length	Constitutive/PGK	ARE-lux A/B + ARE-lux CDE+ftp	Test ligands	[113]
	rat	Full length	Constitutive/GPD	GRE-LacZ	Test ligands	[136]
	human	Full length	Constitutive/GPD	ARE-LUC-slk	Environment	[137]
	human	Full length	Constitutive/GPD	ARE-yEGFP	Test ligands	[44]
	human	Ub-FLAG-AR	Inducible/CUP1	ARE-LacZ and ARE-GFP	Test ligands	[138]
	mouse	lexADBDBD-ARLBD	Constitutive/ADH1	lexA-LacZ	Test ligands	[139]
	human	Full length	Constitutive/PGK	ARE-LacZ	Test ligands	[140]
	human	Full length	n.a.	ARE-LacZ	Environment	[141]
	human	Full length	n.a.	ARE-LacZ	Environment	[142]
	human	GAL4DBD-PPAR+GAL4AD-CBPID	Constitutive/ADH1	MEL1-LacZ	Test ligands	[63]
PPAR	rat	Ub-PPAR + Ub-RXR	Inducible/CUP1	PPARRE-LacZ	Test strain	[143]
RXR	human	RXR-LBD + TIF2-ID	Constitutive/ADH1	GAL1-LacZ	Environment	[144]

(continued)

Table 2 (continued)

Receptor	Species	Receptor structure	Receptor expression/ Promoter	Reporter	Applications	References
AhR	AhR	AhR + ARNT + SRC1a	Inducible/GAL1	MEL1DUAS-DRE-Luc	Environment	[145]
	human	AhR + ARNT	Inducible/GAL1	DRE-LacZ	Test ligands	[146]
	human	AhR + ARNT	Inducible/GAL1	DRE-LacZ	ligands human	[147]
	human	AhR + ARNT	Inducible/GAL1	DRE-LacZ	Environment	[148]
	human	AhR + ARNT	Inducible/GAL1	DRE-LacZ	Environment	[149]
	human	AhR + ARNT	Inducible/GAL1	DRE-LacZ	Environment	[150]
	human	AhR + ARNT	Inducible/GAL1	DRE-LacZ	Environment	[151]
	human	AhR + ARNT	Inducible/GAL1	DRE-LacZ	Test ligands	[152]
	human	AhR + ARNT	Inducible/GAL1	DRE-LacZ	Test ligands	[153]
	human	AhR + ARNT	Inducible/GAL1	DRE-LacZ	Test strain	[29]
	human	AhR + ARNT	Inducible/GAL1	DRE-LacZ	Environment	[74]
	human	AhR + ARNT	Inducible/GAL1	DRE-LacZ	Test ligands	[66]
GR	human	DN	Inducible/GAL1	GRE-LacZ	Test ligands	[154]
PXR	mouse	GAL4-DBD-mPXR+GAL4AD-SRC1 or GAL4AD-RIP140	Constitutive/ADH1	GAL4UAS-LacZ	Environment	[155]
	human	GAL4DBD-TRa1LBD + GAL4AD-RXRaLBD	Constitutive/ADH1	GAL1-LacZ	Test ligands	[156]
VDR	human	GAL4DBD-VDR + GAL4AD-RXRβ	Constitutive/ADH1	GAL1-LacZ	Test ligands	[157]
	human	GAL4DBD-VDR + GAL4AD-hRXRa	Constitutive/ADH1	GAL4UAS-LacZ	Test ligands	[158]

Abbreviations: n.a. not available; DBD, DNA Binding Domain; LBD, Ligand binding Domain; AD, Activation Domain; ID, Interaction Domain; ΔN, truncated N-terminal domain; UAS, Upstream Activating Sequence

This imitates the mechanism by which many hormone-responsive genes are regulated in vertebrates; the fundamental similarity of all eukaryotes ensures that the same mechanism also works in yeast. A comparison between the physiological pathway in vertebrate cells and its engineered yeast version is shown in Fig. 2.

The expression of the reporter gene is monitored by its enzymatic or fluorescent activity by using convenient quantitative techniques. This activity is directly related to the effective concentration of estrogenic compound(s) through a typical dose–response sigmoidal curve. The system does not provide a direct measurement of the molar (or mass) concentration of endocrine disruptors, but of their estrogenic activity. For simplification, results are given in “hormone equivalents”, defined as the amount of a standard ligand (estrogen for the estrogen receptors, progesterone for the progesterone receptors, testosterone or di-hydroxytestosterone for the androgen receptor, etc.) that should be present in a given sample to account for all the observed response.

1.1 The Genetic Setup

1.1.1 Receptors

Yeast lack nuclear receptor homologues; therefore, they have to be introduced in the strain’s genetic make-up. This apparently complicated step is much facilitated by the power of Molecular Biology tools available for *Saccharomyces cerevisiae*,

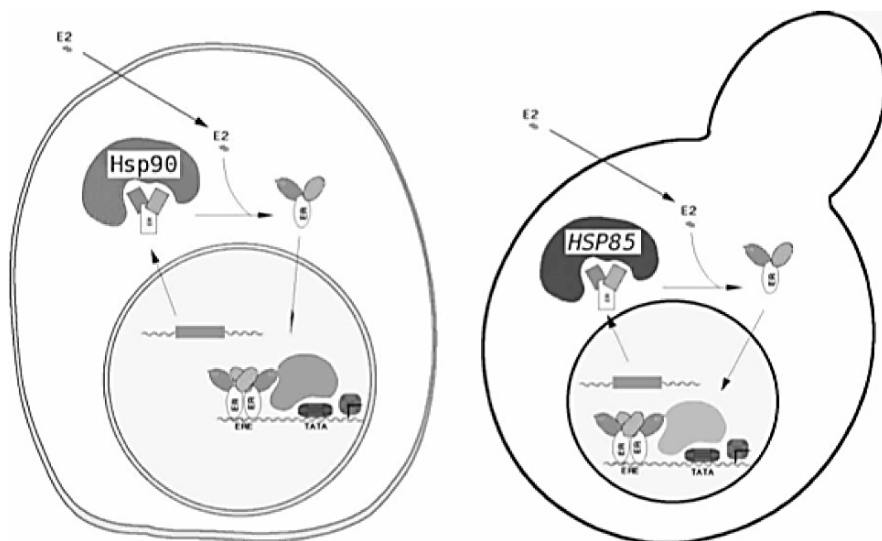


Fig. 2 Schematic representation of the transfer of the mechanism of hormone response from a vertebrate cell (*left*) to yeast (*right*). Note that the receptor itself and the reporter gene are the only two elements that have to be added to the yeast genetic set-up; the rest of the components are provided by the yeast genome

the yeast species almost universally used for RYA assay. Cloning and expressing non-homologous proteins in yeast are standard procedures. In the design of a RYA system, there are at least three choices to make: the receptor to clone, the species to clone it from, and the yeast expression system.

In principle, DNA sequences coding for any desired receptor could be obtained from appropriate tissues of any species. In practice, the methodology (extraction of RNA from the tissue, its retrotranscription into cDNA, and amplification of the specific cDNA molecule encompassing the desired gene) requires not only physiological information of the tissue to start from and, some times, the appropriate conditions of age and maturation of the specimen, but also DNA sequence information to design the appropriate primers and probes. Because of the multi-family nature of nuclear receptors, there are different genes with similar sequences but different functions, the so-called orphan receptors being good exponents of this. A number of different receptors for mammals and teleostei has already been cloned and tested; there is a much shorter list for birds, reptiles and amphibians, and an even shorter one for the rest of animal Phyla. To have a hint of how imperfect our knowledge of the endocrine system in non-vertebrates is, it is illustrative of the fact that we do not have a good understanding of the mechanism of action of the juvenile hormone in insects, despite the massive knowledge on development and molecular biology of *Drosophila* [45, 46].

1.1.2 Yeast Expression Vectors

Expression of any heterologous gene in yeast can be brought about by a handful of well-studied genetic mechanisms (see [47–50] for detailed protocols and methods). The basic strategy is to place the desired DNA coding sequence under the control of a yeast promoter; the addition of a yeast terminator 3' of the inserted gene normally facilitates the post-transcriptional processing of the heterologous transcript and, many times, increases its stability in the cell. The conjunction of promoter-coding sequence-terminator is sometimes described as an “expression cassette”, for it constitutes a genetic unit that directs the expression of the cloned protein (the receptor, in our case) when introduced into the yeast cell. Two main types of yeast promoters are used to express foreign proteins. Constitutive promoters are those that induce expression of contiguous genes permanently, which results in a stable level of transcript. On the contrary, inducible promoters are silent unless a given inducer is present in the cell. Expression vectors activated upon addition of galactose or copper are the most used inducible systems to express foreign proteins in yeast. The use of one or another system is essentially a trial and error decision: some proteins inhibit yeast growth and, therefore, it is better to use an inducible system to propagate yeast cells in their absence and express it only when is needed. However, this introduces a necessary activation step that may increase both variations among experiments and the time required to complete the process.

The combination of promoter-coding sequence-terminator has to be afterwards introduced into a recipient yeast cell, and maintained in a form as stable as possible.

There are essentially three ways of introducing and maintaining foreign DNA sequences in yeast: by integration into the yeast genome, by creating artificial yeast chromosomes, either linear or circular, or by using the naturally occurring yeast plasmid 2 μ [50].

Yeast integrates into its genome linear sequences by homologous recombination, provided their flanking sequences coincide with DNA sequences already present in the genome. As a result, the cell receives a single copy of the introduced sequence. Yeast genome has minimal positioning effects and they are restricted to perfectly documented regions, such as telomeres and subtelomeric sequences [51].

Unlike most eukaryotes, yeast chromosomes have very small and well-defined centromeres, which span less than 1 Kb and that are fully functional when cloned into any DNA fragment. This property can be used to construct artificial DNA elements that segregate into daughter cells with efficiencies similar to the natural chromosomes. In the simplest version, circular molecules encompassing a yeast centromere and an origin of replication (ARS) can be used to carry any desired DNA sequence. These constructs, called ARS/CEN plasmids, are very useful for maintaining DNA fragments up to 10–20 Kb in yeast. Longer sequences require the use of linear constructs that encompass, in addition to centromere and ARS, yeast telomeres at both ends. These constructs are called YAC (for yeast artificial chromosomes), but they are not usually used for the purposes described here. ARS/CEN plasmids are usually maintained in one to three copies in the yeast cell [52–54].

The 2 μ (two-micron) plasmid is a genetic autoreplicative element found in many, but not all, yeast strains, and for which no specific function has been described yet. Its replication is uncoupled from the yeast genomic DNA and it maintains some 200 copies in the cell, being distributed passively into mother and daughter cells. The wild plasmid encodes different enzymes required for its own replication; however, a defective form only encompassing the origin of replication (about 1 Kb) is stable in yeast provided the complete 2 μ version supplies necessary trans elements. The defective 2 μ has been widely used for introducing and maintaining a stable number of copies (from 10 to 20) of chimeric bacterial/yeast constructs, able to propagate in both systems and that can be engineered to carry different sequences, including gene expression cassettes [54].

Expression of foreign proteins confers no selective advantage to yeast strains; on the contrary, it can reduce their fitness. Therefore, receptor-expressing cells would be rapidly overgrown by non-expressing counterparts. For this reason, it is necessary to associate the receptor-expressing cassette with some genetic element that provides the necessary selective advantage. These elements, called markers, exist in two main forms. In the simplest one, the marker provides the recipient cell with enzymes required for its growth in selective media. This is usually achieved by using yeast strains defective for some crucial step in some catabolic process and introducing the missing gene along with (usually, physically linked to) the construct [52]. A second type of marker includes those conferring resistance to antibiotics, like gentamicin or hygromycin. These so-called dominant markers have the advantage of not requiring any specific yeast genetic background, but they do require the continuous presence of the antibiotic to maintain the selective pressure [55].

1.1.3 Reporter Genes

Once the receptor is expressed in the yeast cell, it is necessary to design a method to monitor its binding to any ligand that may exist in the medium. Nuclear receptors are facultative transcription factors, able to recognise specific DNA sequences and to activate neighbouring promoters when the ligand is present. RYA takes profit of this fundamental property by devising the so-called reporter genes, that is, genes encoding proteins easy to detect under the control of a silent promoter complemented with DNA recognition sites for the expressed receptor (HRE, hormone response element). Upon the presence of an appropriate ligand, the receptor-ligand complex would bind to the HRE and activate transcription of the reporter gene; the concentration of the resulting protein would reflect the amount of ligand present (see below).

The most used reporter genes are bacterial enzymes with activities completely absent in natural yeast strains, like β -galactosidase (lacZ) or β -glucuronidase (GUS) [56–58]. The resulting enzymatic activities may be easily monitored by using chromogenic (ONPG, X-gal blue, etc.) [59] or fluorogenic substrates (MUG, MUGal, etc.) [60]. An alternative possibility is to use autofluorescent proteins, like GFP. In this version no substrate is required, as GFP fluorescence can be detected and quantified by irradiating GFP-expressing cells with appropriate wavelengths [61]. Another approach comes from the use of bioluminescent bacteria operons that recapitulate in yeast the bioluminescence phenomenon observed in bacteria upon activation of its expression. The feasibility of these approaches depends on the capabilities of sophisticated fluorescence readers able to differentiate the natural yeast fluorescence from the induced one. Whatever the technique used to measure the expression levels of the reporter gene, it reflects the concentration of the ligand following a sigmoidal dose-response curve. The mathematics underlying this kind of response will be discussed below

1.1.4 Requirement for Cofactors

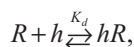
Nuclear receptors differ in their capability to trigger transcriptional activation of reporter genes in yeast. Mammalian ER is a very good activator in yeast, indicating that the yeast transcriptional machinery readily recognises activation domains in the ER as such, in an amazing demonstration of evolutionary conservation of cell regulatory signals. However, this is not always the case, and many nuclear receptors are very poor activators in yeast. In this case, the habitual solution is the use of the so-called “two hybrid strategy”, in which both the receptor and recognised targeted factors are cloned in yeast, in a way that the receptor bears the DNA recognition domain (homologous or heterologous, see below) and the cofactor is supplied with a bona fide yeast activation domain. The presence of the ligand favours the binding of the receptor to DNA and its interaction with the homologous counterpart. The presence of the yeast activation domain transforms this ternary complex into a true yeast activator [62–64].

A particular case occurs when the receptor needs some coactivator to bind DNA. This is the case, for example, of VDR (Table 1), which requires RXR to bind to the

corresponding HRE. The unrelated AhR also requires a cofactor, known as AhR Nuclear Translocator, or ARNT, to bind DNA. In this case, there are at least two possible strategies. An obvious one is to supply the yeast strain with all cofactors required for allowing the receptor to bind DNA and promote transcription. A second possibility is to replace the homologous DNA binding domain (DBD) of the receptor by a heterologous DBD. In many cases, these chimeric constructs maintain most pharmacological features of the homologous system [65], although some fundamental differences on response to different ligands have been reported recently [66].

1.2 Mathematical Modelling

The simplest and most popular model for dose-response curves assumes equilibrium between hormone-free and hormone-loaded hormone receptor molecules in solution,



in which R represents the concentration of hormone-free receptor molecules, h the hormone concentration, hR the concentration of the hormone-loaded receptor molecule, and K_d is the dissociation constant. This particular model assumes a single agonist molecule binding to each receptor molecule, as has been demonstrated by numerous physical and crystallographic data for nuclear receptors [67]. Assuming a hormone concentration much larger than the receptor concentration, the fraction of receptor bound to the hormone Φ_r can be described by the Hill equation:

$$\Phi_r = \frac{[hR]}{R_t} = \frac{1}{1 + \frac{K_d}{[h]}}. \quad (1)$$

In which R_t is the total receptor concentration (bound and free) [68].

The Hill equation only describes the equilibrium for the hormone–receptor interaction. It is frequently assumed that this is the rate-limiting step in the cascade of events going from the presence of a given concentration of hormone and the final physiological effects – expression of β -galactosidase, GUS or GFP fluorescence. K_d represents the dissociation constant of the ligand-hormone complex; its value coincides with EC_{50} , the ligand concentration giving 50% of the maximal response. Apparent K_d values can be calculated for each sample or tested compound by measuring the observed response at different concentrations or dilution factors. There are different ways to calculate apparent K_d values from an experimental dose–response curve. A simple way is to determine the LOD (lower observable dilution) and estimate that it corresponds to EC_{10} , approximately [60]. More rigorously, the mid-point of the resulting dose–response curve (by definition, EC_{50}) can be calculated from the experimental data by non-linear regression methods, assuming

the Hill's equation [60]. Some linear approaches to this method have also been published [69, 70]. Figure 3 shows a typical example of a dose-response graph for the estrogen receptor.

Different K_d values obtained by RYA can be used to estimate the relative ligand potency of a given substance or environmental sample, when compared to a standard ligand we use as reference. The nature of this reference ligand depends on the receptor. In some cases, it is easy to determine which molecule has to be selected, like estrogen for the estrogen receptors, progesterone for the progesterone receptors, testosterone or di-hydroxytestosterone for the androgen receptor, etc. For some receptors, like the PPAR or the AhR, there is no such "natural standard", and some well-known and easily manipulable heterologous chemical (rosiglitazone, β -naphthoflavone) has to be used instead [63, 71–73]. Once the standard ligand has been selected and its EC_{50} values have been established for each receptor, the relative ligand potency of a substance can be then calculated as:

$$\frac{EC_{50}(\text{compound})}{EC_{50}(\text{reference})}. \quad (2)$$

A very similar equation applies to complex samples:

$$\frac{EC_{50}(\text{dilution factor})}{EC_{50}(\text{reference})}. \quad (3)$$

In this case, the relative potency of the sample can be interpreted as the concentration of standard ligand it should contain to elicit the observed response. This approach can be used in the inverse direction; if we know the concentration of different ligands in a sample and their relative potencies, we can predict the expected potency of the sample, following the equation:

$$\text{Total potency} = \sum X_i \varepsilon_i. \quad (4)$$

In which X_i represents the concentrations for each compound calculated from chemical analysis and ε_i the corresponding relative potency factors. The adjustment of this prediction to the observed potency of the sample would indicate that the chemical analysis is able to identify and quantify all relevant ligands for a particular receptor. Significant differences between the two values indicate either the presence of an unchecked ligand (Calculated < Observed) or interferences between different ligands (Observed < Calculated). Examples of such analysis and relative potency values have been already published (Table 3, data from [73, 74]).

1.2.1 Inhibitory Effects

All biological assays are more or less prone to suffer from inhibitory effects that reduce its sensitivity. In the case of RYA, an obvious possibility is that the sample or compound to analyse may be toxic to the yeast cell and therefore it prevents the

Table 3 EC₅₀ values for different chemicals tested with ER-RYA and AhR-RYA systems

COMPOUND	AhR EC ₅₀ (mg/L)	ER EC ₅₀ (mg/L)
17- β -Estradiol	n.d.	$(7.3 \pm 0.4) \times 10^{-5}$
b-Naphtoflavone	0.014 ± 0.02	n.d.
PCB9	0.4 ± 0.2	b.d.l
PCB29	1.1 ± 0.3	8 ± 5
PCB30	b.d.l	63 ± 23
PCB50	b.d.l	7.5 ± 2.3
PCB58	b.d.l	b.d.l
PCB77	0.014 ± 0.005	b.d.l
PCB104	b.d.l	5.3 ± 3.7
PCB126	0.9 ± 0.3	b.d.l
PCB188	b.d.l	b.d.l
HCB	7.7 ± 0.5	b.d.l
pp-DDT	b.d.l	b.d.l
Diuron	0.26 ± 0.10	>200
N-Demethyl diuron	2.7 ± 1.2	b.d.l
1-(3,5-Dichlorophenyl)urea	40.6 ± 0.7	b.d.l
Irgarol	b.d.l	b.d.l
Sea-Nine	b.d.l	b.d.l
BZT-OH	6.9 ± 0.8	>10
BZT	10.2 ± 0.6	5.5 ± 1.3
MetBZT	11.0 ± 0.6	8 ± 3
DEHP	n.d.	2.3 ± 1.5
OP	n.d.	0.19 ± 0.08
Estriol	n.d.	$1.2 \times 10^{-4} \pm 6.4 \times 10^{-5}$
estrone	n.d.	$2.1 \times 10^{-3} \pm 1.2 \times 10^{-3}$
Et-estradiol	n.d.	$2.3 \times 10^{-4} \pm 1.3 \times 10^{-4}$
DES	n.d.	$8.7 \times 10^{-4} \pm 4.4 \times 10^{-4}$
BPA	n.d.	1.6 ± 0.9
BBP	n.d.	5.1 ± 2.2
DEP	n.d.	7.0 ± 2.3
DMP	n.d.	4.1 ± 1.2
BZT	n.d.	5.5 ± 1.3
MeTBZT	n.d.	7.8 ± 3.2
DBP	n.d.	4.0 ± 1.5
HOBt	n.d.	6.3 ± 1.7
NP	n.d.	0.08 ± 0.02
NP1EO	n.d.	3.0 ± 0.4

bdl. below detection limit; nd. not done

Abbreviations: BBP: butyl benzyl phthalate; BPA: bisphenol A; BZT-OH: 3'-hydroxy benzothiazole; BZT: benzothiazole; DBP: dibutyl phthalate; DEHP: bis(2-ethylhexyl) phthalate; DEP: diethyl phthalate; DES: dietilestilbestrol; DMP: dimethyl phthalate; HCB: hexachlorobenzene; HOBt: 1-Hydroxybenzotriazole; MetBZT: 2-methyl benzothiazole; NP: nonylphenol; NP1EO: nonylphenol mono etoxylated; OP: octylphenol; p,p'-DDT: 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane; PCB: polychlorobiphenyl.

required biological response to the presence of ligands. There are several procedures to assess whether or not a negative value is due to toxicity or to the absence of ligand activity. An obvious one is to spike the samples or compounds with a saturating concentration of the standard ligand; toxicity would prevent response whatever the ligand would be. A more sophisticated method is to measure endogenous yeast

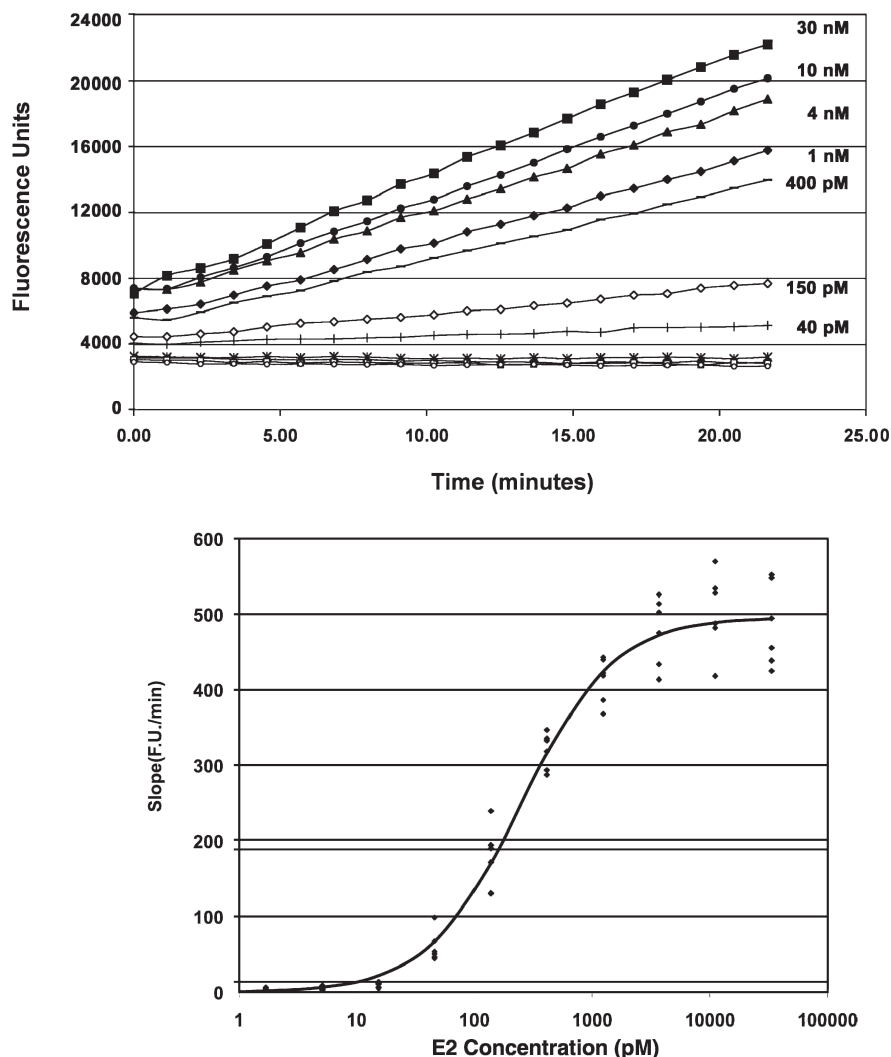


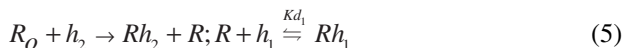
Fig. 3 Response ER-RYA to different concentrations of estradiol (fluorogenic substrate). *Top*, linear plots of the increase of fluorescence (in fluorescence units, FU) with time for different estradiol concentrations in the ER-RYA system. Concentrations of estradiol are indicated on the right margin. *Bottom*, Dose-response curve for estradiol (E2) in the ER-RYA system. The y-axis represents β -galactosidase activity in FU/min, *diamonds* represent values from six independent determinations for each estradiol concentration. Graphs indicate theoretical curves as defined by Hill's equation. The corresponding parameters were calculated by non-linear regression methods. Data from [60]

activity (for example, expression of a reporter gene under a yeast promoter) in the presence and absence of the sample; in this case a quantitative estimation of the toxicity can be obtained [42]. However, there is a radically different mechanism preventing a bona fide ligand to elicit biological response. There are ligands, the binding of

which not only does not elicit response, but it prevents the system from responding to the presence of other ligands present in the same solution. Ligands that elicit the physiological response are known as receptor agonists, whereas those preventing the same response are called antagonists. The term “partial agonists” is used to define ligands able to elicit a limited subset of the physiological responses of pure agonists.

The interplay between agonists, antagonists and partial agonists is central in the physiology and the pharmacology of many endocrine-acting compounds. However, most of this delicate balance is simplified in RYA, as yeast lacks most of the cofactors and interactions intervening in the vertebrate hormonal regulation. As a consequence, most ligands able to bind to a given receptor would be classified as agonists in the yeast assay. However, there are substances acting as antagonists in yeast, and this peculiarity is important in the characterisation of the potential toxicity of a new substance. The antagonistic effect can be precisely studied in yeast, producing data on the inhibitory potential, in a similar way as the ligand potential described before (Fig. 3, [42, 73]). In this regard, one has to consider three mechanisms of mutual interaction among a pure agonistic ligand (h_1) and an inhibitor (h_2).

The competitive inhibition model proposes an equilibrium between free receptor, R , and two ligands that bind alternatively to a single site of the receptor molecule, with dissociation constants K_{d1} and K_{d2} :



$$K_{d1} = \frac{[R][h_1]}{[Rh_1]}, K_{d2} = \frac{[R][h_2]}{[Rh_2]}. \quad (6)$$

The total amount of receptor molecules, free and occupied (R_o), can be expressed as

$$R_o = [Rh_1] \left[1 + \frac{K_{d1}}{[h_1]} + \frac{K_{d1}}{K_{d2}} \frac{[h_2]}{[h_1]} \right]. \quad (7)$$

At any given concentrations of h_1 and h_2 , any target gene would show a fraction of its maximal activation at a saturating concentration of h_1 ($A/A_{\max}$), which that could be expressed as:

$$\frac{A}{A_{\max}} = \frac{[Rh_1]}{R_o} = \frac{1}{K_{d1} + \frac{K_{d1}}{1 + \frac{K_{d2}}{[h_1]}} [h_2]} \quad (8)$$

In this variant of the Hill equation, $A_{\max}$ is independent from h_2 , whereas the apparent EC_{50} for h_1 (EC_{50app}) equals K_{d1} only when $h_2 = 0$. At any other concentration of h_2 , EC_{50app} would equal

$$EC_{50app.} = K_{d_1} + [h_2] \frac{K_{d_1}}{K_{d_2}} \quad (9)$$

And therefore,

$$\frac{EC_{50(h_2)}}{EC_{50(h_2=0)}} = 1 + \frac{[h_2]}{K_{d_1}} \quad (10)$$

in which $EC_{50(h_2)}$ and $EC_{50(h_2=0)}$ correspond to the EC_{50} for h_1 in the presence and in the absence of a given concentration of h_2 . K_{d_2} is then identical to IC_{50} ; its value can be calculated as the inverse of the slope of the plot corresponding to (10).

A second possible method of inhibition implies that the binding of h_2 to the receptor irreversibly inactivates it, reducing the amount of available receptor molecules for binding to h_1 :



If the proportion of receptor molecules becoming inactivated follows a typical logistic function with an inhibitory constant K_i ,

$$\frac{Rh_2}{R_o} = \frac{1}{1 + \frac{K_i}{[h_2]}} ; R = R_o - Rh_2. \quad (12)$$

In this model, the maximal activity at saturating concentrations of h_1 depends on the concentration of h_2 , as follows:

$$h_1 \rightarrow \infty, \frac{A_{\max, h_2} - A_{\min, h_2}}{A_{\max, h_2}} = \frac{R_o}{R} = 1 + \frac{[h_2]}{K_i}. \quad (13)$$

K_i is therefore equivalent to the concentration of h_2 reducing to 50% the maximal activity at saturating concentrations of h_1 , IC_{50} , by definition. This model is indistinguishable from a general toxicity of the tested compound for the yeast cells, with identical parameters.

1.3 Practical Examples

Table 2 shows some examples of how RYA can be used in different ways to monitor putative endocrine disruptors. This kind of study can be divided into at least three groups, relating to their intended purpose. The largest group is constituted by experiments aimed at assessing the potential affinity of a known substance to a

given receptor, as a method to evaluate environmental risks [44, 72, 73, 75]. A derivative of this group is the use of RYA to monitor changes of the affinity upon chemical modification of the ligand, either to evaluate the risk of ligand derivatives appearing in the environment, or to monitor the degradation or inactivation of the ligand by wastewater processing schemes [42, 76]. Finally, RYA can be used to evaluate endocrine disruption activities in environmental or industrial samples, without previous knowledge of the active compounds. Combined with chemical analysis, this system may help in the identification of the possible contaminants with potential endocrine disruption activities and monitor the appropriate remediation programs. As an example of this, Fig. 5 shows the results of a survey on EDC loads in different points along a complete basin in the Spanish Northern Mediterranean area to estimate the contribution of STPs to these loads [77]. In this particular case, chemical analysis data and RYA data showed a quasi-linear correlation, demonstrating the complementarity of both methods. Moreover, the

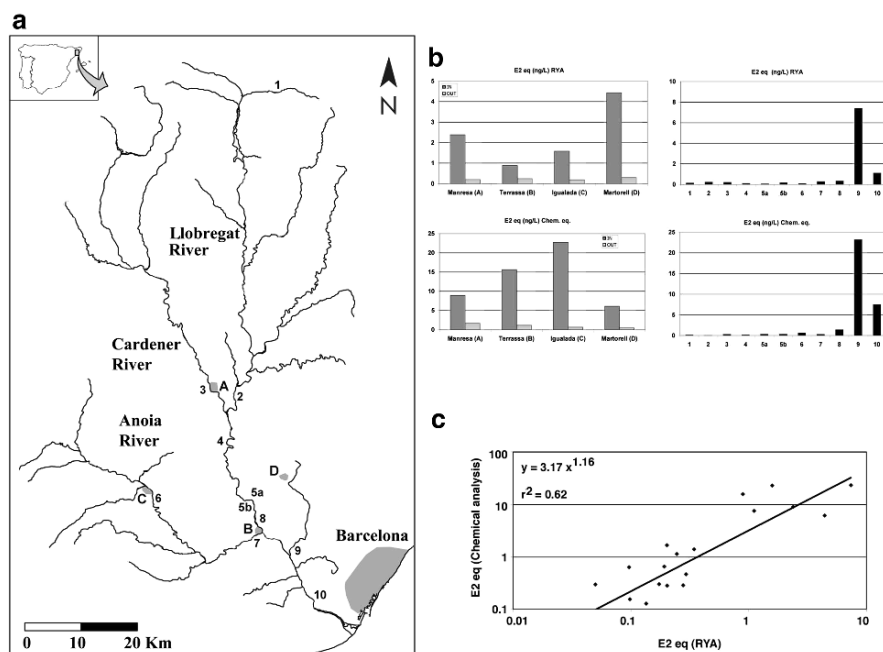


Fig. 4 Monitoring of putative endocrine disruptors in the Llobregat basin. (a) Map of sampling sites in the Llobregat basin. Numbers 1–10 indicate sampling points for river water, letters A–D correspond to STP locations. Grey areas indicate approximate locations of cities near to these STPs: A-Manresa; B-Terrassa, C-Igualada; D-Martorell. The position of the city of Barcelona is given to facilitate localisation of the area in the map. (b) Correlation between total estrogenicity values [(in E2 eq)] obtained from RYA (x-axis) and predicted from the chemical composition of the samples (y-axis). (c) Correlation between total estrogenicity values (in E2 eq) obtained from RYA (x-axis) and predicted from the chemical composition of the samples (y-axis). Note logarithmic scales and the power of regression. Squares represent data points for river samples, solid triangles correspond to SPT influent waters and grey circles show the corresponding values for effluent waters. Data from [60]

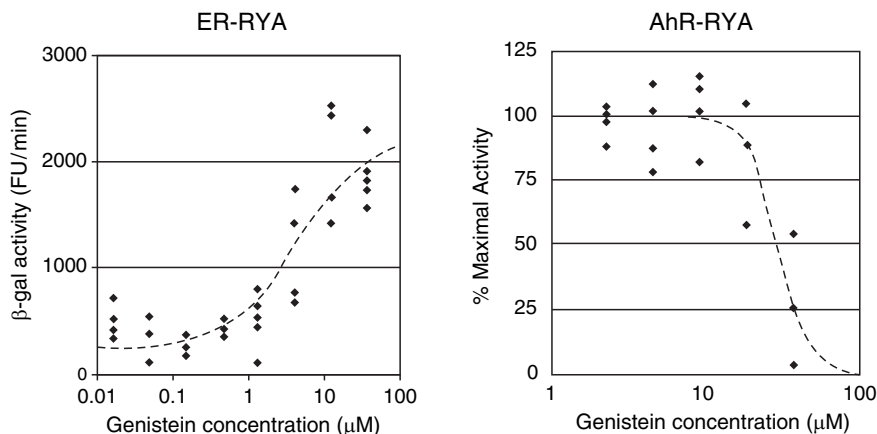


Fig. 5 Measurement of agonist and antagonist effects of genistein in RYA systems. (a) Dose-response curve for genistein in the ER-RYA system, in which it acts as a mild estrogen. (b) Inhibitory effect of genistein in the YCM-RYA (AhR), in which increasing concentrations of genistein resulted in a decrease of transcription of the reporter gene in spite of the presence of a saturating concentration of β -naphthoflavone (0.5 μ M). Data from [60]

concentrations of the analysed compounds were enough to explain the total estrogenicity of water and STP samples. However, RYA systems are able to detect the presence of active substances not specifically targeted by chemical analysis.

The combination of different RYA systems can also help with the characterisation of specific receptor antagonists. In nature, ligand binding to nuclear receptors elicits a delicate imbalance of activatory and inhibitory effects central to the physiological homeostasis of the living being. Antagonist effects could be easily mimetized or masked by cytotoxic effects, which prevent transcriptional activation by inhibiting vital functions. As opposed to cytotoxic substances, true receptor antagonists for a given RYA system would display receptor specificity, that is they should be neutral or activators in a different RYA system. This is the case for the natural phytoestrogen genistein, which acts as an agonist in ER-RYA (Fig. 4), with a calculated EC_{50} value of 2.9 ± 0.7 μ M (Table 3). However, when tested in an AhR-RYA system, genistein acted as an antagonist suppressing activation by β -naphthoflavone (Fig. 4).

2 Gene Expression Biomarkers in the Study of Endocrine Disruption

Biomarkers constitute an integral part of the ecotoxicological evaluation of risk in environmental management. Defined as changes in a biological (molecular) response that can be related to exposure to environmental pollutants, they are sensitive indicators

of toxic effect of pollutants in exposed biota [78, 79]. A reliable biomarker should distinguish between natural variability and contaminant-induced stress and should have some toxicological significance, that is, its response has to be related to the impact to the organism. Biomarkers evaluate the effects of the observed pollution on the exposed biota and provide an early warning in the cases in which anthropogenic impact is suspected but not confirmed. They also provide an excellent instrument to monitor the recovery of former impacted sites in remediation schemes.

The main functional role of nuclear receptors is to modify transcription of the genes constituting their primary response (Figs 1 and 2). This response is thereafter transmitted to behavioural, immunological, metabolic and reproductive levels, with ultimate effects on the population and on the whole ecosystem. Therefore, one can monitor the very early response to a ligand (or a disrupter) by measuring the changes in the transcription rates of the primary hormone response genes. This approach can be utilised in at least two different aspects of the study of endocrine disruption: incorporated into a bioassay scheme, in which laboratory animals are treated with different substances and extracts to analyse their potential as disrupters; or use them as biomarkers in exposed populations to determine the possible impact of endocrine disrupters.

There are many designed biomarkers to detect endocrine disruption. Feminising effects in fish and amphibians can be tested by measuring the levels of female-specific, egg-forming proteins in blood and/or liver from exposed males [80–83], including the egg-yolk precursor protein vitellogenin (Vtg) and several chorion-forming proteins, like the zona radiata proteins (Zrp). In mammals, the standard method is the uterotrophic test, which measures uterus growth in ovariectomised rats or rabbits after injection of a given substance [35, 84]. There are fewer valid tests for androgenic disruption, one of the most reliable is monitoring the expression of the glue protein, spiggin, in kidneys of female sticklebacks (*Gasterosteus aculeatus*) [85, 86]. The presence of AhR ligands is usually revealed by the activation of Type I and II enzymes in liver or other organs or the increase of their associated enzymatic activities, like EROD [13, 87, 88]. Thyroid disruption can be monitored by the levels of thyroid hormones in the blood or by histology of the thyroid gland [15, 17, 89].

Detection of endocrine disruption by biochemical or histological biomarkers usually requires relatively large amounts of sample, obtained either by exsanguination (blood) or by dissection (liver, kidney). In addition, in some cases they require specific antibodies that have to be obtained for each animal group and, sometimes, for each species to analyse. An alternative to these methods is the detection of the corresponding mRNA in the target tissue [42, 90–92] (Fig. 6). These methods require very little sample and benefit from the versatility and sensitivity of polymerase chain reaction (PCR)-based procedures. We would refer to these biomarkers based on measurement of the RNA as gene expression biomarkers.

Specific RNA quantification has become one of the most sensitive tools in Molecular Biology, especially since the introduction of the polymerase chain reaction, which allows detection of truly minimal amounts of RNA molecules by amplification of specific sequences. With appropriate extraction and analytical methodologies, as few as 10–100 RNA molecules can be detected and, with some limitations, quantified. The application of devices able to monitor the process of

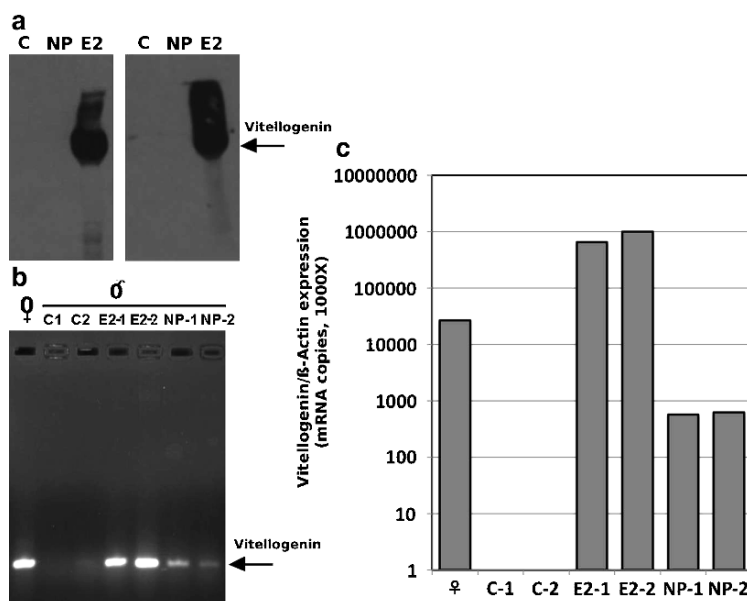


Fig. 6 Comparison of methods to monitor vitellogenin induction in *Fundulus heteroclitus* males upon injection of estradiol. (a) Immunological detection of vitellogenin in blood. (b) Semi-quantitative PCR analysis of vitellogenin expression in liver. (c) Relative quantitation of vitellogenin mRNA levels in liver qRT-PCR. Data from [42]

PCR in real time (quantitative Real-Time PCR, or qRT-PCR), makes more precise and reproducible the quantitation of amplified sequences, and allows processing of large numbers of samples [93, 94]. These characteristics are very useful when dealing with living populations. A high sensitivity allows both the application to small individuals (small animals, for example) and the use of dispensable parts of the body (scales, blood, blubber), avoiding the killing of larger animals [95–99]. On the other hand, the possibility of analysing many individuals allows the study of ecological impacts at the levels of populations rather than at the individual level.

The only true limitation for the application of gene expression biomarkers in biological monitoring is the knowledge of appropriate DNA sequences: any gene can be analysed in any species provided its sequence is known. RNA extraction protocols produce stable RNA solutions that can be stored at -80°C indefinitely and that can be re-tested and re-analysed should a new biomarker appear perhaps years after the original sampling. It is worthwhile considering that 1 mg of a soft tissue (e.g. liver) provides enough total RNA to perform 1,000 qRT-PCR tests; therefore, there is a considerable margin for re-evaluation of old, valuable samples. And lastly, total RNA samples contain information for all genes expressed in the sampled tissue, irrespectively of being enzymes, structural cell components or stress proteins. This allows simultaneous study of multiple effects and correlation with different sources of pollution with the same technology and sample preparation.

2.1 Methodology of the Analysis of Gene Expression

2.1.1 Sampling, Transport and Extraction

One of the critical points for the analysis of RNA from environmental samples is the choice of species and of tissue. Most (if not all, see below) RNA quantification methods rely on the knowledge of the exact sequence of at least a portion of the gene(s) to be monitored. Genomes of very different species are getting sequenced at amazing speed, but they are (and will forever be) restricted to a limited number of species, if we compare with the amazing biodiversity present in many environmentally relevant ecosystems. Even worse, there are entire Phyla and Classes that are severely under-represented in the list of sequencing projects, many of them of great environmental interest (for example, molluscs, annelids, and, among vertebrates, birds, reptiles and amphibians). This situation is bound to improve with time, but it would always be difficult to find one or two sequenced species in a particular environment. A plausible solution is to infer the unknown sequences from homologous genes from a close, better known species (see below); therefore, a suitable approach would be to choose species with well-known close relatives (rodents, salmonids, cyprinids, oysters, drosophilids, etc.) over those for which there are no “standard” relatives (gastropods, cartilaginous fish, annelids). In any case, most of the “orphan” taxa will be covered in the future, given the considerable effort being applied to cover wider portions of the extant biodiversity, especially for animals.

A second level of choice is what part of the organism is to be sampled. Some small organisms, like arthropods or very small fish, may be sampled whole, but in many cases the analysis has to be restricted to a defined organ or structure. This approach takes advantage of the differences in transcriptional regulation of many genes among tissues: a gene may respond to a particular hormone in liver, let's say, but not in brain or muscle —among other things, because the corresponding receptors may be absent. Therefore, limiting the sampling to the gene(s) and the tissues known to respond to a particular response increases the sensitivity and reliability of the results. As a disadvantage, only a fraction (some times minute) of the target tissue can be sampled for large to median-size individuals. Therefore, heterogeneous tissues, like brain, skin or even liver, if infiltrated with pancreas, require a special attention to ensure that the obtained sample is representative.

Nucleic acids in general, and RNA in particular, are highly unstable outside the living cells. This instability is not a consequence of their chemical structure, but of the ubiquitous presence of enzymes specifically designed to degrade them. These enzymes (DNases and RNases) come from a large variety of sources, the most important one being the very same tissue we pretend to extract nucleic acids from. There is only one method to really prevent RNA degradation, which is to get rid of RNases before they act. This is usually achieved by using phenol or/and mixes of chaotropic agents, such as guanidium salts, just after sampling. If this is not possible, liquid nitrogen is also used to quick-freeze samples and to transport them to the lab, where

they can be safely processed. Recently, commercial mixtures of chaotropic agents have been introduced as RNA stabilisers at non-freezing temperatures; these mixtures allow collection and distribution of samples with a minimum of equipment and do not require deep-freezing equipment, like liquid nitrogen tanks, for example.

Once in the lab, there are many protocols to extract RNA from tissues. If an RNA preserver has been utilised, particular care is required to ensure that the extraction protocol is compatible with the preserver. Phenol/chloroform mixtures are probably the most reliable agents to purify nucleic acids; however, some less toxic, chaotropic-based methods are also available. Purity and integrity of samples can be checked by UV spectrophotometry and/or gel electrophoresis; given the small amounts of RNA usually obtained from field samples, miniaturised systems are most preferable (e.g. nanospectrophotometer and micro-fluidic electrophoresis).

2.1.2 Sequence Selection and Primer Design

Once RNA samples are obtained, the task of analysing them is comparatively simple, as far as one knows what genes are to be quantified. Up to now, most work on gene expression biomarkers uses counterparts of well-established biochemical biomarkers, like cytochromes P450 for dioxin-like and other AhR ligands, or vitellogenin or zona radiata proteins for feminising compounds. The rationale underlying these assays is that the increase in expression and/or activity of the biochemical biomarkers should be reflected by—or be a consequence of—changes in the expression of the corresponding genes. This is not necessarily true in all cases, so a previous validation of the intended genetic biomarkers is always required [92].

The requirement for exact sequences is also a limitation for this step. Many genes, including cytochromes and egg-forming proteins constitute multigenic families from which only some of the members may be hormone-responsive. Therefore, the analysis should be selective enough to distinguish very similar gene sequences. This is particularly complicated when the known sequence does not belong to the sampled species, but to a relative. In this case, clustering of homologous sequences from several species from close taxa (same family at least) may allow determining the most conserved regions, which are the ones that would presumably coincide with the sequence of the species of interest. Once the appropriate region of the gene is established, standard primer-design programs (available both on-line and as part of sequence-analysis program packs) will determine primer pairs suitable for amplification. In most cases, it is advisable to amplify (see below) and sequence a relatively large portion—let's say 1–2 Kb—of the actual gene in the tested species, and to redesign primers using the actual targeted sequence [83, 92].

The number of putative biomarkers is going to increase enormously with the advances in genomics and transcriptomics. The deciphering of the regulatory web controlling gene expression at the cellular level would provide a great deal of new hormone-responsive genes in different species.

2.1.3 RNA Quantification Methods

Once separated from the rest of the cellular components, RNA molecules are all very similar, at least from the point-of-view of their chemical and physical properties. This is especially true for the messenger RNA molecules (mRNA), which code for proteins and constitute the primary source for gene expression biomarkers. In fact, the only and crucial difference among them is their own sequence, and this is the character we should focus on to identify and quantify the selected mRNA species. In practice, this represents detecting a particular RNA sequence diluted in a mixture of other RNA molecules in a molar proportion of, at most, $1:10^5$ and, in many cases, several orders of magnitude lower; this can be likened to finding a needle in a haystack.

Two properties of nucleic acids and of nucleic acid-synthesising enzymes are central to the development of RNA (and DNA) quantifying methods. First, the notion of complementary strands: nucleic acid molecules with complementary sequences associate one to each other forming an extremely stable complex. This complex may be detected and quantified by labelling one of the complementary molecules and measuring the corresponding signal, either radioactive, fluorescent or enzymatic. This is the basis of many of the “classical” RNA quantification methods, like the Northern blot or the dot blot. In the first case, RNA molecules are separated by gel electrophoresis, blotted to a convenient membrane and exposed to a solution containing the labelled complementary molecule, usually obtained by cloning the corresponding DNA fragment and growing it in bacteria. The dot blot uses the reverse strategy: the complementary molecule is fixed to a membrane unlabelled, and the sample is the one that is marked with the appropriate labelling. In both cases, the amount of label remaining tightly associated to the membrane is assumed to reflect the relative concentration of the targeted sequence in the RNA sample.

A different strategy for RNA detection and quantification comes from the PCR technique. This method relies on the ability of DNA polymerases to make exact copies of any DNA molecule provided the necessary cofactors (a priming DNA molecule, deoxyribonucleotide triphosphates and magnesium) are present. The discovery of DNA polymerases able to survive temperatures higher than those required to separate the two strands of DNA (about 95°C) allowed the design of an iterative protocol in which a single DNA molecule can be duplicated 20, 30 or more consecutive times, until reaching concentrations detectable by basic DNA fluorescent dyes, like Ethidium bromide or similar ones. Semi-quantitative PCR methods estimate the amount of the original molecule by quantifying the total amount of the amplification product (amplicon) after a sub-saturating number of amplification cycles; in qRT-PCR, the amount of amplicon in each of the amplification cycles is continuously detected by a fluorescence-based monitoring system (Fig. 7). The sequence of the amplicon is determined by the sequence of the primers present in the reaction, which should be complementary to both ends of the sequence to be amplified. To apply this methodology to RNA, it should be first transcribed (technically, retro-transcribed) into a complementary DNA molecule (cDNA); there are different enzymes (retro-transcriptases) catalysing this reaction. The qRT-PCR reaction is summarised in Fig. 7.

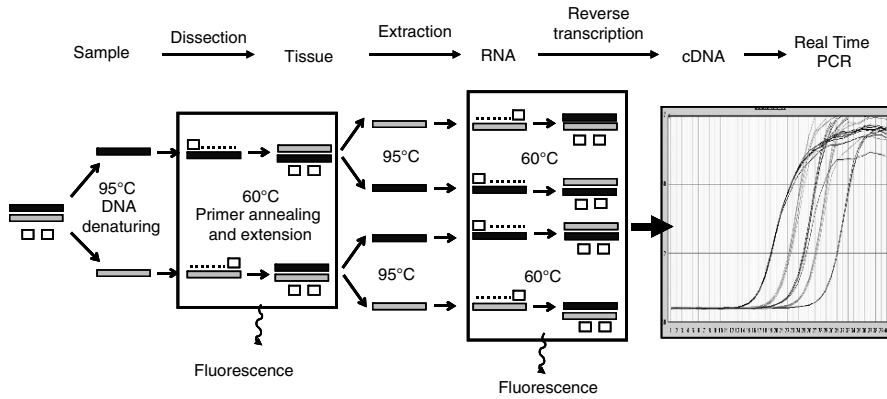


Fig. 7 Process of mRNA quantitation by qRT-PCR. Total RNA is extracted from various tissues (e.g. liver and scales) and copied to cDNA by the reverse-transcriptase reaction. Specific cDNA molecules are selectively amplified by a polymerase chain reaction; the process is monitored in real time by fluorescence methods. The number of cycles required for detection of the amplification product is inversely related to the initial number of molecules of mRNA coding the amplified sequence

2.1.4 Real-Time PCR Quantitation

Quantification of the expression of a particular gene by real-time PCR is based on the exponential relationship between the number of molecules accumulated in the reaction well, N , and the initial amount of gene copies, N_0 , according to (14):

$$N = N_0(1 + E)^c \quad (14)$$

In which E is the efficiency of the amplification reaction and c the cycle number. In optimal conditions, E equals 1.

During the amplification reaction, a fluorescent signal directly proportional to the amount of N is generated and monitored, giving rise to an amplification curve. The number of cycles required to reach the exponential increase in fluorescence is used to determine N_0 [see (14)]. Calculation of this cycle number depends on the real-time PCR platform, and there are essentially two methods: determination at a constant fluorescent level (Threshold Cycle or C_t , Applied Biosystems and Biorad) and calculating the second derivative maximum of the amplification curve (C_p , Roche Applied Science and Corbet Research) [93, 100, 101]. Both methods aim to determine how many cycles are required for each sample to reach the same number of copies of the PCR product (N); this information is used to calculate N_0 according to (14).

Quantification of copy number can be absolute, when relating the PCR signal to input copy number using a calibration curve [102–104], or relative, when measuring the relative changes of copy number between two or more samples. In this case, units used to express relative quantities are irrelevant, and the relative quantities can be compared across multiple real-time RT-PCR experiments. However, there

are many factors that may influence the number of mRNA copies in a given sample and preclude adequate data comparison between samples. Changes in the integrity of the nucleic acid, in the amount of starting material and/or in the efficiency of the reverse transcription reaction may influence qRT-PCR results by even an order of magnitude. Therefore, and in order to compensate for these sources of variation, normalisation by an internal control is required. The most popular strategy is to refer data to a reference gene, whose abundance should be considered uniform across individuals and is not affected by the studied parameters [105].

Probably no gene fulfils completely these requisites in any living species, but a common approach is to select genes implicated in the formation and maintenance of the cell, the so-called “housekeeping” (HKG) genes. They fall mainly in three categories: structural proteins (actins, tubulins, etc.), enzymes involved in the glycolysis (GPD), and proteins involved in the formation and function of ribosomes (ribosomal proteins and translation factors) [102]. However, numerous studies have shown that mRNA levels of many HKG genes may vary under different experimental conditions [106, 107]. Then, the best strategy is to test some of these putative candidates in a relatively large subset of samples to assess which one better reflects the total amount of mRNA actually participating in the PCR reaction [105]. There are software tools capable of evaluating this kind of data and determine the most appropriate reference genes [108, 109]. Table 4 shows a list of reference genes developed for environmentally relevant species.

Amplification of reference genes can be carried out in separate reactions or co-amplified in the same tube (multiplex assay). Normalised copy number (R) of the target gene is calculated according to (15):

$$R = \frac{N_0^{\text{target}}}{N_0^{\text{HKG}}} = \frac{N_{\text{target}} / (1 + E_{\text{target}})^{C_t^{\text{target}}}}{N_{\text{HKG}} / (1 + E_{\text{HKG}})^{C_t^{\text{HKG}}}} \quad (15)$$

For simplicity, this and the following equations are formulated considering C_t values as the output from the qRT-PCR experiment, but they can also be applied to

Table 4 Housekeeping genes used in environmental biomonitoring

Housekeeping gene	Function	References
GAPDH	Important glycolytic pathway enzyme	[159, 160]
b-, g-actins	Essential for the structure and kinetics of the cytoskeleton	[161, 162]
a-, b-tubulins	Essential for the structure and kinetics of the cytoskeleton	
L8, S20	Ribosomal proteins	[160, 163]
16S, 18S rRNA	Ribosomal RNAs	[160, 164]
GAPDH, YWHAZ	Glyceraldehyde-3P-dehydrogenase, tyrosine 3-monooxygenase	[165]
EF1A (A and B isoforms)	Involved in translation by catalyzing GTP-dependent binding aminoacyl-tRNA	[160, 166]

C_p values without modification. Fold changes (RQ) in transcription levels between two samples, are simply calculated from the ratio between normalised copy number of the target gene in both samples, as described in (16).

$$RQ = \frac{R_{\text{sample}}}{R_{\text{control}}} = \frac{\left[\frac{(1 + E_{HKG})^{C_t^{HKG}}}{(1 + E_{\text{target}})^{C_t^{\text{target}}}} \right]_{\text{sample}}}{\left[\frac{(1 + E_{HKG})^{C_t^{HKG}}}{(1 + E_{\text{target}})^{C_t^{\text{target}}}} \right]_{\text{control}}} = \frac{(1 + E_{\text{target}})^{\Delta C_t (\text{control} - \text{sample})}}{(1 + E_{HKG})^{\Delta C_t (\text{control} - \text{sample})}}. \quad (16)$$

Two mathematical models are established to calculate R : the “delta C_t ” or “delta-delta C_t ” method without efficiency correction [110] and the efficiency-corrected methods [111]. “Delta C_t ” methods assume 100% efficiencies for all genes in all samples, simplifying (16) to:

$$RQ = \frac{R_{\text{sample}}}{R_{\text{control}}} = 2^{-(\Delta C_{t,\text{sample}} - \Delta C_{t,\text{control}})}. \quad (17)$$

However, as can be observed from both (15) and (16), the PCR amplification efficiency (E) has an important influence on the accuracy of the quantification results, since small variation in the amplification kinetics will generate false differences in the expression ratio. Therefore, determination of E for all amplification reactions is essential and must be carried out before any relative quantification is done. This value can be estimated from the slope of the linear correlation existing between C_t and C_p values and the logarithm of the amount of template added in a serial dilution scheme, as described in (14). There are a variety of methods to calculate E for any given pair of primers (see <http://efficiency.gene-quantification.info>).

2.1.5 Statistical Analysis

Quantitation of gene expression by qRT-PCR poses several difficulties to traditional statistical analysis. Classical parametric tests (analysis of variance or T-test) assume a normal distribution of data, but this requisite cannot be expected in absolute or relative quantification analysis since quantities obtained are derived from ratios [see (4)]. To solve this problem, the usual approach is to perform these parametric tests on the logarithmic transformations of the same ratios, which follow a normal distribution in most cases—a procedure called normalisation. This is probably the best strategy when multiple sets of data are to be compared by

using ANOVA (analysis of variance) or repeated measures ANOVA. Permutation or randomisation tests, like REST [112], overcome these problems by making no assumptions about the distribution of data. This test is based on the hypothesis that the observed effect in treated samples is as likely to have occurred in a control group. Then, C_t or C_p values for reference and target genes can be jointly reallocated to both groups and the effects on expression ratio are calculated, determining the probability of the hypothesis to be true. To assess the level of significance between control and treated samples REST 2005 software makes 50,000 allocations and calculates the mean and confidence interval of the expression ratio, and also the probability of the treatment effect to be at random (p -value). In addition, REST has the unique property of being capable of analysing multiple data points simultaneously. In this way, the analysis can integrate both variations between technical replicates (that is, independent qRT-PCR reaction from the same mRNA sample) and differences among biological replicates, from independent experiments or individuals. However, REST is not adequate when more than two sets of samples are to be compared. We strongly recommend the double approach (normalisation of data and randomisation tests) for analysing large sets of data from qRT-PCR experiments. There are available software packages to support statistical analysis of quantification results at <http://www.gene-quantification.de/main-bioinf.shtml>.

2.2 *Practical Uses of Gene Expression Biomarkers: What are they Useful for?*

Biomarkers are as diverse as the pathological effects of pollutants. No technique, however powerful, may conceivably cover all possible effects of the exposure of anthropogenic chemicals on biota. In this regard, gene expression biomarkers are restricted to a very specific subset of pollutants, the mode of action of which involves (or induces) changes in transcription of target genes. For this reason this technique is particularly suited for endocrine disruption, as hormone receptors are, almost by definition, regulatable transcription factors.

Within those limits, the application of gene expression biomarkers is extremely flexible. A single sample (a single RNA extraction) can be used to monitor any possible gene being expressed in the sampled tissue; by applying microarray techniques, all of them could theoretically be analysed at the same time. However, for most practical situations, analysis by qRT-PCR of a battery of target and reference genes is the option of choice. This method is relatively fast, inexpensive and precise; once the primers are optimised, the whole analysis could take a couple of hours or even less. The cost runs at under a US dollar for the qRT-PCR reaction, and 96 or 384 reactions (depending on the platform) can be performed simultaneously. It can be applied as part of a bioassay (e.g. exposing laboratory animals to

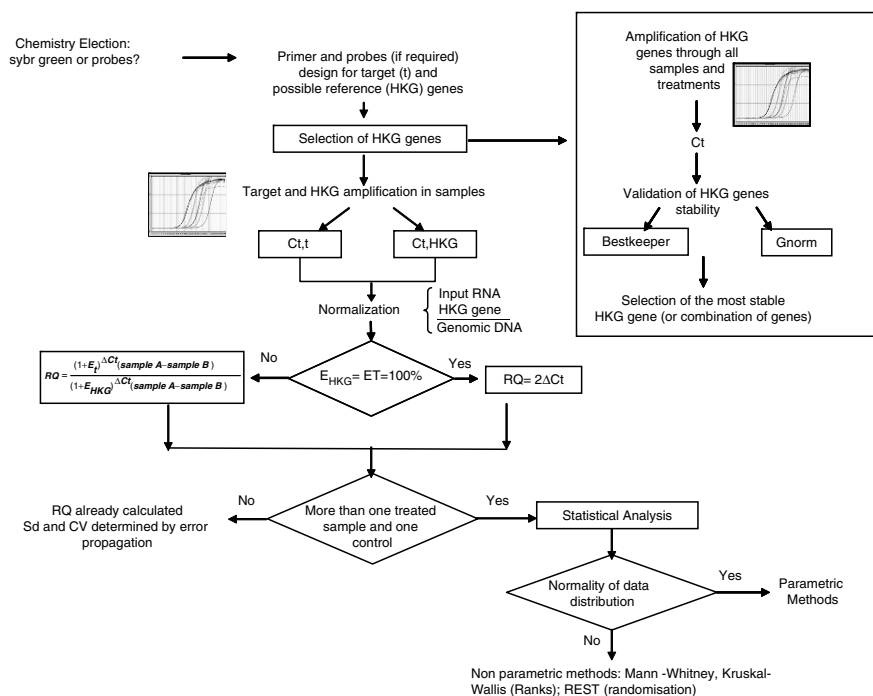


Fig. 8 Flow-chart of options and strategies required to develop a monitoring program based on quantitation of gene expression biomarkers by qRT-PCR

unknown substances or water samples) or as part of a field survey, taking samples of animals (or plants) in the wild, stabilising them with the appropriate solutions and analysing the samples in the lab. In this case, the variability present in nature imposes the requirement for a thorough analysis of the populations: 10–20 individuals per population are normally required for statistically relevant conclusions. It should be stressed here that small or non-vital parts of the specimen are frequently sufficient to perform the survey, allowing sampling of endangered or economically relevant populations. Figure 8 shows a flow-chart of options and strategies required to develop a monitoring program based on quantitation of gene expression biomarkers by qRT-PCR.

The list of available gene expression biomarkers and of species they can be applied to will surely increase enormously with genome sequencing programs and transcriptome analysis of model and non-model species under stress conditions. Table 4 shows a list of references using qRT-PCR techniques for evaluating endocrine disruption, either by analysing samples from the wild or by exposing controlled animals to known or unknown toxicants in the lab and analysing their responses (Table 5).

Table 5 Examples of the use of gene expression biomarkers for detection of endocrine disruption (laboratory experiments)

Species	Pollutant/Area or Condition studied ^{a)}	Genes analysed ^{b)}	Technique ^{c)}	Other methods ^{d)}	References
<i>Carassius auratus</i>	Cd2+, MeHg	ER, IGF-1, MT	RT-PCR	ALP activity, TRAP activity	[167]
<i>Carassius auratus</i>	BNF	CYP1A	qRT-PCR	EROD	[95]
<i>Cyprinodon variegatus</i>	E2	Vtg	DD RT-PCR	not done	[90]
<i>Cyprinus carpio</i>	17 β -estradiol.	Vtg	RT-cPCR	not done	[168]
<i>Danio Rerio</i>	B[a]P	Vtg, CYP1A	Q-RT-PCR	not done	[169]
<i>Fundulus heteroclitus</i>	PAH exposure	CYP1A, A12, XIR, DW	Q-PCR	Pyrene and PAH concentrations (HPLC)	[164]
<i>Fundulus heteroclitus</i>	E2	Vtg	qRT-PCR	None	[42]
<i>Mugil soley</i>	E2	Vtg	Q- RT-PCR	not done	[170]
<i>Oncorhynchus mykiss</i>	BNF	CYP1A	RT-PCR	EROD assay	[171]
<i>Oncorhynchus mykiss</i>	E2, CdCl2	Vtg, ER	RT-PCR	not done	[172]
<i>Oncorhynchus mykiss</i>	4-nonylphenol, EE2	Vtg	Dot blot analysis RT-cPCR	Western blot (Vtg/ZRP)	[91]
<i>Oncorhynchus mykiss</i>	Indole-3-carbinol	CYP1A, Vtg	Q-RT-PCR array	immunoblot analysis (CYP1A/ZR), Vtg by ELISA.	[173]
<i>Oncorhynchus mykiss</i>	a-ZEA, E2	ZR, Vtg	Q-RT-PCR	Vtg/ZR ELISA assay	[161]
<i>Oncorhynchus tshawytscha</i>	pulp mill effluent	CYP1A1	RT-cPCR	not done	[174]
<i>Oreochromis mossambicus</i>	Cd2+	MT	RT-PCR	Metal determination (AAS)	[175]
<i>Oryzias latipes</i>	NP, BPA, EE2	Vtg1, Vtg2, choriogenin, ER	RT-PCR	not done	[82]
<i>Oryzias latipes</i>	E2, NP, AdP, BPA, MBP, DHMS	Vtg1, VtgII, ERa	RT-PCR	not done	[176]
<i>Pimephales promelas</i>	EE2, flutamide	Vtg, ERa, ERb, ERg, AR	Q- RT-PCR	Vtg by ELISA	[163]

(continued)

Table 5 (continued)

Species	Pollutant/Area or Condition studied ^{a)}	Genes analysed ^{b)}	Technique ^{c)}	Other methods ^{d)}	References
<i>Platichthys flesus</i>	Arochlor 1254	CYP1A	Q- RT-PCR	not done	[177]
<i>Pleuronectes americanus</i>	CdCl ₂ , NaAs ₃ +	MT	RT-PCR	MT and Metal determination	[178]
<i>Salmo salar</i>	BNF	CYP1A	RT-PCR Slot-blot analysis Northern blot analysis	not done	[179]
<i>Salvelinus namaycush</i>	BNF	CYP1A1	Q-RT-PCR Northern blot analysis	Immunocytochemistry	[162]
<i>Sparus aurata</i>	TCDD	CYP1A	RT-PCR	EROD assay, Western blot analysis, CYP1A ELISA assay, Immunohistochemistry	[87]
<i>Sparus aurata, Liza aurata</i>	BNF	CYP1A1	RT-PCR	EROD assay, CYP1A ELISA, 2-AA	[180]
<i>Cyprinus carpio</i>	PCBs	CYP1A	Q- RT-PCR	Chemical analysis (GC-MS)	[181]

^{a)} Abbreviations: BNF, β -Naphthoflavone; BPA, Bisphenol A; E₂, 17 β Estradiol; EE₂, Ethynyl estradiol; MeHg, methyl mercury; NP nonylphenol; PAH, polycyclic aromatic hydrocarbons; PCB, polychloro biphenyl benzene

^{b)} Abbreviations: CYP1A, cytochrome P4501A; ER, estrogen receptor; AR, androgen receptor; MT, metallothionein. Vtg, vitellogenine; EST, Expressed-sequence tag (not fully characterised); ZR, *Zona Radiata* protein.

^{c)} Abbreviations: RT, reverse transcription; sqPCR, semiquantitative PCR; qPCR, quantitative Real-time PCR; cPCR competitive PCR.

Table 5 Examples of the use of gene expression biomarkers for detection of endocrine disruption (field surveys)

Species	Pollutant/Area or Condition studied ^(a)	Biomarker ^(b)	Technique ^(c)	Other methods ^(d)	References
<i>Barbus graellsii</i>	River pollution	CYP1A, MT-1, MT-2	qRT-PCR	EROD, heavy metal analysis, MT determination by differential pulse polarography	[182]
<i>Fundulus heteroclitus</i>	Spawning season	MT	RT-PCR QPCR (ECL)	not done	[183]
<i>Gobio gobio</i>	Belgium survey (Cd2+)	MT	qRT-PCR	MT determination (Cd saturation thiomolybdate) Metal determination (AES)	[184]
<i>Gobiomorphus cotidianus</i>	Cu(SO4), Zn(SO4)	MT	qRT-PCR	not done	[185]
<i>Leuciscus cephalus</i>	Metal mix (Ni ²⁺ , Cd ²⁺ , Zn ²⁺), lab exposure (Cu2+, Mn2+, Zn2+)	MT	RT-cPCR real time PCR (RotorGene)	MT determination (capillary electrophoresis)	[186]
<i>Lithognathus mormyrus</i>	no data	CYP1A	RT-cPCR	EROD assay CYP1A ELISA assay	[187]
<i>Lithognathus mormyrus</i>	estrogenic compounds	Vtg	qRT-PCR	not done	[188]
<i>Mullus barbatus</i> , <i>Mugil cephalus</i> , <i>Gobius niger</i> , <i>Anguilla anguilla</i> , <i>Thunnus thynnus</i>	estrogenic compounds	Vtg	RT-PCR	not done	[189]
<i>Oncorhynchus mykiss</i>	BaP; E2, Cd ²⁺ PAHs, heavy metals and estrogenic compounds contamination	CYP1A, MT-B, Vtg	RT-PCR	Chemical analysis (HPLC)	[190]

(continued)

Table 5 (continued)

<i>Species</i>	Pollutant/Area or Condition studied ^{a)}	Biomarker ^{b)}	Technique ^{c)}	Other methods ^{d)}	References
<i>Oncorhynchus mykiss</i> , <i>Oncorhynchus clarkii</i> , <i>Salmo salar</i>	PAHs, heavy metals and estrogenic compounds contamination	CYP1A, MT, Vtg	qRT-PCR	not done	[191]
<i>Platichthys flesus</i>	PAHs, heavy metals and estrogenic compounds contamination	CYP1A, MT, Vtg	qRT-PCR	EROD assay, BILE FACS, VG ELISA assay, Cadmium-saturation assay (MT)	[192]
<i>Salmo salar</i>	PCB contamination BNF	CYP1A	qRT-PCR	not done	[193]
<i>Salmo trutta</i>	Airborne POPs	Cyp1A	qRT-PCR	Chemical analysis (GS-MS)	[194]
<i>Salmo trutta</i>	E2	Vtg	qRT-PCR	Vtg by ELISA	[195]
<i>Trematomus bernacchii</i>	PCB, PAHs	CYP1A1	RT-cPCR	Biliary FACS	[196]

^{a)} Abbreviations: BNF, β -Naphthoflavone; BPA, Bisphenol A; E2, 17 β Estradiol; EE2, Ethynyl estradiol; MeHg, methyl mercury; NP nonylphenol; PAH, polycyclic aromatic hydrocarbons; PCB, polychloro biphenyl benzene

^{b)} Abbreviations: CYP1A, cytochrome P4501A; ER, estrogen receptor; AR, androgen receptor; MT, metallothionein, Vtg, vitellogenine; EST, Expressed-sequence tag (not fully characterised); ZR, *Zona Radiata* protein.

^{c)} Abbreviations: RT, reverse transcription; sqPCR, semiquantitative PCR; qPCR, quantitative Real-time PCR; cPCR competitive PCR.

3 Conclusions

Recent advances in Molecular Biology have provided new approaches to developing bioassays and biomarkers to evaluate the effects of the observed pollution on the exposed biota or to provide an early warning in cases in which anthropogenic impact is suspected but not confirmed. These instruments are particularly valuable in risk-assessment protocols (including QSAR), to monitor the recovery of formerly polluted sites in remediation schemes and to check possible environmental risk associated with any of the thousands of new compounds appearing each year in the market. The main—and perhaps the only—limitation to their development is our limited knowledge of how these compounds affect the physiology, genetics, embryology, and, in general, the biology of the impacted biota. Further research in biochemistry (including metabolism), genomics and transcriptomics would undoubtedly extend their applicability to monitoring of new environmental impacts arising from emerging contaminants and from the impending global change.

Acknowledgements This work has been supported by the Spanish Ministry for Education and Science (MEC, grants BIO2005-00840 and AGL2000-0133-P4-03).

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Biosensors for Aquatic Toxicology Evaluation

Marinella Farré and Damià Barceló

Abstract There is an increasing need for effective tools to estimate the risks derived from the large number of pollutants released to the environment by human activities. These studies need the combination of chemical analysis and ecotoxicological evaluation. Environmental toxicology is the qualitative and quantitative study of the adverse effects of anthropogenic and naturally occurring chemical stressors.

Initial aquatic ecotoxicology studies were based on acute toxicity measurements of vertebrates. However, these methods suffer some standardization problems, are expensive, time-consuming, and moreover, are associated with ethical problems. For this reason new technologies for ecotoxicological studies, such as embryo tests, micro invertebrates, algae, micro organisms, cell lines and tissues, biochemical reactions and nucleic acids, were launched.

Biological tools like bio-analytical systems, bioassays, biomarkers and biosensors provide us with detection systems for signalling a potential damage in the environment. Early recognition will prevent eventual damage to environmental matrices. Ideally, early warning signals in ecosystems using sensing systems (biosensors) and biochemical responses (biomarkers) as well as the classical effect-related bioassays would not only tell us the initial levels of damage, but these signals will also provide us with answers for the development of control strategies and precautionary measures.

Herein new trends in ecotoxicity assessment will be reviewed with special emphasis on principles, advantages and limitations of biosensors for environmental toxicology assessment in the aquatic environment.

Keywords Bioassay, Biosensor, Ecotoxicology, Environmental monitoring, Sensor, Toxicity

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Contents

1	Introduction.....	116
2	Bioassays for Aquatic Ecotoxicity Assessment	118
3	Biosensor Classification.....	123
3.1	Optical Transducers	123
3.2	Electrochemical Transduction.....	125
3.3	Electrical Sensors	126
3.4	Mass-Sensitive Sensors.....	126
3.5	Thermometric Sensors	127
4	Ecotoxicity Biosensors.....	127
4.1	Enzyme Biosensors.....	127
4.2	Estrogen Receptor.....	129
4.3	Immunosensors	131
4.4	DNA Biosensors.....	132
4.5	Whole-Cell Biosensors	134
5	Current Trends and Future Perspectives	145
6	Conclusions.....	147
	References.....	148

1 Introduction

Environmental toxicology is the qualitative and quantitative study of the adverse effects of anthropogenic and naturally occurring chemical stressors.

The publication in 1962 of Rachel Carson's *Silent Spring* [1] catalyzed the separation of environmental toxicology – and, subsequently, ecotoxicology – from classical toxicology.

Initial aquatic ecotoxicology studies were based on acute toxicity measurements of vertebrates such as fish [2–4]. However, these methods suffer some standardization problems, are expensive, time-consuming, and moreover, are associated with ethical problems [2].

The development of *in vitro* technologies occurred for scientific, ethical and economical reasons. Ecotoxicological tests involving higher organisms have been progressively replaced by different technologies, such as embryo tests, micro invertebrates, algae, micro organisms, cell lines and tissues, biochemical reactions and nucleic acids [5, 6].

During the last four decades a wide variety of biological methods have been developed to investigate different end points, sub-lethal effects, and biochemical responses, evaluating: acute toxicity, cytotoxicity, genotoxicity, neurotoxicity or endocrine disrupting effects [5].

About 80,000 chemicals (including 600 pesticides) are currently used, and more than 1,000 enter the market every year [7]. The increasing need for effective tools to estimate the risks from the large number of pollutants released into the environment by human activities has led to the development of sensitive techniques

for toxicological and chemical assessment. In recent years, a growing number of initiatives and legislative actions for environmental pollution control, with particular emphasis on water quality control, have been adopted in parallel with increasing scientific and social concern in this area. These new policies such as the Water Framework Directive (WFD) (Directive 2000/60/EC of the European Parliament and of the Council) of 23rd October 2000, establishing a framework for Community action in the field of water policy [8], forces us to increase toxicological and chemical monitoring studies based on the use of new methodologies able to obtain responses in short times, or even on-line.

Biological tools like bio-analytical systems, bioassays, biomarkers and biosensors provide us with detection systems for signalling a potential damage in the environment. Early recognition will prevent eventual damage to the environmental matrices. Ideally, early warning signals in ecosystems using sensing systems (biosensors) and biochemical responses (biomarkers) as well as the classical effect-related bioassays would not only tell us the initial levels of damage, but these signals will also provide us with answers for the development of control strategies and precautionary measures.

Figure 1 presents a representation of the relationship between environmental distress signal detectability and ecological relevance.

The implementation of safety programs calls for environmental analysis comprising two parts [9, 10]:

Aquatic ecotoxicological studies based on screening methods capable of predicting the possible dangerous biological effects of the cocktail of pollutants;

Analysis of the positive samples with chromatographic methods, such as LC/MS-MS, LC-QqTOF/MS, GC/MS or ICP-MS for the identification of the compounds causing the effects.

Bio-response-linked instrumental analysis combines two processes, biological recognition initiating a biological effect, and chemical analysis. The biomolecular components are used as targets for active substances. Although it is possible to

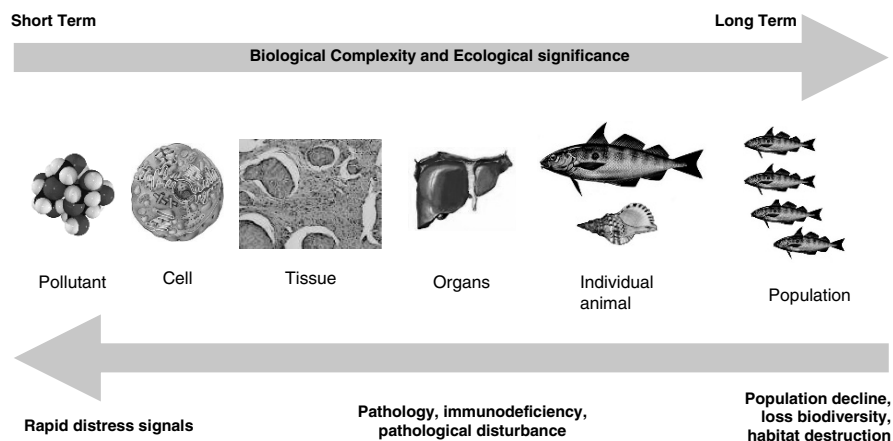


Fig. 1 Scheme of relationship between environmental distress signal detectability and ecological relevance

apply, for example, binding assays that provide effects-linked equivalents, information on the responsible contaminants is only accessible by chemical analysis. Therefore, a subsequent step is provided by the chemical analysis of the substances that are bound by the biorecognition components and which are therefore bio-effective.

In order to allow toxicant identification both effect-directed analysis (EDA) and toxicity identification evaluation (TIE), are shown to be appropriate [10–13]. EDA and TIE are analytical procedures combining biological and chemical analysis with fractionation techniques. While TIE originates from effluent control in a regulatory context in the U.S., EDA is a more scientific approach developed by analytical chemists to identify unknown hazardous compounds in the environment.

In addition to bioassays based on microorganisms, cell lines, recombinant organisms, or biochemical responses, biosensors have been demonstrated to have great potential and thus arise as proposed tools for effective ecotoxicology monitoring programs. According to the IUPAC definition a biosensor is an integrated device that is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor), immobilized onto a transduction element. This is the main difference with bioassays.

The main biosensor advantages are that they allow rapid responses, portable, miniaturized, on-line, on-site, and remote control configurations, and cost-effective measurements. These technologies are excellent alarm tools [14].

Herein new trends in ecotoxicity assessment will be reviewed with special emphasis on principles, advantages and limitations of biosensors for environmental toxicology assessment in the aquatic environment.

2 Bioassays for Aquatic Ecotoxicity Assessment

Among aquatic toxicity bioassays should be emphasized the tests based on invertebrates such as *Daphnia magna* [15], micro algae, such as *Skeletonema costatum* [16] and *S. capricornotum* [17], the marine bacteria *Vibrio fischeri* and *Photobacterium phosphoreum* [18], because these are well-established tests using standardized organisms, and are available from different commercial firms. The use of macro invertebrates, such as *D. magna* or *Ceriodaphnia*, presents many advantages, such as high sensitivity and short reproductive cycle.

Chronic tests with members of the genus *Daphnia* were initiated in the early 1970s by Biesinger and Christensen [] and are currently broadly accepted. Despite the diversity of test species available, in many regulatory schemes, the only, or preferred, invertebrate species recommended for acute or chronic toxicity testing is the cladoceran, *D. magna*. Presumably, the focus on *D. magna* results from its sensitivity to environmental contaminants relative to other invertebrate species [20]. This focus has resulted in a large number of acute and chronic toxicity tests with this species. Nevertheless, the advantages of *D. magna* over other species such as *Ceriodaphnia* has been a widely debated question, and some authors support that the acute and chronic toxicity of a broad range of materials, including metals, organic compounds and effluents, are approximately similar for members of the genera *Ceriodaphnia* and *Daphnia* [21]. These toxicity tests can be carried out for

lethality, growth, reproduction, mobility and population growth rate by exposing the organisms to the tested substances under controlled conditions, and measuring the acute toxicity or the chronic toxicity.

Experiments using *D. magna* are usually carried out using genetically identical populations and using 3rd–5th brood offspring. While the standardized acute lethality toxicity test based on *D. magna* is conducted over 21 days [22], other alternative tests at screening level are carried out with short exposure times, such as the 24–48-h screening test with *Daphnia* [23].

The effects produced by pollutants on *D. magna* at suborganismal and organismal levels, as well as, the implications for population dynamics are of great interest. A high number of studies have established the responses using *Daphnids* to different pollutants using different conditions and end points. Some examples are the studies of pesticides [24, 25], polyaromatic compounds [26] and anionic surfactants [27, 28] in *Daphnia*.

The use of algae as bio-indicators of toxicity is also well established, because they represent an important component of any aquatic community, besides being the lowest link in the food chain. For this reason, a substance toxic for the algae of an ecosystem can produce a strong impact in the ecosystem. Microalgae, such as *Chlorella fusca*, *Selenastrum capricornotum*, *Dunaliellasalina* or *Dunaliella tertiolecta* are used as toxicity indicators because of their sensitivity to pesticides [29, 30] and metals [31].

Hernando et al. [32] reported the utility and validity of toxicity tests based on *V. fischeri*, *S. capricornotum* and *D. magna* for monitoring of wastewater treatments.

Nevertheless, the more widely used bioassays in routine laboratories for evaluating water toxicity are based on inhibition of the bioluminescence of marine bacteria. The better-known species of luminescent marine bacteria are *V. fischeri* and *Photobacterium phosphoreum*, which naturally emit light due to an enzyme, the bacterial luciferase. Any substance that affects the bacterial metabolism produces a proportional inhibition of the luminescence.

The use of luminescence organisms to assess toxicity has been known for more than 40 years [33]. In 1979 a toxicity bioassay using luminescent bacteria was developed by Bulich et al. [34] to assess toxicity of wastewater effluents and industrial discharges. This technique allows the easy screening of large numbers of aqueous samples in a quick, reliable and inexpensive way. This toxicity assay was commercialized for the first time by Microtox and described in Beckman's Operating Manual.

The bioluminescence inhibition of *V. fischeri* has been exploited to investigate organic [35–38] and inorganic pollutants [39, 40], but one of the more relevant applications is for the rapid evaluation of real samples [41–45].

Over the years, many researchers have studied the physiological, biochemical and genetic control of bacterial bioluminescence. These discoveries have revolutionized the area of Environmental Microbiology using luminescent genes as biosensors for environmental studies. The ability to introduce the *lux* phenotype, coupled to specific promoters allowing its expression only in the presence of the proper analyte, into different bacterial species, provides a convenient method to multiply the possibilities for rapid, simple and sensitive screening of the environmental conditions. The *lux* operons, employed in these genetic manipulations, include those encoding for the bacterial luminescent system, the *lux* genes of firefly luciferase as well as those of *gfp*, the green fluorescent protein (GFP) from *A. victoria*.

A number of recombinant plasmids, carrying the *lux* operon expressed constitutively in many Gram- and Gram+ bacteria, have been constructed and used to transform a wide variety of bacteria. A promoter is usually required on the plasmid to obtain a high expression. The possibility to transform in luminescent organisms the bacteria previously isolated from the media under analysis allows one to produce biosensors with the maximum of suitability, since the vitality of the transformed organisms in their environment will be optimal, as well as its response to the presence of extraneous, noxious compounds [46].

A large number of procedures, testing the wastewater toxicity, are based on the GMB Shk1. A quick estimation of the toxicities of organic chemicals with different functional groups has been proposed, developing quantitative structure-activity relationship (QSAR) models, based on the logarithm of the octanol-water partition coefficient, as well as an overall QSAR model without discriminating the functional groups [47]. Model predictions were compared to experimental data and the model accuracy was found to be one order of magnitude from the experimental values.

The performance of the construct obtained by fusing the reporter genes of green fluorescent proteins (*A. victoria gfp*) and bioluminescence (*V. fischeri luxCDABE*) genes, to either SOS (*recA*) or heat shock (*grpE*) promoters, was compared [48]. In both cases, bacterial bioluminescence allowed faster and more sensitive detection. The fluorescent (FL) reporter proteins were much more stable and in long-term tests allowed detection at levels similar to that of the bioluminescence.

To evaluate the nitrification inhibition activity in wastewaters, a recombinant, tryptophan-dependent *Nitrosomonas europaea* luciferase gene, has been employed. In the case where the ammonia monooxygenase activity of *Nitrosomonas* was inhibited, a rapid drop in NADH or ATP concentration occurs. This reduction in the overall energy level of the cell was revealed by the reduction of the light emission intensity.

Various *Escherichia coli* strains, containing plasmid-borne fusions of *V. fischeri lux* to the *recA* promoter-operator region, have been created: (1) modifying the host cell's toxicant efflux capacity via a *tolC* mutation; (2) incorporating the *lux* fusion onto the bacterial chromosome, rather than on a plasmid; (3) changing the reporter element to a different *lux* system (*Photorhabdus luminescens*), with a broader temperature range; (4) using *Salmonella typhimurium* instead of an *E. coli* host [49].

Bacterial bioluminescence can be combined with other techniques to enhance its response in specific conditions. A bioluminescent *Campylobacter jejuni* organism, generated from the fusion of *luxCDABE* genes from *Xenorhabdus luminescens* to the *flaA* promoter of *C. jejuni*, was used in conjunction with two-dimensional gradient gels to map the responses of this organism to pH, NaCl concentration, temperature, and to various concentrations of l-fucose, d-fucose and sodium desoxycholate [50]. To monitor the bio-active fraction of Hg, instead of the total Hg, bioluminescent sensor systems were developed by fusion of a Hg-resistant operon (*mer* operon) from *Pseudomonas* sp. K-6y4 with the *lux* operon from *V. fischeri*. The resulting recombinant plasmids were cloned in *E. coli* cells for environmental applications [51].

A number of genotoxicity assays have been also developed based on the use of recombinant microorganisms. Genotoxicity is associated with different structures,

such as phenols, chlorophenols, polychlorinated biphenyls (PCBs), or polyaromatic hydrocarbons (PAHs), and constitutes an early screening for possible cancer-inducing activity of pollution. The most widespread is the Ames test that was established as a routine method of analysis. It is based on the retro mutation of *Salmonella typhimurium* TA98 (histidine dependent). The umu test is also based on *S. typhimurium*, and the genotoxicity is detected measuring the activation of the bacterial SOS repair response of genetic damage in the bacterium, through measuring β -galactosidase activity [52]. This is a standardized method that is validated for water control. Other genotoxicity bioassays that should be mentioned are those based on *Saccharomyces cerevisiae* [53–56], *Bacillus subtilis* [57–61] or *Escherichia coli* [15, 62–64].

In vitro systems such as primary cells and cell lines are of growing importance in ecotoxicology. Cells from different tissues and species of fish are used for the assessment of toxic action of chemicals and evaluation of environmental samples. For different organic pollutants such as organotins and substituted phenols, it has been found that the in vitro cytotoxicity is positively correlated with the acute toxicity in vivo, and therefore cytotoxicity assays may serve as an alternative for acute fish toxicity testing. A high number of works have been published, and correlation between toxicity, cytotoxicity and estrogenicity with in vivo tests has been presented. The cytotoxicity of eight aromatic products of toluene aerobic biotransformation was investigated by Shen et al. [65] in bluegill sunfish BF-2 cells. The concentrations of these products causing 50% inhibition in cell replication, protein content, uptake of natural red, and colony formation were evaluated and compared. The results of the study indicate a direct relationship between the exposure concentration of these products and observed cytotoxic effects. In descending order of cytotoxicity, the compounds were 3-methylcatechol, 4-methylcatechol, catechol, *o*-cresol, *p*-cresol, *m*-cresol, benzaldehyde and methyl benzoate.

Okamura et al. [66] developed a simple, rapid toxicity test using the suspension-cultured fish cell line CHSE-sp derived from chinook salmon *Oncorhynchus tshawytscha* embryos in order to assess the toxicity of new marine antifouling compounds. Good correlation was found with in vivo tests.

Kannan et al. [67] assessed the genotoxicity and cytotoxicity using an in vitro system with a permanent cell line *Epithelioma papulosum cyprini* (EPC) derived from a skin tumour of carp (*Cyprinus carpio* L.). EPC cells were exposed to different concentrations of organic sediment extracts from the North Sea for 24 h. After incubation the cells were analyzed for viability and DNA strand breaks with the comet assay or single-cell gel electrophoresis (SCGE). The results confirm the sensitivity of this assay.

Vevers et al. [68] studied the intrinsic genotoxic and cytotoxic potential of titanium dioxide (TiO₂) engineered nanoparticles (ENPs). The biological effects were evaluated in a metabolically competent assay based on a fish cell line derived from rainbow trout gonadal tissue (i.e. RTG-2 cells).

In recent years, many natural and synthesized substances have been associated with endocrine disruptions in wildlife, especially aquatic fauna [69]. Several bioassays have been developed to assess the activity of alleged estrogenic substances. In vivo assays are widely used. They are based on a wide variety of end points, including

cell differentiation and enzyme activities. Nevertheless, they are unsuitable for large-scale screening; moreover, in vivo assays are based on complex responses that may be modulated through mechanisms that do not directly involve the estrogenic receptor. In order to carry out environmental monitoring of endocrine disrupting chemicals (EDCs), a great number of in vitro assays have been developed. The assays, based on the strength of binding of a substance to the estrogen receptor (ER), are well established [70]. Their main drawback is the fact that the “binding activity” is not sufficient to determine the estrogenicity of a substance, because that also depends on the ability of the ligand to elicit an estrogenic-receptor response.

Yeast reporter gene assays and MCF-7 cell-based proliferation assays (E-screen) are particularly popular. The E-screen, in which proliferation of human breast cancer cells (MCF-7) is measured as a response to estrogen, has also been used to determine the estrogenicity of sewage effluent and surface water [71]. A correlation of an enzyme-linked receptor assay (ELRA) with a yeast reporter gene assay is shown. Several assays have been reported in the literature, such as the ultra-sensitive luminescent ELRA developed by Seifert [72] with a detection limit of 20 ng L⁻¹ for 17 β -estradiol.

Although these assays are simple, many factors affect the potency of estrogenic substances, such as differences in culture conditions, cell density or cell-line clones, and that makes the standardization of these methods difficult.

The induction of several proteins or enzyme activities has also been used to study estrogenicity (e.g. the increasing levels of alkaline phosphatase, cathepsin D, prolactin and vitellogenin as a consequence of progestogens). However, expression of these proteins or enzyme activities is restricted to specific cell lines and cannot be extrapolated to other tissues or species.

Over the past decade, a great number of recombinant receptor-reporter gene assays have been developed. Basically, these assays are based on simple cell models that express a gene under the control of defined promoters responding to specific substances and that produce an easily quantified signal. These assays are of great interest for rapid screening of the estrogenicity of artificial or natural compounds. In integrated systems, these cell models are very useful to study the synergy or antagonism of different substances and, in the field of environmental research, they are excellent tools to identify EDCs. Some of them are based on the bioluminescent gene reporter assay. Among these, the YES assay using yeast cells [73] should be emphasized.

Those papers mentioned above and additional papers, concerning new ecotoxicity bioassays, are listed in Table 1.

Beyond the practical advantages of new bioassays for toxicity assessment, they should be comparable to conventional analytical systems in terms of reliability and robustness. There is a lack of validation of new bioassays. To date just a few bioassays are standardized; some examples are *D. magna*, *V. fischeri*, *S. capricornutum*, and the umu-test. The implementation of new bioassays therefore needs to be verified and validated before being accepted. The validation procedures can vary depending on the technology that is evaluated and include organization and participation in

Table 1 Organisms widely used for toxicity assessment

Species	Parameter measured	References
Plants and algae		
<i>Selenastrum capricornutum</i>	Growth inhibition	[28–30, 74–77]
<i>Dunaliella tertiolecta</i>	Growth inhibition	[29, 75, 77, 78]
<i>Skeletonema costatum</i>	Growth inhibition	[79, 80]
Daphnids		
<i>Daphnia magna</i>	Immobility	[24–26, 32, 81–83]
<i>Daphnia magna</i>	Mortality	[84–88]
<i>Ceriodaphnia dubia</i>	Immobility	[86, 89]
Microorganism		
<i>Vibrio fischeri</i>	Bioluminescence	[36, 43, 44, 90, 91]
<i>Escherichia coli</i>	Metabolic status	[92–94]
<i>Pseudomonas putida</i>	Metabolic status	[37, 98–100]
<i>Pseudomonas fluorescens</i>	Bioluminescence	[97, 101]
<i>Bacillus subtilis</i>	Growth	[102–105]

interlaboratory trials, use and development of certified reference materials (CRMs) or comparison with conventional chemical analysis. Validation may involve some difficulties since, in addition to general quality parameters, it may be necessary to evaluate some other aspects such as stability of the biological reagents. In addition, there is a lack of guidelines established for validation of different groups of bioassays.

On the other hand, although there are several validation procedures accepted for quantitative methods, in the case of biological methods there are no established guidelines [106, 107].

3 Biosensor Classification

Biosensors are usually classified either according to:

- the transduction element (for example, electrochemical, optical, piezoelectrical or thermal) and bioreceptor (for example, enzymatic, immunoaffinity recognition, whole-cell sensors, or DNA)

3.1 Optical Transducers

Optical transducers are based on various technologies of optical phenomena, which are the result of an interaction of an analyte with the receptor part. The various types of optical transducers exploit properties such as simple light absorption, fluorescence/phosphorescence, bio/chemiluminescence, reflectance, Raman scattering and refractive index. Apart from speed, sensitivity and robustness, other attractive features of optical sensors include their suitability for component miniaturization, remote sensing

and their multi-analyte sensing capabilities. In addition, the increasing market of telecommunications has supported new optical materials research.

This group may be further subdivided according to the type of optical properties which have been applied in sensing:

Absorbance, measured in a transparent medium.

Reflectance is measured in non-transparent media, usually using an immobilized indicator.

Luminescence, based on the measurement of the intensity of light emitted by a chemical reaction in the receptor system.

Fluorescence, measured as the positive emission effect caused by irradiation. Also, selective quenching of fluorescence may be the basis of such devices.

Refractive index, measured as the result of a change in solution composition. This may include also a surface plasmon resonance effect (SPR).

Optothermal effect, based on a measurement of the thermal effect caused by light absorption.

Light scattering, based on effects caused by particles of definite size present in the sample.

The application of many of these phenomena in sensors became possible because of the use of optical fibres in various configurations. Such devices have also been called optodes.

A high number of optical transduction techniques can be used for biosensor development. These may employ linear optical phenomenon, including adsorption, fluorescence, phosphorescence, polarization, rotation, interference, etc., or non-linear phenomena, such as second harmonic generation.

Total internal reflection fluorescence (TIRF) has been used with planar and fibre optic waveguides as signal transducers in a number of reported biosensors. In these transducers, light is propagated down a waveguide which generates an electromagnetic wave (evanescent wave) at the surface of the optically denser medium of the waveguide and the adjacent less optically dense medium. The amplitude of the standing wave decreases exponentially with distance into the lower refractive index material. The fluorescence of a fluorophore excited within the evanescent field can be collected.

Surface plasmon resonance biosensors exploit special electromagnetic waves—surface plasmon polaritons—to probe interactions between an analyte in solution and a biomolecular recognition element immobilized on the SPR sensor surface. A surface plasmon wave can be described as a light-induced collective oscillation in electron density at the interface between a metal and a dielectric. At SPR, most incident photons are either absorbed or scattered at the metal/dielectric interface and, consequently, reflected light is greatly attenuated. The resonance wavelength and angle of incidence depend upon the permittivity of the metal and dielectric (Fig. 2).

The general advantages of optical techniques involve the speed and reproducibility of the measurement, and the main drawback is the high cost of the apparatus.

Most of the works based on SPR have been focused on the rapid identification and quantification of different groups of toxicant pollutants.

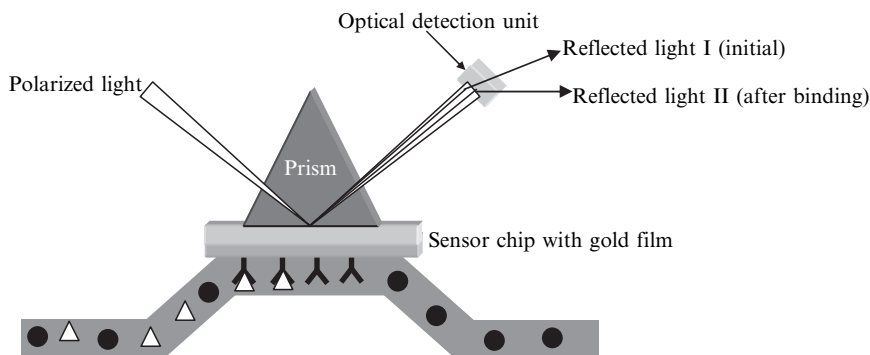


Fig. 2 General scheme of a surface plasmon resonance sensor

3.2 *Electrochemical Transduction*

Electrochemical transduction transforms the effect of the electrochemical interaction between an analyte and the electrode into a primary signal. Such effects may be stimulated electrically or may result in a spontaneous interaction at the zero-current condition. Most of the biosensors described in the literature are electrochemical biosensors. Electrochemistry offers high sensitivity, compatibility with modern micro-fabrication technologies, portability, low cost and minimal power requirements

The following subgroups may be distinguished:

- Voltammetric sensors, including amperometric devices, in which current is measured in the d/c or a/c mode. This subgroup may include sensors based on chemically inert electrodes, chemically active electrodes and modified electrodes. In this group are included sensors with and without (galvanic sensors) an external current source.
- Potentiometric sensors, in which the potential of the indicator electrode (ion-selective electrode, redox electrode, metal oxide electrode) is measured against a reference electrode.
- Chemically sensitized field effect transistor (CHEMFET) in which the effect of the interaction between the analyte and the active coating is transformed into a change of the source-drain current.
- Potentiometric solid electrolyte gas sensors, differing from potentiometric sensors because they work in high temperature solid electrolytes and are usually applied for gas sensing measurements.

Electrochemical biosensors are based on monitoring electroactive species that are produced or consumed by the action of the biological elements (e.g. enzymes), and can be performed basically under potentiometric and amperometric measurements.

The principle operation of amperometric biosensors is defined by a constant potential applied between a working and a reference electrode. The imposed potential promotes a redox reaction, which produces a current. The magnitude of this current is proportional to the concentration of electroactive species present in solution.

Potentiometric biosensors are based on the monitoring of the potential at a working electrode, with respect to a reference electrode.

3.3 *Electrical Sensors*

Electric sensors are based on measurements, where no electrochemical processes take place, but the signal arises from the change of electrical properties caused by the interaction of the analyte. Examples are shown below:

Metal oxide semiconductor (MOS) sensors are used principally as gas phase detectors, based on reversible redox processes of analyte gas components.

Organic semiconductor sensors, based on the formation of charge transfer complexes, which modify the charge carrier density.

Electrolytic conductivity sensors.

Electric permittivity sensors.

3.4 *Mass-Sensitive Sensors*

Mass-change sensors transform the mass change at a specially modified surface into a change of a property of the support material. The mass change is caused by accumulation of the analyte.

Piezoelectric devices used mainly in the gaseous phase, but also in solutions, are based on the measurement of the frequency change of the quartz oscillator plate caused by adsorption of a mass of the analyte at the oscillator.

Surface acoustic wave devices depend on the modification of the propagation velocity of a generated acoustical wave affected by the deposition of a definite mass of the analyte.

The vibration of piezoelectric crystals produces an oscillating electric field in which the resonant frequency of the crystal depends on its chemical nature, size, shape and mass. By placing the crystal in an oscillating circuit, the frequency can be measured as a function of the mass. When the change in mass (m) is very small compared to the total mass of the crystal, the change in frequency (f) relates to m as follows:

$$\Delta f = Cf^2 \Delta m/A$$

where f is the vibration frequency of the crystal in the circuit, A is the area of the electrode and C is a constant determined in part by the crystal material and thickness. Piezoelectric crystals, sometimes referred to as quartz crystal microbalances (QCM), are typically made of quartz and operate at frequencies between 1 and 10 MHz. These devices can operate in liquids with a frequency determination limit of 0.1 Hz, the detection limit of mass bound to the electrode surface is about 10^{-10} to 10^{-11} g.

Microcantilevers: Micromechanical devices comprise emerging sensor platforms with straightforward sensing mechanisms. Molecular adsorption onto the sensing element, typically a cantilever, shifts its resonance frequency and changes its surface forces (surface stress). Adsorption onto the sensing element composed of two chemically different surfaces produces a differential stress between the two surfaces and induces bending. The analyte that induces the mechanical response may be physi- or chemisorbed onto the cantilever in a reversible or irreversible process. Devices that respond to chemical stimuli in this manner are more commonly referred to as microcantilever (MC) sensors. A compelling feature of microcantilever sensors is that they can be operated in air, vacuum or liquid. The rapid growth in microcantilever-based sensor technology parallels advancements in micromachining methodologies and is in response to the need for more sensitive and selective detection of airborne and waterborne toxic and pathogenic substances.

Vertical, lateral or torsional movement of a cantilever changes its position. This movement ranges from several angstroms to a micrometer or more, depending upon the dimensionality of the cantilever and the magnitude of surface stress.

This group of sensors can be based on optical, interferometric, the electrical conductivity of a piezoresistive material when stress is applied to it, or under capacitive modes when the cantilever acts as one of the parallel plates of a capacitor.

3.5 *Thermometric Sensors*

Thermometric sensors exploit the absorption or evolution of heat in biological reactions [108]. This is reflected as a change in the temperature within the reaction medium and is transduced by a change in the resistance of a thermistor, which acts as a temperature transducer.

As reviewed by Ramanathan and Danielsson [108], thermistor-based calorimetric biosensors have mainly been applied to clinical and industrial process monitoring [108].

In the environmental field, only a few applications have been described up to now: several pesticides [109], for example, have been measured by thermometric biosensors.

4 Ecotoxicity Biosensors

The main classes of biosensors for ecotoxicity assessment are summarized in Fig. 3

4.1 *Enzyme Biosensors*

Enzyme biosensors have been described using a range of transduction elements (amperometry, potentiometry, optical and photo-thermal).

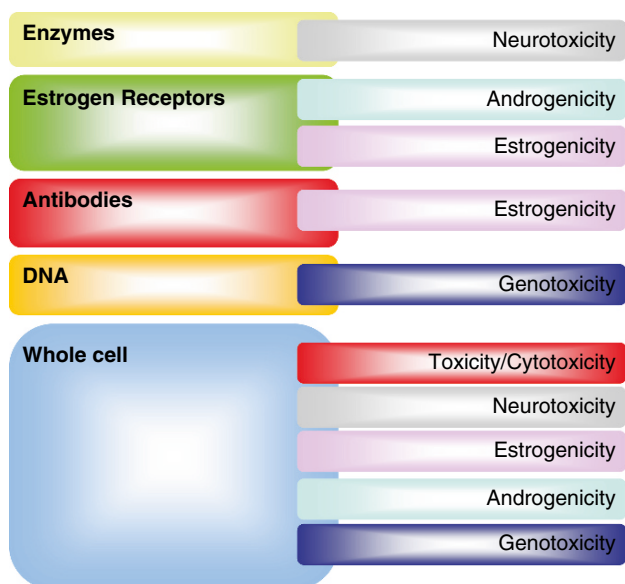


Fig. 3 Summary of main classes of biosensors for ecotoxicity assessment

The first biosensor was described in the literature by Clark and Lyons (1962) based on the use of glucose oxidase with electrochemical detection [110]. This principle has been widely exploited for the detection of some groups of organic compounds, such as phenols [111–115] and pesticides [116–122].

Enzymes typically used in amperometric biosensors are oxidases. The main advantage of this class of transducer is the low cost; a high degree of reproducibility and disposable electrodes are often available. The instrumentation is also very easy to obtain and can be inexpensive and compact; this allows for the possibility of on-site measurements. Limitations for amperometric measurements include potential interferences to the response if electroactive compounds are present in the sample, because they can generate false current values. An amperometric biosensor for hydrogen peroxide can also be used to measure organophosphate (OP) pesticides at concentrations as low as 10^{-9} M.

One of the main contributions of enzyme biosensors to ecotoxicology studies is related to neurotoxins. Neurotoxin detection has been accomplished using acetylcholinesterase [123], acetylcholine receptors, and butylcholine esterase (BchE) [124].

The combined inhibition effects in mixtures of organophosphates and carbamates (such as paraoxon/carbaryl, diisopropyl fluorophosphate (DFP)/carbaryl, paraoxon/DFP/carbaryl) were studied by Simonian et al. [125]. Mutual interactions of various neurotoxins did lead to competition for acetylcholinesterase (AChE) binding sites, and the overall inhibition effects were not additive but dependent on the types of chemicals present.

Neuropathy target esterase is the target protein for neuropathic organophosphorus compounds that produce organophosphorus compound-induced delayed neurotoxicity (OPIDN). Inhibition/aging of brain neuropathy target esterase within hours of exposure predicts the potential for development of OPIDN in susceptible animal models. Lymphocyte NTE has also found limited use as a biomarker of human exposure to neuropathic organophosphorus compounds. Makhaeva et al. [126] developed a highly sensitive biosensor for neuropathy target esterase activity using a tyrosinase carbon-paste electrode for amperometric detection of phenol produced by hydrolysis of the substrate, phenyl valerate.

During the last years new nanotechnological materials have led to the development of improved characteristic biosensors. Some examples are those based on nanoparticles, as a novel electrochemical method for the investigation of pesticides using AChE. The sol–gel-derived silicate network assembling gold nanoparticles (AuNPs–SiSG) provided a biocompatible microenvironment around the enzyme molecule to stabilize its biological activity and prevented them from leaking out of the interface. AuNPs promoted a conductive pathway for electron transfer and improved electrochemical reactions at a lower potential. In this work monocrotophos, methyl parathion and carbaryl were selected for the sensitivity tests [127]. The same group in another work developed a simple immobilization method of AChE on multi-wall carbon nanotube (MWNT)–chitosan composite which was proposed to increase sensitivity and stability of amperometric sensors for quantitative determination of organophosphorous insecticides [128]. In another work by Shulga et al. [129] the electrodeposition of colloidal gold nanoparticles onto planar gold electrodes was used to create a more favourable surface for the attachment of the enzyme AChE.

Several examples of enzyme biosensors have been reported using optical transduction [130, 131]. During the last few years, optodes, such as fibre optic biosensors, have been demonstrated to be of great interest because they provide some advantages, such as no direct electric connection, ease of miniaturization, possibility of remote sensing, and in situ monitoring, and different examples have been reported such as a sol–gel acetyl cholinesterase fibre optic biosensor for organophosphorus neurotoxins [130].

In order to improve the storage stability on enzyme-based biosensors, different immobilizations and electrodes have been assayed, including carbon-paste electrodes (CPEs), solid graphite electrodes [132] and surface-modified electrodes [112].

4.2 Estrogen Receptor

Biosensor estrogenic potency of real samples polluted with endocrine disruptor compounds has been assessed by means of different biosensor approaches based on the immobilization of estrogen receptors.

Steroid hormones induce different effects in mammalian cells after binding to specific intercellular receptors, which are ligand-dependent transcription factors.

Many endocrine disruptors can bind to estrogen receptors either as agonists or antagonists. Thus, the binding ability of the chemicals toward the ER is used to test their potential environmental toxicity. The advantage of receptor assays is that they are quite simple to perform and allow the identification of all endocrine disrupters that act through the estrogen receptor [133]. The natural sensing element most commonly used is the human ER [134]. Some of these ER-based biosensors are reported in Table 2.

By using surface plasmon resonance, Usami et al. [143] developed a simple competitive assay for the evaluation of different chemicals using human recombinant ER. The system measured the binding between estradiol immobilized on the sensor chip and an injected human recombinant ER.

In another example using an optical SPR sensor, and the human estrogen α receptor, Hock et al. [144] performed binding studies with estradiol and xenoestrogens. A rapid enzyme-linked receptor assay using the BIAcore system was developed and validated by Seifert et al. [145] for detection of estrogens and xenoestrogens. ELRA can be used to estimate the estrogenic potential of chemicals, for drinking water control and environmental monitoring.

Sesay and Cullen described the detection of hormone mimics [146] by a portable SPR. Recently, Butala et al. [141] described a fractal analysis used to model the binding and dissociation kinetics between analytes in solution and the estrogen receptor immobilized on a SPR sensor chip.

A novel evanescent-based biosensor [147] was developed with laser-based fibre optics using fluorescent dye-labelled recombinant human estrogen receptor- α

Table 2 ER-based biosensors

Transduction	Analytes	Effect measured	References
Nanomechanical	Diethylstilbestrol, 17- β -estradiol, 17- α -estradiol, 2-OH-estrone, bisphenol A, p,p'-dichlorodiphenyldichloroethylene	Estrogenicity	[135]
Electrophoretic mobility shift assay	17- β -estradiol	Estrogenicity	[136]
Fluorescence	17- β -testosterone	Androgenicity	[137]
Fluorescence	17- β -estradiol	Estrogenicity	[138]
SPR	Estrogens	Estrogenicity	[139]
Fluorescence	Ligands of nuclear hormone receptors	Estrogenicity and androgenicity	[140]
SPR	Estrogens	Estrogenicity	[141]
Cyclic voltammetry	17 β -Estradiol	Estrogenicity	[142]
SPR	Estrogens, progestogens, bisphenol A, 4-nonylphenol and tamoxifen.	Estrogenicity	[143]
SPR	17 β -Estradiol, synthetic estrogens and xenoestrogens	Estrogenicity	[144]
SPR	Estrogens and xenoestrogens	Estrogenicity	[145]

(rhERalpha) and hERbeta as probes. A three-tiered approach evaluating various steps in the formation of the estrogen-receptor complex and its subsequent activity was developed for instrument calibration to detect estrogen mimics in biological samples, water and soil. Using this approach, binding affinities and activities of certain known estrogen mimics were determined for their use as calibrator molecules. Results indicated rhERalpha and rhERbeta may be employed as probes to distinguish estrogen mimics with a broad range of affinities.

In another example, Dutta et al. [135] developed a nanomechanical transducer to detect endocrine disrupting chemicals combining fluidic sample injection and delivery with bioreceptor protein functionalized microcantilevers. The combination of protein receptors, which include estrogen receptor alpha (ER- α) and estrogen receptor beta (ER- β), as well as monoclonal antibodies (Ab), with MC systems employing modified nanostructured surfaces provides for excellent nanomechanical response sensitivity and the inherent selectivity of biospecific receptor-EDC interactions.

Murata et al. [142] proposed a bioaffinity sensor based on the specific binding of estrogens to the receptor immobilized on a gold disk with cyclic voltammetry detection. The biosensor was applied to the detection of estradiol.

Finally, Zhihong et al. [148] developed a new sandwich-type assay for estrogens using a piezoelectric biosensor. The principle of the detection is that the estrogen receptor captures the estrogen and then the complex is bound with an estrogen response element (ERE) immobilized on the sensor.

4.3 Immunosensors

The recognition elements are immunochemical interactions antibody-antigen. This type of device combines the principles of solid-phase immunoassay with physico-chemical transduction elements (electrochemical, optical, piezoelectric, evanescent wave and surface plasmon resonance).

Most of the applications of immunosensors are to determine target analytes or groups of structurally related compounds, in contrast with applications reporting whole biological effects. However, several applications have been developed for estrogenicity. Biomarkers, such as vitellogenin, can be used to show effects of endocrine substances on whole organisms [133]. Vitellogenin (Vtg) is a serum phospholipo-glycoprotein precursor to egg yolk, which is produced in high amounts by fish exposed to estrogenic compounds. Thus, the presence of Vtg in male fish is a useful biomarker to identify estrogenic activity of natural or anthropogenic substances. By using a biosensor to detect vitellogenin in fish, the estrogenicity of surface waters was determined by Kroger et al. [134].

Darain et al. [149] demonstrated a disposable amperometric immunosensor for the rapid detection of carp (*Carassius auratus*) vitellogenin. The sensor was fabricated

based on screen-printed carbon arrays (SPCAs) containing eight carbon working electrodes and an integrated carbon counter electrode. The sensor arrays exhibit a linear range of Vtg concentration from 0.25 to 7.8 ng ml⁻¹ and the detection limit was determined to be 0.09 ng ml⁻¹. Furthermore, the performance of the immunosensor for the determination of Vtg was evaluated by a standard addition method performed in fish serum samples.

4.4 DNA Biosensors

DNA's structure is very sensitive to the influence of environmental pollutants, such as heavy metals [150, 151], polychlorinated biphenyls [152] and polyaromatic compounds [153]. These substances are characterized by a great affinity to DNA, causing mutagenesis and carcinogenesis. So, it is very attractive to use DNA-based biosensors [154, 155] to perform genotoxicity assays or for rapid testing of pollutants for mutagenic and carcinogenic activity. Genotoxicity sensors should thus be considered alternatives to the classical and more involved genotoxicity tests and will simplify the analysis of large numbers of samples.

Most nucleic acid biosensors are based on the highly specific hybridization of complementary strands of DNA and also RNA molecules. In addition, toxicity tests based on the interaction of toxic compounds with DNA molecules have been developed especially for environmental monitoring and toxicity screening assays. In an earlier review by Mascini et al. [156] DNA biosensors were described including biosensors for toxicity screening, PCB and aflatoxin detection with specific discussion of application to food and environmental samples. Improvement of the immobilization of DNA probes onto transducer surfaces has been reported by Tombelli et al. [157].

Electrochemical biosensors based on immobilized DNA and its parts integrate detection sensitivity with a high specificity of biomolecules. This reduces the consumption of DNA and gives rise to the development of modern methods of analysis of DNA effectors, including toxic ones, in environmental and biological objects [158, 159].

Disposable electrochemical DNA-based biosensors have been used for the determination of low-molecular weight compounds with affinity for nucleic acids and for the detection of the hybridization reaction. The rapid screening of genotoxic compounds using the molecular interaction between surface-linked DNA and the target pollutants or drugs has been applied in different configurations.

The determination of genotoxic compounds was measured by their effect on the oxidation signal of the guanine peak of calf thymus DNA immobilized on the electrode surface and investigated by chrono-potentiometric analysis by Mascini et al. [156]. This type of DNA biosensor is able to detect known intercalating compounds, such as daunomycin, polychlorinated biphenyls, aflatoxin B1 and aromatic amines. Applicability to river and wastewater samples is also demonstrated [160]. Disposable electrochemical sensors for the detection of a specific sequence of DNA were realized by immobilising synthetic single-stranded oligonucleotides onto a graphite screen-printed electrode. The probes became hybridized with different

concentrations of complementary sequences present in the sample. The hybrids formed on the electrode surface are evaluated by chrono-potentiometric analysis using daunomycin as indicator of the hybridization reaction.

An interesting article by Lu et al. investigates the use of catalytic RNA and DNA for the detection of metals and their potential application in environmental and pharmaceutical fields [161]. Lu et al. analyzed 27 different metals ranging from Ag to Zn with a DNA-based sensor in an effort to develop broad range sensing elements for environmental analysis [161].

In another example the electrochemical detection of cadmium using surface-immobilized DNA was reported by Wong et al. [162].

Recently, a dsDNA-electrochemical in situ biosensor, employing differential pulse voltammetry, was used for the in situ evaluation of Pb^{2+} , Cd^{2+} and Ni^{2+} interaction with dsDNA. The results confirm that Pb^{2+} , Cd^{2+} and Ni^{2+} bind to dsDNA, and that this interaction leads to different modifications in the dsDNA structure. These modifications were electrochemically recognized as changes in the oxidation peaks of guanosine and adenosine bases [163].

Chiti et al. described a chrono potentiometric DNA biosensor for amine detection. The sensor was able to quantify concentrations of those molecules with two or more aromatic rings in the submicromolar range after only two minutes of accumulation. Wastewater samples were tested and biosensor signals compared favourably with classical genotoxicity tests like the Toxalert 100 based on bioluminescence signals [164, 165].

In a similar approach, Oliveira-Brett et al. utilized a DNA biosensor for the detection of pesticides using ten derivatives of the 1,3,5-triazine herbicides as model analytes. A time-dependent interaction of all herbicides with the immobilized DNA was observed with the electrochemical sensor [166].

A variety of optical DNA and RNA biosensors using surface plasmon resonance have been reported. DNA or RNA aptamers are single-stranded oligonucleotide sequences, which can be produced to bind to various molecular targets such as small molecules, proteins, nucleic acids, and even cells, tissues and organisms [123]. Moreover, the synthesis of aptamers is straightforward and reproducible. However, few examples of application reported the use of SPR biosensors for toxicological measurements such as genotoxicity.

So et al. [167] recently presented a simultaneous optical and voltammetric detection of bioactivated genotoxicity for the first time employing ultrathin films of DNA, model metabolic enzymes, and electrochemiluminescence (ECL) generating metallopolymer $[Ru(bpy)_2PVP_{10}]^{2+}$ on pyrolytic graphite (PG) electrodes. Cytochrome P450_{cam} and myoglobin were used as model monooxygenase enzymes to mimic in vivo processes. Sensor film growth and component amounts were monitored using a quartz crystal microbalance. Subsequent to the enzyme reaction, DNA damage in the sensor films was measured simultaneously using a simple apparatus combining a standard voltammetry cell coupled with an optical fibre and photomultiplier tube.

Recent improvements in DNA biosensors arise from the use of nanomaterials for the biosensors construction, and several examples have been published during recent years. The highly sensitive and sequence-specific detection of single-stranded oligonucleotides using non-oxidized silicon nanowires (SiNWs) has been demonstrated

by Zhang et al. [168]. To maximize device sensitivity, the surface of the SiNWs was functionalized with a densely packed organic monolayer via hydrosilylation, subsequently immobilized with peptide nucleic acid (PNA) capable of recognizing the label-free complementary target DNA. Because of the selective functionalization of the SiNWs, binding competition between the nanowire and the underlying oxide is avoided.

Gold nanoparticle/polyaniline nanotube membranes on the glassy carbon electrode (Au/nanoPAN/GCE) were used by Feng et al. [169] to construct electrochemical sensors. The properties of the Au/nanoPAN/GCE, the characteristics of the immobilization and hybridization of DNA were studied by cyclic voltammetry, differential pulse voltammetry and electrochemical impedance spectroscopy. According to the results of this work the synergistic effect of the two kinds of nanomaterials, nanogold and nanoPAN, could enhance dramatically the sensitivity for the DNA hybridization recognition.

4.5 Whole-Cell Biosensors

Main classes of whole-cell biosensors applied to ecotoxicological studies are:

Bacterial biosensors

Algal biosensors

Yeast biosensors

Fish-cell biosensors

Because of their use as genotoxicity and toxicity sensors, biosensors based on whole cells as the biorecognition element form the largest group among the publications targeting ecotoxicity.

Good general review articles providing an overview about the technology and publications can be found [170].

Genetic engineering of bacteria or yeast cells to bear the *lux* or *luc* gene operon, expressing luminescence proteins such as the green fluorescence protein (GFP), and similar techniques have been investigated for years. Good review articles giving an overview of this technology in recent years can be found, which focus on hydrocarbon pollutants [171], stress [172, 173], or include descriptions of other signal reporter systems such as bacterial luciferase, insect luciferase, β -galactosidase, green fluorescent protein, alkaline phosphatase etc. Depending on the genetic engineering approach and the cell type chosen, biosensors specific for one analyte can be constructed, or those that exhibit general stress response such as toxicity and genotoxicity sensors. A few examples of the different types of biosensors and the different analytes targeted are given in the following discussion.

Fabrication of a whole-cell biosensor requires immobilization of microorganisms on transducers. Since whole-cell biosensor response, operational stability and long-term use are, to some extent, a function of the immobilization strategy used, immobilization technology plays a very important role and the choice of immobilization

technique is critical. Microorganisms can be immobilized on transducer or support matrices by chemical or physical methods [174–176].

Chemical methods: Chemical methods of bacteria immobilization include covalent binding and cross-linking [175–177]. Covalent binding methods are based on a covalent bond between functional groups of the microorganism's cell wall components such as amine, carboxylic or sulphhydryl and the transducer such as amine, carboxylic, epoxy or tosyl. To achieve this goal, whole cells are exposed to harmful chemicals and harsh reaction conditions, which may damage the cell membrane and decrease the biological activity. For that reason, this method has not been successful for immobilization of viable microbial cells [174].

Cross-linking involves bridging between functional groups on the outer membrane of the cells by multifunctional reagents such as glutaraldehyde and cyanuric chloride, to form a network. This method has found a wide acceptance for immobilization of microorganisms. The cells may be cross-linked directly onto the transducer surface or on a removable support membrane, which can then be placed on the transducer. The ability to replace the membrane with the immobilized cells is an advantage of the latter approach.

While cross-linking has advantages over covalent binding, the cell viability and/or the cell membrane biomolecules can be affected by the cross-linking agents. Thus, cross-linking is suitable for constructing microbial biosensors where cell viability is not important and only the intracellular enzymes are involved in the detection [178].

Physical methods: Adsorption and entrapment are the two widely used physical methods for microbial immobilization, because they produce a relatively small perturbation of microorganism native structure and function [174, 179].

Physical adsorption is the simplest one. Typically, a microbial suspension is incubated with the electrode or an immobilization matrix, such as alumina and glass bead [175, 177, 178, 180, 181], followed by rinsing with buffer to remove unadsorbed cells. The microbes are immobilized due to adsorptive interactions such as ionic, polar or hydrogen bonding and hydrophobic interaction. However, immobilization using adsorption alone generally leads to poor long-term stability because of desorption of microbes.

The immobilization of microorganisms by entrapment can be achieved by either retention of the cells in close proximity of the transducer surface using dialysis or filter membrane or in chemical/biological polymers/gels such as (alginate, carrageenan, agarose, chitosan, collagen, polyacrylamide, polyvinylalcohol, poly(ethylene glycol), polyurethane, etc. [174, 182]. A major disadvantage of entrapment immobilization is the additional diffusion resistance offered by the entrapment material, which will result in lower sensitivity and detection limit.

4.5.1 Bacterial Biosensors

Most reported transduction elements in bacterial biosensors are electrochemical and optical.

Amperometric whole-cell biosensors: The most relevant application is the determination of the biochemical oxygen demand (BOD) [183–185]. However, bacterial biosensors using amperometry as the transduction method have also been relevant in other fields such as environmental toxicity assessment.

Neurotoxic organophosphate compounds have found wide application as pesticides and insecticides in agriculture and as chemical warfare agents in military practice [186].

Amperometric biosensors based on genetically engineered *Moraxella* sp. and *Pseudomonas putida* with surface-expressed organophosphorus hydrolase (OPH) have been developed by Lei et al. [186, 187] for the detection of organophosphorus pesticides. The biosensor consisted of recombinant PNP-degrading/oxidizing bacteria *P. putida* JS444 anchoring and displaying organophosphorus hydrolase on its cell surface as the biological sensing element and a dissolved oxygen electrode as the transducer. Surface-expressed OPH catalyzed the hydrolysis of fenitrothion and EPN to release 3-methyl-4-nitrophenol and p-nitrophenol, respectively, which were oxidized by the enzymatic machinery of *P. putida* JS444 to carbon dioxide while consuming oxygen, which was measured and correlated to the concentration of organophosphates. Under the optimum operating conditions, the biosensor was able to measure as low as 277 ppb of fenitrothion.

Recently, a novel single-walled carbon nanotube (SWNT) based biosensor for real-time detection of organophosphate has been developed by Liu et al. [188]. Horizontally aligned SWNTs were assembled to desirable electrodes using AC dielectrophoresis technique. Organophosphorus hydrolase immobilized on the SWNTs by non-specific binding triggers enzymatic hydrolysis of organophosphates, such as paraoxon, consequently causing a detectable change in the conductance of the SWNTs. The conductance change is found to be correlated to the concentration of organophosphate.

Amperometric bacterial biosensors based on monitoring of cell respiration have been used for surfactants analysis. Some examples are the use of surfactant-degrading bacteria [189, 190], or *Acetobacter peroxydans* [191].

The characterization of wastewater toxicity by means of a whole-cell bacterial biosensor using screen-printed electrodes with *E. coli* was reported by Farré et al. [93]. In another study, the same group determined the acute toxicity of wastewaters using *P. putida* electrodes, in conjunction with chemical analysis [192].

Potentiometric bacterial biosensors: Conventional potentiometric microbial biosensors consist of an ion-selective electrode (pH, ammonium, chloride and so on) or a gas-sensing electrode ($p\text{CO}_2$ and $p\text{NH}_3$) coated with an immobilized microbe layer. Microbe consuming analyte generates a change in potential resulting from ion accumulation or depletion. Potentiometric transducers measure the difference between a working electrode and a reference electrode, and the signal is correlated to the concentration of analyte. Because of a logarithmic relationship between the potential generated and analyte concentration, a wide detection range is possible.

However, this method requires a very stable reference electrode, which may be a limitation of these transducers. A few examples of biosensors based on potentiometric transducers have been developed for toxicological purposes in environmental

research. This type of biosensor has been applied for neurotoxicity evaluation. Several microbial biosensors based on modification of a glass pH electrode with genetically engineered *E. coli* expressing organophosphorus hydrolase intracellularly and on the outer surface of cells and wild-type organophosphorus pesticides degrading bacteria *Flavobacterium* sp. have been reported [193–195].

Conductimetric biosensors: Many microbe-catalyzed reactions involve a change in ionic species. Associated with this change is a net change in the conductivity of the reaction solution. Even though the detection of solution conductance is non-specific, conductance measurements are extremely sensitive. Nevertheless, few applications have been developed. An example is the single-use conductivity and microbial sensor developed by Bhatia et al. [196] to investigate the metabolic activity of *E. coli*. This sensing system combines physico-chemical and biological sensing and greatly increases the ease with which comparative data could be assimilated.

The modulation in optical properties such as UV-VIS absorption, bio- and chemi-luminescence, reflectance and fluorescence brought by the interaction of the biocatalyst with the target analyte is the basis for optical microbial biosensors. Optical-based biosensors offer advantages of compactness, flexibility, resistance to electrical noise, and a small probe size.

Bioluminescent bacteria systems: Most of the systems developed for ecotoxicity assessment using whole-cell biosensors and optical transduction have been based on the use of luminescence (natural or transgenic microorganisms), because bacterial luciferase immobilized systems can offer a general tool, and when the luminescent reagents and enzymes, or the whole cells, are immobilized on solid supports their peculiarities change, usually in a positive way.

Several solid supports and procedures are available to immobilize macromolecules or whole cells. The chemical methods give better yields, in terms of active luciferase, than the physical ones. Nevertheless, chemical procedures involve covalent coupling, and usually lead to some protein inactivation. Agarose, collagen, epoxy methacrylate and nylon have proved to be the most effective among the different solid supports that have been investigated. The gel entrapment technique has the advantage of better protein stability and enables the co-immobilization of luciferase and other enzymes with their substrates through an easy process. An important, general, feature of these immobilized enzymes is the possibility to incorporate them into flow cells, used for multiple assays, to be recycled and reused in automated devices. The continuous flow format offers greater possibilities than a single-batch system, and it leads to rapid and sensitive assays. Flow assays are characterized by extremely accelerated kinetics: a very high surface-area-to-volume ratio is obtained and the reactions do not have to rely on passive diffusion to bring reagents together. Many analytes can be detected at pmol levels, with good precision and a wide range of linearity [197]. The immobilization of biological components on a solid support is quite regularly the preliminary step to the creation of a biosensor.

In fact, biosensors are generally described as probe-type devices made up of a selective biological layer, with very sharp molecular recognition capacity, and of a physicochemical transducer, often an optical system. Among these transducers,

the (bio- or chemi-) luminescent ones have the advantages that they do not require light sources and monochromators [198].

Any kind of luminescent bacteria can undergo immobilization, including the genetically modified ones. A systematic review of the methods to immobilize the different luminescent enzymes and microorganisms (natural and genetically engineered) for use in biosensors is that of Kratasyuk and Esimbekova [197].

An immobilized film of *Photobacterium phosphoreum* has been used to prepare a sensor based on the determination of acute toxicity effects of molecules that are difficult or even impossible to be measured by traditional analytical chemistry methods [199].

Recently, a biodetector was designed based on a bioluminescence test with the bacterium *V. fischeri* performed in a liquid continuous flow-through system. In this system a new flow-cell holder and a new case including a “top cover” to connect the flow cell with the waste and the incubation capillary in a light-proof manner are described [200].

Genotoxic agents, such as mitomycin C, have also been studied by a stable dark variant of *P. phosphoreum* (A2), fixed in agar-gel membranes immobilized onto the exposed end of optic fibres, linked with a bioluminometer [201]. A bio-MEMS based cell-chip can be fabricated by immobilizing bioluminescent bacteria in a microfluidic chip. The pattern of the recombinant *E. coli* strain GC2 was successfully generated by photolithography, utilizing the PVA-SbQ (polyvinyl alkyl-styrylpyridinium) polymer, as the immobilization material [202]. A hydrogen peroxide detection system was obtained simply immobilizing bioluminescent bacteria, the DK1 strain, which increased bioluminescence in the presence of oxidative damage in the cells [203]. *Pseudomonas fluorescens* HK44, which emits light when in contact with naphthalene and its metabolites, has been immobilized into a silica matrix, on glass slides, by the sol-gel technique [204]. The test slides could be used for multiple determinations, since the bacteria responded to the inductor at least 8 months after immobilization, and to more than 50 induction cycles.

A series of two-stage bioreactor systems, connected by a fibre optic probe to a luminometer, have been assembled to set up a multi-channel system for continuous monitoring and classification of toxicants. Each channel was used for cultivating different recombinant bacterial strains: TV1061 (*grpE::luxCDABE*), DPD2794 (*recA::luxCDABE*) and DPD2540 (*fabA::luxCDABE*), which are induced by protein-, DNA- and cell-membrane damaging agents, respectively. GC2(*lac::luxCDABE*) is a bacterium expressing bioluminescence constitutively [205]. Each channel showed a specific bioluminescent response according to the chemicals contained in wastewater samples, while GC2 showed a general response to cellular toxicity [205]. A portable format of the previously described biosensor consists of three parts, a freeze-dried biosensing strain within a vial, a small lightproof test chamber, and an optic-fibre connecting the sample chamber to a luminometer. It can be used for field sample analyses and monitoring on-site of various water systems. A different arrangement of genetically engineered luminescent organisms has been used in a bacterial cell chip, creating the possibility to emit light of different colours from each well, by using quantum dots (QDs). Quantum dots have several advantages such as broad absorption spectrum, narrow emission spectra and stability [206].

Each well gives off the specific signal (light) according to the recombinant strain exciting the quantum dots. A multi-strain bacterial cell array chip has been developed also by Lee et al., as a revealing test for the presence of reactive oxygen species (superoxide radical and hydrogen peroxide), generated by different chemicals in the sample [207]. The previously described multi-channel system has been successfully implemented in the form of computer-based data acquisition. The bioluminescent signatures are delivered from four channels by switching one at once, while the data are automatically logged to a personal computer [208]. Improvements of the system have been the manipulation of the dilution rate and the use of *thermo-lux* fusion strains. The system is now being implemented in a drinking water reservoir and river for remote sensing as an early warning system [208].

The use of quantum dots opens a new window in biosensor development. Another example is an electrogenerated precursor that has been developed for green synthesis of highly luminescent aqueous CdTe quantum dots with unique quantum yield and strong electrogenerated luminescence, which can access cellular targets via specific binding and have potential application as biolabels in highly sensitive biosensing and cell imaging [209].

The papers reported above and additional papers concerning biosensors based on luminescent and fluorescent bacteria are listed in Table 3.

Table 3 Bioluminescence and fluorescence bacterial biosensors

Target	Bacteria	Natural or induced luminescence	Transducer	References
Acute toxicity	<i>Photobacterium phosphoreum</i>	Natural	Bioluminescence	[202]
Acute toxicity	<i>Vibrio fischeri</i>	Natural	Bioluminescence	[210]
Genotoxicity	<i>Photobacterium phosphoreum</i>	Dark variant	Bioluminescence	[204]
Genotoxicity	<i>Escherichia coli</i>	GC2	Luminescence	[205]
Acute toxicity	<i>Pseudomonas fluorescens</i>	10568	Fluorescence	[211]
Acute toxicity	<i>Vibrio fischeri</i>	Natural	Bioluminescence	[211]
Acute toxicity	<i>Escherichia coli</i>	HB101	Luminescence	[211]
Acute toxicity	<i>Pseudomonas putida</i>	TVA8	Luminescence	[211]
Acute toxicity	<i>Escherichia coli</i>	DH5 α	Luminescence	[211]
Toxicity and genotoxicity	<i>Escherichia coli</i>	DPD2511, DPD2540, DPD2794 and TV1061	Luminescence	[212]
Genotoxicity	<i>Pseudomonas fluorescens</i>	HK44	Luminescence	[207]
Acute toxicity	<i>Photobacterium phosphoreum</i>	Natural	Bioluminescence	[213]
Genotoxicity	<i>Escherichia coli</i>		Luminescence	[214]
Genotoxicity	<i>Escherichia coli</i>	recA':lux V. fischeri	Luminescence	[215]
Genotoxicity	<i>Escherichia coli</i>	DPD1718 recA':lux	Luminescence	[216]
Genotoxicity	<i>Salmonella typhimurium</i> TA1535	TL210 and TL210ctl	Luminescence	[217]

Different examples of the application of biosensors based on bioluminescent bacteria for wastewater monitoring have been reported. The construction of whole cell, genetically modified, bioluminescent biosensors and their immobilization on thin films of poly(vinyl alcohol) cryogels has been carried out by Philp et al. [218]. The biosensor was designed for use in monitoring the toxicity of industrial wastewaters containing phenolic materials. It has been proved that they operate predictably with pure toxicants, within the wastewater treatment plant [218]. Several recombinant bioluminescent bacteria have been employed to set up a multi-channel, continuous, water toxicity monitoring system. That system developed by the research group of Kim was based on channels, each one hosting a different recombinant bacterial strain, and was composed of two mini-bioreactors, to enable a continuous operation, that is without system interruption due to highly toxic samples [219]. The luminescent strains were: DPD2540 (*fabA::luxCDABE*), DPD2794 (*recA::luxCDABE*) and TV1061 (*grpE::luxCDABE*), induced by cell membrane-, DNA- and protein-damaging agents, respectively; GC2 (*lac::luxCDABE*) was a constitutive strain. Field samples were waters discharged from a nuclear power plant and a thermoelectric power plant. Each channel showed specific luminescent response profiles and by comparing the luminescent bacteria signals of the standard chemicals with those of discharged water samples, the equivalent toxicity of the field water could be estimated [219].

A procedure developed for rapid toxicity finger printing of polluted waters involves a single constitutive *lux* bacterial biosensor, thanks to the fact that the different toxicants elicit highly characteristic light response curves in the biosensor. The technique can be applied to any pollutant or pollutant mixture that elicits a toxic response in the biosensor.

Two bioluminescent bioassays, one based on lyophilized marine luminous bacteria, the *Microbiosensor*-B17-677F, and the other on a genetically modified luminous strain of *E. coli*, the *Microbiosensor*-ECK, have been employed to reveal areas of impaired water quality in the river and sewage waters of different regions of Siberia, showing the same dependence on the concentration of the toxicants. Nevertheless, the sensitivity to phenol compounds of the *Microbiosensor*-ECK was higher, and corresponded to that determined on intact cells of *P. phosphoreum* and of various hydrobionts.

Aqueous extracts of soils have been tested by the *lux*-marked bacterial biosensor *E. coli* HB101, to reveal the presence and toxicity of four commonly used herbicides (atrazine, diuron, mecoprop and paraquat) [220]. Toxic responses, for all four herbicides, were stronger in the extracts than in the corresponding spiked water samples, suggesting that intrinsic soil factors may be altering the bio available fraction of herbicides, making them more toxic than equivalent concentrations in water.

Recently, two toluene bacterial biosensors have been reported. The toluene bacterial biosensors comprised of two reporter genes, *gfp* and *luxCDABE*, characterized by green fluorescence and luminescence, respectively, and their abilities were compared to detect bioavailable toluene and related compounds. The bacterial luminescence biosensor allowed faster and more sensitive detection of toluene; the fluorescence biosensor strain was much more stable and thus more applicable for

long-term exposure. Both biosensors were field-tested to measure the relative bioavailability of BTEX in contaminated groundwater and soil samples [221].

4.5.2 Algal Toxicity Biosensors

Fluorescence transduction has been widely applied in ecotoxicity biosensors using immobilized microalgae. Bozeman et al. [222], in a pioneering work, compared the toxicity of seven pollutants of different origin (Cd, Cu, Glyphosate, Hydrothol, Paraquat, pentachlorophenol and sodium dodecyl sulphate) to free and immobilized cells of the green microalga *Selenastrum capricornutum*, suggesting the possibility of the use of immobilized systems in in situ toxicity experiments. Following those authors, differences in toxicity for free and immobilized algae varied from no significant differences for copper and pentachlorophenol to nearly four times more sensitive for free cells in the case of Glyphosate or Paraquat. Admiraal et al. [223] performed an experiment on sand and natural glass-attached microbenthic assemblages of algae and bacteria in a metal polluted stream in the river Dommel system (Belgium). The authors compared the sensitivity of those assemblages to zinc, finding different sensitivities as a function of the origin of the assemblages (the most polluted origin, the lower sensitivity).

Protection against toxicity in immobilized cells is reported in different works [224]. Awasthi and Rai [225] demonstrated lower inhibition of nitrate uptake in *Scenedesmus quadricauda* immobilized (with respect to free cells) when exposed to Ni, Zn or Cd. In this work, no metal measurements were performed in the media, and the easiest explanation is the removal of part of the metals by the entrapping matrix, being thus less available for cells. But removal of toxicants by immobilizing matrixes would not explain all cases of less toxicity of immobilized cells. Surfactants are not so selectively adsorbed by Ca-alginates. Moreno-Garrido et al. [226] found less toxicity for immobilized cells of *Phaeodactylum tricornutum* exposed to sediments spiked with surfactant lineal alkylbenzene sulphonate (LAS) than for free cells.

Immobilization by gel entrapment is also a very interesting topic for in situ microalgal experiment design because it provides protection to microalgae from-grazers [227]. Microalgal grazers cannot be easily eliminated from waters or sediments due to the small size that those organisms can achieve: nematodes and, over all, amoebas or ciliates can predate on microalgal cells slightly smaller than them.

Twist et al. [227] developed a method of in situ biomonitoring using Ca-alginate immobilized *Scenedesmus subspicatus* for the assessment of eutrophication in surface waters.

A clear advantage of this technique is that local flora can be isolated and incorporated into the biomonitor. Of course, when Ca-alginate is used in a natural environment, a limitation of the technique is degradation of the beads. In freshwater streams the timescale for bead degradation seems to be a couple of weeks. In marine environments the timescale is quite a lot shorter (a few days) [228]. Improvements in bead fabrication are being made in an effort to avoid these problems [229].

Frense et al. [230] used *S. subspicatus* immobilized on filter paper and covered with alginate in an optical biosensor based on the chlorophyll a fluorescence as the biomarker for pollutants in water and soil extracts, as a pre-selection protocol before sending samples to high-cost standard analysis. *C. vulgaris* has also been used in optic biosensors in order to determine the toxicity of herbicides [231]. Algal immobilization was performed on GF/C Whatman filters. Filter paper disks immobilizing algae have also been used by Sanders et al. [232] in biosensors designed to detect chemical warfare agents.

Electrochemical transduction has also been applied in some examples. An amperometric algal-based sensor has been designed by Shitanda et al. [233], taking advantage of variations in the photosynthetically produced oxygen.

Another example is the development of novel conductimetric biosensors based on immobilization of the microalgae *C. vulgaris* by Chouteau et al. [234]. In this study, the detection of the local conductivity variations caused by algae enzymatic reactions could be achieved. The inhibition of *C. vulgaris* alkaline phosphatase activities in the presence of cadmium ions was measured. These results were compared with measurements in bioassays. It finally appeared that conductometric biosensors using algae seemed more sensitive than bioassays to detect low levels of cadmium ions (the detection limit for the first experiment was 1 ppb of Cd^{2+}).

Recent efforts have been made in the field of co-immobilization [233]. De-Bashan et al. [235] co-immobilized *Chlorella* with a microalgae growth-promoting bacterium (*Azospirillum brasiliense*) in Ca-alginate beads. This bacterium is not able to remove nutrients from wastewaters, but enhances growth of immobilized algae.

Limitations of toxicity testing using free or immobilizing microalgae are restricted to those toxicants that affect structures present in the algal cells. But these types of biosensor are highly sensitive to toxicants affecting photosynthesis (such as copper ions or herbicides).

4.5.3 Yeast-Based Biosensors

Most whole-cell sensors have used bacterial cells as the sensing element. Fungal cells, however, can provide all of the advantages bacterial cells offer but in addition they can provide information that is more relevant to other eukaryote organisms. These cells are easy to cultivate and manipulate for sensor configurations and are amenable to a wide range of transducer methodologies. An overview of the use of yeast and filamentous fungi as the sensing element of some biosensors is presented in this section.

There are several reports on the use of wild-type fungal cells and yeast to detect toxicity. As explained above the use of eukaryote cells as the detection element is preferable because their response to the toxin more accurately predicts the response in plant and animal cells than does the prokaryote response. The naturally bioluminescent fungi *Armillaria mellea* and *Mycena citricolor* were used as the sensing element in toxicity bioassays [236], and the basis for the development of toxicity

biosensors. The bioluminescence inhibition during 60 min exposure to the toxin was monitored to provide EC_{50} values.

Immobilized *Sacharomices cerevisiae* was used to assess the toxicity of four free, and three conjugated cholanic acids and a toxicity scale was constructed from the results [237]. The authors concluded that this sensor could therefore be considered a valid instrument for the preliminary evaluation of the toxicity of organic compounds or drugs. Campanella et al. also used *S. cerevisiae* as the sensing element of four general toxicity biosensors. Toxicity was determined by detecting the retardation of metabolic activity.

Many of the current yeast sensors are for genotoxicity and are based on genetically modified *S. cerevisiae*. Other genetically modified yeast sensors have been constructed to detect specific molecules or groups of molecules. Some modified cell sensors function as model eukaryote cells to detect toxicity and in particular genotoxicity, either of single specific molecules or of mixtures of molecules such as in environmental samples. While their use in the laboratory is in general not problematic, the use of genetically modified sensors outside the laboratory, for example for on-site environmental monitoring, may be restricted by biosafety requirements in some jurisdictions.

The green fluorescent protein reporter gene (GFP) has been incorporated into a plasmid, yEGFP, to optimize its expression in *S. cerevisiae*. This reporter gene was fused to the promoter region of the yeast gene, RAD54, to create a yeast genotoxicity biosensor. The reporter responds to the genetic regulation of DNA repair by RAD54 but does not respond to chemicals that delay mitosis and thus is specific for DNA damaging molecules [238].

Further work incorporated a human cytochrome P450 gene into the modified *S. cerevisiae* cells. Exposure of these cells to genotoxic molecules causes in vivo metabolic activation of promutagens, replicating the human response to these agents more accurately than the Salmonella-based Ames test [239].

The construction and characterization of dual stress-responsive bacterial biosensors was reported by Mitchel et al. [240] Using the genes for the green fluorescence protein and *Xenorhabdus luminescens* luciferase operon and the promoters for the *recA* and *katG* genes, two stress-responsive *E. coli* biosensor strains have been constructed that can individually or concurrently respond to oxidative and genotoxic conditions.

A yeast-based promoter-reporter biosensor was constructed by Benton et al. [241] for the detection of genotoxic compounds within a cell's local environment. In this work a fusion containing the HUG1 promoter and GFP was performed and it was incorporated into the yeast genome creating a stable, sensitive genotoxicity indicator.

Firefly luciferase has been cloned into *S. cerevisiae* to create a bioluminescent yeast strain. The presence of any toxic chemical that interferes with the cell's metabolism results in a quantitative decrease in bioluminescence. Hollis et al. demonstrated that the luminescent yeast strain senses chemicals known to be toxic to eukaryotes in samples assessed as non-toxic by prokaryotic biosensors [242]. The authors noted that this biosensor complements the GFP biosensor designed by

Walmsley et al. in that it responds to a wide range of toxins, not just genotoxic agents.

Terziyska et al. [243] used a *S. cerevisiae* mutant to increase the sensitivity of this organism to mutagens and carcinogens present in environmental samples. The cells carry a mutation which increases the permeability of the outer mannoprotein layer of the cell wall. The responses of these cells to environmental samples were compared to the Ames test (*Salmonella typhimurium*) and the D7 test of Zimmerman (*S. cerevisiae*). The authors conclude that their test (D7ts1) detects mutagenic/carcinogenic activity undetectable by the D7 test. Furthermore, all samples positive in the Ames test were positive in the D7ts1 test and some samples negative in the Ames test were D7ts1 positive.

Other genetically modified yeast have been constructed to detect a specific molecule or group of molecules. A strain of *S. cerevisiae* that lacked the gene for dihydrofolate reductase (DHFR) was complemented with mouse DHFR containing a ligand-binding domain inserted in a flexible loop. This construct was used to identify mutations in the ligand-binding region. Additionally, the cells could discriminate between analogues of the ligand, in this case oestrogen, and was used as a screening protocol to identify the various analogues [244].

Sakaki et al. [245] constructed a modified strain of *S. cerevisiae* by cloning rat CYP1A1 and CYP1A2 genes to create recombinant yeast cells capable of metabolizing polychlorinated dibenzo-*p*-dioxins (PCDD's).

Lehmann et al. [246] have constructed sensors for copper by transforming *S. cerevisiae* with a plasmid containing the Cu²⁺-inducible promoter of the CUP1 gene of *S. cerevisiae* fused to the lacZ gene of *E. coli*. The constructed lac+ve yeast responded quantitatively to the presence of copper. The response to copper was detected by monitoring the catabolism of lactose.

Webb et al. [247] transformed *A. pullulans* with a vector containing the GFP reporter gene. The level of fluorescence was directly related to the number of live cells. Exposure of the organism to biocides such as sodium hypochlorite and 2-*n*-octylisothiazolin-3-one (OIT) caused a rapid loss of fluorescence that was highly correlated with a decrease in the number of viable cells.

A yeast-based cytotoxicity and genotoxicity assay for environmental monitoring using novel portable instrumentation was presented by Knight et al. [248]. This assay uses eukaryotic (yeast) cells, genetically modified to express a green fluorescent protein whenever DNA damage, as a result of exposure to genotoxic agents, is repaired. A measure of the reduction in cell proliferation is used to characterize general toxicity producing lowest effect concentration (LOEC) and EC₅₀ (50% effective concentration) data. The results of sensitivity to a wide range of substances and effluents suggested that the assay can be useful for environmental toxicity monitoring.

Cell suspension, membrane entrapment and cell immobilization are all used in the construction of yeast biosensors. The choice of cell location is dependant on the biosensor format. For example, where the cell response to the analyte is slow, incubation of the cells with the substrate could be separated from the transducer whereas if the cell response is rapid, cells may be situated on the transducer surface. Cells situated on the surface of the transducer may be immobilized on a membrane that is attached

to the surface, immobilized in a double membrane sandwich that is attached to the transducer surface or immobilized in a polymer directly on the surface. Membranes used to retain cells at the transducer surface include dialysis membranes, membrane filters of various materials such as cellulose acetate, nitrocellulose, polycarbonate and Teflon. The pore size can be relatively large given the size of yeast cells and fungal filaments.

Various polymers have been used to entrap yeast either directly on the transducer surface, on the surface of a membrane or to form structurally robust beads, discs, etc.

4.5.4 Fish-Cell Biosensors

Cell cultures, in particular those derived from fish, have been successfully employed as a biological alternative to the use of whole animals in ecotoxicity studies. The use of fish cell lines in conventional bioassays such as neutral red retention assays is however labour intensive, lengthy and costly. To date several transduction elements have been explored, however, most biosensor schemes are based on optical transduction. While vertebrate cell lines in biosensor configurations can be monitored electrochemically [249] stability is poor, compared with microbial cells. This may reflect stressor damage to the cells during immobilization, or adverse sensitivity to the mediator regimen, or both. Therefore, an alternative approach to monitor cellular activity using luminescence reported genes is being developed. Different works report biosensors using this strategy. Fibroblastic cells of blue gill sunfish (BF-2) have been transfected with a plasmid containing the luciferase gene *Luc*, which allows the luminescence of the transgenic cells following environmental challenge to be monitored via an optical transducer [250, 251].

Fish chromatophores have been shown to be promising biosensors for the detection of hostile agents in the environment [252, 253].

5 Current Trends and Future Perspectives

Biosensors and especially bio-effect related sensors like whole-cell sensors and receptor sensors constitute a very promising field of research that has been transformed over the past two decades through advancements such as new material technologies, novel transduction approaches, and powerful computer software to control devices. However, in order to exploit the principal advantage of biosensors as early warning systems for ecotoxicological purposes, standardization and harmonization are needed.

Current research activities to improve and to obtain more reliable devices are focused on continuous monitoring systems and remote controlled devices, to obtain more stable bio-receptors, more robust transduction elements, and miniaturized devices.

Some systems have been developed for use as continuous monitoring systems that can provide easy, rapid and on-site measurements. They may also be useful for mapping of contamination when it is important to obtain rapid results in the field,

such as after accidental spills or pollution events. Gu et al. [254] have recently applied a novel early-warning protocol for monitoring the toxicity of the effluents of a water treatment plant.

The emergence of large-scale widely deployed chemo/biosensing networks for environmental monitoring will grow from the top-down, with initial activity focusing on relatively sophisticated platforms deployed at relatively large distances (km). Clusters of these devices may be located at particular pollution hotspots, but the realization of a densely deployed heterogeneous sensor network requires significant effort to understand the level of information content that can be acquired from relatively dumb sensors used in a collaborative manner. On the other hand, developments in wearable sensors will occur much more quickly, as issues like power maintenance, length of deployment, communication distance and availability of a reliable platform infrastructure are more solvable [255, 256].

Nanotechnology is also playing a relevant role in the development of biosensors. Different studies reported the improvement of sensitivities and robustness in final biosensors developed using these new materials [257–259, 260].

Self-assembly of biomaterials, such as proteins, lipids or nucleic acids, has inspired the development of new biosensors, and molecular self assembly has been proposed for the synthesis of nanostructures capable of performing unique functions.

Several research groups have begun to explore alternative strategies for the development of optical SPR biosensors based on the extraordinary optical properties of metal nanoparticles.

Endo et al. [261] reported a novel label-free cell-based assay that is based on a localized surface plasmon resonance (LSPR) biosensor, which is excited using a core-shell structured nanoparticle layer substrate. The detection of the cell metabolites was achieved in this work using the isolated cells from mouse thymus. For detection of the cellular metabolites, the refractive index change by the specific interaction between the antigen and antibody was detected on the antibody immobilized LSPR-based biosensor.

Cao et al. [262] showed how oligonucleotides and presumably other biomolecules can be used to modify the surfaces of such particles, thereby imparting useful biorecognition properties to them. In another example, Banerjee et al. [263] presented a novel and simple cell-based detection system with a collagen-encapsulated B-lymphocyte cell line as a biosensor for rapid detection of pathogens and toxins. This approach is based on a cell-based sensing system using collagen-encapsulated mammalian cells for rapid detection of pathogenic bacteria or toxins, and demonstrates a potential for on-site use as a portable detection system.

Souza et al. [264] recently reported a novel approach for fabrication of spontaneous, biologically active molecular networks consisting of bacteriophage (phage) directly assembled with gold (Au) nanoparticles. The spontaneous organization of these targeted networks can be manipulated further by incorporation of imidazole, which induces changes in fractal structure and near-infrared optical properties. The networks can be used as labels for enhanced fluorescence and dark-field microscopy, surface-enhanced Raman scattering detection, and near-infrared

photon-to-heat conversion. Together, the physical and biological features within these targeted networks offer convenient multifunctional integration within a single entity with potential for nanotechnology-based biomedical and environmental applications.

Newly developed methods for spatially resolved immobilization of biomolecular recognition elements in transducer surfaces are playing an important role in the development of multi-analyte devices.

In addition, the advances in microelectronics and microfluidics allowed the miniaturization of analytical systems, reducing sample volumes and reagent consumption. Biosensor miniaturization has a particular significance in *in vivo* monitoring [265–268].

The current generation of biosensors is taking advantage of novel biosensing materials based on gene engineering. Gene engineering focuses on two main fields in the area of biosensors: genetically transformed cells and genetically engineered receptor molecules.

Modified cell biosensors are being constructed by fusing a reporter gene to a promoter element that is induced by the presence of a target compound. In the case of bioluminescence sensors, the recombinant bacteria employed contain bacterial luminescence genes and gene fusions between the regulatory regions of the *mer* operon (*merR*) that respond to contaminants. Many biosensors based on this principle have been already discussed.

6 Conclusions

Biosensors have great potential as smart, cost-effective devices for on-time and on-site determinations, especially at the screening level.

Biological approaches applied in conjunction with chemical analysis give more complete information about the possible biological impact of complex, polluted effluents.

Nevertheless, validation of new biosensors is an important issue for their progressive integration in pollution-control programs, and biological detector systems, such as biosensors and related techniques, must overcome a number of obstacles, such as the limited life times for some biorecognition components, and the lack of validation and correlation studies.

In addition, new environmental issues call for on-time analysis, miniaturized equipment and more robust methods. Many technical obstacles have been overcome thanks to the progress in optics, microelectronics, and nanotechnology that should permit new improvements in detection systems, such as the miniaturization of diode lasers and a decrease in component costs.

However, stabilization of the properties of biorecognition elements continues to be a crucial aspect in future development of biological techniques.

Furthermore, the development of genetic engineering offers the possibility of obtaining genetically engineered receptor molecules and genetically transformed cells.

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Genetically Engineered Bacteria for Genotoxicity Assessment

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Abstract Ever since the introduction of the *Salmonella typhimurium* mammalian microsome mutagenicity assay (the *Ames test*) over three decades ago, there has been a constant development of additional genotoxicity assays based upon the use of genetically engineered microorganisms. Such assays rely either on reversion principles similar to those of the Ames test or on promoter–reporter fusions that generate a quantifiable dose–dependent signal in the presence of potential DNA damaging compounds and the induction of repair mechanisms. While some of these assays were only briefly described in the scientific literature, others have been developed all the way to commercial products. Out of these, only one, the *umu*-test has been fully validated and ISO- and OECD-standardized. Herein we review the main directions undertaken in the construction and testing of bacterial-based genotoxicity bioassays, including the attempts to incorporate at least a partial metabolic activation capacity into the molecular design. We list the genetic modifications introduced into the tester strains, compare the performance of the different assays, and briefly describe the first attempts to incorporate such bacterial reporters into actual genotoxicity testing devices.

Keywords Biosensors, Genetically engineered bacteria, Genotoxicity, Promoter-reporter fusions, Reversion assays

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Contents

1	Introduction	162
2	The Promoter–Reporter Concept	162
3	Sensing Elements	163
3.1	SOS Promoters	163
3.2	Non SOS Promoters	166
4	Reporter Systems	166
4.1	Colorimetric and Electrochemical (<i>lacZ</i> , <i>phoA</i>)	166
4.2	Bioluminescence (<i>lux</i> , <i>luc</i>)	167
4.3	Fluorescent Protein Genes	168
5	Cytotoxicity Controls	169
6	Genotoxicants Detection Performance	170
7	Sensitivity Enhancement and Expansion of the Response Spectrum	171
7.1	Introduction of Host Strain Mutations for Enhanced Sensitivity	174
7.2	Altering the Sensing Element: Manipulation in Regulatory Sequences	174
7.3	Introduction of Metabolic Activation Enzymes	174
8	Tests that Do not Involve Promoter–Reporter Fusions: Reversion Assays	177
9	Reversion Tests Using Reporting Genes/Selection Markers	177
10	Devices Incorporating Bacterial Genotoxicity Reporters	178
11	Summary and Outlook	179
	References	179

1 Introduction

The increasing need to assay and monitor the potential genotoxic effects of an ever growing number of chemicals and environmental samples is countered by the logistic, economical, and ethical constraints imposed by the use of animal-based test systems. Consequently, ever since the introduction of the revolutionary *Salmonella typhimurium* mammalian microsome mutagenicity assay (the *Ames test*) over three decades ago [1], continuous efforts have been directed towards the development, improvement, and implementation of additional bacterial-based genotoxicity assays. Several such assays employ genetically engineered microorganisms, “tailored” to generate a quantifiable signal that reflects the genotoxic potency of the tested sample. Such assays share several significant advantages including rapid response times, high reproducibility, facility of use, and low operational cost. Yet, bacterial-based assays can not carry out the complex biochemical reactions collectively known as “metabolic activation” which take place mainly in mammalian liver cells, in which many xenobiotics are transformed into genotoxic forms. Herein we review the main directions undertaken in the construction and testing of bacterial-based genotoxicity bioassays, including the attempts to incorporate at least a partial metabolic activation capacity into the molecular design.

2 The Promoter–Reporter Concept

As will be discussed below, several genetic engineering approaches have been employed over the years in the construction of bacterial reporter strains that respond to the presence of genotoxic compounds. Many of these share the same

basic principle: the fusion of a gene promoter, known to be activated by the presence of genotoxic chemicals, to a gene or a group of genes the activity of which can be monitored quantitatively, preferably in real time [2]. The gene promoter acts as the sensing element, which – upon activation – drives the transcription of the downstream reporter gene(s). Consequently, the gene promoter will dictate the response spectrum of the construct and, to some extent, its sensitivity. The reporter genes determine the nature of the generated signal (bioluminescence, fluorescence, etc.) and thus also the instrumentation required for its acquisition. The host cell, the third major component in the construction of a genotoxicity reporter strain, is selected for ease of genetic manipulation, for its relevance, and – most importantly – for its effects on detection sensitivity and threshold.

3 Sensing Elements

In the selection of sensing elements to be used for the construction of genotoxicity reporter systems, the most promising candidates are promoters of genes involved in DNA repair. Such genes, induced in response to either actual DNA damage or to the presence of DNA damaging agents, are mainly part of two inducible systems, the *recA*-dependent, *lexA*-controlled SOS response and the *recA*-independent, *ada*-controlled adaptive system induced in response to alkylation damage of DNA. The latter system responds specifically to the presence of methylated phosphotriesters generated by DNA alkylation that activate the *ada* gene product which, in turn, triggers the transcription of genes such as *ada*, *alkA*, *alkB*, and *aid* [3, 4].

The SOS response is under the control of the LexA protein that binds to the SOS box in the promoter region of the regulon genes, repressing their expression. Derepression occurs when the RecA protein binds to single-stranded DNA at replication forks that are blocked by DNA damage, forming RecA-ssDNA nucleoprotein filaments [5]. Once bound to DNA, the RecA protein changes conformation and acts as a coprotease in the cleavage of LexA, thus allowing transcription of the SOS genes [6–8]. Among these are genes such as *uvrA*, *recA*, *recN*, or *umuCD*, responsible for DNA repair, and others such as *sulA*, that couple DNA damage to cell division [7, 9]. Expression of a given SOS gene depends on the specific LexA-binding properties of its promoter, determined by the sequence of the LexA-binding sites (SOS boxes), their number and arrangement [10–12].

3.1 SOS Promoters

Several gene promoters from over 30 known SOS regulon genes induced in times of DNA damage were used for the construction of genotoxicity sensors, as is briefly described below.

3.1.1 *umuDC*

The two proteins coded by this operon, UmuD and UmuC, are induced under DNA damage conditions by the LexA- and RecA-dependent transcriptional upregulation of the SOS regulon operon. They first form a UmuD₂C complex, which acts as a checkpoint inhibitor of cell division until repair can address the original inducing DNA damage signal. After RecA–ssDNA mediated UmuD cleavage, these proteins form a UmuD’₂C complex (DNA polymerase V), which carries out the error-prone replication of damaged DNA (SOS mutagenesis; for a review see [13]).

The first description of a *umuC’–lacZ* fusion coded on plasmid pSK1002 in *S. typhimurium* for the detection of genotoxic agents was published by Oda et al. [14], and has since been recognized as the “*umu*-test.” The *S. typhimurium* strain carrying this fusion (TA1535) has undergone several modifications including excision repair deficiency (*uvrB*), an *rfa* deletion which increases permeability to many chemicals, and a deletion of the natural *lac* operon. The *umu* test was standardized according to DIN (DIN 38415-3 [15]) and the International Standardization Organization (ISO) (ISO/CD 13829 [16]). It is now a part of the set of tools available to authorities and researchers for the investigation and monitoring of genotoxicity of environmental samples. The system was adapted to a 96-well microtiter plate format [17] and has been used, for example, to detect a wide range of carcinogenic mutagens [14, 18–21], as well as genotoxic activity in disinfectants [22], complex mixtures [23, 24], environmental pollutants [25], river waters and industrial wastewaters [17, 26, 27].

3.1.2 *sulA* (*sfiA*)

The SulA protein, produced in large amounts during the SOS response, halts cell division in *E. coli* by binding to the tubulin-like GTPase, FtsZ [28]. It has been used as a sensing element in genotoxicity detection in several cases, most notably in the colorimetric SOS-chromotest [29], commercialized in 1984. The *E. coli* PQ37 tester strain used in the SOS-chromotest harbors a *sfiA’::lacZ* fusion and carries a deletion of the normal *lac* region, so that β -galactosidase activity is strictly dependent on *sfiA* expression. Similarly to the *umu*-test bacterium it is mutated in *uvrA* to hinder DNA repair, and in *rfa* to increase permeability [30]. A different colorimetric assay based on a plasmid-borne *sulA’::lacZ* fusion in *S. typhimurium* TA1538 was proposed by El Mzibri et al. [31]; a procedure that includes metabolic activation based on S9-mix has been described as well.

3.1.3 *recN*

Another *E. coli* SOS gene promoter fusion that has been developed into a commercial product (VITOTOX®) is based on *recN*, coding for a protein that is involved in double-stranded DNA break repair. *E. coli* and several *S. typhimurium* strains (TA98, TA100,

TA104) are used as bacterial hosts. A multicopy plasmid harboring a fusion of the *recN* promoter to the *Vibrio fischeri luxCDABE* genes drives the emission of light in response to the presence of DNA damaging agents [32], allowing real-time monitoring of the bacterial response. The VITOTOX[®] test strains were tested with a variety of chemicals [32, 33] as well as river waters [34], ground water [35], and air samples.

3.1.4 *recA*

The gene of the RecA recombinase, which plays a key role in the SOS response by its coprotease activity on the LexA repressor, has been the basis of several attempts for the construction of genotoxicity sensors. Nunoshiba and Nishioka [36, 37] described the *E. coli* colorimetric “Rec–lac test,” based on the GE94 strain that carries the *recA–lacZ* fusion gene and its DNA repair-deficient derivatives such as KY946 (*uvrA*), KY945 (*recA*), and KY943 (*lexA*). The system was tested against 4-nitroquinoline-*N*-oxid (4-NQO), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), mitomycin C (MMC) and UV radiation, as well as with hydrogen peroxide (H₂O₂), formaldehyde (CH₂O), *tert*-butyl hydroperoxide, cumene hydroperoxide, and streptonigrin.

A different system, the *V. fischeri luxCDABE* genes, was used by Vollmer et al. [38] to generate several *E. coli* reporter strains, one of them (DPD2794) carrying a *recA'::luxCDABE* fusion on the multicopy plasmid pUCD615. These bioluminescent fusions allow real-time visualization of the transcriptional responses induced by DNA damage, without the need for cell-free enzyme assays or the exogenous addition of luciferase substrates. To make full use of these advantages, Polyak et al. [39] have alginate-immobilized a similar *recA'::lux* harboring strain to the tip of an optic fiber, the other end of which was connected to a photon counter. The instrument allowed a real-time determination of genotoxicity by dipping the bacteria-clad end into a sample.

3.1.5 *Cda*

The colicin D gene *cda*, a constituent of the ColD plasmid [40], also served as a basis for a bioluminescent genotoxicity sensor using *Photobacterium leiognathi luxCDABE* as a reporter. This “SOS-*lux*” test responded sensitively to diverse genotoxins, such as MMC, MNNG, nalidixic acid (NA), dimethylsulfate (DMS), H₂O₂, CH₂O, and UV and γ radiation [41]. This assay was later combined with the GFPuv-based Lac-Fluoro test to generate a combined toxicity–genotoxicity sensor [42].

Four different SOS promoters (*recA*, *umuCD*, *sulA*, and *cda*) were compared by Norman et al. [12] using the same fluorescent reporter (*gfp mut3**, in plasmid pANO1). The differences between the constructs were evaluated after the exposure of host cells harboring the fusion plasmids (MG1655/pANO1::SOS promoter) to the known genotoxicant *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). A *tolC* mutation enhanced the sensitivity to this agent; no other chemicals were tested in this study. Performance of the *cda*-based sensor in response to MNNG clearly surpassed the

other three with respect to the SOS-induction factor, as a result of high rates of gene expression combined with a low background activity of the *cda* promoter. Thus, the *cda* promoter was selected for the further development of the GenoTox test [43].

3.2 Non SOS Promoters

3.2.1 *alkA*

Several bacterial DNA protection and repair systems that are independent of the SOS regulon have been described, one of which, most efficiently induced by alkylating agents, has been generally termed the “adaptive response.” Several genes of this system have been characterized, including *alkA*, which encodes a repair glycosylase (N3-methyladenine DNA glycosylase II; [3]). A promoter of this gene has been fused by Vollmer et al. [38] to the *V. fischeri luxCDABE* genes. The construct displayed a very strong response to the alkylating agent MNNG, the magnitude of which was attributed to a very low background bioluminescence; the responses of an equivalent *lacZ* fusion were much more moderate.

3.2.2 *nrdA*

The expression of the *E. coli nrdA* gene, which encodes for a ribonucleoside diphosphate reductase, is strongly affected by DNA damage, induced for example by an exposure to UV irradiation, but is independent of LexA [44]. The *nrdA* promoter was fused by Hwang et al. [45] to *Photorhabdus luminescens luxCDABE* genes. *E. coli* strain BBTNrdA carrying this plasmid-borne fusion responded to the DNA damaging agents NA, MMC, MNNG, and 4-NQO and hydrogen peroxide but not to other oxidants or phenolic compounds [45].

4 Reporter Systems

The spectrum of reporter systems available for monitoring gene expression by transcriptional fusions is continuously expanding, as is the instrumentation for signal detection and quantification. Colorimetric, fluorescent, bioluminescent and (to a small extent) electrochemical detection of genotoxicity have been described, and will be briefly outlined below.

4.1 Colorimetric and Electrochemical (*lacZ*, *phoA*)

The β -galactosidase gene, *lacZ*, has been used as a gene expression reporter for several decades. The most common substrates employed for assaying the activity of this enzyme are *o*-nitrophenyl β -D-galactopyranoside (ONPG) and 5-bromo-4-

chloro-3-indolyl β -D-galactoside (X-gal) for colorimetric detection, 4-methylumbelliferyl- β -D-galactopyranoside (MUG) for fluorimetry, 1,2-dioxetane substrates for luminescence, and *p*-aminophenyl- β -D-galactopyranoside (PAPG) for electrochemical analysis. The advantages of colorimetric assays lie in their simplicity and rapidity, but the need for improved sensitivity, faster response times, a broader dynamic range and the capability of real-time monitoring has led to a continuous search for alternatives [46]. The *umu*-test [14], SOS-chromotest [29], *sula*-test [31] and Rec-lac test [37] were all developed using *lacZ* as the reporter and ONPG as the substrate. Several remedies have been proposed to overcome interferences by colored samples, such as the inclusion of a washing step after the exposure of the bacteria to the samples [19, 47], or a post-treatment dilution and reincubation [17, 18]. The latter procedure was reported to enhance the sensitivity of the *umu*-test to genotoxicants in environmental samples in a high-throughput microtiter plate system [23]. Oda et al. [48] achieved higher sensitivity by using a different substrate, chlorophenol red- β -D-galactopyranoside (CRPG). The red reaction product after cleavage by the β -galactosidase has a longer life time than *o*-nitrophenol, the ONPG reaction product.

Similar modifications were also introduced to the SOS-chromotest [49]. The original colorimetric procedure of the assay [29] was successfully changed to a fluorimetric one by using the fluorescent substrate 4-methylumbelliferyl- β -D-galactopyranoside (MUG) [50].

A different approach was proposed by Matsui et al. [51], who provided the *umu*-test bacteria (TA1535/pSK1002) with the substrate PAPG, the end product of which (*p*-aminophenol) can be monitored electrochemically. This approach, utilized earlier for other bacterial sensor systems [52–55], requires the addition of an external substrate but does not involve lysis or permeabilization of the cells. Similarly to bioluminescence, it is thus suitable for continuous online measurement of enzymatic activity, even in turbid solutions and under anaerobic conditions. Matsui et al. [51] have demonstrated this by scanning electrochemical microscopy (SECM) in a specialized glass biochip configuration, using 5 nl cell aliquots immobilized in collagen gel. Overall, lower limits of detection of 2-aminofluorene (2-AF), MMC and 2-aminoanthracene (2-AA; +S9-mix) were obtained by the microbial chip as compared to the conventional *umu* tests, but it should be noted that the definition of the detection limit was different and exposure times were longer.

4.2 Bioluminescence (*lux*, *luc*)

The reaction by which photons are released by living organisms, bioluminescence, appears in numerous groups of organisms including bacteria, protozoa, fungi, insects, and fish. In all cases the reaction is catalyzed by an enzyme termed luciferase that oxidizes a substrate known as luciferin, but the chemical and enzymatic nature of both entities depends on the organism from which the system is derived. The two bioluminescent systems most commonly used as reporters of gene activation are of bacterial and insect (firefly) origin. Firefly luciferase, coded by the *luc* gene, is a 62-kDa monomeric protein, and its activity is oxygen- and ATP-dependant. Its luciferin,

benzothiazoyl-thiazole, has to be added externally when *luc* is used as a reporter gene. Bacterial luciferase catalyzes the oxidation of a reduced flavin mononucleotide (FMNH₂) by a long-chain fatty aldehyde to FMN and the corresponding fatty acid in the presence of molecular oxygen. All bacterial luciferases are heterodimeric proteins composed of two subunits, α (40 kDa) and β (37 kDa), encoded by the *luxA* and *luxB* genes of the *lux* operon. The three other genes in this operon (*luxCDE*) encode the synthesis and recycling enzymes of the fatty acid aldehyde [56]. Constructs carrying just *luxA* and *luxB* are sufficient to generate a bioluminescent signal, but necessitate the external addition of the aldehyde substrate. The commonly used luciferases of *V. fischeri* and *Vibrio harveyi* have limited upper temperatures of 30°C or 37°C, respectively. In recent years, *Photobacterium luminescens lux* genes have thus often been used due to the higher upper temperature limit (45°C) of their gene products [57]. The noninvasive protocol using *lux* fusions allows real-time reporting of the transcriptional activation of the monitored gene promoters. As described above, the VITOTOX[®] test uses the *V. fischeri luxCDABE* operon under the control of the *recN* promoter [32, 33], and the SOS-*lux* test employs the *lux* operon (*luxCDABFE*) of *P. leiognathi* under control of the *cda* gene promoter of the plasmid ColD [41]. Vollmer et al. [38] have fused the *E. coli recA*, *uvrA*, and *alkA* promoters to the *V. fischeri luxCDABE* operon. Further modifications to the same system [58, 59] included integration of the *recA'::lux* fusion into the *E. coli* chromosome, a change of the reporter system to *P. luminescens lux*, and the use of either *S. typhimurium* or a *tolC E. coli* mutant as alternative hosts. Application of the *P. luminescens* reporter, which allowed a working temperature of 37°C, resulted in a more rapid response to various genotoxic chemicals and UV.

The *luxCDABE* genes of *V. fischeri* were also fused to the *recA* promoter of *Pseudomonas aeruginosa* [60]. As a soil and freshwater bacterium, *P. aeruginosa* was presented as a good candidate to serve as a sensor for the state of natural bacterial communities of both pristine and polluted habitats. Light production in response to UV exposure was monitored in this strain as part of a study of UV effects on natural bacterial populations.

To increase the sensitivity of the *umu*-test and to expand its detection capabilities, two groups independently replaced its β -galactosidase reporting gene by either bacterial [61] or insect [62] luciferase. In both cases, improvements in performance were reported, including enhanced sensitivity, improved signal-to-noise ratios, stronger signals, and a better neutralization of color interferences.

4.3 Fluorescent Protein Genes

The highly stable green fluorescent protein, GFP, of the jellyfish *Aequorea victoria* was the first fluorescent protein the gene of which was utilized as a molecular reporter [63]. It was soon followed by additional fluorescent protein genes isolated from various marine organisms as well as by mutated forms with improved performance [64–70]. The GFP protein has a high quantum yield and can be

expressed in both prokaryotic and eukaryotic systems with no need of a substrate or cofactor [71]. To increase the sensitivity of assays based on the GFP reporter system, several green fluorescent protein mutants were constructed [65, 72–74].

Arai et al. [75] have modified the *umu*-test by replacing the *lacZ* gene with a DNA fragment encoding for EGFP (enhanced green fluorescent protein). This construct was tested in *E. coli* strain KY706 with 3 $\mu\text{g/ml}$ 4-NQO, a concentration that strongly induced β -galactosidase activity in the *umu*-test. The GFP reporter system was equivalent to the β -galactosidase reporting system with respect to its detection sensitivity only after inserting additional modifications to the plasmid. These modifications include utilization of tandem *lacUV5* and chimeric *trp/umu* promoters, and coexpression of the *E. coli* *recA5327* mutant. An additional construct that harbors the fusion of the *umuCD* promoter to the *gfp* gene was generated by Justus and Thomas [76], who reported an overall poorer performance compared to the *lacZ*-based assay.

Other fluorescent genotoxicity sensors were constructed by fusing the *gfp* and *gfpmut3* reporting genes to the *recA* promoter [77]. GFPmut3 is a mutant that is approximately 20-times more fluorescent than wild-type GFP and only weakly excited by UV-light [72]. The use of the wild-type *gfp* yielded dose-dependent but weak results, while with *gfpmut3* the detection thresholds for MMC, MNNG, NA, hydrogen peroxide, and formaldehyde were comparable to the SOS-chromotest [29], the *umu*-test [19], and the SOS-*lux* test [41].

The three fluorescent protein genes coding for EGFP, GFPuv, and DsRed (fluorescent protein derived from the sea anemone *Discosoma* sp. [66]) were similarly fused to the *recA* promoter [78], and the responses to nalidixic acid were compared to a luminescent *recA'::lux* strain. Performance was usually poorer compared to bioluminescent *recA*-based reporters: lag times were longer and detection thresholds were higher, unless incubation times were very long. The *recA'::DsRed* plasmid, hosted in *E. coli* UTL2, was used in order to monitor antigenotoxic activity of plant extracts and exhibited some protection against MMC, NA, and hydrogen peroxide [79].

The use of fluorescent proteins as reporters has been characterized as superior in terms of stability but inferior to enzyme-based reporters in terms of sensitivity and response kinetics [78, 80]. Norman et al. [81] have demonstrated that these drawbacks can be circumvented by the use of flow cytometry, showing that the response threshold of strain *cda'::gfpmut3* to MNNG was 5 nM, tenfold lower than the minimal detectable concentration (MDC) of the *umu*-test [17]. Moreover, the experimental procedure enabled the detection of MMC in spiked soil.

5 Cytotoxicity Controls

As samples or chemicals suspected of DNA damaging activity are likely to also be cytotoxic, genotoxicity assays often incorporate suitable controls to neutralize or correct for the effects cell damage and death may have on assay results. One simple

measure is an optical determination of cell growth in parallel to assaying reporter gene activity [82]. However, this solution is limited, since optical density does not necessarily reflect the viability status of a cell suspension. A different approach is based on the inclusion of an additional, constitutive reporting strain or enzyme which serves as a “light off” sensor: a decrease in its signal indicates a toxic effect of the sample. This approach, for example, was adopted in the VITOTOX[®] test that introduced a constitutive light-producing strain with a *lux* operon under the control of the strong promoter, *prl* [33].

The SOS-*lux* test similarly incorporated a cytotoxicity reporting strain harboring a constitutive *lac*-GFPuv plasmid in the same *S. typhimurium* host strain [42]. In a further development of this system a *SWITCH* plasmid was added, combining the SOS *lux* plasmid pPLS-1 and the LAC-*Fluoro* plasmid pGFPuv [83].

Using a different approach, the tester strain in the SOS-chromotest was made constitutive for alkaline phosphatase synthesis [84]. This enzyme, noninducible by DNA-damaging agents, is assayed in parallel to β -galactosidase and the ratio of the two activities is taken as a measure of the specific activity of β -galactosidase [29].

The toxicity of a sample can also be evaluated with promoters that are induced by a broad spectrum of environmental insults and are thus good indicators of toxic cellular stress, such as the *grpE* promoter, a component of the functionally cooperating chaperone network in *E. coli* [85, 86]. The use of two strains, one harboring the plasmid *recA*::GFPuv and the other *grpE*::*lux* allowed an assessment of the toxicity of the sample along with its genotoxicity [78]. A dual-function toxicity/genotoxicity bioreporter system was reported by Hever and Belkin [87] who described a plasmid containing both *recA*::EGFP and *grpE*::DsRed fusions. A somewhat different double reporter concept was demonstrated by Mitchell and Gu [88], who presented a strain containing a fluorescent genotoxicity reporter fusion (*recA*::*GFPuv4*) and a bioluminescent oxidative stress reporter (*katG*::*luxCDABE*).

6 Genotoxics Detection Performance

As described in detail above, numerous genotoxicity bioassays based on genetically engineered bacteria have been presented over the years. While some of them, such as the *umu*-test and the SOS-chromotest, have undergone intensive validation and have been tested against hundreds of compounds, others have only been briefly described along with their responses to a very limited range of chemicals. Quite clearly, therefore, pending further validation of the latter group, the validated tests are of a much higher value for routine testing and their results receive higher credibility. Detailed reports of an extensive testing of these assays and their comparison to the Ames test can be found in Nakamura et al. [19], Reifferscheid and Heil [20], and Quillardet and Hofnung [89]. The *umu*-test has been standardized and

accepted as an ISO and a DIN test for waste water quality (ISO/CD 13829, DIN 38423-5) [15, 16].

Table 1 summarizes the detection thresholds to selected genotoxicants exhibited by many of the assays described in the present review. Possibly the major factor which stands out from even a brief look at Table 1 is that only two systems, the *umu*-test and the SOS-chromotest, were challenged with the required spectrum of genotoxic chemicals necessary to demonstrate their applicability to environmental testing. All the others were only preliminarily challenged with a very limited number of compounds. In fact, this Table only lists chemicals that have been tested by at least one bioassay in addition to the *umu*-test and the SOS-chromotest; it thus does not contain the detection thresholds for hundreds of other compounds that have been reported for these two tests [14, 19, 20, 29, 49, 89, 94, 99]. On the basis of this limited comparison it may also be observed that detection thresholds vary greatly between the different assays, sometimes by several orders of magnitude. Other factors such as response times, detection spectra or facility of use, which have not been compared in Table 1, confer additional advantages to some of the reporter strains.

A further comparison of the performance of some of these bioassays has been performed in several hands-on workshops conducted in Belgium (Mol TECHNOTOX; [90, 100]) and in the USA (Eilatox-Oregon; [101-104]). In addition to highlighting differences in response characteristics, such workshops help to emphasize the difficulties encountered when taking a newly developed test out of the lab and into the field.

7 Sensitivity Enhancement and Expansion of the Response Spectrum

Very early in the short history of genetically engineered bacterial reporters it became apparent that simple promoter-reporter fusions may be sufficient to demonstrate the applicability of the concept for genotoxicity testing, but that additional molecular manipulations are required in order to turn them into efficient tools for routine use. Such manipulations have taken several forms including modification of the sensing elements, introducing mutations into the reporter strains for enhanced sensitivity and permeability, and the incorporation of metabolic activation capabilities.

Table 2 lists some of the genetic manipulations introduced into the *E. coli* or *S. typhimurium* host strains and their reported effects. The modifications can be divided into two classes: deficiencies that reduce the ability of the cells to defend against DNA damaging agents and new or enhanced abilities of bacterial cells to metabolically activate progenotoxic compounds, thus at least partially mimicking the metabolic pathways such compounds may undergo in mammalian systems.

Table 1 Published detection thresholds of selected chemicals by genetically engineered bacterial genotoxicity reporters

		SOS-lux test		SOS-chrom		SOS-lux test		sulA-test		recA'::lux		nrdA'::lux [45]		recA'::gfp		cda'::gfp		recA'::DsRed	
Promoter	Reporter gene(s)	umu-test	otest	umuCD	lacZ	sfIA	lacZ	recN	recA	luxCDABE V. fischeri	luxCDABE P. luminescens	recA	gfpmut3	gfpmut3	Cda (ColD)	gfpmut3	DsRed2	recA	DsRed
Host strain		S. typhimurium TA1535 E. coli PQ37 S. typhimurium TA1535 S. typhimurium TA1535 S. typhimurium TA1535 E. coli RFM443 E. coli RFM443 S. typhimurium TA1535 E. coli UTTL2																	
Compound		MDC- Minimal detectable concentration [μM]																	
Fungal toxins and antibiotics																			
MMC	0.01 [19]	0.016 [29]	0.016 [29]	4.3 × 10 ⁻³	0.046	5.3 × 10 ⁻³	2.9 × 10 ⁻⁴	0.93	0.012	9.1 × 10 ⁻³	0.011								
Doxorubicin	1.1 [19]	0.41 [93]	0.9	0.69	0.43														
NA	2.4 [19]	NA	4.6	0.69															
Bleomycin	0.04 [19]	22.5 [93]	0.35																
Esters																			
MMS	150 [17]	63 [29]	73	2,061	117														
EMS	1.8 × 10 ³ [19]	10 ⁴ [94]	2,061	3,414															
DMS	300 [19]	6.7 [29]	7.5																
Nitroso-, nitro-																			
DEN	5 × 10 ⁴ [19]	199 [29]	0.45 [94]	0.6	2,349	1.5 × 10 ⁴	0.763	0.16											
MNNG	3.0 [23]	0.45 [94]			0.40	1													
NPAHs																			
4-NOPD	32 [14]	ND [49]	10.4	0.06	10.4														
3-NFA	0.04 [95]	0.25 [96]	0.06	0.004	0.06														
4-NQO	0.1 [19]	0.02 [29]	0.02 [29]	0.042	0.004	0.018													
Furazolidone	0.22 [47]	<0.44 [49]	0.002		0.002														
HAs																			
2-AA	1 [17]	3 [94]	1.08	0.037	1.1														
2-AF	0.4 [17]	< 274 [94]																	0.08

<i>PAHs</i>						
B[a]P	4 [19]	2.33 [29]		0.79	0.58	5 × 10 ⁻⁵
Fluoranthene	ND [19]	ND [97]		15.3		
Chrysene	65 [19]	<87 [49]		21.9		
<i>Acridine</i>						
Acridine orange	12 [19]	75 [29]			0.83	
ICI 191	0.5 [19]	0.7 [94]		0.68	0.3	
9-aminoacridine	46 [19]	ND [49]			5	
<i>Miscellaneous</i>						
H ₂ O ₂	1,322 [19]	1,000 [94]	50	59	84	23.5
CH ₂ O	623 [19]	>1,000 [94]	6,500			1,470
Epi	649 [19]	3,300 [94]		1,383		286
EtBr	127 [19]	1,165 [30]				305
						24.7
						141
						588
<i>Metals</i>						
K ₂ Cr ₂ O ₇	258 [19]	68 [98]	0.68	14		
CdCl ₂	ND [20]	ND [30]		ND		0.99

MDC The lowest concentration at which the response is systematically over twice the background; *ND* Not detected; *NPAH* Nitrated polycyclic aromatic hydrocarbons; *HA* Heterocyclic amines; *PAH* Polycyclic aromatic hydrocarbons; *MMC* Mitomycin C; *NA* Nalidixic acid; *MMS* Methyl methanesulfonate; *EMS* Ethyl methanesulfonate; *DMS* Dimethylsulfate; *DEN* Diethylnitrosamine; *MNNG* *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; *4-NOPD* 4-Nitro-*o*-phenylenediamine; 2-NFA 2-Nitrofluoranthene; 4-*NQO* 4-Nitroquinoline-*N*-oxide; 2-AA 2-Aminoanthracene; 2-AF 2-Aminoflouren; *B[a]P* Benzo[a]pyrene; *H₂O₂* Hydrogen peroxide; *CH₂O* Formaldehyde; *Epi* Epichlorohydrin; *EtBr* Ethidium bromide

7.1 Introduction of Host Strain Mutations for Enhanced Sensitivity

As listed in Table 2, several mutations have been introduced into genotoxicity reporter strains to enhance their sensitivity and thus lower their detection thresholds. While some mutations, such as *tag* or *oxyR* have only been reported once (for the SOS-chromotest, [89]), others have become almost a prerequisite in microbial genotoxicity reporters. Most notable in the latter group are *uvrAB* mutants deficient in excision repair, and *rfa* mutations that, by increasing membrane permeability, allow higher intracellular concentrations of the tested chemicals [111, 112].

7.2 Altering the Sensing Element: Manipulation in Regulatory Sequences

Molecular manipulations of the DNA fragment harboring the sensing promoter element in order to improve the bacterial response to the target chemicals have also been described. The most notable example is the VITOTOX[®] strain [32]. In addition to the wild-type *recN* promoter, two different promoter mutants were constructed and tested singly and in combination: a deletion of one of the LexA binding sites, and a “promoter up” mutation where a consensus nucleotide was introduced in the -35 region. Each of the single mutants was superior to the wild type in at least one respect, but the double mutation resulted in poorer performance compared to the wild type. Another successful effort was done by Arai et al. [75], who improved the performance of a *umuCD'::gfp* construct by replacing the wild-type -35 promoter sequence with the -35 sequence of the highly active *trp* gene promoter.

7.3 Introduction of Metabolic Activation Enzymes

To at least partially alleviate the lack of metabolic activation potential in prokaryotes and correspondingly reduce the dependency on external metabolic activation by rodent-derived cytochrome P450 (S9) preparations [1, 113], several attempts have been made to genetically engineer bacterial cells to incorporate some of the enzymatic activities involved in the activation process of xenobiotics. Some of these efforts are summarized in Table 2, clearly demonstrating the viability and potential of this approach. Nevertheless, while the study of single enzymes is a necessary step in improving our understanding of the activation of promutagens to mutagens [114], it should be remembered that the cyt P-450 complex is composed of a plurality of activities that are not very likely to be engineered into a single reporter strain in the near future.

Table 2 Molecular modifications introduced into genotoxicity reporter strains to enhance sensitivity, expand the response spectrum and incorporate metabolic activation capabilities

Manipulation	Test	Strain	Modified capabilities	Effect	Reference
<i>uvrAB</i> mutation	SOS-chromotest	PQ37/ <i>sfIA::Mud(Ap lac) cts</i>	Deficiency in nucleotide excision repair (NER)	Increased sensitivity toward certain genotoxics	[1, 14, 29, 31, 37, 42, 43]
	<i>umu</i> -test	TA1535/ <i>umuCD'::lacZ</i>			
	GenoTox	TA1535/ <i>cda'::gfp</i>			
	SOS- <i>lux</i>	TA1538/ <i>cda'::lux</i>			
	VITOTOX®	TA104/ <i>recN2-4::lux</i>			
<i>tag</i> mutation	<i>sulA</i> -test	TA1538/ <i>sulA'::lacZ</i>			[89, 105]
	Rec- <i>lac</i> test	KY946 ϕ (<i>recA-lacZ</i>)			
	SOS-chromotest	PQ243/ <i>sfIA::Mud(Ap lac) cts</i>	Inactivation of the constitutive 3-methyl-adenine DNA glycosylase I	Response to lower concentrations of alkylating agent as MNNG, MMS, etc.	
<i>oxyR</i> mutation	SOS-chromotest	PQ300/ <i>sfIA::Mud(Ap lac) cts</i>	Depletes the oxidative stress responses under the control of OxyR transcription regulator	More sensitive to various classes of peroxides and compounds generating peroxides	[89, 93]
<i>rfa</i> mutation	SOS-chromotest	PQ37/ <i>sfIA::Mud(Ap lac) cts</i>	Mutation in the core enzymes of the Lypopolysaccharide (LPS) biosynthesis. Incomplete LPS composed of the ketodeoxy-octanoate-lipid core	Higher permeability to substances, especially important with larger hydrophobic genotoxins	[1, 14, 30, 31, 33, 43, 106]
	<i>umu</i> -test	TA1535/ <i>umuCD'::lacZ</i>			
	GenoTox	TA1535/ <i>cda'::gfp</i>			
	SOS- <i>lux</i>	TA1538/ <i>cda'::lux</i>			
	VITOTOX®	TA104/ <i>recN2-4::lux</i>			
<i>tolC</i> mutation	<i>sulA</i> -test	TA1538/ <i>sulA'::lacZ</i>			[12, 58, 106]
	GenoTox	N43/ <i>cda'::gfpmut3</i>			
	SOS- <i>lux</i>	PB3/ <i>cda'::lux</i>			
	<i>recA'::lux</i>	DE112/ <i>recA'::lux</i>			
	<i>umu</i> -test	NM1011/ <i>umuCD'::lacZ</i>			
<i>S. typhimurium</i> NR overexpression			Inactivation of the efflux, outer membrane transporter- TolC	Limited efflux capability, increases sensitivity to genotoxins	[95]
			High nitroreductase activity	Highly sensitive towards many nitroarenes as 2-NF, 1-NP, etc.	

(continued)

Table 2 (continued)

Manipulation	Test	Strain	Modified capabilities	Effect	Reference
<i>S. typhimurium</i> O-AT overexpression	<i>umu</i> -test	NM2009/ <i>umuCD'::lacZ</i>	13-fold higher isoniazid-N-acetyltransferase activity	High sensitivity toward nitro- and dinitro- containing compounds, as well as arylamines, aminoazo and HAS	[107, 108]
<i>S. typhimurium</i> O-AT and NR overexpression	<i>umu</i> -test GenoTox	NM3009/ <i>umuCD'::lacZ</i> TGO2/ <i>cdd'::gfp</i>	High O-AT and NR activity	Increased sensitivity to aromatic amines and nitroarenes with/without external MA (S-9)	[43, 48, 108]
Human CYP1A2 and NADPH-P450 reductase expression in <i>S. typhimurium</i>	<i>umu</i> -test	OY1001/ <i>umuCD'::lacZ</i>	7-Ethoxyresorufin O-deethylation and NADPH-cytochrome c reductase activity	Detection of some carcinogenic HAS, without the addition of metabolic activation system (S-9)	[109]
Human CYP1A2 and NADPH-P450 reductase with O-AT expression in <i>S. typhimurium</i>	<i>umu</i> -test	OY1002/ <i>umuCD'::lacZ</i>	7-Ethoxyresorufin O-deethylation and NADPH-cytochrome c reductase activity, joint with high O-AT activity	More sensitive to HAS than the previous strain, without external MA. Detects the mutagens APNH and APH	[48, 110]

CYP1A2 Cytochrome P450 1A2; O-AT O-acetyltransferase; NR nitroreductase; HA heterocyclic amines; MA metabolic activation; APNH aminophenylharman; APH aminophenylharman

8 Tests that Do not Involve Promoter–Reporter Fusions: Reversion Assays

The second major group of bacterial test systems for the assessment of genotoxicity is based on the reversion of auxotrophic bacterial strains to prototrophic strains by mutation events that occur more frequently in the presence of genotoxic compounds. This basic concept was described by Ames and Hartmann already in the 1970s [1, 111]. In this system the target gene sequence for mutagenesis is the histidine operon (*hisLGDCBHAFI*). The *Salmonella typhimurium* tester strains do not proliferate in the absence of histidine due to mutations in the *his* operon but can recover histidine synthesis by remutations. Different strains are available allowing a determination of the type of mutation that is caused by the genotoxicant. Mostly used are the *S. typhimurium* strains TA98, sensitive to +1 and –2 frameshift mutations, and TA100, responsive to base substitutions. The sensitivity of these strains to genotoxic compounds is enhanced by *uvrB* and *rfa* mutations (see above). Further milestones were the addition of S9-preparations to the assay in order to mimic the metabolism of xenobiotics in eukaryotes and the introduction of the *mucAB* genes via the plasmid pKM101 [115].

Many attempts have since been reported to further improve the performance of the Ames test, such as the already discussed introduction of genes coding for enzymes involved in the activation of xenobiotics in order to enhance the metabolic competence of the bacterial cells. An interesting approach was described by Aubrecht et al. [116] with the cloning of the *P. luminescens luxCDABE* genes into strains TA98 and TA100. Bioluminescence, which is coupled to the metabolic state of the cell, is used as a sensitive sensing element allowing the identification of histidine-independent revertants in a high-throughput fashion.

The basic concept of growth recovery after reversion was adopted to rationally designed *lacZ* mutants of *E. coli* that are sensitive for defined mutation events [117, 118]. After reversion the test strains grow on lactose minimal medium because of the regained enzymatic activity of β -galactosidase [119, 120].

9 Reversion Tests Using Reporting Genes/Selection Markers

An attractive alternative to the traditional reversion test is its combination with reporter genes and/or selection markers. Ulitzur et al. isolated a spontaneous dark variant of *P. leiognathi* and showed that its reversion frequency to the luminescent wild type is increased by the presence of mutagenic compounds [121]. The Mutatox test was developed using this approach [122]. A further example for this strategy is the usage of a dim *luxE* mutant of the marine bacterium *V. harveyi* [123–127].

Other approaches utilize the gain of resistance to antibiotics induced by genotoxins for the assessment of the mutagenic potential of a compound. The “MutaGen” assay uses a reversibly knocked-out beta-lactamase gene coding for ampicillin

resistance. Revertants are able to grow in the presence of ampicillin whereas nonreverted bacteria disappear due to lytic death. The *lacZ* gene under the stringent control of the tetracycline repressor provides a sensitive detection system for revertants [128, 129].

Another approach applying an antibiotic selection marker quantifies the appearance of neomycin-resistant mutants of the marine bacterium *V. harveyi* [130, 131]. To improve performance, a mutant with enhanced UV-sensitivity was generated by transposon mutagenesis and a plasmid containing the *mucAB* genes was added.

10 Devices Incorporating Bacterial Genotoxicity Reporters

Several reports describe genetically engineered bacteria incorporated into specially designed hardware to generate dedicated genotoxicity biosensors. Polyak et al. [39] have immobilized the *E. coli* strain DPD1718 [58, 59] that contains a chromosomally integrated *recA':lux* fusion in sodium alginate onto the tip of an optical fiber. The luminescent signal induced in the bacteria by the presence of genotoxins was collected by the fiber and electronically amplified. Sensor strains embedded in alginate retained their sensitivity following a 2-month incubation, but at the cost of a significantly delayed response [58]. A biosensor composed of a high-density living bacterial cell array was fabricated by depositing single *E. coli* cells carrying a *recA':gfpmut2* into a microwell array formed on one end of an imaging fiber bundle [132]. Each fiber in the array had its own distinct light pathway, enabling thousands of individual cell responses to be monitored simultaneously with both spatial and temporal resolution. The biosensors demonstrated an active sensing lifetime of more than 6 h and a shelf lifetime of 2 weeks. An on-chip whole-cell genotoxicity bioassay was developed [133], using a three-dimensional microfluidic network system composed of one perforated microwell chip bound in two microchannel chips, in which the sensor cells were immobilized in agarose. The bioluminescent responses to MMC of the sensor strain, SOSluc, in a wild-type *E. coli* or its *tolC* deficient derivative were measured by a charge-coupled camera (CCD). The immobilized cells were stable for at least 1 week at 4°C.

An electrochemical mutagen screening based on the *umu*-test was performed on a microbial chip combined with a scanning electrochemical microscopy (SECM) device [51]. The microbial chip was fabricated by embedding 5 nl of collagen-immobilized genetically engineered *S. typhimurium* strain (TA1535/pSK1002) in a microcavity on a glass substrate. β -Galactosidase activity was monitored by electrochemical determination of the concentration of *p*-aminophenol (PAP), the enzymatic hydrolysis product of PAPG. Although such biosensors open potential horizons for both field applications and laboratory high-throughput screening systems, their current status requires much additional development before they become available for routine use. Such future development should take into account the necessity of including metabolic activation as an integral part of the process.

11 Summary and Outlook

Genetically engineered bacterial sensor systems display a central element in effect-directed analysis of environmental contaminants and the assessment of the DNA damaging potential of chemicals. The basis for a successful construction of bacterial sensors consist of three elements: (a) a bacterial tester strain that offers an appropriate genetic background facilitating high permeability for chemicals, an appropriate DNA-repair capacity and negligible background activity of the reporter gene; (b) a sensitive promoter that offers well-balanced repressor binding properties for sensitive induction of the reporter gene; and (c) a sensitive, fast responding reporter system with a broad dynamic range and preferably the capability of real-time monitoring.

The discovery and the understanding of the bacterial SOS-system, a regulatory pathway that is mainly responsible for inducible DNA repair and induced mutagenesis in bacteria, opened a wide variety of possibilities to genetically tailor bacteria for the specific, sensitive, and fast detection of genotoxic contaminants. The fusion of SOS gene promoters to reporter genes that generate quantifiable signals allows one to easily detect the genotoxic potency of a sample. Herein we have described the current stage of development of the sensing systems by discussing the molecular, biochemical, and physico-chemical characteristics of the different promoters and suitable reporter genes based on colorimetric, luminometric, fluorimetric, and electrochemical detection. The advantages compared with classical test systems were discussed.

The potential of future sensor developments with enhanced external and/or internal metabolic competence is immense. Against the background of a worldwide increasing freshwater demand and the need for reclamation of process water as a drinking water resource, bacterial sensors can play a crucial role in risk minimization. To fully reach this objective, additional progress needs to be made in several directions including enhancement of sensitivity, expansion of the response spectrum, stabilization of the more sensitive reagents and, most importantly, the introduction of broad-spectrum metabolic activation capabilities into the sensor strains.

Acknowledgements The authors are grateful for funding provided by the German BMBF and the Israeli MOST in the framework of project WT601 ("DipChip") of the binational Water Technology Program.

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Adverse Effects in Aquatic Ecosystems: Genotoxicity as a Priority Measurement

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Abstract Some chemical substances are capable of modifying genetic material and thereby also damaging it. Aquatic genotoxicity testing represents an important contribution to environmental research today.

Sensitive testing methods for practical applications have been developed with reasonable certainty, which allow genotoxic (mutagenic) agents to be discovered in the environment. Risk levels for humans and the environment may be reliably assessed and the resulting consequences predicted.

Keywords Bacterial test systems, Eukaryotic indicator tests, Genotoxicity, Test strategies

Contents

1	Introduction.....	188
2	Methods of Aquatic Genotoxicity Testing	189
2.1	Bacterial Assays.....	189

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2.2	Eukaryotic Assays.....	192
3	Examples of Practical Applications	195
3.1	A Battery of Tests.....	197
4	Future Prospects.....	198
4.1	Implementation.....	199
4.2	Identification of Key Biological Mechanisms Associated with Specific Contaminants	199
5	Summary	200
	References.....	200

1 Introduction

The ever-increasing volume of regulations for water protection and drinking water standards in industrialized countries has led to a shift in focus from microbial problems to various kinds of chemical contamination of aquatic systems. Such chemical contamination is generalized under terms like “adverse or novel effects”, and includes effects such as genotoxicity, immunotoxicity, neurotoxicity and reproductive toxicity.

Environmental contaminants that are suspected of being genotoxic take on special precedence on the basis of their biological consequences in a so-called reactionary chain of “exposure and effect”. Genotoxic compounds (synthetic or naturally occurring) are substances that are capable of altering DNA and bringing about genetic changes, which usually result in adverse biological effects. Genotoxic substances can cause hereditary genetic mutations, cancer and embryonic malformations in humans.

Investigations on the influence of genotoxic threats to the population in an ecosystem have identified the following relevant effects (just to name a few): Decrease in vitality and a reduction of genetic diversity, which is associated with a reduced fitness of the population as a whole (Fig. 1).

The identified risks and consequential reaction in environmental and healthcare policy make the handling of the topic “genotoxicity” a priority research area, and demonstrate an immediate need for regulatory measures with the aim of minimizing exposure to genotoxic compounds or, when technically possible, avoiding exposure altogether.

The first significant step in this daunting task is the identification of genotoxic impact and the recognition of substance-specific mechanisms. Investigations of complex exposures in a low-dose-range, which are designed to reflect real conditions of exposure in the environment, entail quite a few methodological difficulties.

Nevertheless, several aquatic genotoxicity testing systems are available today that sufficiently provide systematic, needs-based genotoxicity testing, and under appropriate conditions allow for a reliable detection of potentially genotoxic substances.

Key questions of aquatic genotoxicity testing:

1. What substances are in the water, and in what concentrations do they occur?
2. What mechanisms of action do such substances have?

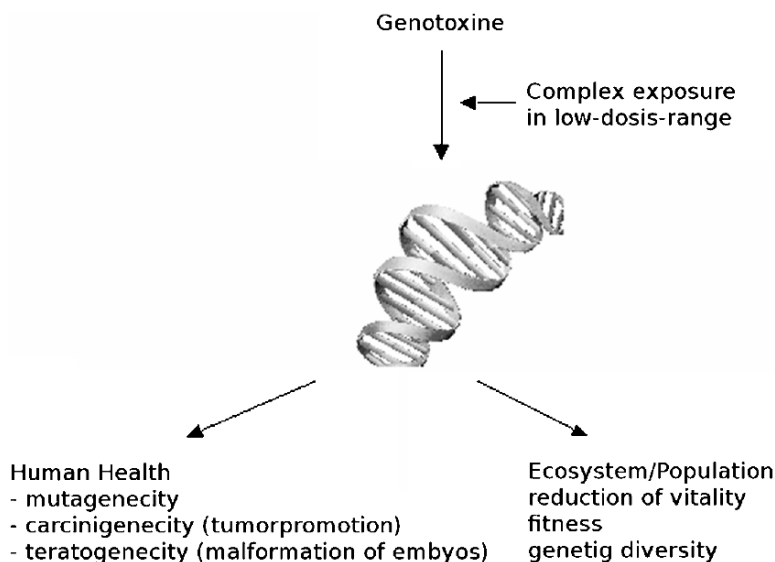


Fig. 1 Genotoxic effects and biological consequences: human health and environmental integrity

3. In order to keep health risks as low as possible, what are acceptable levels of potential exposure (threshold values)?

2 Methods of Aquatic Genotoxicity Testing

In genotoxicity testing a distinction is made between indicator and mutation testing (or assays). An indicator test demonstrates damage to the molecular structure of the DNA. The indicated DNA damage can result in a genetic mutation. A mutation assay, on the other hand, indicates already existing mutations (e.g. gene or chromosome mutations), which are presumably caused by exposure to genotoxic agents.

In the following we shall take a detailed look at such genotoxicity testing systems as they have been applied to “water” as a testing matrix, and in some cases been developed to address relevant issues effecting aquatic organisms, out of which primary cells or cell lines have been derived for research purposes (Table 1).

2.1 Bacterial Assays

Bacterial assays belong to the basic set of tools for genotoxicity testing of chemicals and environmental samples. There are several established methods that are easy to

Table 1 Genotoxicity and test organism

Test system	Test organism
Alkaline filter elution	Mussels (<i>Corbicula fluminea</i>) Algae (<i>Chlamydomonas reinhardtii</i>) [1]
DNA-unwinding assay	Fish cell line (RTG-2, PLHC-1) Fish larvae (<i>Danio rerio</i> , <i>Oncorhynchus mykiss</i>) Crustaceans (<i>Astacus astacus</i> , <i>Corophium volutator</i> , <i>Gammarus pulex</i>)
Comet assay	Mussels (<i>Dreissena polymorpha</i>) [2] Fish cell line (RTG-2, PLHC-1) Primary hepatocytes (<i>Danio rerio</i> , <i>Oncorhynchus mykiss</i>) Protozoa (<i>Acanthamoeba castellanii</i>) Algae (<i>Chlamydomonas reinhardtii</i>) Mussels (<i>Corbicula fluminea</i>) Fish (<i>Danio rerio</i>) [3, 4]
UDS assay	Primary hepatocytes (<i>Oncorhynchus mykiss</i>) [5, 6]
In vitro micronuclei assay	Fish cell line (RTG-2, PLHC-1) [7]

administer and usually generate reproducible results. Of the higher eukaryotic cells, bacterial cells are distinguished by their genome organization and metabolic mechanisms. In higher organisms such as mammals, a sophisticated and highly variable xenobiotic metabolism relies on a system of enzymes to remove unwanted contaminants from the body. Through the addition of this enzyme system (such as the S9 extract from a rat or mouse liver) to the bacterial assay, the activation potential of indirect mutagens in the mammalian organism may be partially reconstructed in vitro. However, lessons learned from the bacterial assay may not always be directly transferred to eukaryotic systems, and require additional analysis.

Examples of bacterial indicator tests for screening mutagenicity are the umu-test, the SOS-Chromotest and the LUX-FLUORO-Test. These tests use the bacteria *Salmonella typhimurium* and *Escherichia coli* as test organisms. The tests are based on the measurement of a genotoxically determined induction of a group of regulated genes, or so-called SOS regulons. The SOS functions are complex biochemical processes, which counteract the degradation of bacterial genes and allow the survival of the cell. These include a somewhat fallible enzymatic DNA repair system, the formation of an excision repair complex and an inhibitor of cell division. The turning-on of an SOS regulon is in effect the cell's physiological response to a DNA-damaging impact. In all three tests the induction of an SOS-regulated promoter is measured with the help of a hidden (genetically engineered) reporter gene. The Umu and SOS Chromotests are mostly concerned with isolating the lacZ gene, which codes for the β -Galactosidase enzyme found in *E. coli*, and is easily identified by a colour reaction. In the LUX-FLUORO-test, the magnitude and kinetics of the SOS induction are determined by measuring the induced bioluminescence. In this case, the lux-Operon is the reporter that codes for the enzyme Luciferase and its substrate (Fig. 2).

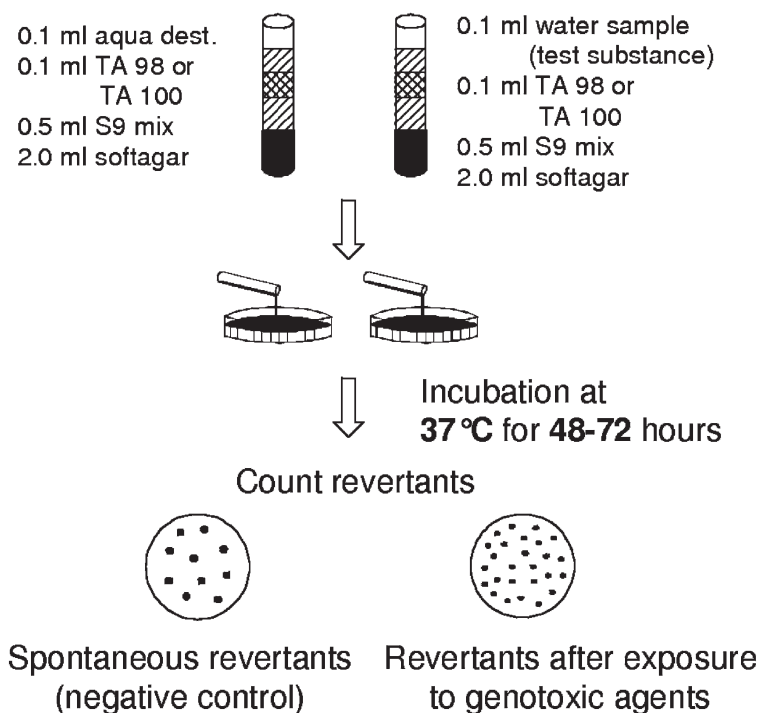


Fig. 3 Flow chart of the *Salmonella*/microsome test (AMES test)

research purposes as well as official governmental monitoring [9]. Through its mention in the Waste Water Ordinance (AbwV, 2002), the umu-test has also found its way into legal regulatory frameworks.

A true mutation test is the *Salmonella typhimurium* reverse mutation assay (Ames test, Fig. 3). Because of mutagenetic changes, the salmonella strains used in this test require the amino acid histidine for growth (his⁻). In addition, these strains contain mutations that increase the bacteria's sensitivity to some genotoxic agents. The rfa-mutation increases the cell membranes' permeability against larger, particularly hydrophobic molecules. The uvrB-mutation causes the failure of an enzyme system that resects certain DNA damages. In the presence of genotoxic agents, reverse mutations to the wild-type (his⁺-phenotype) frequently occur. These reverse mutations grow on agar plates containing mere traces of histidine, and can grow up to colonies, which can then be counted. The number of reverse mutations serves as a measure of genotoxicity. Different salmonella strains were used to identify different types of mutagens or mutation types. In the standardized version of the *Salmonella typhimurium* reverse mutation test, strain TA 98 is used to detect frameshift mutations, while strain TA 100 is used to detect base substitution mutations. In addition to the umu-test, the Ames test has also been standardized according to DIN and ISO norms for aquatic screening [10].

2.2 *Eukaryotic Assays*

There are a number of available methods to practically identify genotoxicity in eukaryotic cells and aquatic organisms at various endpoints, such as DNA strand breaks, DNA repair and micronuclei. Methods for identifying DNA strand breaks include the Comet assay, the base filter elution test and the DNA alkaline unwinding assay. In these tests, single and double-strand breaks as well as alkaline or abasic points converted to strand breaks are summarily detected. Moreover, all three methods allow such endpoints to be identified in different cell types and tissues, regardless of the cell cycle. Strand breaks reduce the length of chromosomal DNA. Numerous genotoxic substances can cause DNA strands to break indirectly. The strand breaks occur (intermittently) with more frequency if the damaged DNA sites are repaired by base and nucleotide excision. The DNA damage is first visible when DNA repair is initiated in the test.

The Comet assay (or Single Cell Gel Electrophoresis assay) uses the different migration behaviours of intact and damaged DNA in an electric field [11]. In the Comet assay, single cells (either from permanent cell cultures, or isolated from the organism itself) are embedded in agarose on laboratory slides and lysed under alkaline conditions or in high salt concentrations. DNA is thereby released and then electrophoretically unravelled in a strong alkaline environment and tinted with specialized fluorescent dyes. The damaged DNA is then stretched into comet-shaped strands in the electric field, revealing a characteristic comet tail, after which the test is named (Fig. 4). The length of the comet tail and its genetic contents are dependent on the extent of the existing strand breaks or the frequency of alkali-labile sites in the DNA. In contrast, intact DNA does not migrate; rather it remains in the form of a circular core area. The extent of DNA damage is thus mostly determined by the DNA migration in the electric field, or, by the length of the comet's tail. In addition to tail length, fluorescence intensity, and therefore the amount of DNA in the head and tail areas, may be measured with the help of appropriate image evaluation technology.

The base filter elution test also confirms evidence of such DNA lesions. In this test reduced lengths of chromosomal DNA due to strand breaks can be detected [12]. In a strongly alkaline elution solution, damaged DNA fragments pass more rapidly through the filter (with defined pore size) than the longer, intact DNA strands. The eluate containing DNA is thus captured in fractions. While many short pieces of severely damaged DNA fall out in the first eluted fractions, only a few pieces are initially eluted in the case of marginally damaged DNA. The elution rate of the DNA is calculated according to a defined time. In addition, one can fluorometrically determine the DNA content in the eluate fractions as well as the amount of DNA remaining on the filters. Under the given experimental conditions, the alkaline elution of DNA almost appears as a first-order of kinetics. The decline of DNA on the filter, per unit of time, is proportional to the frequency of single-strand breaks. Damaged and undamaged DNA may therefore be distinguished by the different (negative) slopes of their elution curves. This procedure has been greatly simplified in recent years, so that measurements are also possible with microplates.

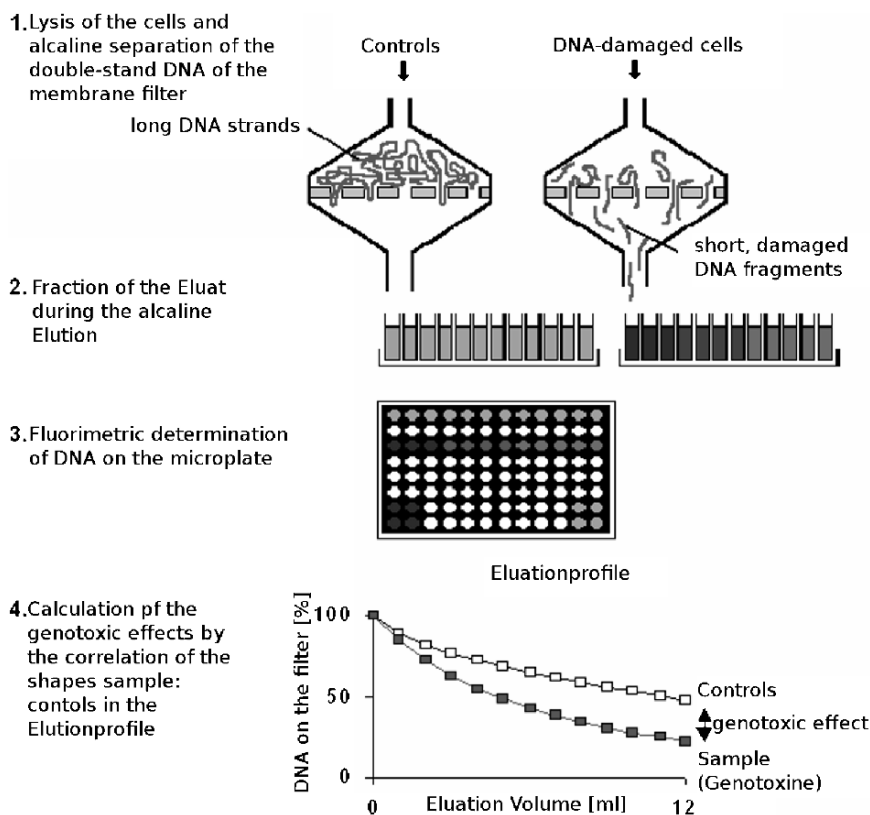


Fig. 4 Flow chart of the Alkaline Filter Elution (AFE test)

The DNA alkaline unwinding assay exploits the fact that DNA strand breaks reveal additional unwinding sites in an alkaline environment, while the number of unwinding sites of intact DNA mainly reflects the number of DNA strand ends and physiologically determined topoisomerase interfaces along the DNA molecule. During an alkaline lysis, DNA with strand breaks tends to unravel faster than undamaged DNA because of the additional unwinding sites present. The default unwinding rate is measured over time, usually 30 min. After single and double-stranded DNA are separated from each other with the hydroxylapatite ion chromatography method and quantitatively calculated in the fluorometer, the relative fraction of double-stranded DNA (*F*-value) remaining after this given time period may then be determined [2].

The measurement of DNA repair synthesis (UDS) is a widely accepted indicator test for genotoxicity, and represents an important part of testing strategies for chemicals and pharmaceuticals [13]. The UDS test was developed with many different variations, using primary cells and cell lines such as lymphocytes, epithelial cells and liver cells. It covers a wide spectrum of applications, for example, using

liver cells from rodents. Cells from aquatic organisms such as trout or zebra fish have also been used in water and wetland protection screening research.

Generally a cell is capable of detecting lesions in the DNA early on, excising the damaged sections and replacing these through neosynthesis. This process is called unscheduled DNA synthesis (UDS). The principle of UDS testing is based on the fact that physico-chemical contaminants can induce or activate DNA-Excision Repair. In so doing, the introduced cells, used together with the test substance or test mixture, will be provided with radioactive thymidine. This is formed during the repair synthesis in the newly synthesized section of DNA. The amount of built up radioactivity is for the most part autoradiographically determined. It is a measure to confirm that repairs have taken place. After the photochemical developing and staining of specimens, a semi- or fully automatic microscopic analysis of the cells follows. The number of silver grains on the nucleus region is calculated, as well as about one to three large plasma areas of similar size for comparison. The net silver rate is the average difference between the silver grain frequency (NNG) and the nuclei and the cytoplasm area. The greater the net silver rate of the sample, the more likely the sample should be evaluated as genotoxic.

The *in vitro* micronucleus test represents a relatively simple procedure for detecting chromosome damage [14, 15]. Micronuclei are structures containing chromatin in the cytoplasm. While formation *in vivo* and *in vitro* frequently occurs under exposure to physical or chemical contaminants, spontaneous formation occurs rather infrequently. Micronuclei arise during mitosis, when a chromosome or chromosome fragment fails to be incorporated into one of the daughter nuclei during cell division. When cells with chromosomal damage form in the anaphase of cell division, fragments lacking centromeres cannot reach the terminal ends of the cell and remain in the cell, outside the teleophase nucleus. Another cause of micronuclei formation is an abnormal function of the spindle apparatus, or centromere region of the chromosome, in which whole chromosomes in the spindle apparatus can no longer migrate to the prospective region of the nucleus. The chromosomes, or fragments thereof, are not incorporated in the daughter cell's nuclei during cell division and go on to develop their own nuclear membrane, resulting in what appears to be a tiny autonomous nucleus. Since it is presumed that the formation of micronuclei occurs during mitoses, a corresponding testing method for divisible eukaryotic cells is made available. On account of the possibility of automated image analysis, micronucleus testing, with its use of cell lines or cultured cells taken from (exposed) organisms, has become increasingly important in ecotoxicological screening for potential genotoxic substances.

3 Examples of Practical Applications

Modern chemical analysis has allowed environmental contaminants to be identified in the nano-, pico- and even femtogramme ranges. To what extent analytically detectable contaminants are also toxicological threats, must be confirmed by data

from biological testing systems. The evaluation of potential genotoxic risks in complex environmental media and their impact on humans and the environment cannot be limited to the consideration of individual substances. In addition to considerations about reasonable operation ease and expense, important criteria for the selection of relevant testing systems include the specificity and sensitivity to pollutant mixtures. In the following example, by using data from analytical measurements and biological tests (*Salmonella*/microsomal test), an attempt was made to establish justified principles for the application-specific assessment (concerning necessary remediation) of the potential risks of military waste deposits (typical explosive compounds, STV), as they occur as complex mixtures in water. STV causes the formation of methaemoglobin, reproductive disorders, mutations and cancer. Genotoxic effects of this substance class are represented by the nitro-functional group, which demonstrates an active structural principle of genotoxicity. Initial analytical measurements at the site of a former military arms manufacturer pointed to a diffuse and widespread contamination of adjacent surface waters, including nitro-aromatic STV from military waste deposits. In a 1-year measurement program, genotoxicity could be decidedly detected in monthly water screening tests (with reproducible results) only in samples with certain nitro-aromatic concentrations. Through this specific case study, a concentration-dependent correlation between complex chemical exposure and biological effects could be made. The combination of chemical analysis and biological testing led to a usage-based prioritization of pending remediation measures and, ultimately, to ecologically and economically acceptable proposals for an acute environmental problem.

The DNA unwinding test serves as a further example of an application-based genotoxicity testing procedure in the environment. In particular, its current range of applications includes monitoring of coastal and inland waters as an adjunct research interest of chemical residue testing, as well as the evaluation of damaged fish larvae as a consequence of UV-B exposure. The effects of biotoxins (such as the neurotoxins domoic acid and saxitoxin) can likewise be detected at the genetic level, both in the case of exposed individual organisms as well as populations and ecosystems. In addition to chemical residue testing data, monitoring studies on the Elbe River have delivered important data on genotoxic potential, as gathered in DNA unwinding tests on exposed zebra mussels (*Dreissena polymorpha*), which have led to important specifications for remediation of certain parts of the river. With the help of DNA unwinding tests on zebra mussels, a colour-coded genotoxicity table for water quality classification could be developed. This work will be included in the Federal Water Quality Atlas, conventional water quality maps and corresponding follow-up documentation for the European Water Framework Directive (WFD).

As an important part of sediment monitoring and the evaluation of impacts to estuaries and inland waters, DNA unwinding tests on long-term exposed mussels have helped provide important information about the environmentally safe deposition or disposal of contaminated sediments.

In situ exposure studies of UV-B radiation (from sunlight) on pelagic fish eggs have proven to be especially useful in the verification of genotoxic impacts on the

ecosystem. DNA unwinding tests on fish eggs allow for quantitative registration of Thymindimere and DNA damage, in addition to deformity rates at the genetic level.

Similar to the DNA unwinding test and Comet assay, the base filter elution test can also be carried out on different test organisms from aquatic habitats. A few years ago, on commission from the State Rheinland-Pfalz, Ministry of the Environment, the base filter elution test was carried out to detect DNA strand breaks in golden orfe (*Leuciscus idus melanotus*) in connection with the fish-monitoring test (golden orfe test). After 14 days of exposure in the Rhine, golden orfe from the Rhine water station in Mainz were tested for genotoxic potential using the base filter elution test. The target tissue was taken from the liver of the fish. The golden orfe, a vertebrate, was later replaced by the freshwater clam, *Corbicula fluminea*, for ethical reasons. In this case the target tissue was taken from the gills. In order to compare test results gleaned from different test organisms, results were referenced to the genotoxic substance Benzo[a]pyrene (B[a]P), and specified in GSP (genotoxic impact potential, 1 GSP Ψ 0.2 μg B[a]P part per Litre).

Average genotoxicity levels in the Rhine near Mainz decreased almost continuously from 1987 to 1995. Over the course of the study, genotoxic potentials were measured with increasing infrequency, while the overall amount of the measured effects declined as well. Over the 8-year research period, the lowest genotoxic impact levels were recorded in 1990. During the same period, the percentage of adsorbable organic halides (Adsorbierbare organisch gebundene Halogene, AOX) also steadily declined. These parallel developments suggest a link between the two parameters, in that the reduction of AOX is consequentially accompanied by a reduction in genotoxic potential.

3.1 A Battery of Tests

Discussions in the field of chemical and pharmaceutical screening over the past 10–20 years have shown that there is no single ideal testing system for the detection of genotoxicity. However, a definitive testing strategy consisting of three consecutive steps has been generally accepted. The test assumes that two to three in vitro methods with several different biological endpoints are sufficient for achieving a satisfactory result in the first phase of testing contaminants for possible genotoxic effects. Environmental monitoring also seems to follow the uncontested dictum that a hierarchy of testing stages is necessary for the detection of genotoxicity. The aim of the first stage (the basic test) must be the positive detection of genotoxic potential in the water sample by using two to three short-term procedures. For qualitative screening, a crucial selection criterion in the choice of testing systems is a heightened sensitivity of the system. Special consideration is given to the fact that substance mixtures with unknown genotoxic potential will be tested.

The objective of the basic test is achieving qualitative conclusions on the genotoxicity of a sample. For this reason, in vitro methods are usually used in the first stage of genotoxicity testing. Literature indicates a high level of reliability in the

Table 2 Exposure scenarios, action plans and target in the aquatic genotoxicity assessment

Scenario	Action plans/Target values	Measures
Impact monitoring, waterways and lakes	Documentation of the status and trends of the waterways and lakes	Planning concepts and long-term decision support for water authorities and municipalities
Acute disasters	Real-time risk assessment, Risk communication	Risk management monitoring indicators and remediation strategies
Emissions and genotoxic effects in the recipient waters	Assessment and evaluation of genotoxic effects (dose-response relationship and characterisation of the effects and mechanism, Impact identification	Eco-toxicological and toxicological evaluation of the impact (risk description, risk estimation, risk characterisation)

informational value of in vitro testing [16]. The methods described above are carried out relatively quickly and can easily differentiate between genotoxic (yes) and non-genotoxic (no) results.

While standardized test protocols are already available for the microbial test systems (Umu and Ames tests), the current methodological validation and standardization of eukaryotic testing are in drastic need of improvement. Ultimately, these test versions are also to be defined and integrated in the battery of tests.

As long as such essential prerequisites (such as the procurement of quantitative evidence and the detection of specific mechanisms or impacts) have not been met, the decisive yes/no (genotoxic/non-genotoxic) result of the basic testing phase cannot be regarded as a definitive, scientifically validated risk assessment.

The basic test, with its precautionary character and qualitative predictive value, makes an important contribution towards protecting the environment from potentially genotoxic substances, and respectively minimizing their effects with reasonably cost-effective measures.

The basic test phase prescribes no specified testing strategy. The heterogeneity of environmental problems certainly does not allow this. Rather, the testing strategies are to be methodically adapted to the specific environmental question at hand, in accordance with existing exposure scenarios and related scientific knowledge (Table 2).

While the basic test may be considered adequate enough for emissions testing, it is clearly insufficient for timely risk assessments, the scientific presentation of evidence in the case of environmental damage, and in the evaluation of continuous emissions of substances with suspected genotoxic effects. It will be necessary to supplement the basic test with other testing systems (e.g. cytotoxicity or apoptosis assays) that can both quantitatively and qualitatively determine the respective impact potential of the substances in question.

In any case, the basic test will likely remain the cornerstone for initial assessments of potential genotoxic risks. The nature and extent of the necessary tests for risk assessment indicate the structure of the battery of tests thus far, and provide incentive for further testing strategies.

4 Future Prospects

At the current stage of development, two tasks must be considered with highest priority for the future:

- Implementation of existing methodological instruments into current environmental monitoring programs;
- An efficient integration of basic research (e.g. identification of biological target structures) and application-based developments in chemical analysis to identify key biological mechanisms associated with specific contaminants.

4.1 Implementation

The current practice in water monitoring shows that aquatic genotoxicity testing is still in a developmental stage that largely precludes a routine broad-based deployment. With knowledge of the uncertainties in the use of the battery of tests, methodological development and the collection of data should be otherwise undertaken.

The medium-term goal of current scientific work should therefore be to update and develop the available testing methods in order to process as much of the spectrum of specific issues in the aquatic field as possible and to be able to incorporate these into regulatory requirements. Despite existing objections to the integration of additional parameters into existing screening programmes, the current research concept aims at keeping testing programmes financially feasible, enabling innovative new developments to come about and thus creating opportunities for sustainable water protection in the context of potential health problems of the future.

4.2 Identification of Key Biological Mechanisms Associated with Specific Contaminants

Environmental toxicology greatly benefits from rapid advancements in molecular biology. The use of molecular biological methods will trigger a sea change in environmental risk analysis and assessment, at which point it is worth mentioning: contaminant-associated effects must be understood at the molecular level, if they are to be minimized or prevented.

A significant goal of current research activities is thus to adapt the instrumental equipment of analytical nuclear technologies to the latest achievements in biological and medical research (*the molecular approach*) as soon and as best as possible. The integral analytical procedures common to the fields of nucleic acid-, protein- and analytic function research undoubtedly possess enormous potential for development and application in the field of environmental toxicology.

The fascination with these technologies may not, however, obstruct the question of where the use of these new methodological approaches in environmental fields may lead.

For one thing, the accurate measurement of exposure proves to be a crucial criterion for the successful application of the procedures. In any case, it is a matter of one exposure in the context of complex mixtures, which can lead to the induction of complex toxicological effect profiles.

On the other hand, the allocation of macromolecules places enormous demands on the analytical method - from the collection of the sample (yield), to the sample preparation (stability of the effect complex), to the actual analytical procedure (sensitivity and specificity).

It is also to be made clear, that the substantiated interactions between biological target structures and environmental contaminants usually provide no proof of the biological relevance of such a finding. In most cases, causal chains of effects must be additionally determined in respective biotests.

5 Summary

The occurrence of genotoxic substances in aquatic systems is a serious problem because of the risk to both human and ecosystem health. The far-reaching consequences of possible genotoxic impacts (in situ) for environmental policy, trying to discourage exposure to genotoxic substances, require a refined experimental concept.

The bioassays, umu-test, Ames-test, Comet assay (in protozoans, green algae, mussels, primary fish hepatocytes, fish and mammalian cell lines), the DNA unwinding test (in fish larvae, crayfish, mussels and fish cell lines), the alkaline filter elution (in mussels and green algae), the UDS-test (in primary fish hepatocytes) and the micronucleus test (in fish and mammalian cell lines and primary hepatocytes) are sensitive enough to detect genotoxic effects in complex mixtures in a low dose range.

These tests seem to be suitable for routine testing and should therefore be used in the first stage of a graduated battery of tests for the detection of genotoxicity in aquatic systems in combination with effect-oriented chemical analysis.

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Genotoxicity in the Environment (Eco-Genotoxicity)

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Abstract Genotoxicity is a priority measurement for assessment and communication of genotoxic effects (DNA strand breaks) in the water matrix and the trophic levels of the ecosystem (Eco-Genotoxicity). Genotoxicity induction with single substances and surface water samples shows clearly the good sensitivity and reproducibility of the DNA-unwinding assay. The DNA alkaline unwinding assay detects levels of DNA fragmentation; the relative numbers of DNA strand breaks are described as the “ $-\log F$ -value.” The results of DNA fragmentation in genotoxin-exposed cells of a RTG-2 cell line, the mussel *Dreissena polymorpha*, and fish larvae of the fish species *Danio rerio* are discussed. The early life stages of the fish species *D. rerio* show the significant high sensitivity and reproducibility of the DNA alkaline unwinding assay. As a reference method for the validation of genotoxicity pulsed-field gel electrophoresis (PFGE) was investigated. The LOEC (Lowest Observed Effect Concentration) of the genotoxin 4-nitroquinoline-*N*-Oxid (4-NQO) was $78 \mu\text{g l}^{-1}$ for both test organisms, cell lines and fish larvae. Native surface water samples from representative stations on the River Rhine and the River Elbe significantly induced genotoxicity in the RTG-2 fish cell line and in fish larvae from *D. rerio*. An increased sensitivity was measured

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in *D. rerio* fish larvae 48 h after hatching during 3 h-exposure. The LOEC of the mussel *D. polymorpha* exposed in river water was less sensitive in the DNA alkaline unwinding assay: LOEC = 250 $\mu\text{g l}^{-1}$ 4-NQO. In coastal waters the marine mussel *Mytilus edulis* is a good indicator organism for the detection of genotoxic effects and biotoxins. Beside immunotoxicity also genotoxic effects (DNA strand breaks) are found especially in UV-B (ultraviolet-B) exposed mussels (Luckas, B., J. Dahlmann, K. Erler, G. Gerdt, N. Wasmund, C. Hummert, P.-D. Hansen (2005) An Overview on Key Phytoplankton Toxins and their recent occurrence in North Sea and Baltic. Environmental Toxicology 20(1):1–17). To validate the influence of UV-B on exposed mussels (hepatopancreas and mussel hemocytes) UV-B exposure was investigated (Figs. 1 and 2) to induce and demonstrate eco-genotoxic effects in mussels and fish embryos. The fish eggs of the marine fish species *Limanda limanda* were experimentally exposed to UV-B radiation in a solar radiation simulator onboard the research vessel RV Walther Herwig III. The experimental design attempted to simulate present and future conditions in the context of eco-genotoxicity and increased UV-B exposure due to northern hemisphere ozone loss. The impact of low dosages of UV-B indicates an important genotoxic and ecological threat concerning ecosystem integrity status at the population level. In our experiments embryos of *L. limanda* taken from sites located along a gradient of chemical stress in the German Bight were exposed to solar ultraviolet-B light and allowed to recover for a defined period. The involved research groups from different disciplines and research organizations showed a significant time and dosage-dependent influence of UV-B radiation on mortality and sublethal eco-genotoxic effects (DNA damage in the environment). Our hypothesis was that there might be an interaction between exposure to chemical contaminants and the ability of embryos to recover from UV-induced genotoxic effects by repairment.

Keywords Alkaline unwinding assay, Biotoxins, *Danio rerio*, DNA, *Dreissena polymorpha*, Early Life Stages, Genotoxicity, *Limanda limanda*, RTG-2, Thymine dimers, UV-B radiation

Contents

1	Introduction.....	205
2	Genotoxicity and Organisms for Assessment	206
2.1	Chemicals and Environmental Samples	206
2.2	Fish Cell Line RTG2.....	207
2.3	Mussels: <i>Dreissena polymorpha</i>	207
2.4	Fish Larvae (Yolk Sac), <i>Danio rerio</i>	208
3	DNA-Unwinding Assay for Monitoring	208
3.1	Statistical Analyses	211
3.2	Genotoxicity and Repairment: Influence of Solar Ultraviolet-B on Pelagic Fish Embryos: DNA Fragmentation and DNA Thymine Dimers.....	213
3.3	Fish Embryos: <i>Limanda limanda</i> and UV-Exposure	214
3.4	Embryonal Development and UV-Exposure	218
3.5	Mussels: <i>Mytilus edulis</i>	220
4	Concluding Discussion and Future Trends for Real-Time Assessment.....	221
	References.....	224

1 Introduction

The estimation of environmental pollution in respect of genotoxicity effects on aquatic organisms is relevant for monitoring studies and risk assessment and risk management. More than 200 genotoxicity assays are available [1]. The main emphasis of genotoxicity assays is the detection of mutations as well as the induction of carcinogenic processes in order to estimate human risk [2, 3]. An environmental task of importance is the determination of ecotoxicological (eco-genotoxic) end-points [5–11]. With regard to waste water and effluent assessment, bacterial test systems, i.e., the Ames-assay [4, 5, 12], the SOS-Chromotest [13, 14], and the umuC assay [9, 15], are the most applied assays for the detection of genotoxins.

Monitoring studies used in genotoxicity assessment of environmental matrices like surface water, sediments, and the organismic level [11, 16] require the development and implementation of standardized and internationally harmonized eukaryotic bioassays. Prokaryotic genotoxicity assays offer an excellent chance for real-time biosensor development (see Fig. 6). This will enhance the relevance at the population and ecosystem level. In eukaryotic cells, DNA strand breaks lead to the formation of DNA fragments that behave differently in electrophoresis than the entire chromosome. Differences in DNA migrations can be detected with the comet assay in single eukaryotic cells [7, 18, 19]; however, the comet assay is only an indicator assay. An alternative method for the assessment of primary DNA damage in tissue from exposed aquatic test organisms, which are of higher organized evolutionary order, is the DNA alkaline unwinding assay. The DNA of an exposed eukaryotic organism shows a higher quantity of DNA-unwinding points in comparison to untreated DNA. As a consequence, the DNA-unwinding process under alkaline conditions ($\text{pH} > 12$) is enhanced. A successive hydroxyapatite chromatography technique is used to separate single-strand (ss-DNA) and double-strand (ds-DNA) DNA fractions. The basics of this procedure were obtained from [20, 21]. This method has been commonly used for monitoring DNA fragmentation potential using various biological systems in ecotoxicological and toxicological research fields [19, 22].

For the assessment of eco-genotoxicity after environmental pollution, impacts of genotoxins as well as biotoxins, the DNA alkaline unwinding assay is a relevant tool for field and laboratory work. [7]. Fish species notably expressed high sensitivity for the detection of genotoxic damage [23], and enhanced effects can be detected when larval stages are investigated [24, 25].

Various studies were carried out with the cyprinidae *Danio rerio* Hamilton-Buchanan as a model organism. Effects of chemical-induced embryo–larval toxicity are measured by incubating newly fertilized eggs from *D. rerio* to test environmental samples [26]. Analogue applications have been developed for testing teratogenicity caused by sewage water. The test species *D. rerio* was used for monitoring genotoxicity and related effects, including tumor formation in adult stages of *D. rerio*, in comparison with mutagenicity effects [27]. The influence of Aflatoxin B1 metabolism on the formation of DNA adducts was studied in *D. rerio* [28] as well as TCDD modulation effects in this context [29]. The endpoints of the exposed marine fish embryos (*Limanda limanda*) were mortality, malformations, changes in buoyancy

of embryos measured as changes in osmolarity of the perivitelline fluid [24], permeability and genotoxicity and repairment (DNA unwinding and Thymine dimers).

2 Genotoxicity and Organisms for Assessment

The DNA alkaline unwinding assay has been applied to the research field of toxicology testing of chemical products in cell cultures such as mouse lymphoma [30], rat and mouse liver tissue [31], human lymphocytes [22], and diploid fibroblasts [32]. In addition, there are many studies in the literature related to the use of the DNA alkaline unwinding assay for testing rainbow trout gonadal cell (RTG-2) permanent cultures. The first investigations were described by [33].

Filter-feeding organisms, such as the fresh water mussel *Dreissena polymorpha* [34] and the marine mussel *Mytilus edulis* [18, 35], are suitable for monitoring the biological impact of xenobiota and genotoxins. *D. polymorpha* [34, 36] is sessile, widely distributed in Europe and North America and expresses a relative resistance against environmental contaminants. The Zebra mussel is a commonly used test organism for passive and active biomonitoring for the detection and quantification of different environmental contaminants like heavy metals, chlorinated hydrocarbons, polycyclic aromatic compounds, and radionuclides as well as to assess their bioavailability in the ecosystem branches [34, 37]. This mussel was used in monitoring studies from the ARGE ELBE in the River Elbe and for analogue studies in the River Rhine [36]. The technique of the DNA alkaline unwinding assay is well adapted to exposure studies to describe environmental scenarios using the following test organisms and suborganismic cells of different trophic levels:

- Rainbow trout gonadal cell (RTG-2) permanent cultures
- The mussel *D. polymorpha* (soft tissue)
- Early life stages of *D. rerio*

In an extended study these three organism levels were investigated to measure the genotoxic potential of the relevant reference substances 4-NQO, 2-acetaminofluorene (2-AAF), benzo(a)pyrene (B(a)P), *N*-nitrosodimethylamine (NDMA), and nitrofurantoin (NF). The laboratory and field trial also investigated different representative surface water samples of the River Rhine and the River Elbe. For the measurement of different fractions of single-strand DNA and double-strand DNA from RTG-2 and *D. polymorpha* tissue homogenates, the method was modified with Bisbenzimidazole fluorescence dye, whereas the recorded low levels of DNA concentrations measured in *D. rerio* yolk sac larvae requires fluorometric measurement with the new Picogreen® fluorescence dye.

2.1 Chemicals and Environmental Samples

The surface water samples tested are from the German rivers Rhine and Elbe. The test program was continued over a period of 1 year (from February to June).

The samples were centrally collected and distributed. Transported at 4°C the samples reached the participants of this trial within 24 h. The reservoir “Wahnbachtalsperre” was studied as a reference station (negative control) and the River Wupper (Gas Works = positive control) for the water samples. The Lake Gatow was a reference station for the mussels *D. polymorpha*.

2.2 Fish Cell Line RTG2

RTG-2 is a permanent cell line derived from embryonic gonad tissue from rainbow trout (*Oncorhynchus mykiss*). This cell line was obtained from the “German Collection for Cell Cultures; DSMZ Braunschweig.” The cultivation of the RTG-2 cell line has to be carried out in flasks at 20°C. Monolayer cultures with a confluence grade of 90% were collected after enzymatic dissociation. Therefore, the cells were washed with PBS (200 mg l⁻¹ KH₂PO₄, 8,000 mg l⁻¹ NaCl, 1,150 mg l⁻¹ Na₂HPO₄; pH 7.2 ± 0.2), overlaid with trypsin solution (0.05% trypsin in PBS/0.02% EDTA) and incubated for 10 min. RTG-2 culture medium (MEM with Earle’s salts, without sodiumbicarbonate, without L-glutamine) was enriched with 4,766 mg l⁻¹ HEPES, 294 mg l⁻¹, 850 mg l⁻¹ NaHCO₃, 50 mg l⁻¹ neomycin sulfate, and the pH was adjusted to 7.2 ± 0.2. After sterile filtration with 0.2-µm pore-filters, 10% FKS was added and the osmolarity was adjusted to 300 ± 20 m Osmol. The FKS was added to inhibit the trypsin activity. For the exposition experiments, a cell concentration of 2 × 10⁵ cells ml⁻¹ was used. Three parallels per test; two independent tests were carried out for each single substance dose, surface water and dilutions, respectively. Cell suspension (10 ml) was placed in Petri dishes and incubated overnight at 20°C. The culture medium was removed and the single substance solved in water or water samples mixed with 2× concentrated medium were placed onto the cells. Substances that were not well soluble in water were solved in DMSO. The maximum DMSO concentration on the cells was below 0.1%. For the single substance exposition for 1 h at 20°C was applied. During the water sample exposition experiments, the exposition time was increased to 2 h. Cells were incubated with 325 µg l⁻¹ 4-NQO as the positive control. After the exposition, the test solutions were removed and the cells overlaid with 4-ml ice-cooled Dulbecco’s PBS (DPBS; 132 mg l⁻¹ CaCl₂·2H₂O, 200 mg l⁻¹ KCl, 200 mg l⁻¹ KH₂PO₄, 100 mg l⁻¹ MgCl₂·6H₂O, 8,000 mg l⁻¹ NaCl, 1,150 mg l⁻¹ Na₂HPO₄). The single layer cells were scratched from the Petri dishes using a wiper and the cell solution placed in a tube (on ice). Afterwards the cells were centrifuged for 5 min at 200× g at 4°C, the supernatant was removed and the cells were resuspended carefully into 200 µl DPBS.

2.3 Mussels: *Dreissena polymorpha*

The reference station for mussel species *D. polymorpha* is Lake Gatow in Lower Saxonia, Germany. The age of the organisms was 3–4 years. After transport the

mussels were maintained at 5°C in a refrigerator under aeration conditions and fed with green algae, if the maintenance was longer than 4 weeks. In a preadaptation period before the exposure experiment starts, the mussels were maintained for 5 days in ISO water (ISO 7346/3) at 22°C with aeration. For each exposition experiment, 9–12 mussels were exposed for 72 h at 22°C in 1 l exposition medium or surface water samples. As positive control, the mussels were exposed in 500 µg l⁻¹ 4-NQO (in 0.1% DMSO in ISO water). After the exposition, the mussels were anaesthetized for 10 min on ice and the whole tissue was pooled from three mussels. From each exposition pool, three parallels were tested. Two independent experiments were carried out for each single substance dose and surface water. Gills were analyzed after in vitro-exposition for 1 h pooling three gill pairs per test in 200 µl homogenization buffer without a homogenization step.

2.4 Fish Larvae (Yolk Sac), *Danio rerio*

The adult zebrafish, *D. rerio* were obtained from the Umweltbundesamt (Federal Environment Agency) Berlin. The adult fish (one female and two male) were maintained in ISO-water at 26–28°C with a 12-h light/dark-cycle. For the production of eggs, plant dummies on grade cover, settled on a glass container, were introduced into the aquarium. Eggs were incubated in ISO-water at 28°C for 50–54 h for embryonic development of fish larvae. Hatched fish larvae were maintained under the same conditions prior to exposition experiments.

For each exposition experiment, ten larvae (48 h after hatching) were used. Each experiment was carried out as two independent experiments in three parallels. The ISO-water was replaced by the exposition medium and the test organisms were exposed for 3 h at 28°C. As the positive control 625 µg l⁻¹ 4-NQO was used.

3 DNA-Unwinding Assay for Monitoring

For testing cytotoxicity in the RTG-2 cell line, the neutral red test was applied. 100 µl of cell suspension (3×10^4 cells) was placed in the wells of a microtest plate. The plates were incubated overnight at 20°C. The medium was removed and 200-µl exposition medium (single substances and surface water samples, respectively) was placed onto the cells in three parallels. The concentration of fetal calf serum was 2% and 1× medium concentration was adjusted.

To avoid evaporation the plates were closed with a plate sealer and were incubated for 1 h at 20°C. The exposition time in the cytotoxicity test was the same as in the DNA alkaline unwinding assay. After the exposition time (1 h) the exposition medium was removed from the cells and overlaid with MEM including Earle's salts containing neutral red solution 0.005%. The plates were incubated for 3 h at 20°C. The medium was removed and the cells were washed with 100-µl Dulbecco's PBS

(DPBS). The plates were incubated for 15 min at room temperature after 150- μ l acetic acid (1% in 50% ethanol) was placed in the wells. The optical density is measured at 540 nm against a reference wavelength of 690 nm. Exposition-induced inhibition of cell growth is calculated in percent.

The cytotoxic effect of the mussels was estimated visually during the exposure experiment. Mussels that have maximal opening of their shells and which express no reaction after mechanical stimulation were removed from the exposition test. Cytotoxicity effects in fish larvae were tested at the end of the exposition experiments. The mobility of the larvae was tested and toxic effects as well as lethality effects were documented.

All steps of the DNA-Unwinding assay were carried out on ice. After the preparation of tissue samples, the 3-fold volume of homogenization buffer (13.8 g l⁻¹ Tris, 4.5 g l⁻¹ NaCl, pH 8.5) was added immediately and the samples were homogenized. The cell debris sedimented for 15 s and 0.2 ml of the homogenized sample was filled in new tubes (control: 0.2 ml of homogenization buffer).

For the lysis 0.5-ml lysis buffer (0.15 mol l⁻¹ NaOH, Titrisol® Merck; 7.1 g l⁻¹ Na₂HPO₄; 58.44 g l⁻¹ NaCl) was added slowly. The samples were incubated for 30 min in the dark (cell lines, mussels, fish larvae).

Afterwards the 0.5-ml neutralization buffer (0.18 mol l⁻¹ HCl; 5.0 mg l⁻¹ phenol red) and 0.5-ml 0.5% SDS (RTG-2) or 0.5-ml 0.5% SLS with 7.46 g l⁻¹ Na-EDTA (aquatic organisms), respectively were added. This was followed by an ultrasound treatment with a tip sonicator for 10 s (40 W).

For the DNA separation process, hydroxyapatite (BioRad DNA-Grade Bio Gel HTP; HAP; 1 Vol. HAP + 2 Vol. 10-mM potassium phosphate buffer) was heated for 30 min at 100°C shortly before use to eliminate CO₂. The stock solution of potassium phosphate buffer included 68.05 g l⁻¹ KH₂PO₄ and 87.09 g l⁻¹ K₂HPO₄. Afterwards 400 μ l of cooled hydroxyapatite solution was added to the samples. With an interval of 3 min the samples were resuspended four-times on a vortex-shaker. The samples were centrifuged quickly at 3,000 $\times$ g for one second. The pellet was washed twice by resuspending in 1.25-ml washing buffer (10 mM potassium phosphate buffer; 20% formamide) under the shaking and centrifuging conditions mentioned above.

Purification of formamide before use: 10 g charcoal plus 50 g mixed-bed ion exchanger were added to 1 l formamide. The solution was stirred for 2 h at 4°C and filtered through a double paper filter. The pH value of a 1:2 dilution with water should be below 7.5 and the conductivity below 40 μ S. Otherwise the procedure has to be repeated.

For the elution of DNA from the hydroxyapatite, 1.0-ml elution buffer I (125-mM potassium phosphate, 20% formamide) was added to the HAP-DNA pellet. With an interval of 3 min the samples were shaken with a vortex-shaker and centrifuged quickly at 3,000 $\times$ g for one second. Afterwards 0.1 ml (cell lines, mussels), 50 μ l of the supernatant (ss-DNA from fish larvae) respectively, were filled in four wells (four parallels) of a microtest plate. The remaining HAP-DNA pellets were eluted with 1-ml elution buffer II (500-mM potassium phosphate, 20% formamide) under the same elution and centrifuge conditions as described for the elution of ss-DNA. Afterwards 100 μ l of the supernatant (ds-DNA from cell lines and

mussels), 50 µl (ds-DNA from fish larvae) respectively, were filled in four microplate wells (four parallels).

The DNA concentration is estimated by Bisbenzimidazole fluorescence staining (cell lines and mussels). The microplates are closed by films and incubated 5 min at 80°C in a water bath. The plates are cooled immediately in ice water. Then 200-µl Bisbenzimidazole solution (250 µM Bisbenzimidazole, dissolved in 0.05 M potassium phosphate buffer enriched with 2.2 M NaCl, pH 7.4) is added. After 45 min incubation at room temperature the fluorescence is measured with a fluorimeter (excitation: 360 nm, emission: 450 nm).

The DNA concentration for the tests with fish larvae is estimated by Picogreen® fluorescence staining. Therefore, the DNA is pretreated with RNase (DNA-free, Boehringer Mannheim). 25 µl of firstly prepared RNase solution (2 µl ml⁻¹ RNase in TE-buffer (10 mM Tris, 1 mM EDTA, pH 7.5) is added to the DNA and the plates are incubated for 15 min at room temperature. The microplates are closed by films and incubated for 5 min at 80°C in a water bath. Afterwards 25-µl Picogreen solution (1% Picogreen® ds-DNA Quantitation Reagent, Molecular Probes, INC, Göttingen in TE-buffer) is added to the microplates. The plates are incubated for 5 min at room temperature and the fluorescence is measured with a DYNATEC Laboratories, Type Fluorolite 1000 fluorescence photometer (excitation: 480 nm, emission: 520 nm).

Calculation of the – log F-value

After subtraction of the blank-fluorescence signal, the relative fluorescence values of the elute fractions were calculated. This *F*-value represents a measure of the remaining ds-DNA compared with the total DNA amount (ds + ss-DNA) in a sample:

$$F = \frac{ds - DNA}{(ss - DNA + ds - DNA)}$$

ds = double stranded

ss = single stranded.

The negative logarithm of the value *F* (–log *F*) is proportional to the relative number of the DNA-unwinding points. By calculation of the factor *p* value from the proportion of the –log-values of exposed and control organisms, it is possible to quantify the DNA-fragmentation potential:

$$\text{Factor } p = \frac{-\log F(\text{exposed organisms})}{-\log F(\text{control organisms})}$$

For measurements using Picogreen® fluorescence staining, the fluorescence signal of the ss-DNA is not proportional to that of the ds-DNA. For the calculation of the –log *F* values the blank-corrected values of the ds-DNA have to be corrected with the factor 1.13.

3.1 Statistical Analyses

Both the experiments with single substances as well as those with surface water samples were carried out in two independent experiments.

The results in genotoxicity from single-substance experiments were evaluated by application of the following factor of significance (SF), calculated by the following analysis of variance:

$$\text{Factor of significance: } SF = 1 + t \frac{\sqrt{\frac{s_{\text{sample}}^2 (n-1) + s_{\text{control}}^2 (m-1)}{n+m-2}}}{\sqrt{\frac{n \cdot m}{n+m}}}$$

The value t represents the 97.5%-percentile of the t distribution (degrees of freedom $n + m - 2$). SF represents the upper limit of the 97.5% for the difference of the arithmetic mean of the induction factors in the DNA-unwinding assay (factor p values), if sample and control variations are normally distributed. The probability that $\overline{IF}_{\text{control}} \leq SF$ is 95% was calculated to indicate a significant genotoxic result.

Statistical significance of genotoxicity effects in surface water samples:

Statistical significance of surface water samples, calculated by comparing the results for $-\log F$ -values of the exposed and nonexposed organisms (controls) were analyzed separately for each experimental run by the Dunnett-Test. The $P < 0.05$ level was considered significant in a single experiment. Taking into account the results of the two independent experiments, samples are defined as genotoxic, if at least one experiment expresses a significant positive result and/or a twofold-enhanced $-\log F$ value above the standard deviation of the control $-\log F$ values is detected in combination with at least one significant result.

3.1.1 Results of the Study

To examine the inducibility of genotoxic effects caused by single substances, the DNA alkaline unwinding assay was performed with the cell line RTG-2, *in vitro* and *in vivo* assays with *D. polymorpha* (gills and soft tissues), and with *D. rerio* fish larvae (Table 1).

3.1.2 Exposure Experiments with the Fish Cell Line (RTG-2)

With exception of 4-NQO (with a detection limit of $78 \mu\text{g l}^{-1}$), genotoxicity effects induced by single substances in the RTG-2 cell line were detected in higher concentration ranges ($>7.8 \text{ mg l}^{-1}$ for 2-AA; 5 mg l^{-1} for B(a)P). The highest detection limit was measured with the single substance DMNA ($>125 \text{ mg l}^{-1}$; Table 1).

Table 1 Genotoxins and LOEC data for RTG-2, *D. polymorpha*, and fish larvae (yolk sac) of *D. rerio* by the DNA-unwinding assay

Test organism	Single substance				
	2-AAF	B(a)P	DMNA	NF	4-NQO
RTG-2 (1 h exposition)	7,812.5	5,000	125,000	7,800	78
<i>Dreissena polymorpha</i> - Gills (1 h in vitro exposition)	n.m.	25*	n.m.	n.m.	n.d.
<i>Dreissena polymorpha</i> -soft tissue (72 h in vivo exposition)	(25,000)*	n.m.	(10,000)*	n.m.	250
<i>Danio rerio</i> -yolk egg stages, static experiments (3 h exposition)	250	100	n.d.	1,250	78

Concentrations are given in [$\mu\text{g l}^{-1}$]

Abbreviations: 2-AAF 2-Acetaminofluorene; B(a)P Benzo(a)pyrene; DMNA N-nitrosodimethylamine; NF Nitrofurantoin; 4-NQO 4-Nitroquinoline-N-oxide

n.d.: not detectable, n.m.: not measured, *: $P < 0.05$ (Dunnett-Test)

The detection limits on the basis of the significance factors (95%-Percentile) for the tests with the cell line RTG-2; *Dreissena polymorpha* and *Danio rerio* (yolk sac larvae stages; 48 h after hatching) with $N = 2$ independent experiments with $N > 3$ parallels per test were calculated. The *in vitro* experiments with gills from *Dreissena polymorpha* analyzed with the DNA alkaline unwinding assay were statistically evaluated with $P < 0.05$ in the Dunnett-test for both experiments

*Not evaluated by the significance factor SF, statistically significant with $P < 0.05$ in the Dunnett-test

3.1.3 Exposure Experiments with the Mussel: *D. polymorpha*

In the *in vitro* exposition experiments with gills of the mussel *D. polymorpha* the highest sensitivity was measured with the single substance benzo(a)pyrene with a detection limit of $25 \mu\text{g l}^{-1}$. In the *in vivo* exposition experiments, the single substance 4-NQO induced genotoxicity with $250 \mu\text{g l}^{-1}$ in a good dose-response relationship. With benzo(a)pyrene and nitrofurantoin no enhanced DNA fragmentation was observed in soft tissues after *in vivo* exposition. In addition, significant enhanced DNA fragmentation was detected with 2-acetaminofluorene in high concentration ranges $\geq 20 \text{ mg l}^{-1}$ (resp. N-nitrosodimethylamine $\geq 10 \text{ mg l}^{-1}$). Enhanced sensitivity was detected in the *in vivo* exposition experiments in soft tissue in comparison with the *in vitro* experiments with gills (Table 1).

3.1.4 Exposure Experiments with Fish Larvae (Yolk Sac Larvae): *D. rerio*

The single substance 4-NQO induced genotoxicity in *D. rerio* yolk sac fish larvae with $78 \mu\text{g l}^{-1}$ whereas 2-acetaminofluorene was genotoxic in concentration ranges $\geq 250 \mu\text{g l}^{-1}$ (Table 1). The substance benzo(a)pyrene induces enhanced DNA fragmentation from $100 \mu\text{g l}^{-1}$ and the antibiotic nitrofurantoin expressed genotoxicity with $\geq 1,250 \mu\text{g l}^{-1}$.

In all chosen test organisms, 4-NQO expressed the highest genotoxic effect in a good dose-response relationship (Table 1).

3.1.5 Genotoxicity Induced by Surface Water Samples

To test genotoxicity in different surface water samples, the results of the DNA alkaline unwinding assay using the test systems with the RTG-2 cell line, the mussel *D. polymorpha* (soft tissues), and yolk sac fish larvae (*D. rerio*) were compared. To measure genotoxicity, surface water samples were analyzed from February to November. Two genotoxic samples were evaluated with the RTG-2 cell line (River Elbe, Schnackenburg in April and the positive control at the Gas Works, a gasoline influenced station). The cell line RTG-2 expressed high sensitivity to genotoxic load in surface water.

Two samples from the River Rhine (Karlsruhe, July) and the supplementary sample from the River Elbe (Schmilka, April) expressed significant enhanced DNA fragmentation in *D. polymorpha* soft tissue.

In summary, eight water samples from stations express enhanced DNA fragmentation. Low variation coefficients compared with high sensitivity of test organisms in the presence of genotoxic load (surface water) were measured and confirmed by the calculated factors of significance ($SF = 1.38$; $N = 65$). The best conditions regarding the exposition time of *D. rerio* larvae was estimated to be 3 h when 48 h old larvae were used in the exposure.

The standard deviation was taken low. No false-positive results were seen.

3.2 Genotoxicity and Repairment: Influence of Solar Ultraviolet-B on Pelagic Fish Embryos: DNA Fragmentation and DNA Thymine Dimers

To investigate genotoxicity and repairment in environmental samples we collected embryos of the fish species *L. limanda* from sites in the zone of influence of the Rivers Rhine and Elbe, and from relatively unpolluted coastal and offshore sites. There are two main fluvial discharges into the German Bight: the Rivers Rhine and Elbe. Both rivers pass through heavily populated and industrialized watersheds and consequently, have long associations with contaminant loadings (references). Secondary fluvial discharges into the German Bight include the Rivers Eider and Weser. Chemical stressors delivered to the sea via those fluvial sources have been associated with impacts in pelagic and bottom-feeding fish [19, 24]. Our hypothesis was that there might be an interaction between exposure to chemical contaminants in the zones of influence of the Rivers Rhine and Elbe and the ability of embryos to repair UV-induced DNA damage. In which case, embryos from the chemically stressed sites would be expected to have more UV-induced DNA damage as reflected by the formation of thymine dimers and DNA strand breaks. The natural unwinding of DNA's double helix starts at either strand ends or at strand nicks, which are generated by topoisomerases. Additional unwinding points occur at apurinic and apyrimidinic (AP) sites, strand breaks (SBs), and repair patches. Unwinding can also start at reactive oxygen species (ROS) induced lesions in the

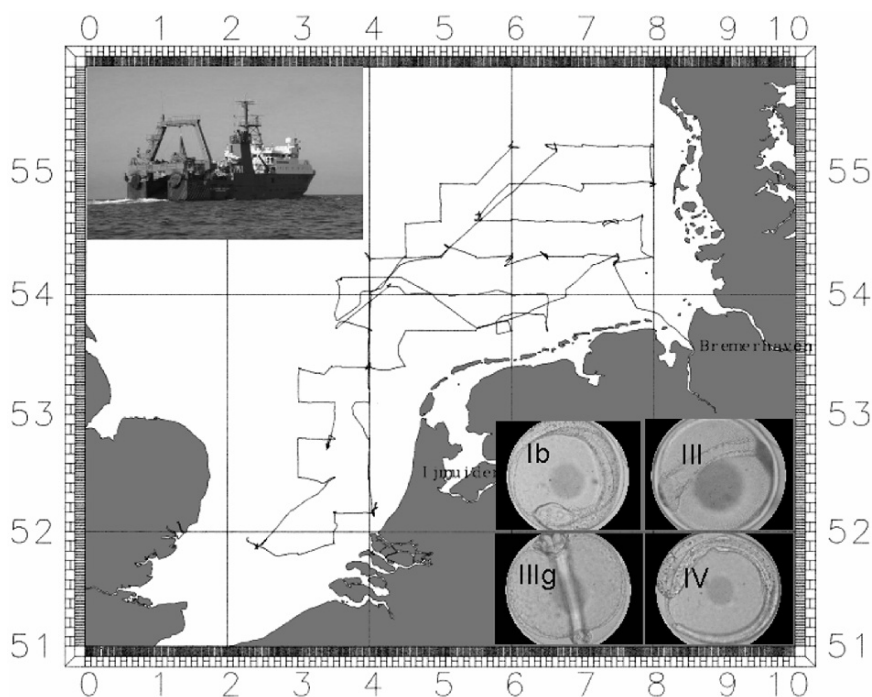


Fig. 1 Cruises of the Research Vessel RV Walther Herwig III and sampling stations. *L. limanda*: ontogenetic stages Ib-IV, 9 days, UV-B exposure und incubation for genotoxin monitoring in the Southern North Sea

DNA-double helix or in the genetic code. Those additional unwinding points combine to enhance DNA's normal unwinding rate under alkaline conditions. DNA damage in the form of thymine dimers differs distinctly from DNA-strand breaks. Thymine dimers, which tend to be stable, form when the H-bonding bridge between thymine and adenine is replaced by a cyclobutane covalent bridge between two adjacent thymine molecules.

Fish embryos (*L. limanda*) were experimentally exposed to different amounts of UV-B irradiation in a sunshine simulator and in exposure experiments onboard of a research vessel (Figs. 1, 2). The experiments were carried out with defined and realistic irradiation qualities and quantities. The following endpoints of the exposed fish embryos were studied: mortality, malformations, and changes in buoyancy of embryos measured as changes in osmolarity of the perivitelline fluid. The permeability was measured and the genotoxicity by DNA unwinding and the thymine dimers by an ELISA.

3.3 Fish Embryos: *Limanda limanda* and UV-Exposure

To obtain information concerning relevant sublethal effects for the fish-egg embryos beside the DNA damage (Fig. 3) the uptake of fluorescein, the accumula-

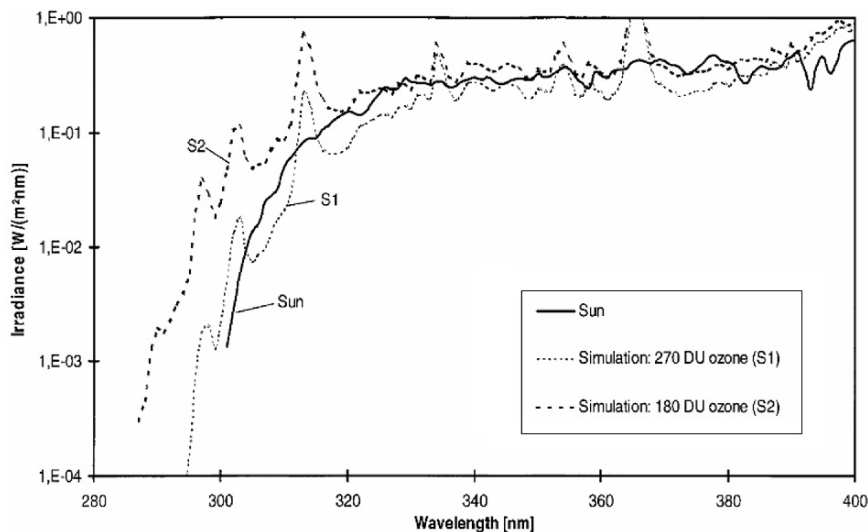


Fig. 2 Sunshine simulators (SONSI) S1 and S2 and adjustment over three cruises with comparison to the solar reference spectrum measured onboard RV Walther Herwig III at 54.5°N, 7.0°E

tion of Rhodamin B (Rh B) combined with the specific ionic blocker, Verapamil (Ver) were used to estimate the export activity of MXRtr in fish eggs. The developmental stages of fish-egg embryos and the uptake of fluorescein were investigated as a parameter to quantify the permeability by the load of fluorescein in the fish-egg embryos (green fluorescence, 450–490 nm). The penetration of fluorescein (Fig. 4) was measured by fluorescent microscopy and microfluorometry. The red fluorescence of accumulated Rh B in the eggs was measured as well by contact microfluorometry (excitation 510–560 nm, dichroic mirror 575 nm, barrier filter >590 nm) with a 16× objective and mirror (25 µm in diameter). The fish-egg embryos were incubated with 0.5 µM Rh B and 10 µM of Ver for 10 min. The mean difference between Rh B fluorescence with and without inhibitor Ver is proportional to the amount of Rh B eliminated by the MXRtr system per hour.

In the present study, an optimized version of the method of [19] was investigated to measure unwinding sites in the DNA of UV-exposed embryos. The embryos were homogenized and then lysed under alkaline conditions, after which the suspension of partially unwound DNA was rapidly neutralized, dispersed, and then ultrasonicated to prevent renaturation. SS-DNA and ds-DNA were then separated by ion exchange chromatography on hydroxyapatite. The DNA contents of the ss- and ds-fractions were quantified by DNA-specific fluorescence. The degree of unwinding was calculated as F (the proportion of double-stranded DNA), and most commonly expressed as $-\log F$, which is proportional to the number of unwinding points. Increases in the $-\log F$ value of embryos from treatment sites compared to control site values are indicative of genotoxic impact. The genotoxic agent

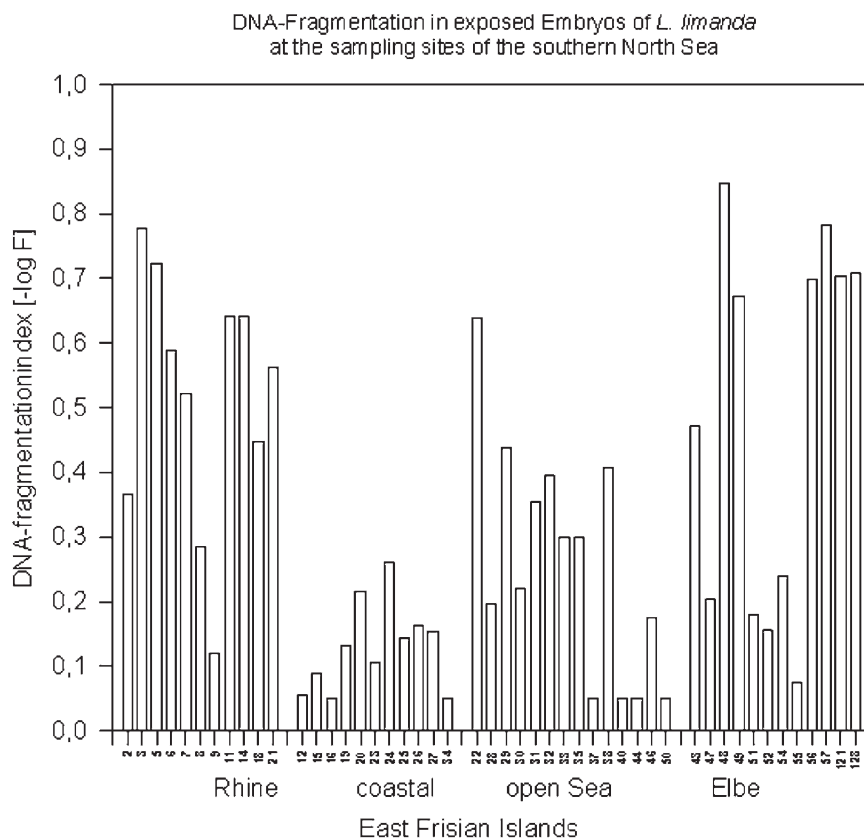


Fig. 3 DNA-fragmentation in exposed embryos of *L. limanda* at the North Sea sampling sites (see Fig. 1) in the Rhine and Elbe Estuary, coastal areas, and open sea

4-nitroquinoline-*N*-oxide or 2-aminoanthracene was the positive control, and a reagent blank was processed with each sample set.

The eco-genotoxic impact on stressed fish-egg embryos exposed to UV light was assessed by DNA-Unwinding. The unwinding of the DNA double helix starts at natural unwinding points which are strand ends and strand nicks caused by enzymes, i.e., topoisomerases. Additional unwinding points can be found in exogenously damaged DNA. These can be apurinic/apyrimidinic sites, strand breaks, and repair patches. They enhance the average unwinding rate under controlled conditions. After a defined time, alkaline lysates of tissue homogenizates or cells are neutralized, and the single-stranded and double-stranded fractions are separated by ion exchange chromatography using hydroxyapatite.

The DNA concentration of each fraction is quantified by DNA-specific fluorescence stain in a microplate fluorometer.

The enzymes DNA-ligases and DNA-polymerases activated by UV-light are responsible for repair of DNA strand breaks. These enzymes are monitoring the

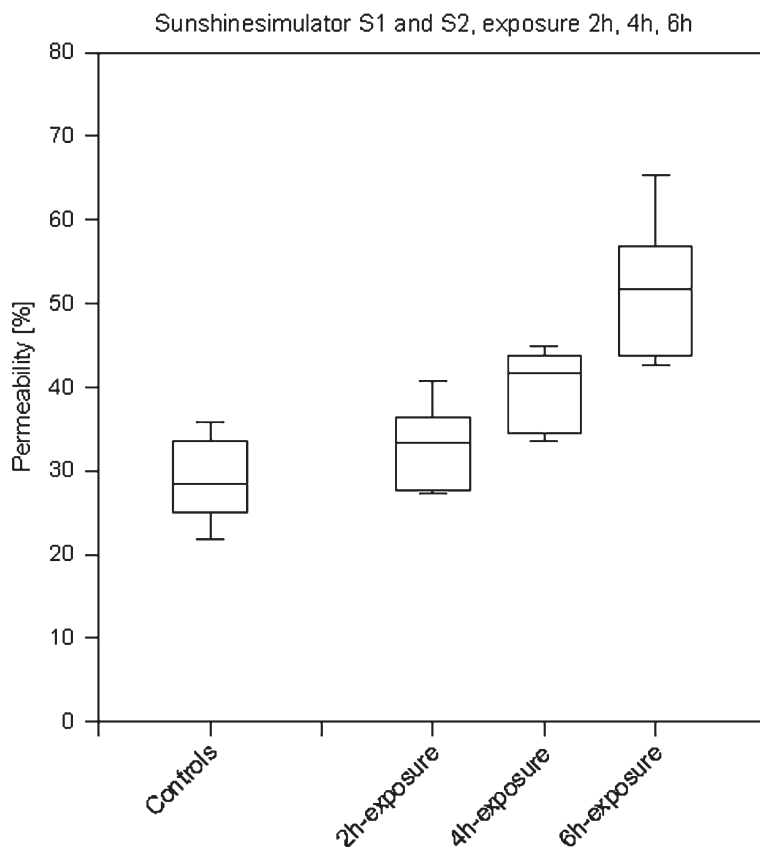


Fig. 4 Permeability [%] of exposed fish eggs (*L. limanda*) after 2 h, 4 h, 6 h

DNA-molecule to check the H-bridging covalent binding sites and the nucleoside gaps to replace these gaps by the corresponding nucleotide. In general there are lesions on the DNA-double helix or in the genetic code because of the ability of the reactive oxygen species including some oxygen-centered free radicals to modulate genes and protein expression and alter DNA polymerases.

Thymine dimers cause no lesions in the DNA-molecule like DNA-strand breaks. The formation of thymine dimers is a result of the breaking of H-bridging covalent binding sites between thymine and adenine and replacement by a covalent binding cyclobutane thymine dimer between two thymine molecules, which are located side-by-side at the DNA strand. The cyclobutane-thymine dimers are very stable. The repairment is improved by special enzymes like DNA-polymerases and topoisomerases that perform the genetic code repair.

To measure possibly UV-mediated DNA damage in fish-egg embryos both procedures, the DNA-unwinding assay and the assay for the determination of thymine

dimers, were applied. The DNA-Unwinding Assay (DNA alkaline denaturation assay) monitors the genotoxicity in the eukaryotic tissues of the fish-egg embryos. In this study the assay was optimized and applied to homogenized embryos of *L. limanda* (for a detailed method see [19]).

The protocol for the alkaline unwinding assay was originally derived from procedures published in [20]. The alkaline DNA unwinding assay has three analytical steps (see Sect. 3); the actual unwinding of the DNA in the fish-egg embryo under controlled alkaline conditions, the separation of single-stranded DNA from double-stranded DNA by ion exchange chromatography, and the quantitative measurements of the DNA fractions by fluorometry. After the lysis and the time-related defined end of the unwinding process by neutralization of the lysate the samples are sonicated to prevent rejoining of complementary DNA strands. For positive controls and internal standard a mixture of homogenized tissues of fish eggs and a dissolved genotoxic substance, e.g., 4-nitroquinoline-*N*-oxide or 2-aminoanthracene were used. Natural unwinding points are the DNA strand ends, topoisomerase nicks, and spontaneous DNA lesions.

The biological significance of UV-mediated DNA lesions are their premutagenic and carcinogenic potential. The DNA unwinding is measuring genotoxicity, i.e., DNA-damage and DNA repair. Examples of DNA-damage are single- and double-strand breaks, DNA-crosslinks, oxidative lesions (ROS), loss of bases, and adduct-formation. UV-B and UV-C light generate lesions in DNA like cyclobutyl pyrimine dimers and 6–4 pyrimidione dimers which block DNA replication and transcription. Because of the importance of these premutagenic lesions and their lethal consequences and carcinogenic potential if unrepaired an immunochemical method (ELISA) was developed to measure the thymine dimers.

After lyses of the fish-embryos the samples were stored at -80°C . The DNA was purified by RNases degradation, phenol-extraction, extraction by chloroform and isoamylalcohol and finally by precipitation in ethanol. The analytical steps are the denaturation of the DNA and a competitive ELISA with thymine dimer antibodies [25]. Beside damage to the DNA by increasing strand breaks and photoproduction of dimers, increase of the transcriptional processes in the fish-egg embryo may also lead to an increase in DNA repair.

3.4 Embryonal Development and UV-Exposure

Extended experiments were carried out with different developmental stages of the fish-egg embryos to determine UV-light stress-related change of the permeability (fluorescein uptake). There was a high deviation of 5–40% in the early stages especially in stage Ia, a lower deviation of the embryonal stages Ib, II α , II β , II γ , and III α (around 30%). The mean permeability of stage III β was approximately 20%. The permeability of the later stages III γ , IV α , and IV β increased to 35%.

The DNA unwinding results were calculated as *P*-values ($P = -\log Fx / -\log F0$) and only results >1 are discussed as effects (see Sect. 3). Most of the investigated samples

for genotoxicity from 1997, 1998 and 1999 are individual samples so there is no knowledge about the range of $-\log F$ values and the deviation of thymine dimers in the controls and the exposed samples. Analytical studies in 1999 completed the measurements and determined the analytical range of the controls and the dosage data [25].

In the final phase of the experiments the results demonstrated the effect of the UV light in increasing DNA repair (Fig. 5b) through the increase in transcriptional processes in the fish-egg embryos. The developmental stage of the fish-egg

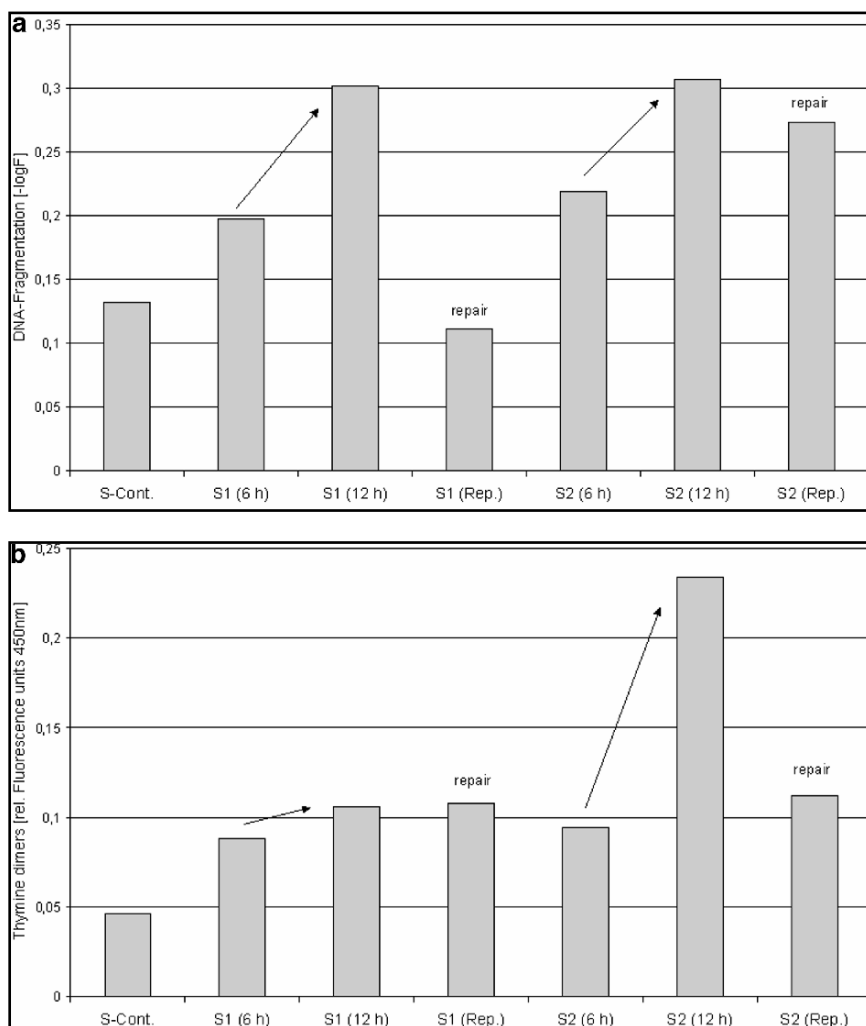


Fig. 5 (a) Sunshine simulator (SONSI) S1 and S2: DNA-fragmentation of sequential UV-B-exposed embryos (*L. limanda*) and repair. (b) Sunshine simulator (SONSI) S1 and S2: DNA thymine dimers of sequential UV-B-exposed embryos (*L. limanda*), early ontogenetic stages Ib-II, two-times 6 h in total 12 h exposure and 18 h repairment

embryos at the beginning of the experiment was $Ib\beta$ and at the end of the experiment $III\beta$ - $III\gamma$ in the controls.

The data show permeability (Fig. 4) of the embryos and DNA damage (Figs. 3 and 5a) measured by DNA unwinding [19] and thymine dimers (Fig. 5b) measured by ELISA. The results showed a time and dosage-dependent influence of UV-B radiation on the sublethal parameter genotoxicity (DNA damage). The repairment of the DNA strand breaks and the thymine dimers were investigated in [38] though the results are questionable (postreplication repair in embryo cells).

3.5 Mussels: *Mytilus edulis*

Some algal toxins such as saxitoxins (STX) and microcystin LR may strongly induce the repair mechanisms of DNA strand breaks at low concentrations. Conversely, another algal toxin, domoic acid (DA), led to clear increases in DNA strand breaks at similar concentrations [35]. Biotoxins (Red Tide) represent an ecological and economic problem concerning aquaculture and tourism in coastal areas world wide [39]. The genotoxic, neurotoxic, and immunotoxic effects by domoic acid are produced by the diatom *Pseudonitzschia multiseries* and the biotoxin saxitoxin causes paralytic shellfish poisoning (PSP) and is produced by *Alexandrium catenella* and *Alexandrium ostenfeldii*. In addition, the flagellate *Dinophysis acuta* causes diarrhetic shellfish poisoning (DSP). The need for increasing quality control at different critical steps of the food production chain has to be addressed by developing a set of rapid biomarker and sensor systems. The subacute effects of the toxins on the immune defense mechanisms of hemocytes and DNA fragmentation in cells of gills and hepatopancreas of several commercially relevant mussel species have been investigated. The PSP toxins were quantitatively analyzed by the Institute for Nutrition in Jena (Germany). Little is known about the metabolism and accumulation pathways of saxitoxins in mussels. The acute toxic effect of saxitoxins on bivalves has also rarely been investigated. In this study the neurotoxic, genotoxic, and immunotoxic effects of saxitoxins on different tissue of the blue mussels *M. edulis* were investigated under laboratory conditions. The neurotoxic, immunotoxic, and genotoxic effects of domoic acid on *M. edulis* were also investigated by laboratory exposure experiments. Mussels were intramuscularly injected with DA at concentrations 1, 10, 100, and 500 ng g⁻¹ body weight (bw). After incubation eco-toxic parameters including cholinesterase (ChE) activity in the gills of the mussels, phagocytosis activity of mussel hemocytes, and DNA strand breaks (DNA unwinding) in the hepatopancreas of the mussels were investigated. PSP toxins were extracted from the digestive glands of the mussel *M. edulis* to check the immune defense mechanisms of the hemocytes and DNA fragmentation in cells of gills and hepatopancreas. The toxins caused no change in the number of hemocytes, but there was an alteration of the membrane integrity. Phagocytosis activity of the hemocytes was strongly suppressed by PSP toxins. PSP toxins diminished physiological DNA fragmentation in gills and hepatopancreas of mussels

due to induction of the repair pathways for primary DNA damage. The causes and effects of biotic and abiotic parameters, i.e., UV-B exposure, were discussed [39]. The final aim was to develop a bivalve toxicity assay as a commercial immunotoxicity test kit for algal toxins.

Primary DNA damage in the blue mussel *M. galloprovincialis* can be reduced through antioxidants like alpha-tocopherol. The heterogeneity of DNA strand breaks in animals analyzed by field experiments are obviously based on the dose, synergetic, or antagonistic impacts of chemical contaminants as well as individual alteration in the activity of repair pathways of DNA damage.

Surprisingly, fish and mussel samples collected in relatively clean areas, e.g., the Islands of Helgoland and Spiekeroog (Germany), indicated also relatively high genotoxic responses. Although the greatest level of most chemical parameters was observed in soft tissue of *M. edulis* from the estuary of the Elbe River, mussels from the “reference site” the Island of Helgoland have been moderately contaminated with a number of chemical compounds. In particular, residues with relatively high genotoxic potency such as polychlorinated biphenyl (PCB) congeners were determined in mussel and sediment samples at the Island of Helgoland, which correlates with genotoxic stress of mussels at this site.

4 Concluding Discussion and Future Trends for Real-Time Assessment

There are a number of different assays that measure DNA strand breakage in alkalis including alkaline elution, DNA alkaline unwinding [18], and the microgel electrophoresis “comet assay,” for application in chemical testing as well as environmental research fields. In the present study, different optimization steps led to sufficient sensitivity regarding the detection of genotoxicity and decreased detection limits (LOEC values) in tests of single substances. For the single substance 4-NQO, Duis et al. [33] described a detection limit of $156 \mu\text{g l}^{-1}$ in comparison with $78 \mu\text{g l}^{-1}$ in the present study (both exposition of mono layer).

Different test organisms react with different sensitivity when exposed to genotoxic substances (the detection limit for 4-NQO was $78 \mu\text{g l}^{-1}$ for RTG-2 and *D. rerio* fish larvae compared with $250 \mu\text{g l}^{-1}$ when *D. polymorpha* was chosen). Different physiological and molecular causative relationships have an influence on the sensitivity of test organisms including detoxification, accumulation of contaminants, excretion, and biotransformation as well as the activation of DNA repair mechanisms.

The analyzed surface water samples had been judged for genotoxicity in *D. polymorpha* with the pulsed-field gel electrophoresis method in parallel experiments. With this method, false-positive genotoxic effects could be excluded. On the other hand, additional water samples were judged as positive in the PFGE compared with the DNA alkaline unwinding assay. These observations suggest the possibility of “borderline effects” especially in the DNA alkaline unwinding test

procedure with the mussel *D. polymorpha*. The use of the mussel *D. polymorpha* as a test organism for long-term monitoring experiments is indicated because of high correlation between the parameter "DNA fragmentation potential" measured by the DNA alkaline unwinding assay when compared with chemical residues of Hg(II) and the sum of HCH isomers in soft tissue during an eight-week exposition interval, estimated by Spearman rank-correlation analysis.

Contrary to these observations, no correlation relationship was found when the DNA fragmentation potential ($-\log F$ values) from surface water exposition was compared with chemical parameters of chlorophenols, trihalogenemethanes, and HCH isomers in the surface water. Residue contents of contaminants in *D. polymorpha* tissue seem to be more suitable parameters for correlation studies of genotoxicity due to accumulation events. In addition, correlation studies with the parameter "DNA alkaline unwinding" using identical protocols with *D. polymorpha* and the parameter AOX did not express a significant relationship between the data. This test organism has a number of defense mechanisms to prevent exposure to pollutants, such as closing the shell, metabolization rate, and excretion; kinetics modifies the amount of genotoxicity. The mussels have an important role in their trophic level as part of the aquatic food chain for surface water systems. Laboratory exposure data for *D. polymorpha* had shown a relatively higher variability compared with cell-culture tests (RTG-2) and *D. rerio* early life stages. The variability of test replicates under laboratory conditions was higher compared with cell cultures (RTG-2) and *D. rerio*. For exposure monitoring experiments a control station with comparable environmental conditions (climate, nutrient content) is indicated. Recent test results from single substance and surface water samples of different pollution grade can be demonstrated by the application of the DNA alkaline unwinding assay to real-world samples to assess quality criteria in genotoxicity for surface and tap water in regulatory processes. To monitor genotoxicity in aquatic systems, especially the early life stages of the fish larvae *D. rerio* show a relatively high sensitivity and reproducibility with the DNA alkaline unwinding assay and should be adapted for application to long-term exposures. A combined test battery using cell lines, mussels, and fish stages allows a more complete characterization of amount of genotoxic pollution in surface waters.

The use of test organisms of different trophic levels introduced in the DNA alkaline unwinding assay allows a differential exposition procedure to assess acute and chronic effects in relation to physiologic adaptation and accumulation in aquatic environments. The detection of DNA strand breaks by alkaline unwinding followed by hydroxyapatite batch elution is a rapid and inexpensive technique especially for analysis of a large batch elution of organisms in field experiments. The lack of a nonpolluted reference site as a control and great variance of DNA strand breaks in analyzed tissues of test animals from selected sampling sites makes it necessary to analyze the physiological range of variance in control test organisms from nonpolluted areas, i.e., in the North Sea and Baltic Sea.

Immune defense reactions in the hemolymph of mussels include the proliferation of hemocytes and their mobilization from certain tissues to the contaminated periphery corresponding with modulation of phagocytosis activity. The immune

defense response of *M. edulis* to domoic acid contamination at a concentration of $1 \mu\text{g g}^{-1}$ bw included the rapid proliferation of hemocytes within 48 h of incubation. The immune defense activity of the Pacific oyster (*Crassostrea gigas*) was also induced by 4 h exposure of the mussels to domoic acid and the diatom *P. pungens* f. *multiseries*. With high doses of cadmium and fluoranthene the number of hemocytes increased significantly. Conversely, the injection of PSP toxins led to increasing heterogeneity of hemocyte number and significant suppression of their phagocytosis activity [35]. Significant suppression of phagocytosis activity in the hemolymph of mussels was also noted following an exposure to phenol or high doses of selected heavy metals.

Saxitoxins have been seen to induce the DNA repair system at concentrations of 100 and 500 ng g^{-1} bw. The main results are the apparently significant induction of the DNA repair mechanism following short-term exposure of mussels to saxitoxins, domoic acid, and microcystin-RL. An isolate of saxitoxins from the gills, digestive organs, and adductor muscle of mussels (*M. chilensis*) received from the Medical Institute of the Salud Aysen in the XI. region of Chile was used for investigations and reference. The chemical differentiation of the isolates carried out in the Institute of Food Chemistry of the Friedrich-Schiller-University in Jena suggested the presence of different saxitoxin and gonyautoxins compounds. After chemical analysis and quantification of the saxitoxins, a stock solution of the isolate was prepared containing 1 mg ml^{-1} saxitoxins. The chemical instrumental analysis shows clearly an extremely high level of saxitoxin equivalents.

Exposure experiments with fish embryos were carried out to demonstrate the effect of UV light in increasing DNA repair through an increase in transcriptional processes in the fish-egg embryos. We employed two main scenarios, a reduction to 270 (S1) and to 180 (S2) Dobson units (DU) in single or repetitive exposure of 2, 4, or 6 h. The developmental stage of the fish-egg embryos at the beginning of the experiment was Ib and at the end of the experiment III β -III γ in the controls. In SONSII 2 the development of the fish-egg embryos was slightly delayed to II β -III α . In summary there are sublethal effects at the molecular level that can be demonstrated by DNA unwinding and thymine dimers. However, in the experiments with only one exposure in the sunshine simulator and in field experiments where radiation was low there was not always enough information for a clear interpretation. In future bio-analytical work the deviation of the data in the matrix fish-egg embryo will be investigated.

Monitoring the effects of environmental genotoxic substances has gained increasing importance in recent times. Assessment of environmental samples for the presence of genotoxins (mutagens and carcinogens) has a high priority in protecting inland and coastal waters. Many tools have been meanwhile standardized and harmonized by [17] – determination of genotoxicity using the umu-test, [40] – determination of the genotoxicity of water – *Salmonella*/microsome test (AMES-test), [41]– Evaluation of genotoxicity by measurement of the induction of micronuclei – Part 2: “Mixed population” method using the cell line V 79 and many other indicator assays like the comet assay but the missing tools are the real-time assays in genotoxicity. In the field of bio-analytical approaches [42] promising on

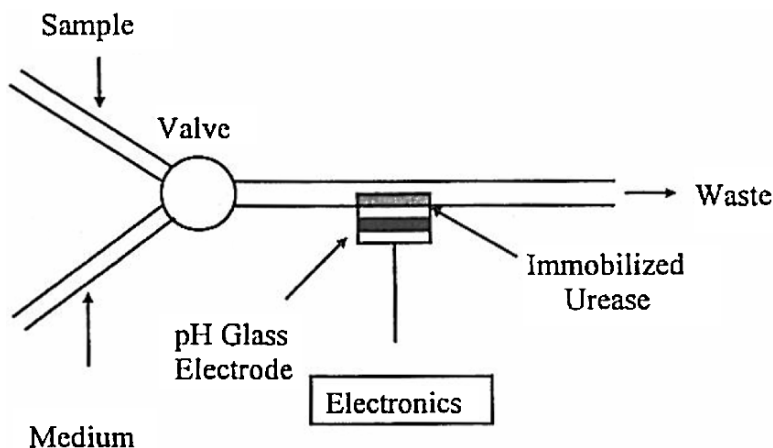


Fig. 6 Whole-cell biosensor for the assessment of a genotoxic potential modified after [2, 17]

line formats are discussed and in progress. There are already some robot systems according to [17] on the market and flow-through concepts (Fig. 6) are on the way. New testing strategies and concepts will provide information on genotoxins in the environment. Extended exposure of organisms to environmental genotoxins results in physiological disorder such as reproduction impairment and other related abnormalities.

Acknowledgments The authors wish to thank the Federal Ministry for Science and Technology (BMBF) in Germany and the European Commission for funding the case studies 1, 2, and 3 via the projects ALGAETOX (ENV4-CT98-0784), SANDRINE ENV4-CT98-0801), BIOMAR EV5V-CT94-0550), BEEP (EVK3-CT2000-00025), and CITY FISH (EVK1-CT1999-00009). We thank Birgit Fischer for technical assistance and Jürgen Büsing from the European Commission for his competent contributions in stimulating many frontier approaches und ideas.

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Liquid Chromatography–Mass Spectrometry Methods for Analysis of Endocrine-Disrupting Chemicals in Wastewaters

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Abstract Endocrine disrupters (EDs) continue to be the focus of extensive research. Their effects and mode of action as well as their occurrence and fate in the aquatic environment are still largely unknown and need investigation. Wastewater treatment plant effluents are for most of them the main source of contamination in water bodies. A large number of EDs are relatively polar compounds that pass easily wastewater treatments and reach the aquatic environment, where they can exert undesirable effects on both wildlife and humans. Their removal during wastewater treatment, very often inefficient, is usually estimated from the comparison of the concentrations detected at the influent and the effluent and the technique of choice for their analysis is in many cases liquid chromatography–(tandem)mass spectrometry (LC–(tandem)MS).

Herein we review the analytical methods, including sample preparation and analysis, based on LC–(tandem)MS described in the literature for the determination of different classes of EDs (pesticides, phthalates, bisphenol A, alkylphenolic compounds, perfluorinated compounds, pharmaceuticals, and estrogens) in wastewater and their occurrence in this kind of matrix.

Keywords Analysis, Emerging contaminants, Endocrine disrupters, LC-MS, Wastewater

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Contents

1	Introduction.....	230
2	Specific Groups of EDs.....	232
2.1	Pesticides	232
2.2	Phthalates	232
2.3	Bisphenol A.....	233
2.4	Alkylphenolic Compounds	233
2.5	Perfluorinated Compounds	234
2.6	Pharmaceuticals	235
2.7	Estrogens.....	236
3	Analysis of EDs in Wastewater.....	236
3.1	Sample Preparation	236
3.2	Instrumental Analysis	249
4	Levels in Wastewater	256
4.1	Pesticides	256
4.2	Phthalates	256
4.3	Bisphenol A.....	258
4.4	Alkylphenolic Compounds	258
4.5	Perfluorinated Compounds	259
4.6	Pharmaceuticals	261
4.7	Estrogens.....	261
5	Conclusions.....	262
	References.....	263

Abbreviations

ACN	Acetonitrile
AcOH	Acetone
AMPA	Aminomethylphosphonic acid
APCI	Atmospheric pressure chemical ionization
APs	Alkylphenols
APECs	Alkylphenoxy carboxylates
APEOs	Alkylphenol ethoxylates
API	Atmospheric pressure ionization
BBP	Butyl benzyl phthalate
BEHP	Bis(2-ethylhexyl) phthalate
BIO	Biochanin-A
BPA	Bisphenol A
CNS	Central nervous system
COU	Coumestrol
DAI	Daidzein
DBP	Di- <i>n</i> -butyl phthalate
DCM	Dichloromethane
DEHP	Di-(2-ethylhexyl) phthalate
DEP	Diethyl phthalate
DES	Diethylstilbestrol
DMP	Dimethyl phthalate
DOP	Di- <i>n</i> -octyl phthalate

DPP	Dipentyl phthalate
E1	Estrone
E1S	Estrone-sulfate
E2	Estradiol
E3	Estriol
ED	Endocrine disrupter
EE	Ethynyl estradiol
EI	Electron ionization
EO	Ethoxylate
EPI	Enhanced product ion
ESI	Electrospray
ETAc	Ethyl acetate
Et ₂ O	Diethylether
FA	Formic acid
FASs	Fluorinated alkyl substances
FOR	Formononetin
6:2 FtS	6:2-Fluorotelomer-sulfonate
GC	Gas chromatography
GEN	Genistein
GLY	Glycitein
GPC	Gel permeation chromatography
HE	Hydroxyl estrone
HOAc	Acetic acid
IT	Ion trap
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MeOH	Methanol
MES	Mestranol
MIPs	Molecularly imprinted polymers
MM	Mixed mode
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MTBE	Methyl- <i>tert</i> -butylether
NH ₄ Ac	Ammonium acetate
NH ₄ HCO ₂	Ammonium formate
NI	Negative ionization
NP	Nonylphenol
NPECs	Nonylphenoxy carboxylates
NPE1C	Nonylphenoxy monocarboxylate
NPE2C	Nonylphenoxy dicarboxylate
NPEOs	Nonylphenol ethoxylates
NP1EO	Nonylphenol monoethoxylate
NP2EO	Nonylphenol diethoxylate
NSAID	Nonsteroidal antiinflammatory drugs
OP	Octylphenol

OPE2C	Octylphenoxy dicarboxylate
OPEOs	Octylphenol ethoxylates
PAEs	Phthalic acid esters (phthalates)
PCPs	Personal care products
PFASs	Perfluorinated alkyl substances
PFBS	Perfluorobutane sulfonate
PFCs	Perfluoroalkyl carboxylates
PFCAs	Perfluorocarboxylic acids
PFO	Perfluorooctane
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonate
PFOSA	Perfluorosulfonamide
PFOSAA	<i>N</i> -ethyl perfluoro-octanesulfonamidoacetate
PFSs	Perfluoroalkyl sulfonates
PFSAs	Perfluorosulfonic acids
PI	Positive ionization
Q	Single quadrupole
QqQ	Triple quadrupole
Q-TOF	Quadrupole-time of flight
Q-trap	Quadrupole-ion trap
RP	Reverse phase
SEC	Size exclusion chromatography
SIM	Single ion monitoring
SPE	Solid-phase extraction
SRM	Selected reaction monitoring
STP	Sewage treatment plant
TCAA	Trichloroacetic acid
TEA	Triethylamine
TET	Triethyl tin
TFA	Trifluoroacetic acid
TOF	Time of flight
TRI	4',6,7-Trihydroxyisoflavone
TrBA	Tri- <i>n</i> -butylamine
UPLC	Ultra-performance liquid chromatography
WWTP	Wastewater treatment plant
XAPECs	Halogenated alkylphenoxy carboxylates
XAPs	Halogenated alkylphenols

1 Introduction

Endocrine disruptors (EDs) are exogenous agents that interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior.

Under the category of EDs there are a wide variety of chemical substances that include both natural but mainly anthropogenic compounds. Over the years various national and international governmental and nongovernmental organizations have compiled lists of EDs. These lists together with information extracted from the scientific literature have formed the basis of the inventory created by the Institute for Environment and Health [1], which includes a total of 966 compounds or elements purported to be EDs.

Herein we focus on seven compounds or compound classes, which are amenable to LC-MS analysis: (1) pesticides, (2) phthalates, (3) bisphenol A (BPA), (4) alkylphenolic compounds, (5) perfluorinated compounds, (6) pharmaceuticals, and (7) estrogens. These compounds or compound classes have been associated to a variety of effects (persistent estrus, mammary, testis and thyroid tumors, reduced fertility, alteration of the luteinizing hormone, cortisol, insulin, and/or testosterone, etc.) but most of them are suspected to have estrogenic and/or androgenic activity (phthalates, bisphenol A, alkylphenols, estrogens) or to disrupt thyroid function (some pesticides, pharmaceuticals, and perfluorinated surfactants). Nevertheless, their effects and mode of action are still largely unknown, as is the occurrence and fate in the aquatic environment of those compounds, such as pharmaceuticals, that have been only recently discovered or recognized as environmental contaminants (known as emerging contaminants).

Some of the studied compounds are included in the list of priority substances in the field of water policy and are thus subject to environmental quality standards in surface waters [2]. This is the case of the pesticides alachlor, atrazine, chlorfenvinphos, chlorpyrifos, diuron, endosulfan, simazine, and trifluralin, the alkylphenolic surfactants nonylphenol (NP) and octylphenol (OP), and the phthalate di(2-ethylhexyl)-phthalate (DEHP). Others, such as the pesticides bentazon, dicofol, mecoprop, glyphosate and AMPA (glyphosate degradation product), bisphenol A, and the perfluorinated compound perfluorooctane sulfonate (PFOS) are included in the list of substances subject to review for possible identification as priority substances or priority hazardous substances [2].

For most of the reviewed compounds wastewater treatment plant (WWTP) effluents are the most important source of contamination in water bodies [3]. Most of them are relatively polar substances that pass easily wastewater treatments and natural filtration systems. Their removal during wastewater treatment, very often inefficient, is usually estimated from the comparison of the concentrations detected at the influent and the effluent. However, wastewater samples are very complex matrices and the determination of microcontaminants at very low concentrations in them is a difficult task. In addition, since some EDs have been recently discovered as environmental contaminants, analytical methods for their determination in the various water bodies are scarce.

The following sections discuss the main properties, uses, and effects of the compound classes under review, the procedures described in the literature for their analysis in wastewaters, distinguishing between sample preparation and analysis, and the levels reported in both influent and effluent wastewater samples.

2 Specific Groups of EDs

2.1 *Pesticides*

Many pesticides and metabolites are suspected or claimed to be EDs. Residues of these chemicals and their transformation products are likely to occur in the environment as some of them are persistent and the ones that are relatively labile are extensively used and may end up in the aquatic media through direct run-off, leaching, careless disposal of empty containers, equipment washing, and effluents [4, 5].

According to the EDs compilation report made by the Institute for Environment and Health [1] a total of 228 biocidal products, such as insecticides, herbicides, and fungicides are established or potential EDs. These compounds belong to very different chemical classes; however, the majority of them correspond to the chemical group of the persistent organochlorine pesticides and metabolites, such as DDT and its metabolites, endosulfan, aldrin, dieldrin, heptachlor, chlordane, metoxychlor, mirex, lindane, hexachlorobenzene, and chlordane (kepone), among others.

The next most important group of pesticides in terms of endocrine-disrupting activity comprise the organophosphorous compounds. Inside this group common use pesticides and insecticides such as chlorfenvinphos, chlorpyrifos, diazinon, dichlorvos, dimetoate, fenitrothion, malathion, and parathion are included.

Different types of herbicides belonging to various chemical classes have also shown endocrine-disrupting effects. These substances are phenoxyacid compounds (2,4,5-T and 2,4-D), amides (acetochlor and alachlor), triazines (atrazine, cyanazine, and simazine), dinitroanilines (trifluraline), triazoles (amitrole), phenylureas (diuron), and ureas (linuron), among others.

Pesticides in general have been subjected to regulation and control for many years [6]. In the EU, for instance, the maximum admissible concentration of any individual pesticide (including the active substances and their relevant metabolites, degradation and reaction products) in drinking water and ground water is $0.1 \mu\text{g L}^{-1}$. For surface waters, the European Parliament has recently adopted environmental quality standards (EQS), which include both annual average (AA) and maximum allowable concentrations (MAC) [2]/105/EC). These EQS are very low for some compounds such as endosulfan (AA concentration of 5 ng L^{-1} in inland surface water and 500 pg L^{-1} in other surface waters) and less restrictive for other compounds such as alachlor, atrazine, diuron or simazine, with AA concentrations of 0.3, 0.6, 0.2, and $1 \mu\text{g L}^{-1}$, respectively, in both inland and other surface waters.

2.2 *Phthalates*

Phthalic acid esters (phthalates, PAEs) are widely used as polymer additives in the manufacture of plastics, rubber, and styrene to make them flexible and, with minor applications, in cosmetics, medical products, and insecticide carriers. PAEs can enter the environment through losses during manufacturing processes or by leaching from

final products since they are not chemically bonded to the polymeric matrix [7]. Phthalates have low acute and chronic toxicity, which generally decreases with increasing alkyl chain length [8]. The reproductive system, and in general the endocrine system, has been described to be affected by these kinds of contaminants [9, 10]; the endocrine-disruptive potency (relative to 17 β -estradiol) for a large number of phthalates ranges from 10⁻⁴ to 10⁻⁷ in in vitro and in vivo assays [11, 12]. The massive use, environmental ubiquity, and estrogenic character of phthalates have inspired disposal criteria, occupational exposure limits, and air and water concentration regulations. The US Environmental Protection Agency (EPA) has established a maximum admissible concentration in water of 6 $\mu\text{g L}^{-1}$ for di-(2-ethylhexyl) phthalate (DEHP) [13]. In surface water, the EU has established an annual average concentration of 1.3 $\mu\text{g L}^{-1}$ for the same compound [2].

2.3 *Bisphenol A*

Bisphenol A (4,4'-isopropylidenediphenol) is used for the production of polycarbonate plastic and epoxy resins, both utilized in a wide variety of applications in food and drink packaging, as powder paints, as additives in thermal paper, in dental fillings and as antioxidants in plastics [14]. It has also been used as an inert ingredient in pesticides, as a fungicide, antioxidant, as a flame retardant, rubber chemical, and polyvinyl chloride stabilizer. Human exposure to bisphenol A eventually released from these materials poses a health risk of increasing concern due to its endocrine-disrupting activity. The estrogenic as well as antiandrogenic activity of BPA has been shown both in in vitro and in vivo experiments [15–22]. BPA is approximately 10,000-fold less potent than 17 β -estradiol [23, 24]. In addition, some studies indicate that humans might be more sensitive than lab rats to bisphenol A: Human liver microsomes do not glucuronidate BPA as extensively as the rat liver microsomes do, and therefore, estrogen target tissues in humans may be subject to greater exposure to BPA [25].

2.4 *Alkylphenolic Compounds*

Alkylphenol ethoxylates (APEOs) are a class of low-cost nonionic surfactants, which have previously been used extensively in both households and industry. But, because of environmental considerations, they have voluntarily been phased out of the majority of household products during the last few decades.

However, as they are low-cost and are multifunctioning (detergents, deemulsifiers, solubility-, defoaming- and dispersion agents) they are still used in large quantities in the industry. Their primary use is in aqueous solutions and they enter easily into the environment through wastewater discharges, often through WWTPs. Alkylphenolic compounds with longer ethoxylate chains have not demonstrated estrogenicity, but are readily transformed into their shorter chain metabolites or carboxylates in the environment or in the WWTPs [26, 27].

Nonylphenol and octylphenol, along with their carboxylates and ethoxylates (with 1–2 ethoxylate units) have all been shown to be weak estrogenic compounds with an estrogenic effect 10^4 – 10^6 -times lower than that of 17β -estradiol [28–30].

However, as concentrations generally are 10^4 – 10^5 -times higher than endogenous estrogens, alkylphenolic compounds often have significant effect on the estrogenicity of natural water samples [31, 32, 33] and are as such of major environmental concern.

2.5 Perfluorinated Compounds

Fluorinated alkyl substances (FASs) are chemicals that contain atoms of both carbon and fluorine. Because of their unique properties, FASs compose a family of manmade chemicals that have been used for decades to make products that resist heat, oil, stains, grease, and water. FASs include perfluoroalkyl sulfonates (PFSSs), nonionic PFSSs, and perfluoroalkyl carboxylates (PFCs) (Table 1).

Table 1 Perfluorooctane sulfonate and related substances

Designation	Name	Formula
PFSSs	Perfluoroalkyl sulfonates	
PFBuS	Perfluorobutane sulfonate	$C_4F_9SO_3^-$
PFHxS	Perfluorohexane sulfonate	$C_6F_{13}SO_3^-$
PFOS	Perfluorooctane sulfonate	$C_8F_{17}SO_3^-$
PFDS	Perfluorodecane sulfonate	$C_{10}F_{19}SO_3^-$
Nonionic PFSSs	Nonionic perfluoroalkyl sulfonates	
PFOSA	Perfluorooctanesulfonamide	$C_8F_{17}SO_2NH_2$
<i>N</i> -EtFOSA	<i>N</i> -ethyl perfluorooctanesulfonamide	$C_8F_{17}SO_2NHC_2H_5$
<i>N</i> -MeFOSA	<i>N</i> -methyl perfluorooctanesulfonamide	$C_8F_{17}SO_2NHCH_3$
<i>t</i> -Bu-PFOS	<i>N</i> -tert-butyl perfluorooctanesulfonamide	$C_8F_{17}SO_2NHC_4H_9$
<i>N</i> -EtFOSE	2-(<i>N</i> -ethylperfluoro-1-octanesulfonamido)-ethanol	$C_8F_{17}SO_2N(CH_2CH_3)CH_2CH_2OOH$
7:1 FTOH	Pentadecafluoro-1-octanol	$CF_3(CF_2)_6CH_2OH$
PFCs	Perfluoroalkyl carboxylates	
PFBA	Perfluorobutyric acid	$C_3F_7CO_2H$
PFPeA	Perfluoropentanoic acid	$C_4F_9CO_2H$
PFHxA	Perfluorohexanoic acid	$C_5F_{11}CO_2H$
PFHpA	Perfluoroheptanoic acid	$C_6F_{13}CO_2H$
PFOA	Perfluorooctanoic acid	$C_7F_{15}CO_2H$
PFNA	Perfluorononanoic acid	$C_8F_{17}CO_2H$
PFDA	Perfluorodecanoic acid	$C_9F_{19}CO_2H$
PFUnA	Perfluoroundecanoic acid	$C_{10}F_{21}CO_2H$
PFDoA	Perfluorododecanoic acid	$C_{11}F_{23}CO_2H$
PFTDA	Perfluorotetradecanoic acid	$C_{13}F_{27}CO_2H$
PFHxDA	Perfluorohexadecanoic acid	$C_{15}F_{31}CO_2H$
PFOcDA	Perfluorooctadecanoic acid	$C_{17}F_{35}CO_2H$
8:2 FTCA	2H,2H-perfluorooctanoic acid	$C_6F_{13}CH_2CO_2H$
10:2 FTCA	2H,2H-perfluorododecanoic acid	$C_{10}F_{21}CH_2CO_2H$
8:2 FTUCA	2H-perfluoro-2-decenoic acid	$C_8F_{16}CHCO_2H$
10:2 FTUCA	2H-perfluoro-2-dodecenoic acid	$C_{10}F_{20}CHCO_2H$

Because of their high production worldwide, in October 2000 the US EPA proposed a significant new rule (SNUR) for 88 perfluorooctane (PFO)-related substances. Perfluorooctane sulfonate (PFOS) and related substances have also been on the agenda of the Organization for Economic Cooperation and Development (OECD) since the year 2000 [34]. Some legislation that generally applies to the release of substances to the environment may be relevant for PFOS. Thus, the IPPC Directive 96/61/EC includes fluorine and its compounds in the “indicative list of the main polluting substances to be taken into account if they are relevant for fixing emission limit values.” Very recently, PFOS has been included in the list of substances subject to review for possible identification as priority substances or priority hazardous substances [2]. PFOS is suspected to be a thyroid and reproductive disrupter.

Many of the degradation products of FASs have been found in the environment throughout the world, but PFOS and perfluorooctanoic acid (PFOA) are the compounds more widely detected and these compounds are environmentally persistent substances that have been detected worldwide in human blood, surface water, wastewater, soils, sediments, air, and biota samples. The origin of FASs in WWTPs is unclear, but it is possible that these chemicals are introduced into WWTPs through the cleaning and care of surface treated products, and their use in industrial processes.

2.6 *Pharmaceuticals*

During the last decade pharmaceutically active compounds (PhACs) have been considered to constitute a health risk for humans and aquatic ecosystems. Drugs are continuously being introduced in the environment, first of all, because variable amounts, up to 95% of the administered dose [35, 36], can be excreted unmetabolized and discharged into domestic wastewater. Unwanted or expired medications can be improperly disposed of directly in wastewater.

Several pharmaceuticals can therefore reach WWTPs in substantial amounts and a portion of them can be released into surface water. Moreover, several pharmaceuticals are excreted as conjugates and can release the active moiety by cleavage during treatment processes [37]. Pharmaceuticals are designed to have a specific action on humans and life organisms. Thus, their presence in natural waters can be expected to induce an undesirable effect on nontarget organisms exposed to them. In most cases, a clear cause-effect relationship has not been established yet, but subtle effects have been attributed, for instance, to tricyclic antidepressants (effects on development, spawning, and a wide array of other behaviors in shellfish, ciliates, and other aquatic organisms) and calcium-channel blockers (dramatic inhibition of sperm activity in certain aquatic organisms). Examples of pharmaceutical EDs are alprazolam (benzodiazepine, inhibits corticotrophin secretion), carbamazepine (anticonvulsant, affects circulating thyroid hormones), diazepam

(benzodiazepine, affects male reproductive organs), and sulfamethoxazole (antibacterial and antipneumocytic, alters thyroid function).

2.7 Estrogens

Estrogens are female steroid sex hormones based on a cholesterol structure and are mostly naturally excreted as their biologically inactive glucuronide and sulfate derivatives. Besides the main endogenous hormones estradiol, estrone, and estriol, other compounds have been synthesized to mimic their method of action, usually for medical reasons (e.g., ethynilestradiol and diethylstilbestrol). In addition several compounds naturally occurring in some plants have been found to cause estrogenic response, the so-called phytoestrogens.

The fate of estrogens during wastewater treatment has been investigated in several studies as WWTP effluents are the major source of contamination by estrogens in urban surface waters. In order to correctly estimate this burden a variety of analytical methods have been developed to assess the levels at which those compounds are present in the WWTP influents and effluents. Currently LC–(tandem)MS preceded by SPE (solid-phase extraction) is the most frequently applied technique [38].

3 Analysis of EDs in Wastewater

3.1 Sample Preparation

3.1.1 Pesticides

Occurrence of endocrine-disrupting pesticides in sewage water has been rarely studied as compared to other environmental matrices such as ground and surface waters. The analysis of these compounds in water is usually done with multiresidue methodologies that determine simultaneously different classes of organic pollutants. An overview of the methodologies reported to analyze endocrine-disrupting pesticides in sewage water based on liquid chromatography–mass spectrometry (LC–MS) is shown in Table 2.

Residues of persistent organochlorine pesticides have been analyzed so far in sewage water samples by gas chromatography (GC) coupled to mass spectrometry or electron-capture detectors (ECD) [40, 44–46]. Because of their physico-chemical properties these chemicals are not amenable to LC–MS analysis, therefore, they are not further discussed.

Table 2 Overview of LC-MS analytical methodologies developed for determination of endocrine-disrupting pesticides in sewage water

Reference	Sample preparation			LC		MS		Observations
	Volume	Method	Cartridge/load- ing column	Chromatographic column	Mobile phase	Detector (interface)	Acquisition mode	
[39] ^a	1 mL	On-line SPE	Hypersil GOLD (20 × 2.1 mm, 12 µm)	Hypersil GOLD TM (50 × 2.1 mm, 3 µm)	Water (0.1% FA)–MeOH	QqQ (ESI)	SRM	Matrix effects study wastewater samples Quantitation by internal standard addition 5 OC pesticides detected by GC–MS Matrix effects study Occurrence in real wastewater samples Quantitation by internal standard addition Matrix effects study
[40] ^b	300 mL	SPE	Strata C-18	Uptispher C18 3HDO (100 × 2 mm, 3 µm)	Water (10 mM NH ₄ Ac) – MeOH	QqQ (ESI)	SRM	
[41] ^c	200 mL	Automated SPE	Oasis HLB (200 mg)	Zorbax, SB-C18 (250 × 3 mm, 5 µm)	PI mode: Water (0.1% FA) – ACN, NI mode: Water – ACN PI mode: Water (0.1% FA) – ACN, (5% water + 0.1% FA) NI mode: Water (0.05% NH ₄ HCO ₃) – ACN	QTRAP (ESI) TOF (ESI)	SRM/EPI SCAN	Occurrence in real wastewater samples TOF analysis used for extra confirmation and identi- fication of unknowns

(continued)

Table 2 (continued)

Reference	Volume	Sample preparation		LC		MS	
		Method	Cartridge/ loading column	Chromatographic column	Mobile phase	Detector (interface)	Acquisition mode
[42] ^d	1 L	SPE	Oasis HLB (60 mg)	Neutral pesticides:	Neutral pesticides:	QqQ (ESI)	SRM
				Nucleodur C18 Gravity (125 × 2 mm, 3 µm)	Water (0.1% FA) – MeOH(0.1% FA)		
				Acidic pesticides: Polymer coated C18 (150 × 2 mm, 3 µm)	Acidic pesticides: Water (0.6% FA) – MeOH(0.6% FA)		
[43] ^e	400 mL	Automated sequen- tial SPE	Isolute C-18 ec (500 mg)	Superspher 100 RP-18 (250 × 2 mm, 4 µm)	Water (0.1% Acetic acid) – ACN	Q (ESI)	SIM
	300 mL		Oasis HLB (200 mg)				

ED pesticides included in the different multiresidue methods:

^aAtrazine, Simazine, Cyanazine, 2,4-D

^bDimethoate, 2-Imidazolidinethione, Tebuconazole, Hexaconazole

^cAtrazine, Simazine, Chlorfenvinphos, Diuron

^dAtrazine, Diazinon, Diuron, Metolachlor, Simazine

^eAtrazine, Simazine, 2,4-D, 2,4,5-T, Carbaryl, Diuron, Linuron, Trifluralin, Chlorfenvinphos, Chlorpyrifos, Alachlor, Propanil

FA formic acid; ACN acetonitrile; MeOH methanol; NH₄HCO₂ ammonium formate; NH₄Ac ammonium acetate; QqQ triple quadrupole; QTRAP quadrupole-ion trap; TOF time-of-flight; Q single quadrupole; SRM selected reaction monitoring; SIM single ion monitoring

The technique most routinely used to preconcentrate this type of pesticides is solid-phase extraction. Prior to the SPE procedure, sewage water samples are decanted [43] or filtrated through 0.45–0.7 μm glass fiber filters [39–42] in order to avoid clogging of the SPE cartridges by suspending matter. In some published methodologies the concentration and clean-up procedures are performed automatically using devices such as the ASPEC XL [41] and the AutoTrace [43]; however, a fully automated on-line method based on column-switching has been recently developed by Viglino et al. [39].

C18 or polymeric sorbents are usually the materials used in the different SPE procedures. Optimum sample volumes loaded into SPE cartridges range between 1 mL (in the on-line approach developed by Viglino et al. [39]) and 1 L [42, 47].

3.1.2 Phthalates

The very low levels of PAEs and the complex matrix of many environmental samples, including sewage water, make sample preconcentration necessary for a reliable determination of these compounds [48]. Several preconcentration techniques such as liquid–liquid extraction (LLE) and, especially, solid-phase extraction, have been developed. Using a hemicelles-based solid-phase extraction before liquid chromatography–atmospheric pressure chemical ionization–ion trap–mass spectrometry (LC–APCI–IT–MS) separation and quantitation, high concentration factors (up to 2,500) and thus low detection limits were achieved by López Jiménez et al. [13] for butyl benzyl phthalate (BBP), DBP, and DEHP in sewage water (see Table 3). A priority goal when analyzing phthalates is to reduce sample treatment to the minimal steps in order to avoid or minimize PAE contamination through laboratory materials like tubing, pipette tips, septa, solvents, etc. [13, 52, 53]. In recent years, solid-phase microextraction (SPME) has been extensively investigated to simplify sample treatment prior to GC analysis

Table 3 LC–MS methods for quantitative determination of phthalate esters in wastewaters

Matrix	Compound	Extraction	Separation and detection	LOD (ng L ⁻¹)	Reference
Industrial effluent	DBP, DMP, BEHP	SPE, Lichrolut EN	LC–APCI–MS	60–100	[49]
Urban waste-waters	DEP, DBP, DMP, DEHP	SPE, Lichrolut RP-18	LC–ESI–MS	10–110	[50]
Wastewaters	DEHP, BBP, DBP	SPE, Hemimicelles	LC–IT–MS	0.035–0.080	[13]
Urban waste-waters	DEP, BEHP, DOP, DMP, DBP, BBP	SPE, Lichrolut RP-18	LC–APCI–MS	12–57 ^a	[51]

^aLOQ Limit of quantification

LOD limit of detection; *DEHP* di-(2-ethylhexyl) phthalate; *DEP* diethylphthalate; *BBP* butyl benzyl phthalate; *DBP* di-*n*-butyl phthalate; *DPP* dipentylphthalate; *BEHP* bis(2-ethylhexyl) phthalate; *DOP* di-*n*-octylphthalate; *DMP* dimethyl phthalate

Table 4 LC-MS methods for quantitative determination of BPA in wastewaters

Matrix	Extraction	Separation and detection	LOD (ng L ⁻¹)	Reference
Urban wastewaters	SPE LiChrolut C18	LC-MS	52 ^a	[51]
Sewage treatment plant and receiving waters	SPE LiChrolut RP C18	LC-MS	50	[50]
Wastewater	SPE Oasis HLB	LC-MS/MS	2	[56]
Textile industry and corresponding WWTP effluents and receiving rivers	SPE Oasis HLB	LC-MS/MS	2	[57]

^a LOQ limit of quantification; LOD limit of detection

of PAEs [54, 55]. The use of SPME reduces the risk of secondary contamination during sample handling, however, some drawbacks such as low extraction yields, long extraction times, and/or poor precision still remain [13].

3.1.3 Bisphenol A

There are several analytical methods based on LC-MS for the determination of BPA among other organic contaminants and/or EDCs in water. Some of them, applied specifically in wastewater matrices, are gathered together in Table 4. Sample pre-treatment previous to LC-MS analysis usually comprises sample preconcentration and clean-up SPE. As is shown in Table 4, silica-based C18 and polymeric HLB are the sorbents commonly selected for SPE.

3.1.4 Alkylphenolic Compounds

Alkylphenolic compounds have been analyzed by a wide range of chromatographic analysis techniques, ranging from GC-MS over normal-phase liquid chromatography with fluorescence detection to reverse-phase liquid chromatography with MS or MS/MS detection. An overview of the sample pretreatment in a number of selected methods dealing with the analysis of wastewater is presented in Table 5. The majority of methods use solid phase extraction as preconcentration. However, in two methods liquid-liquid extraction (LLE) with dichloromethane was chosen, because of higher observed recoveries.

For the SPE methods, wastewater was filtered through 0.45 µm membrane filters, before loading onto cartridges. A variety of solid phases and eluents have been used, but the majority employ C₁₈ (Lichrolut or Isolut) with Oasis HLB as a close second.

3.1.5 Perfluorinated Compounds

One of the major problems associated with FAS analysis is background contamination in the analytical blanks. The use of glass bottles for the water sample collection

Table 5 LC–MS and LC–MS/MS methods used to determine alkylphenolic compounds in wastewater samples

Compounds	Extraction method	Separation and detection method	LC column	Mobile phase	MS system	LOD (ng L ⁻¹)	Reference
NPEOs, NPECs	SPE: ENVI-Carb; acidified	RP-LC-ESI-MS/MS [M+NH ₄] ⁺ , [M-H] ⁻	Zorbax C ₈ (150 × 4.6 mm, 5 μm)	MeOH + 0.1% formic acid/H ₂ O + 10 mM NH ₄ Ac	Quattro Ultima (Micromass)	10–50	[58]
APs, APEOs, APECs	SPE: poly(styrene–divinylbenzene) LLE; dichloromethane (APECs)	MM-LC-ESI-MS/MS	Mixed mode (size exclusion and RP)				[27]
APs, APECs, XAPECs, XAPs, APEOs, XAPEOs	SPE: LiChrolut C ₁₈ ; MeOH	RP-LC-ESI-MS [M+Na] ⁺ , [M-H] ⁻	LiChrospher 100 C ₁₈ (250 × 4 mm, 5 μm)	ACN–H ₂ O (NI) MeOH–H ₂ O (PI)	HP 1100 (Agilent)	20–100	[59]
APs	SPE: Isolute C ₁₈ ; propan-2-ol	RP-LC-ESI-MS/MS [M-H] ⁻	Nucleosil C ₈ (250 × 2 mm, 5 μm)	ACN–H ₂ O	Quattro LC (Micromass)	20–80	[60]
APEOs	LLE: dichloromethane	RP-LC-ESI-MS	Phenomex Luna (250 × 2 mm, 5 μm)	MeOH–H ₂ O (both with 5 mM NH ₄ Ac + 0.05 mM TCAA)	Esquire	1,000	[61]
APs, APEOs, APECs	SPE: Isolute C ₁₈ ; MeOH	RP-LC-ESI-MS [M+Na] ⁺ , [M-H] ⁻	Purospher STAR RP-18 (125 × 2 mm, 5 μm)	MeOH–H ₂ O	HP 1100 (Agilent)	5–100	[62]
APs, APECs, APEOs	SPE: Oasis HLB; MeOH/AcOH/ETA _c + 0.1% formic acid	RP-LC-ESI-MS/MS [M+NH ₄] ⁺ , [M-H] ⁻	Synergi Polar-RP (150 × 2 mm, 4 μm)	ACN–H ₂ O (NI) ACN–H ₂ O + 20 mM NH ₄ Ac	Quattro Micro (Micromass)	1–100	[57]
APEOs	SPE: Oasis HLB; MeOH	RP-LC-ESI-MS/MS [M+Na] ⁺ , [M-H] ⁻	Purospher STAR RP-18 (125 × 2 mm, 5 μm)	MeOH–H ₂ O	Quattro LC (Micromass)	2–50	[56]
APs, NPEOs	SPE: LiChrolut C ₁₈ ; MeOH/DCM	RP-LC-ESI-MS [M+Na] ⁺ , [M-H] ⁻	LiChrospher 100 C ₁₈ (250 × 4 mm, 5 μm)	ACN–H ₂ O (NI) MeOH–H ₂ O (PI)	HP 1100 (Agilent)	60–120	[50]

(continued)

Table 5 (continued)

Compounds	Extraction method	Separation and detection method	LC column	Mobile phase	MS system	LOD (ng L ⁻¹)	Reference
APs, APEOs, APECs	SPE: Oasis HLB: MeOH + DCM	RP-LC-ESI-MS/MS [M+NH ₄] ⁺ , [M-H] ⁻	Zorbax Eclipse XDB-C ₈ (150 × 2.1 mm, 5 µm)	MeOH/H ₂ O (both with 1.5 mM NH ₄ Ac)	API 3000 (Applied Biosystem)	0.04–12	[157]
APEOs, APECs, CAPECs	SPE: black carbon: MeOH + DCM/MeOH	RP-LC-ESI-MS	Altima (150 × 4.6 mm, 5 µm)	ACN-H ₂ O (both with 0.1 mM TEA)	–	–	[63]
APs, APEOs	SPE: Oasis HLB: MeOH-Et ₂ O	RP-LC-ESI-MS [M+Na] ⁺ , [M-H] ⁻	Hypersil Green ENV (150 × 4.6 mm, 5 µm)	ACN/MeOH-H ₂ O (both with 0.5% HOAc + 0.1% TEA)	SSQ-7000 (Finnigan)	100–500	[64]
APs, APEOs	SPE: C ₁₈ : AcOH + MeOH/MTBE	RP-LC-ESI-MS/MS	–	–	–	10–20	[65]
APs	SPE: LiChrolut EN: ACN	RP-LC-ESI-MS [M-H] ⁻	Lichrospher 100 RP-18 (250 × 4 mm, 5 µm)	ACN-H ₂ O + 0.5% HOAc	HPL100 (Agilent)	8–300	[66]
APs	SPE: Oasis HLB: 2-propanol-MTBE	RP-LC-ESI-MS/MS [M-H] ⁻	Phenomex Luna (100 × 2 mm, 3 µm)	ACN-H ₂ O (both with 1 mM NH ₄ -formate)	1100 LC-MSD Trap	1–20	[67]
APs, APECs	SPE: SDS hemicelles-based: MeOH	RP-LC-ESI-MS [M-H] ⁻	Hypersil AV-1634 C ₁₈ (150 × 4.6 mm, 5 µm)	MeOH-H ₂ O	1100 LC-MSD Trap	99–165	[68]
APs	SPE: LiChrolut C ₁₈ : Et ₂ O:MeOH	RP-LC-APCI-MS [M-CH ₃] ⁺	Gemini C ₁₈ (150 × 4.6 mm, 5 µm)	ACN-H ₂ O + 1% HOAc	ZQ 2000 (Micromass)	26–28	[51]

ACN acetonitrile; AcOH acetic acid; APs alkylphenols; APECs alkylphenoxy carboxylates; APEOs alkylphenol ethoxylates; DCM dichloromethane; ESI electrospray; EtAc ethyl acetate; Et₂O diethyl ether; HOAc acetic acid; LC liquid chromatography; MeOH methanol; MM mixed mode; MS mass spectrometry; MTBE methyl-*t*-tert-butylether; NH₄Ac ammonium acetate; NI negative ionization; PI positive ionization; RP reverse phase; SPE solid-phase extraction; TCAA trichloroacetic acid; TEA triethylamine

must be avoided because the target analytes in aqueous solutions may bind to the glass. The samples are usually collected in polyethylene or propylene bottles rinsed with different solvents prior to use [69–71]. Filtration of water is not considered a viable sample preparation step because, as reported in several studies [71, 72], some filters remove the analytes from water and some filters themselves present levels of perfluorinated alkyl substances (PFASs). In order to solve the drawbacks presented by filtration, centrifugation has been selected as an alternative pretreatment step. SPE is the technique most extensively used for the extraction of PFASs from environmental water samples. SPE extraction has usually been carried out with cartridges in the off-line mode. Because of the different polarities of the analytes, their extraction requires different strategies. The ionic short chain (C4–C6) compounds [perfluorocarboxylic acids (PFCAs) and perfluorosulfonic acids (PFSAs)] require moderately polar media (Oasis wax SPE) for efficient extraction. For longer chains, less polar or nonpolar SPE phases (C18 and Oasis HLB) may be applied. The nonionic compounds have usually been extracted from the matrix by nonpolar media (C18), and also by moderately polar media (Oasis HLB and Oasis WAX SPE) [69, 70]. LLE has also been used for extraction of FASs from wastewater samples. By using LLE, it is possible to extract samples with or without prior filtration and hence it is possible to determine concentrations in the aqueous fraction or the whole sample [73]. The extraction procedures described above are often time consuming and imprecise, as additional clean-up steps are usually required. To overcome these drawbacks and simplify the sample pretreatment procedures, direct LC–MS/MS analysis of municipal wastewater has been tested by Schultz et al. [71]. These authors discarded the use of SPE as a sample concentration step because critical SPE experiments with C18 cartridges yielded low recoveries for some target analytes. This technique allowed an increase in the sensitivity that permitted the detection of FASs at environmentally relevant levels in influent and effluent wastewaters.

3.1.6 Pharmaceuticals

Several methods have been developed for the determination of drugs and their metabolites in the lower ng L⁻¹ range. Most of them use SPE followed by analysis by GC–(tandem)MS or LC–(tandem)MS. Several review articles have been published on this topic: [74–76].

The vast majority of the methods developed in the past focus on specific therapeutic classes [77–82], with antibiotics being the most studied ones due to the concern about their potential for antibiotic resistance [83–85]. Because of the elevated polarity of some groups of pharmaceuticals, LC–MS and LC–(tandem)MS is often the technique of choice for analysis of these compounds in wastewater since it avoids the derivatization step required in GC–MS methods, specially for acidic drugs.

A survey of the most recent and representative multiresidue LC–MS/MS methods developed for the determination of regularly used pharmaceuticals in wastewater is given in Table 6.

Table 6 Summary of the most representative multiresidue LC-MS/MS methods for quantitative determination of pharmaceuticals in wastewater

Compounds	Matrix	Pretreatment	Extraction	Elution solvents	Column	Mobile phase	MS system	LOD (ng L ⁻¹)	Reference
30 Compounds: Antibiotics, anticancer, antiinflammatories, bronchodilator; cardiovascular, CNS, gastrointestinal drugs; diuretics, estrogens, and lipid regulators	Urban wastewater	Two methods: (1) neutral drugs at pH = 7, (2) another group at pH = 2	(A) Lichrolut EN (B) Oasis MCX	(1) MeOH/ethyl acetate (2) MeOH/NH ₄ OH and NaOH in MeOH	C8	ESI(+) Aq. formic acid/ACN ESI(-)Aq. TEA/ACN	Triple quadrupole	30–70	[86]
16 Compounds: Antibiotics, β -blockers, psychiatric drugs, antiinflammatories	Hospital wastewater	pH adjustment (pH 7)	Oasis HLB	MeOH	C18	ESI(+) Aq. formic acid/ACN ESI(-) Water/CAN	Triple quadrupole ESI	0.3–5.6 (LOQ)	[13]
28 Compounds: Antiinflammatories, lipid regulators, anti-ulcer agents, antihistaminics, antibiotics, and β -blockers	Urban and industrial wastewater	Natural water pH	Oasis HLB	MeOH	Acquity UPLC™ BEH C18	ESI(+) ACN/MeOH (2:1)/NH4Ac/HOAc ESI(-) MeOH/ H2O	Triple quadrupole ESI-TOF	100–250	[87]
28 Compounds: Antiinflammatories, lipid regulators, anti-ulcer agents, antihistaminics, antibiotics, and β -blockers	River and wastewater	Natural water pH	Oasis HLB	MeOH	C18	ESI(+) ACN/MeOH (2:1)/NH4Ac/HOAc ESI(-) MeOH/ H2O	Triple quadrupole ESI	1	[88]

12 Compounds: Hormones, hormone conjugates and mac- rolide antibiotics	Wastewaters	No pretreat- ment	DVB-phobic Speedisk + clean- up with SEC	SPE: Tert- butyl methyl ether/ MeOH	RP-MAX RP-18	MeOH/H ₂ O (hormones) NH ₄ Ac aq./ ACN (anti- biotics)	Triple quadru- pole ESI	25–50 (LOQ in WW)	[89]
11 Compounds: Antibiotics – tetra- cyclines and sulfona- mides	Wastewaters	Na ₂ EDTA and citric acid pH < 3	Oasis HLB	MeOH	C18	ESI(+) 0.1% aq. HCOOH/ ACN	Triple quadru- pole and ion trap ESI	20–50	[90]
12 Compounds: Antiinflammatorys, lipid regulators and triclosan	Surface and wastewa- ters	Acidified pH = 2–2.5	Oasis HLB	MeOH	Phenyl– Hexyl	ESI(–) MeOH/ H ₂ O // sol- vent A with TrBA and acetic acid	Triple quadru- pole and ion trap ESI	0.1–5.2 (LOQ)	[82]
14 Compounds: Lipid regulators, analgesics and antiinflammato- ries, antineoplastics, vasodilators, and antiepileptic	Wastewaters	Acidic comp. (anal., antiinfl., lipid regula- tors) pH = 2 Basic comp. pH = 7.5	Lichrolut RP-18 (acidic) Supelco LC-18 (basic)	MeOH (acidic and basic)	RP-18 (acidic and basic)	ESI(–) (acidic) ACN/ MeOH (40:60)// 20 mM NH ₄ Ac ESI (+) (basic) ACN in aq.	GC-MS (acidic) and triple quadropole ESI (acidic and basic)	4–47	[91]
27 Compounds: Neutral and acidic pharma- ceuticals, EDCs and PCPs	Surface and wastewa- ters	Acidified pH = 2	Oasis HLB	MeOH/ MTBE (10:90, v/v)/ MeOH	C12	ESI(+) and ESI(–) Aq. formic acid/ MeOH	Triple quadru- pole ESI/ APCI	15–500	[92]
Ten compounds: Antiphlogistics, anti- inflammatorys, anti- diabetics, antiulcer, and psychiatric drugs	River and wastewa- ters	pH adjust- ment (pH = 7–7.5)	Isolute C18	MeOH	RP-18	ESI(+) 20 mM NH ₄ Ac in H ₂ O/ACN	Triple quadru- pole ESI	1–60	[78]

(continued)

Table 6 (continued)

Compounds	Matrix	Pretreatment	Extraction	Elution sol- vents	Column	Mobile phase	MS system	LOD (ng L ⁻¹)	Reference
19 Compounds: Antibiotics - penicil- lins, chloramphenicol, sulfonamides, and macrolides	Surface and wastewa- ters	Acidified (pH = 3)	Lichrolut EN and C18	MeOH	RP-18	TET 20 Mm aq. oxalic acid/CAN The others 10 mM aq. NH ₄ Ac/ ACN	Triple quadru- pole ESI	0.6–15 (LOQ)	[83]
Four Beta blockers, an antiepileptic drug and three fluoroquinolone antibiotics	Surface and wastewa- ters	Natural water pH	Oasis HLB	MeOH	XDB-C18	ACN and 1% acetic acid	Triple quadru- pole ESI	3.5 ng L ⁻¹ in sew- age infl- uent	[93]
29 Multiclass pharma- ceuticals: NSAIDs, lipid regulators, psy- chiatric drugs, anti- histaminics, antitumor agent, antibiotics, and β-blockers	Surface and wastewa- ters	Natural water pH	Oasis HLB	MeOH	RP-18	NI mode, (A) MeOH (B) water PI (A) a mixture of ACN- MeOH (2:1) (B) NH ₄ Ac 5 mM/HAc at pH 4.7	Triple quadru- pole ESI	LOQ 1–160 ng L ⁻¹	[87]
38 Pharmaceuticals and 10 of their most fre- quent metabolites, six pesticides, and two disinfectants	Wastewater	pH 8	Oasis HLB	MeOH	RP-18	PI (A) CAN (B) water with 0.1% formic acid NI (A) ACN (B) Water	LC-QTRAP-MS	0.04 and 50	[41]

Nine Basic pharmaceuticals	Wastewater	pH adjustment (pH = 7–7.5)	Speedisk phenyl and a NH ₂ solid-phase extraction tube for sample clean-up	MeOH	Pentafluorophenyl column (PFP-column)	Eluents were H ₂ O/ACN [95]:5, by vol. (A) and 5:95, by vol. (B)]	Triple quadrupole ESI	LOQ 0.05–10	[94]
12 Antidepressant compounds	Wastewater	Acidification	Oasis HLB	70% methanol in 2% acetic acid	XDB-Phenyl column	Water with 0.1% (v/v) formic acid * ACN	Triple quadrupole ESI	0.19–0.45	[95]
20 Pharmaceutical compounds	Wastewater	pH 4	Strata-X cartridges	MeOH	RP-18	(A) 20:80 ACN:water with 0.1% ammonium acetate (v/v) at pH 6.2 (B) 80:20 ACN:water with 0.1% ammonium acetate (v/v)	Triple quadrupole ESI	0.002–0.855 in influent and 0.001–2.478 in effluent	[96]

The most recent analytical methodologies address the simultaneous extraction of all target compounds in a single SPE step using Oasis HLB cartridges working in general at neutral pH. The main advantages of the extraction with polymeric sorbents at neutral pH are: (1) simplified sample handling and no clean-up for the removal of humic and fulvic acids, (2) less risk of acidic hydrolysis of other compounds in multianalyte analysis procedures, (3) and possibility of on-line extraction using large sample volumes.

C18 cartridges have been also widely used. However, when using this material, depending on the nature of the compounds investigated, sample pH adjustment prior to extraction is usually required.

Less common cartridges used are Lichrolut ENV+, Oasis MCX, and StrataX. The first one is generally recommended for the extraction of polar organic compounds at low pH values, but it can also retain neutral drugs at pH 7, such as carbamazepine (antiepileptic) and macrolides, through hydrophobic interactions. Oasis MCX has been used to extract acidic, basic, and neutral compounds at low pHs. In this material, basic compounds are retained through cation exchange mechanisms and acidic and neutral ones through reversed-phase interactions. Finally, StrataX, as Oasis HLB, can retain a wide spectrum of analytes through hydrophilic and hydrophobic interactions.

However, the simultaneous extraction of several groups of compounds often requires a compromise which does not obtain the best conditions for each compound. An alternative is to use two or more SPE materials operating in series to classify target analytes according to their physico-chemical properties. The advantage of these procedures is that better conditions are achieved for the various target analytes, but they are time consuming.

Other relevant improvements in the extraction of pharmaceuticals from wastewater come from new materials such as the molecularly imprinted polymers (MIPs). An example is the method recently developed by Gros et al. [97] to determine eight β -blockers in wastewaters. The advantages offered by MIPs in terms of selectivity and specificity were compared with the polymeric material most commonly used Oasis[®] HLB. Even though recoveries achieved with both sorbents were similar, MIPs provided lower LODs than Oasis[®] HLB, due to their specificity for the target analytes and closely related analogues.

Another attractive process is on-line SPE. In on-line SPE the whole sample is transferred to the chromatographic system thus increasing the method sensitivity and reducing the volume of sample required. Other advantages are high reproducibility and less risk of contamination. An example is that presented by Quintana et al. [98] for analysis of nonsteroidal antiinflammatory drugs (NSAID) in surface water, wastewater, and urine.

3.1.7 Estrogens

Wastewater samples are complex matrices composed of organic matter, bacteria, and all kinds of chemical pollutants which are present at high concentrations and may interfere during all steps of the analytical procedure. The pretreatment of the sample is thus very important, especially when very low concentrations have to be detected, as is the case for estrogens.

Table 7 shows examples of complete analytical methods based on LC-MS used for the analysis of estrogens in wastewater samples. As can be seen, the most studied compounds in wastewater samples are the natural hormone estradiol (E2) and its metabolites, estrone (E1) and estriol (E3). A variety of glucuronide and sulfate derivatives (conjugates) of the previously mentioned hormones, hydroxyl estrone (HE) and the synthetic compounds ethynyl estradiol (EE), diethylstilbestrol (DES), and mestranol (MES) have also been analyzed. Concerning phytoestrogens, daidzein (DAI), genistein (GEN), and biochanin-A (BIO) have been reported in a few studies, whereas only one work has addressed the analysis of 4',6,7-trihydroxyisoflavone (TRI), formononetin (FOR), coumestrol (COU), glycitein (GLY), and the two conjugates daidzin and genistin.

Extraction of these compounds from environmental samples can be performed by sorptive or adsorptive techniques. Adsorptive SPE is very versatile and allows good extraction recoveries with cartridges containing varying adsorbents. Polymeric (e.g., Oasis HLB), modified silica-based (e.g., C18) and graphitized carbon-based (e.g., Carboglyph) adsorbents are commonly used for wastewater samples (see Table 7). An important advantage of this technique is the possibility of automation, yielding highly robust and sensitive methods [119]. Adsorptive disks have also found application [89, 104, 105]. Some authors include an additional clean-up procedure after SPE (e.g., GPC, SPE, LLE) to obtain more pure extracts. One example of the application of a sorptive extraction technique is that reported by Mitani et al. [122] based on fully automated in-tube solid-phase microextraction coupled with LC–MS/MS with which limits of detection (LOD) in the low ng/L range for the main estrogens have been achieved.

3.2 Instrumental Analysis

3.2.1 Pesticides

The procedures for chromatographic separation are very similar and most of them rely on the use of reversed-phase C18 columns and the application of a gradient binary mobile phase (see Table 2). Ionization of the compounds is performed with an electrospray ionization source (ESI) working in the negative ionization (NI) mode when analyzing acidic pesticides such as phenylureas and phenoxyacid compounds, and in the positive ionization (PI) mode when analyzing the rest.

The main drawback of using this interface is the enhancement or suppression of the analyte MS signal due to matrix effects. Each pesticide may be affected to a different extent by this phenomenon in complex matrices such as sewage water, even from sample to sample; therefore quantitation of the compounds by the internal standard method [39, 42] or by the standard addition method [40] is indispensable to achieve reliable results [42]. Most of the peer-reviewed methodologies evaluate matrix effects in wastewater [39–42]. Ion suppression occurs for practically all analytes investigated, reaching up to 65% in the case of diazinon [42]; signal enhancement has been reported by Baugros et al. [40] for a series of endocrine-disrupting fungicides and OP compounds.

Table 7 LC–MS and LC–MS/MS methods for analysis of estrogens in wastewater influent (I) and effluent (E) samples

Compounds	Matrix	Extraction SPE		LC separation		Mobile phase	Interface	Detection	Reference
		material	Clean-up	Column					
E1, E2, E3, EE	E	Oasis HLB	–	Zorbax Extended-C18 (50 × 2.1 mm, 5 µm)		ACN/Water (10 mM NH ₄ OH)	ESI(NI)	MS/MS	[99]
E1, E2, E3, EE, EIS	I, E	tC18	GPC-SEC + SPE (NH ₂)	Gemini C18 (100 × 2 mm, 3 µm)		MeOH/Water (0.1% NH ₄ OH)	ESI(NI)	MS/MS	[100]
E2, EE, E1	E	Oasis HLB	–	Acquity UPLC BEH C18 (100 × 1 mm, 1.7 µm)		ACN/Water (0.1% formic acid)	ESI(PI)	MS/MS	[101]
E1, E2, E3, EE	E	Oasis HLB	–	Synergi RP-C12 (250 × 4.6 mm, 4 µm)		MeOH/Water (0.1% formic acid)	APCI(PI)	MS/MS	[102]
E1, E2, E3, EE, DAI, GEN, BIO	I, E	IST C18 + Sep-Pak®Plus NH ₂ Propyl	–	Purospher STAR-RP-18e (125 × 2 mm, 5 µm), and Acquity C18 (50 × 2.1 mm, 1.7 µm)		ACN/Water	ESI(NI)	MS/MS	[103]
E1, E2, E3, EE, HE, MES, +3 conj	E	DVB-phobic (Speedisk)	GPC-SEC	Synergi RP-MAX (150 × 2 mm, 4 µm)		MeOH/Water	APCI/ESI(NI)	MS/MS	[89, 104]
E1, E2, E3, EE	E	PolarPlus C18 (Speedisks)	–	BetaBasic C18 (150 × 2.1 mm, 3 µm)		ACN/Water (10 mM <i>N</i> -methylmorpholine)	ESI(NI)	MS/MS	[105]
E1, E2, E3, EE	I, E	Carbograph-4	–	Alltima C-18 (250 × 4.6 mm, 5 µm)		ACN/water (+MeOH/NH ₃ post column)	ESI(NI)	MS/MS	[106, 107]
E1, E2, EE	I, E	Bakerbond C18	SPE (silica gel)	RP-C8 Hypersil MO5 (100 × 2.1 mm, 5 µm)		ACN/(MeOH)/water	ESI(NI)	MS/MS	[108]
DAI, GEN, BIO, FOR, GLY, TRI, COU + 2 conj	I, E	Oasis HLB	–	Alltima C-18 (25 × 4.6 mm, 5 µm)		ACN/water (10 mM TFA)	ESI(PI)	MS/MS	[109]
E1, E2, E3, EE + 5 conj	I, E	Sep-Pak Vac tC18	–	Synergi 4 µ Hydro-RP (75 × 2 mm, 4 µm)		MeOH/water	ESI(NI)	MS	[110]

6 conj	I, E	Oasis HLB	Anion exchange (Nucleogen 60–7, 125 × 4.6 mm)	Zorbax Extend-C18 (100 × 2.1 mm, 3.5 μ m)	ACN/water (13.5 mM NH ₄ OH)	ESI(NI)	MS/MS	[111]
E1, E2	I, E	LiChrolut EN + C18	Immunoaffinity extraction	Betasil C18 (150 × 2.1 mm, 3 μ m)	ACN/water	ESI(NI)	MS	[111, 112]
E1, E2, E3, EE + 9 conj	I, E	Oasis HLB	SPE (Sep-Pak Plus Florisil and NH ₂)	Zorbax Extend-C18 (150 × 2.1 mm)	ACN/water (1 mM NH ₄ OH)	ESI(NI)	MS/MS	[113]
DAI, GEN, BIO	I, E	Oasis HLB	–	Alltima C-18 (25 × 4.6 mm, 5 μ m)	ACN/water (10 mM TFA)	ESI(PI)	MS/MS	[114]
E1, E2, E3, EE	I, E	Oasis HLB	–	Alltima C-18 (25 × 4.6 mm, 5 μ m)	ACN/water (+NH ₃ post column)	ESI(NI)	MS/MS	[114]
E1, E2 (α + β), E3, EE + 10 conj	E	Autoprep EDS-1	SPE (Florisil)	Zorbax Extend-C ₁₈ (150 × 1 mm, 3.5 μ m)	ACN/water (100 mM TEA)	ESI(NI)	MS/MS	[115]
E1, E2, EE	E	C18	LLE + SPE (Florisil)	Hypersil BDS C ₁₈ (250 × 2.1 mm, 3 μ m)	ACN/water	ESI(NI)	MS/MS	[116]
E1, E2, E3 + 8 conj	I, E, U	Carbograph 4	–	Alltima C-18 (25 × 4.6 mm, 5 μ m)	ACN/water (10 mM for- mic acid)	ESI(NI)	MS/MS	[117]
E1, E2, E3, EE + 8 conj	I, E	Carbograph-4	–	Alltima C18 (250 × 4.6 mm, 5 μ m)	ACN/water (+NH ₃ post column)	ESI(NI)	MS/MS	[118]
E1, E2, E3, EE, DES, MES	E	on-line PLRP-s	–	LiChrospher 100 RP-18 (250 × 4 mm, 5 μ m)	ACN/water	ESI(NI/PI)	MS	[119]
E1, E2, E3, EE, DES	I, E	LiChrolut RP-18	–	LiChrospher 100 RP-18 and 60 RP-Select B (250 × 4 mm, 5 μ m)	ACN/water	ESI(NI/PI)	MS	[120]
E1, E2, E3, EE	I, E	ENVI-CARB	–	Alltima C-18 (250 × 4.6 mm, 5 μ m)	ACN/water	APCI(PI)	MS/MS	[121]

GPC Gel permeation chromatography; SEC Size exclusion chromatography

Mass spectrometric detection of these substances in sewage water is mainly done with triple quadrupole analyzers (QqQ) operating in the selected reaction monitoring (SRM) mode. Some authors have also used single quadrupole instruments to identify the target analytes [43, 123]; however, these instruments do not offer enough selectivity to determine trace levels in such complex matrices.

Martinez-Bueno et al. [41] developed an analytical methodology based on the use of a hybrid quadrupole-ion trap instrument (QTRAP) operating under SRM and enhanced product ion (EPI) mode for target analyte identification and unequivocal confirmation of simazine, atrazine, diuron, and chlorfenvinphos, and a time-of-flight (TOF) spectrometer for extra confirmation and identification of nontarget or unknown metabolites.

3.2.2 Phthalates

The most common technique for PAE determination in environmental samples has been gas chromatography with detection through electron capture (ECD) and/or mass spectrometry. Reversed phase LC with UV or MS detection, combined with SPE, has been also extensively used for PAE analysis [13, 124, 125] (see Table 3). LC-ESI-MS based on the formation of sodium adducts can provide molecular ion information and has been found to be a reliable tool for quantitative analysis of phthalate esters in various matrices [53]. Phthalate esters show a characteristic peak at m/z 149 (protonated phthalic anhydride), except for the dimethyl phthalate (DMP) that shows the most abundant fragment at m/z 163 ($[M+H-2CH_3]^+$) [51–53]. An LC-ESI-MS/MS study showed that the two major pathways of phthalate fragmentation in ESI(+)-MS/MS are similar to those occurring in electron ionization (EI)-GC-MS [53]. One reaction is dominated by the loss of one of the substituents leading to the formation of monoester sodium adducts and another by the formation of sodiated phthalic anhydride ions $[(C_8H_4O_3Na)^+]$ with m/z 171. Study with H^+ as mobile phase modifiers showed that protonated molecular ions are more reliable for the identification of phthalates by the MS-MS technique [53].

3.2.3 Bisphenol A

The methods most frequently used for the determination of BPA in water samples are GC and LC, both coupled to MS. In recent years, different bioassays have also been developed as a cheap and quick alternative to traditional chromatographic methods for high-throughput analysis or real-time monitoring. Some of these bioassays have been applied to the determination of the potential biological impact of bisphenol A, based on the measurement of toxicity [126] or estrogenicity [127], whereas other studies are based on immunoassays [128, 129], and immunosensors [130, 131].

LC-MS analytical methods described for the determination of BPA and other organic contaminants in wastewater matrices are listed in Table 4. Particularly interesting is the analytical method developed by Zafra-Gomez et al. [51] for the simultaneous determination of different EDCs including bisphenol A and its

chlorinated derivatives (monochloro, dichloro, trichloro and tetrachlorobisphenol A). BPA shows a base peak at m/z 227 which corresponds to the molecular ion $[M-H]^-$ measured in negative ion mode. Using an ESI interface, the main fragment ion, in addition to the base peak, is observed at m/z 212, which results from the cleavage of one of the CH_3 groups. Using APCI [132], additional fragments are observed at m/z 211 $[(C_6H_4)C_3H_6(C_6H_4OH)]^-$ and m/z 133, which is assigned to $[(C_6H_4)C(CH_3)CH_2]^-$. Quantitative LC–MS/MS analysis of BPA is carried out in the SRM mode with two characteristic precursor–product ion transitions: $227 \rightarrow 133$ and $227 \rightarrow 211$ [56, 57]. Method detection limits achieved with LC–MS and LC–MS/MS-based methods have been around 2 and 50 ng L⁻¹, respectively.

3.2.4 Alkylphenolic Compounds

All but one of the methods reviewed employ reverse-phase (RP) chromatographic separation for analysis of alkylphenolic compounds in wastewater (see Table 5). The remaining method uses a mixed mode column utilizing both size exclusion and reverse-phase principles.

When working with reverse-phase separation it is not possible to separate APEOs with different numbers of ethoxylate (EO) units, but separation between OP and nonylphenol NP as well as their ethoxylates and carboxylates is obtained. Normal-phase chromatography, on the other hand, can separate APEOs as a function of their number of ethoxylate units, but can not separate the different alkylphenol species.

Many methods report the sum of long-chained APEOs instead of the single species, obtaining higher intensities and thus sensitivity. But even though the APEOs coelute, it is still possible to extract individual peaks when using MS or MS/MS detection.

ESI has been used almost exclusively because it has demonstrated better sensitivity than APCI towards the short-chain APEOs and the alkylphenols (APs) themselves. When using ESI in positive mode the APEOs almost solely form sodium adducts and these are often chosen as the parent ion. Unfortunately there is still rather poor sensitivity towards alkylphenol mono- and di-ethoxylates (APEO₁₊₂) which is often improved by measuring $[M+NH_4]^+$ instead of $[M+Na]^+$.

Limits of detection are reported from 1 to 1,000 ng L⁻¹ with the lowest obtained in the SRM mode, using MS/MS.

3.2.5 Perfluorinated Compounds

As mentioned before, sample contamination can occur during sample pretreatment and also depends on the instrumental technique used. It is recommended to replace the PTFE HPLC tubing with stainless steel and PEEK [71]. According to the literature of wastewater reviewed, LC separation of fluorinated compounds has been mainly carried out with C18 columns; only one study reported the use of C8 columns.

Regarding mobile phases, mixtures of acetonitrile-water and methanol-water, often modified with ammonium acetate (2 mM) to improve LC separation and MS sensitivity have usually been used. LC in combination with MS or tandem MS has been the technique used for analysis of FASs in wastewater samples. MS detection is performed with an ESI interface operating in the negative ion mode. In the fragmentation pattern of FASs, the deprotonated molecules $[M-H]^-$ are the predominant ions. Typical ions and fragmentations monitored for PFOS and related substances correspond to $[SO_3]^-$, $[FSO_3]^-$, and $[M-SO_3]^-$ ions. For perfluorosulfonamide (PFOSA) and PFOA, $[SO_2N]^-$ and $[MCOOH]^-$ ions are the most abundant ones, respectively [69, 70, 73]. Today it is widely accepted that ESI ionization is susceptible to matrix effects. Matrix effects result from coeluting residual matrix components affecting the ionization efficiency of the target analytes and can lead to erroneous results. Wastewater matrixes are complex and their analysis typically requires sample enrichment and reduction of particle load. In the quantitative analysis by LC–(tandem)-MS, several strategies will be appropriate to diminish the analytes signal suppression of the analyte. The use of internal standards compensates well the matrix effects [70, 71, 73]. Other methodologies such as dilution of the extracts and adding a filter step before LC–MS analysis have been tested in the analysis of FASs in wastewater samples, but these methodologies did not compensate the matrix effects in influent wastewater samples [69].

3.2.6 Pharmaceuticals

LC–(tandem)MS via atmospheric pressure ionization (API) interfaces, especially ESI, has become the analytical technique of choice for analysis of pharmaceuticals in wastewater, since most of them are polar compounds, not amenable to GC analysis.

Chromatographic separation is in general achieved by means of C18 columns using acetonitrile, methanol, or mixtures of both solvents, and water as the mobile phase. To increase retention efficiency and MS sensitivity, mobile phase modifiers, buffers and acids, are recommended and widely used in concentrations from 2 to 20 mM (see Table 6), since it has been observed that higher concentrations can lead to a reduction of the signal intensities [133].

Generally, for acidic pharmaceuticals, which are determined in the NI mode, organic solvents and ultrapure water or aqueous ammonium acetate are preferred; whereas for neutral and basic compounds, analyzed in the PI mode, the use of buffers or modifiers is more common at neutral and acidic pH. When mobile phases are the same for both ionization modes, which are specific cases, pH of the mobile phases are usually adjusted to acidic or neutral conditions.

A critical aspect in quantitative analysis with LC–(tandem)MS is the occurrence of matrix effects producing ion suppression due to the presence of organic and inorganic components in the sample that coelute with target analytes. Since matrix components can be rather variable between samples—in nature and in amount—their effects are difficult to be predicted. Matrix effects may be reduced with proper

pretreatments and efficient chromatographic separation, but the best manner to compensate them is using internal standards or standard addition. These methods, however, increase significantly the cost and time of analysis.

With respect to the type of MS detector, QqQ instruments have been by far the most widely used analyzers for quantitation of pharmaceuticals in wastewater. Nevertheless, in recent years, hybrid quadrupole-linear ion trap (Q-trap) and quadrupole-time of flight (Q-TOF) detectors have also been tested for this purpose (see Table 6).

Q-trap instruments outperform the conventional ion trap systems and deliver the functionality of QqQ [41, 95].

The main advantage of the TOF analyzers is the availability of full-scan mass spectra throughout each chromatogram and the accurate mass measurements that provide qualitative information for unequivocal identification of analytes present in the samples [134]. The most important drawback for quantitative measurements is the effective linear dynamic range which is significantly lower than the quadrupole instruments dynamic range (typically four orders of magnitude). Marchese et al. [80] compared the performance of a QqQ and a hybrid Q-TOF instrument for the determination of analgesics in water. The LOQs obtained with the TOF experiment were approximately three to five times higher than those obtained using the QqQ system operating in SRM mode.

More recently, Petrovic et al. [87] have developed a new multiresidue method based on ultraperformance liquid chromatography (UPLC–Q–TOF–MS/MS) for screening and confirmation of 29 pharmaceutical compounds. In another work by the same group, 32 compounds were included and the LOQs ranged from 0.1 to 15 ng L⁻¹ in tap water, and from 2 to 300 ng L⁻¹ in wastewater [135].

3.2.7 Estrogens

The analysis of estrogens by LC–(tandem)MS allows their selective and sensitive determination without previous derivatization (as in GC–MS). Separation of the analytes is mostly performed using C18-based reversed-phase columns. The mobile phase usually consists of acetonitrile or methanol with water. Some authors prefer methanol over acetonitrile affirming that a significant increase of signal intensity occurs when using methanol [100, 111, 118], although in most cases acetonitrile is reported to give better results. As the interface to MS detection ESI in NI has found larger application than APCI operated in PI (see Table 7). However, several authors have reported problems with matrix effects and prefer to employ APCI [89, 102, 121]; unfortunately APCI does not ionize the more polar estriol and sulfate derivatives efficiently [89].

The addition of basic or acidic compounds to the mobile phase is performed in about half of the methodologies reviewed. Ionization of estrogens using ESI in the NI has shown an improvement by the postcolumn addition of a weak base or weak basic conditions during chromatography [99, 100, 105–107, 111, 113–115, 118].

The LC of phytoestrogens can be performed with trifluoroacetic acid (TFA) [109, 114] or without acid addition [103].

Regarding MS detection, most works rely on the use of triple quadrupole tandem mass spectrometry due to its high selectivity and sensitivity when working in SRM mode. Detection limits are in the low ng/L range (commonly below 20 ng L⁻¹) even for wastewater influent samples. In a few cases TOF and Q-TOF instruments have been employed successfully to identify the compounds using their exact mass [103, 111].

4 Levels in Wastewater

4.1 Pesticides

There are very few data on the presence of endocrine-disrupting pesticides in sewage water and the compounds targeted vary considerably from one study to another.

In a study conducted in Spain, where atrazine, simazine, chlorfenvinphos, and diuron were investigated in the effluents of five sewage treatment plants (STPs), diuron was found to be the most ubiquitous and one of the most abundant compounds (average concentration 163 ng L⁻¹) and atrazine the least frequent and least abundant compound (average concentration 9 ng L⁻¹). Chlorfenvinphos and simazine were positively identified in 20% of the collected samples with average concentrations of 163 and 16 ng L⁻¹, respectively [41]. Similar trace levels of atrazine to those found in Spain have been determined in influent and effluent STP samples in the area of Montreal in Canada [39].

In the north of Italy, atrazine (41 ng L⁻¹) and diuron (165 ng L⁻¹) were the only disrupting pesticides detected in one of the effluent samples investigated in the course of a study conducted in three STPs [43].

In France the endocrine-disrupting fungicide tebuconazole and the insecticide dimethoate were frequently found in STP influents with levels ranging from 2 to 43 ng L⁻¹ and from 2 to 20 ng L⁻¹, respectively. Another fungicide, hexaconazole, was found in treated sewage water at levels between 26 and 32 ng L⁻¹ but was below the method limit of quantification in influent water. In the same study, a distinctly high concentration (2,287 ng L⁻¹), but in only one of the influent samples investigated, was detected for the carbamate pesticide degradation product 2-imidazolidinethione [40].

4.2 Phthalates

Phthalates have low water solubility and when released into the aquatic environment, they tend to absorb strongly to suspended particles and sediments [7, 11].

Biodegradation is expected to be the dominant degradation removal mechanism for phthalates entering STPs or released to surface waters, since a wide range of bacteria and actinomycetes can degrade phthalates under both aerobic and anaerobic conditions. Ultimate degradation of phthalates with sewage sludge inocula ranged from >50 to 99% in a study published by Staples et al. [8], while primary degradation half-lives in surface waters were estimated to range from less than 1 day to 2 weeks. Their degradation rate appears to decrease with increasing alkyl chain length because of the corresponding increase in $\log K_{ow}$ (which ranges from 1.46 to 13.1) [7]. DEHP and diethylphthalate (DEP) constitute the most ubiquitous phthalates found in wastewater, which correlates with their high production, widespread use and physico-chemical properties (low solubility and relatively high K_{ow}). There are several studies about the occurrence of phthalates in the aquatic environment. The most systematic of them reported levels of DEHP and DBP for 116 surface water samples, 35 sediments from rivers, lakes and channels, 39 sewage effluents and 38 sewage sludges collected in Germany [7]. The phthalate burden was mainly from DEHP, whilst di-*n*-butyl phthalate (DBP) was found in minor concentrations and BBP at concentrations near the detection limit. The concentrations found ranged from 0.3 to 98 g L⁻¹ (surface water), 1.7–182 g L⁻¹ (sewage effluent), 28–154 mg kg⁻¹ (sewage sludge), and 0.2–8.4 mg kg⁻¹ (sediment). Table 8 brings together environmental phthalate levels found for the case of different wastewaters, which is one of the major sources of these contaminants in the aquatic environment.

Table 8 Levels of phthalates in WWTPs

Compounds	WWTP influent (µg/L)	WWTP effluent (µg/L)	Geographical location	Reference
DEHP	Na	1.74–182	Germany	[7]
DMP	Na	0.6–3	Portugal	[49]
DEP	9.08–16.2	13.3	Spain	[50]
DBP	<LOD- 22	<LOD- 1.9	Spain	[13]
BBP	<LOD- 1.2	<LOD- 0.76		
DEHP	1.1–2.9	<LOD		
DEP	0.15–0.98	na	Spain	[51]
DEHP	0–0.15			
DOP	0–0.1			
DEP	0.059–0.389	na	Australia	[136]
DBP	0.025–0.138			
BBP	0.006–0.062			
DEHP	<LOD- 20.3			
DPP	0.07	0.008	Denmark	[137]
DBP	1.03	0.91		
BBP	0.39	0.13		
DEHP	35.4	0.96		

na Not analyzed

4.3 Bisphenol A

BPA can enter the environment through leaching from final products and during manufacturing processes [7]. WWTPs appear to be one of the major sources of pollution of BPA, which have led to elevated levels in both surface waters and sediments [138]. Table 9 shows the concentration values of BPA detected in different WWTPs. Despite the high removal rate in WWTPs, BPA is nevertheless released into the environment and commonly found in river waters [7, 138, 144–147] and occasionally in ground waters and drinking waters [145, 146, 148]. Some studies show that BPA may decompose microbially in water, with a half-life between 2.5 and 4 days [14], although persistence and bioaccumulation of BPA has also been reported [149]. Other studies pay attention to the chlorination derivatives formed during chlorination of drinking waters and wastewaters [51].

4.4 Alkylphenolic Compounds

During the last decade a large number of monitoring studies have been carried out in order to determine the fate of alkylphenols in wastewaters. Table 10 gives an overview of the results for the nonylphenol species found in such studies.

During the wastewater processes long-chain APEOs are degraded into short-chain APEOs (mainly di and mono ethoxylates) and the corresponding carboxylates (APECs) and then finally into NP or OP, which are not readily degradable.

In correspondence with this theory it is observed that influent concentrations of nonylphenol ethoxylates (NPEOs) are significantly higher than effluent concentrations. It can also be seen that the concentrations of nonylphenoxy carboxylates (NPECs) are not reduced from influent to effluent, which is attributed to the circumstance that NPECs are formed as rapidly as they are removed. The concentrations of nonylphenol are also reduced during the wastewater processes; however, this is believed to be due to adsorption to sludge more than elimination.

Table 9 Levels of BPA in WWTPs

WWTP influent ($\mu\text{g L}^{-1}$)	WWTP effluent ($\mu\text{g L}^{-1}$)	Geographical location	Reference
na	0.702	Germany	[7]
na	2.01–24–32	Spain	[139]
149.20	1.080	Canada	[140]
1.55	<LOD	Portugal	[56]
na	0–0.006	Belgium and Italy	[57]
0.542–3.01	0.162–0.258	Germany	[141]
0.378–0.890	0.012–0.028	UK	[142]
<LOD– 2.14	<LOD–1.10	Greece	[143]
0.06–1.51	<LOD –0.27	Spain	[50]

LOD limit of detection; na non analyzed

Table 10 Overview of environmental levels of alkylphenols in the last decade

Compounds		Country	Conc. ($\mu\text{g L}^{-1}$)	Reference
NPEO	Influent	Austria (NP1EO)	2.6–35	[65]
		Austria (NP2EO)	1.2–5.8	[65]
		Italy	29–221	[150, 151]
		Spain	27–2,120	[152–154]
		Spain (NP1EO)	0.54–125	[62]
		Spain (NP2EO)	0.71–33	[62]
		Switzerland	96–430	[155]
		USA Sum(0–3EO)	47.6–262	[27]
		USA Sum(4–16EO)	265–745	[27]
		Denmark	6.5–344	[61]
	Effluent	Croatia	5–392	[156]
		Germany (NP1EO)	0.072–0.175	[157]
		Germany (NP2EO)	0.09–0.21	[157]
		Spain (NP1EO)	0.73–156	[62]
		Spain (NP2EO)	0.12–4.4	[62]
		USA Sum(0–3EO)	1.58–32.3	[27]
		USA Sum(4–16EO)	0.042–8.42	[27]
		Denmark	4.67–444	[61]
NPEC	Influent	Spain	0.16–450	[62]
		USA	0.28–3.23	[27]
		Croatia (NPE2C)	0.001–4.37	[156]
	Effluent	Croatia (NPE1C)	0.001–3.2	[156]
		Spain	1.3–307	[62]
		USA	7.58–99.3	[27]
NP	Influent	Germany (NPE1C)	0.8–1.12	[157]
		Belgium	0.4–219	[158]
		Italy	0.352–40	[60]
		Spain	0.5–251	[62, 153, 159]
		Austria	1.05–8.6	[65]
		The Netherlands	0.1–40	[160]
	Effluent	Croatia	0.46–4.4	[156]
		Norway	0.002–5.2	[161]
		Germany	0.14–00242	[157]
		Italy	0.081–0.88	[60]
		Spain	0.22–5	[62]

OP and OP with two carboxylate units (OPE2C) are found at concentrations 10-times lower than the corresponding nonalkylphenols. This reflects the fact that octylphenol ethoxylates (OPEOs) only make up for 10% of the overall use of alkylphenols.

4.5 Perfluorinated Compounds

Discharge of municipal wastewater effluents is one of the principal routes for introducing organic chemicals that are used in domestic and industrial settings into aquatic

environments. The literature on FASs levels in wastewater samples is scarce. The main FASs reported in wastewater are the PFCs perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), PFOA, perfluorononanoic acid (PFNA) and perfluorodecanoic acid (PFDA), the PFSs perfluorobutane sulfonate (PFBS), perfluorohexane sulfonate (PFHxS), PFOS, perfluorodecane sulfonate (PFDS) and 6:2 fluorotelomer-sulfonate (6:2 FtS), and the nonionic PFSs perfluorooctanesulfonamide (PFOSA), *N*-ethyl perfluorooctanesulfonamide (N-Et-FOSA), 2-(*N*-ethylperfluoro-1-octanesulfonamido)-ethanol (N-Et-FOSE), and *N*-ethyl perfluoro-octanesulfonamidoacetate (PFOSAA) (see Table 1 for acronyms). Figure 1 shows ranges of concentration found in wastewater samples reported in the literature reviewed. Most of the analytes determined correspond to short-chain compounds because of the possible biodegradation of potential precursors entering the wastewater treatment plants. The highest concentrations were exhibited by PFOS, 6:2 FtS, and PFOA in both influent and effluent samples. The decrease of PFOS observed in WWTPs can be attributed to sorption into sludge [162]. The opposite situation occurs with PFOA, which is found at higher concentrations in effluents than influents [69, 71]. Biodegradation of precursors (fluorotelomer alcohols) could be responsible for the observed increases [163]. The distribution and fate of FASs during wastewater treatment are not well-known. More studies are needed in order to understand the behavior of FASs in WWTPs.

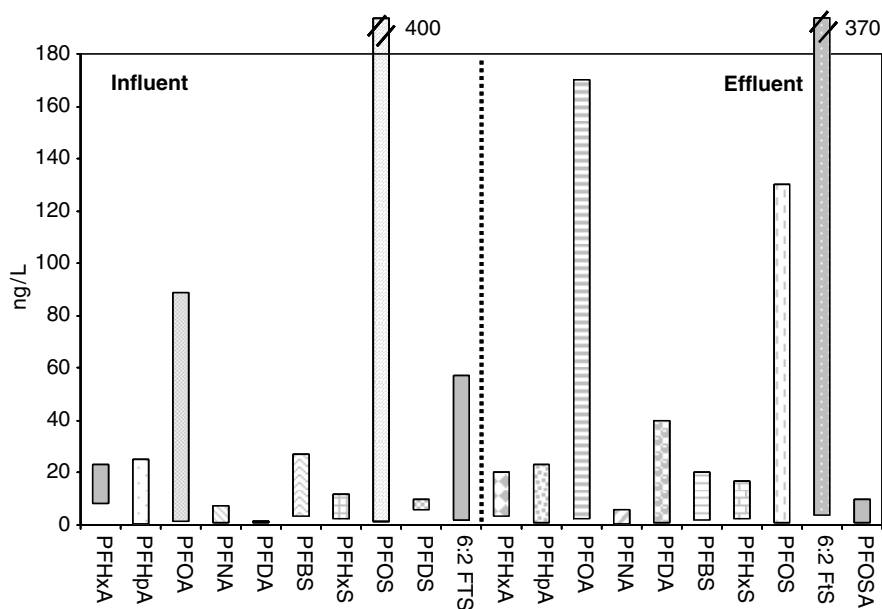


Fig. 1 Levels of fluorinated compounds in wastewater samples

4.6 *Pharmaceuticals*

Human and veterinary drugs are continuously being released into the environment mainly as a result of manufacturing processes, the disposal of unused or expired products, and excreta. The amount of pharmaceuticals and their bioactive metabolites being introduced into the environment is likely low. However, their continuous environmental input may lead to a high long-term concentration and promote continual, but unnoticed adverse effects on aquatic and terrestrial organisms. Numerous papers have reported levels of pharmaceuticals in wastewaters and aqueous and solid environmental matrices, the most ubiquitous ones in both surface and wastewaters being analgesics and antiinflammatories, antibiotics, lipid regulators, psychiatric drugs, β -blockers, and others such as ranitidine and omeprazole (ulcer healing), salbutamol (β -agonist), and iopromide (X-ray contrast media).

Different studies across Europe have reported medium concentrations for effluents of WWTPs from 0.3 to 2 $\mu\text{g L}^{-1}$, lipid regulators in the range from 0.1 to 2 $\mu\text{g L}^{-1}$, antiepileptic drugs such as carbamazepine in medium ranges between 0.7 and 2 $\mu\text{g L}^{-1}$, and in lower concentrations antibiotics in the range of 0.05–0.3.

The human health and ecological significance of pharmaceutical residues in water resources is subject of on-going studies. To date, drug residues in drinking water have not been shown to adversely impact human health. There is concern, however, that antibiotics in surface water are playing a role in the ability of disease-causing pathogens to develop resistance to the drugs used to fight them. Additionally, numerous studies provide compelling evidence that hormonal drugs in surface water are causing adverse impacts to some fish populations.

Many believe that of all the emerging contaminants, antibiotics are the biggest concern because of the potential for antibiotic resistance [83–85]. The increasing use of these drugs in livestock, poultry production, and fish farming during the last five decades has caused a genetic selection of more harmful bacteria, which is a matter of great concern. However, other pharmaceutical compounds, especially polar ones, such as acidic antiinflammatory drugs and lipid regulators also deserve particular attention. Elimination of acidic pharmaceuticals in WWTPs has been found to be rather low and consequently sewage effluents are one of the main sources of these compounds and their recalcitrant metabolites. Because of their physico-chemical properties (high water solubility and often poor degradability) they are able to penetrate through all natural filtration steps and enter groundwater as well as drinking water.

4.7 *Estrogens*

Estrogens are mainly excreted as their less active sulfate, glucuronide and sulfo-glucuronide conjugates [164]. However, in WWTPs, as well as in the environment, these conjugates may suffer deconjugation and act as precursors of the corresponding free steroids. Thus, an appropriate evaluation of their

Table 11 Levels (ng L^{-1}) of free estrogens detected in wastewater

E2	E1	E3	EE	Reference
4.7–25	25–132	24–188	0.4–13	[106]
n.d.-21	10–57	27–220	n.d.	[113]
10–31	16–60	23–48	n.d.	[114]
3–22	8–52	n.a.	n.a.	[165]
n.d.-234	9.4–232	n.d.-108	2.4–138	[107]
17	<0.2–58	n.a.	<0.2–114	[166]

n.d. not detected; *n.a.* not analyzed

occurrence and impact requires the analysis of both free and conjugated estrogens.

Most of the studies dealing with the investigation of estrogens in wastewaters have been performed in WWTPs receiving urban/domestic discharges and concentrations reported have been most usually in the ng/L range. E2 and E1 have been the free estrogens most frequently found whereas E3 has been studied and detected only occasionally. However, E3 concentrations, when detected, have been usually higher than those of E2 and E1. In general, estrogen concentrations increase in the order $\text{E3} > \text{E1} > \text{E2}$ (see Table 11 for examples). Thorough revision of all data available situates mean and median concentrations in the range of 9–20 ng L^{-1} for E2, 20–55 ng L^{-1} for E1, and 45–75 ng L^{-1} for E3 [3].

The most studied synthetic estrogen, EE, has been either not detected [110, 113, 114] or detected at concentrations in general much lower than the other estrogens [100, 106, 108] (see Table 11). Levels higher than 100 ng L^{-1} have been only occasionally reported (e.g., 155 ng L^{-1} [167] and 138 ng L^{-1} [107]).

As regards conjugated estrogens, mostly sulfates and glucuronides of E1, E2, and E3 have been included as target analytes and detected at similar levels as the free estrogens [3].

5 Conclusions

LC–(tandem)MS is a very valuable tool for analysis of endocrine-disrupting compounds in wastewater since many classes of EDs are polar, nonvolatile compounds. Electrospray-tandem mass spectrometry with triple quadrupole analyzers operating in the SRM mode is by far the configuration most commonly used for quantitation of EDs in wastewater. The main advantages of this technique are sensitivity (achieving LOD in the ng L^{-1} or even pg L^{-1} range after sample preconcentration) and selectivity and confidence in compound identification (achieving a minimum of three identification points when acquiring two SRM transitions per compound). The main drawback, associated to the complexity of the samples, is the likely existence of matrix ionization effects; this makes recommendable the use of matrix-matched calibration, standard addition, or isotopically labeled surrogate standards

for quantitation. The application of this methodology to elucidate the presence of EDs at the entrance and exit of WWTPs is primordial to know the efficiency of current wastewater treatments and check advanced, alternative ones for a better protection of the environment and human health from EDs effects.

Acknowledgments This work has been supported by the EU projects MODELKEY (GOCE 511237) and INNOVA MED (INCO-2006–517728) and by the Spanish Ministry of Science and Innovation (CTM2006–26227-E/TECNO, CTM2007–30524-E, and CGL2007–64551/HID). This article reflects the authors view alone. The EU is not liable for any use that may be made of the information contained in it. Cristina Postigo acknowledges the European Social Fund and AGAUR (Generalitat de Catalunya, Spain) for their economical support through the FI predoctoral grant.

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Index

A

2-Acetaminofluorene, 212
Acetochlor, 232
Acetylcholine receptors, 128
Acetylcholinesterase (AChE), 128
Acridine orange, 173
Aflatoxins, 132
AhR nuclear translocator (ARNT), 80
Alachlor, 42, 232, 238
Aldehyde dehydrogenase, 51
Aldrin, 232
ALGAETOX, *xi*
Algal biosensors, 134, 141
Alkaline phosphatase (AP), 51
Alkaline unwinding assay, 203
Alkylphenol ethoxylates (APEOs), 233, 258
Alkylphenolic compounds, 233, 253
 wastewater, 258
Alkylphenols, ethoxylates, 52, 253
Alprazolam, 235
Ames Test (ISO 16240), *x*, 121, 143, 144, 161, 170, 177, 192, 198, 205, 223
2-Aminoanthracene, 167
2-Aminofluorene (2-AF), 167
p-Aminophenol (PAP), 178
p-Aminophenyl- β -D-galactopyranoside (PAPG), 167
Amitrole, 232
Amoxicillin, 61
Amperometric biosensors, 47, 51
Ampicillin, 177
 milk, 61
Anabolic androgenic steroids (AAS), 52
Anilides, 39
Anti-atrazine antibodies, *xi*
Antibiotics, 47
 resistance, 79
Antibodies, 47, 48
Antidepressants, tricyclic, 235

Antiinflammatory drugs, 261
Anti-simazine antibodies, *xi*
Aquatic ecotoxicological studies, 117
Aquatic genotoxicity testing, 189
Armillaria mellea, bioluminescent, 142
Aroclor 1248 PCB, 54
Aryl hydrocarbon receptor (AhR), 72
Atrazine, *x*, 16, 54, 140, 232, 238, 252, 256
 RIANA, 37, 44
Automated water analyser, 33
Automated Water Analyser Computer Supported System (AWACSS), 24, 33
Automatisation, 39
AWACSS, achievements, 35
 advanced features, 36
 marketing aspects, 40

B

Bacteria, genetically engineered, 161
Bacterial assays, 189
Bacterial biosensors, 134
Bacterial genotoxicity reporters, 178
Bacterial test systems, 187
Benzaldehyde, 121
Benzo[a]pyrene, 212
Benzo[a]pyrene-BSA, 63
Benzodiazepine, 235
Benzothiazole, 83
BIAcore system, 130
Bioassays, 69, 115
 aquatic ecotoxicity assessment, 118
Biochanin-A, 249
Biochemical oxygen demand (BOD), *xi*, 136
Biofunctional surfaces, 48
Biological oxygen demand (BOD), *xi*, 21, 136
Bioluminescence, 80, 119, 123, 139, 143, 165, 167, 190
 inhibition, 119

- Bioluminescent bacteria systems, 80, 137
 Bioluminescent fungi, 142
 Biomarkers, 69, 115
 Biosensor wireless networks, autonomous, 22
 Biosensors, 2, 7, 47, 115, 161
 amperometric, 51
 classification, 123
 DNA, 17, 132
 ecotoxicity, 127
 electrochemical, 7, 50
 environmental monitoring, 11
 enzyme, 11, 127
 estrogen receptor, 129
 evanescent wave optical principle, 57
 immunosensors, 131
 impedimetric, 47
 lactate, 15
 localized surface plasmon resonance (LSPR), 146
 nanomechanical, 62
 optical, 8, 56
 overview, 128
 plasmon resonance, 59
 research funding, 4
 thermistor-based calorimetric, 127
 whole-cell, 21, 134, 224
 Biosensors for Environmental Technology (BIOSET), *ix*
 Biotin, 20
 Biotoxins, 196, 203, 205, 220
 Bisbenzimidazole fluorescence staining, 210
 Bisphenol A, 16, 39, 54, 60, 233, 252
 wastewater, 258
 Bleomycin, 172
 β -Blockers, 47, 248, 261
 BOD, *xi*, 21, 136
 BOD5, *xi*
 BPA-ovalbumin, 60
 Bulk acoustic (BA) devices, 63
 Butylcholine esterase (BchE), 128
- C**
- Cadmium, 133, 142, 223
 Calcium-channel blockers, 235
 Carbamates, 128, 256
 Carbamazepine, 235, 248, 261
 Carbaril, 18
 Catechol, 121
Ceriodaphnia dubia, 118, 123
 Certified reference materials (CRMs), 123
 Chemically sensitized field effect transistor (CHEMFET), 125
 Chloramphenicol, 60
 Chlordane, 232
 Chlordecone (kepone), 232
 Chlorfenvinphos, 232, 256
 Chlorophenol red- β -D-galactopyranoside (CRPG), 167
 Chlorophenols, 222
 Chlorotriazine, *xi*, 44
 Chlorpyrifos, 232
 Choline esterase, 11
 Cholinesterase biosensor, *xi*
 COLUMBUS/BIOLAB, *xi*
 Computer supported system, 33
 Copper, 141, 142, 144
 Coumestrol, 249
 Cresols, 121
 Cyanazine, 232
 Cyclodiene, 16
 Cytotoxicity controls, 169
- D**
- 2,4-D, 15, 42, 232
 Daidzein, 249
Danio rerio, 203, 208
Daphnia magna, 118, 123
 DDT, 83, 232
 DEHP, 257
 Di-(2-ethylhexyl) phthalate (DEHP), 233
 Diarrhetic shellfish poisoning (DSP), 220
 Diazepam, 235
 Diazinon, 232, 249
 3,5-Dichlorobiphenyl, 60
 Dichlorvos, 232
 Dieldrin, 232
 Diethylphthalate (DEP), 257
 Diethylstilbestrol (DES), 71, 130, 249
 Diisopropyl fluorophosphate (DFP)/carbaryl, 128
 Dimethoate, 256
 Dimethyl phthalate (DMP), 252
 Dimethylsulfate, 165
 Dimethoate, 232
 Dinitroanilines, 232
 Diuron, 140, 232, 256
 DMNA (*N*-nitrosodimethylamine), 211
 DNA, 203
 DNA amperometric detection, 13
 DNA biosensors, 17, 132
 DNA fragmentation, 213
 DNA lesions, UV-mediated, 218
 DNA thymine dimers, 203, 213, 217
 DNA-unwinding assay, 208
 Domoic acid, 220
 Doxorubicin, 172

Dreissena polymorpha, 203, 207
Dunaliella tertiolecta, 123

E

Early life stages, 203
 Eco-genotoxicity, 203
 Ecotoxicity biosensors, 127
 Ecotoxicology, 116
 EDs, wastewater, analysis, 236
 Effect-directed analysis (EDA), 118
 Electrochemical biosensors, 50
 Electrochemical impedance spectroscopy (EIS), 54
 Electrochemical magnetoimmunosensing, 53
 Electrochemical transduction, 125
 ELRA, *xi*, 122
 Embryonal development, UV-exposure, 218
 Emerging pollutants, 3
 Endocrine disrupters, instrumental analysis, 249
 sample preparation, 236
 Endocrine disrupting compounds (EDCs), *xi*, 10, 34
 Endocrine disruption, 69
 Endosulfan, 232
 Environmental monitoring, 2, 33, 116
 Environmental Sensor Networks (ESN), 2
 Environmental Technology Verification (ETV) system, 6
 Enzyme biosensors, 127
 Enzyme-linked receptor assay (ELRA), *xi*, 122
 ER-RYA, estradiol, 84
 17 β -Estradiol (E2), 60, 73, 83, 102, 122, 130, 233, 249
 Estrogen receptor, 122, 129
 Estrogen response element (ERE), 131
 Estrogens, 122, 236
 wastewater, 261
 WWTP effluents, 236
 Estrone, RIANA, 37, 39, 44
 Ethoxylate, 253
 Ethylene diamine tetra-acetate (EDTA), 21
N-Ethylperfluorooctanesulfonamide (*N*-Et-FOSA), 260
 2-(*N*-Ethylperfluoro-1-octanesulfonamido)-ethanol (*N*-Et-FOSE), 260
N-Ethylperfluorooctanesulfonamidoacetate (PFOSAA), 260
 Ethynilestradiol, 236
 Ethynyl estradiol (EE), 249
 EuCyanobacteria Electrode, *x*
 Eukaryotic assays, 192
 Eukaryotic indicator tests, 187
 Evanescent wave, 47

F

Fenitrothion, 136, 232
 Firefly luciferase, 119, 143, 167
 Fish cell line RTG2, 207
 Fish embryos, 214
 Fish larvae (yolk sac), 208
 Fish-cell biosensors, 134, 145
 Florefenicol (FF), 60
 Florefenicol amine (FF amine), 60
 Fluoranthene, 173, 223
 Fluorescence, 10, 124, 139
 Fluorescence immunoassay, *xi*
 Fluorescence labelling, *xi*
 Fluorescent (FL) reporter proteins, 120
 Fluorescent protein genes, 168
 Fluorinated alkyl substances (FASs), 234
 6:2Fluorotelomer-sulfonate (6:2 FtS), 260
 Formononetin, 249
 Furazolidone, 172

G

β -Galactosidase (lacZ), 80
 Gene expression, 69
 analysis, 91
 Gene expression biomarkers, endocrine disruption, 88
 practical uses, 97
 Genistein, 249
 agonist/antagonist effects, 88
 Genotoxicants, detection performance, 170
 Genotoxicity, 161, 187, 203
 repairment, 213
 Genotoxicity tests, *x*
 Gentamicin, 79
 GFP-expressing cells, 80
 β -Glucuronidase (GUS), 80
 Glycitein, 249
 Glyphosate, 141, 231
 Gold nanoparticle/polyaniline nanotube, 20
 Gonyautoxins, 223
 Green fluorescent protein (GFP), 119, 134, 143, 168

H

Halogenated bisphenols, 60
 Hapten-protein conjugates, 36
 HCH, 222
 Heavy metals, 17, 132
 Heptachlor, 232
 Herbicides, *xi*, 18, 133, 140, 142, 232
 Hexachlorobenzene, 232
 Hexaconazole, 256

Hormone receptors, 69, 71
 Hormone response element, 80
 Hormones, 47
 Horseradish peroxidase (HRP), 51
 Host strain mutations, enhanced sensitivity, 174
 Hydrolases, 11
 Hydrothol, 141
 4-Hydroxy-3,5-dichlorobiphenyl
 (4-OH PCB 14), 60
 Hydroxyl estrone, 249
 Hygromycin, 79

I

2-Imidazolidinethione, 256
 Immunoassay, 33
 Immunosensors, 15, 48, 131
 piezoelectric, 63
 Impedimetric biosensors, 47, 54
 Insecticides, 232
 Interdigitated microelectrodes, 54
 Iopromide, 261
 Isoproturon, 18
 RIANA, 37, 39, 44

J

Japan Fund for Global Environment (JFGE), 5

L

Laccase, 11, 51
 β -Lactamase, 177
 β -Lactams, 61
 Lactate oxidase, 15
 LC-MS, endocrine disrupters, 228
Limanda limanda, 203
 UV-exposure, 214
 Limit of detection (LOD), 38
 Limits of quantification (LOQ), 38
 Lindane, 232
 Lineal alkylbenzene sulfonates (LAS), 141
 Linuron, 232
 Lipid regulators, 261
 Luciferase, 134
 Luciferin, 167
 Luminescence, 10, 124, 139

M

Mach-Zehnder interferometry (MZI), 9, 58
 Malathion, 232
 Maximum residue limits (MRLs), 50
 MCF-7 cell-based proliferation assays
 (E-screen), 122

Mecoprop, 140
 Membrane receptors, 71
 Mestranol, 249
 Metabolic activation, 162
 enzymes, 174
 Metal oxide semiconductor (MOS) sensors, 126
 Methyl benzoate, 121
N-Methyl-*N'*-nitro-*N*-nitrosoguanidine
 (MNNG), 165
 Methylcatechol, 121
 4-Methylumbelliferyl- β -D-galactopyranoside
 (MUG), 167
 Metoxychlor, 232
 Microcantilevers, 47, 62, 127
 Microcystin, 220
 Microtox, *xi*
 Minimum required performance limits (MRPL),
 50
 Mirex, 232
 Mitomycin C, 138, 165, 173
 Motes, 24
 Multi-analyte determination, 38
 Multi-wall carbon nanotube (MWNT)-
 chitosan, 128
 Mussels, 207, 220
 Mutatox test, 177
Mycena citricolor, bioluminescent, 142
Mytilus edulis, 207, 220

N

Nalidixic acid (NA), 165
 Nanobiotechnology, 47
 Neomycin-resistant mutants, 178
 Network systems, 33, 39
 Neurotoxin detection, 128
 Nitrofurantoin, 212
o-Nitrophenyl β -D-galactopyranoside
 (ONPG), 166
 4-Nitroquinoline-*N*-oxid (4-NQO), 165, 211
 Non SOS promoters, 166
 Nonsteroidal antiinflammatory drugs (NSAID), 248
 Nonylphenol, 18, 231, 234
 Nonylphenol ethoxylates (NPEOs), 241, 258
 Nonylphenoxy carboxylates (NPECs), 258
 Nuclear hormone receptor superfamily, 72
 Nucleic acid biosensors, 93, 132

O

Octylisothiazolin-3-one (OIT), 144
 Octylphenol, 83, 231, 234
 Octylphenol ethoxylates (OPEOs), 259
 Omeprazole, 261
 Optical biosensors, 56

Optical immunosensor, 33
Optical transducers, 123
Optical waveguide lightmode spectroscopy (OWLS), 9, 58
Organophosphates (OP), 13, 17, 22, 128, 136
Organophosphorus compound-induced delayed neurotoxicity (OPIDN), 129
Organophosphorus hydrolase (OPH), 22, 136
Orphan receptors, 78
Ovulation indicator, 44
Oxidases, 128
Oxidoreductases, 11, 51

P

Paralytic shellfish poisoning (PSP), 220
Paraoxon, 22, 128, 136
Paraoxon/carbaryl, 128
Paraoxon/DFP/carbaryl, 128
Paraquat, *xi*, 42, 140
Parathion, 14, 129, 232
PCR, 69, 89, 93, 94
 quantitative real-time, 69, 98
Penicillins G/M, 61
Pentachlorophenol, 141
Perfluorinated compounds, 234, 253
 wastewater, 259
Perfluoroalkyl carboxylates (PFCs), 234
Perfluorobutane sulfonate (PFBS), 260
Perfluorodecane sulfonate (PFDS), 260
Perfluorodecanoic acid (PFDA), 260
Perfluoroheptanoic acid (PFHpA), 260
Perfluorohexane sulfonate (PFHxS), 260
Perfluorohexanoic acid (PFHxA), 260
Perfluorononanoic acid (PFNA), 260
Perfluorooctane (PFO), 235
Perfluorooctane sulfonate (PFOS), 235, 260
Perfluorooctanesulfonamide (PFOSA), 260
Perfluorosulfonamide, 254
Peroxidase, 11
Persistent organic pollutants (POPs), 70
Pesticides, 18, 47, 116, 232
 thermometric biosensors, 127
 wastewater, 256
PFOS, 235, 260
PFSS, 260
Pharmaceutical active ingredients and personal care products (PPCPs), 49
Pharmaceuticals, 235
 wastewater, 261
Phenols, halogenated, 60
Phenylureas, *xi*, 39, 232
Photobacterium phosphoreum, 118, 123
Phthalates, 232, 252
 wastewater, 256

Phthalic acid esters (phthalates, PAEs), 232
Phytoestrogens, 88, 236, 249, 256
 genistein, 88
Piezoelectric crystals/devices, 10, 47, 126
Piezoelectric immunosensors, 63
Plasmon resonance, 47
 biosensors, 59
Polychlorinated biphenyls (PCBs), 17, 54, 63, 100, 102, 121, 132
 Helgoland, 221
Polychlorinated dibenzo-*p*-dioxins (PCDDs), 63, 144
Polyclonal antibodies, 36
Polycyclic aromatic hydrocarbons (PAHs), 16, 17, 63, 102, 121
Potentiometric solid electrolyte gas sensors, 125
PRENDISENSOR, *xi*
Progesterone, 42, 44, 52
Promoter-reporter fusions, 161
Propanil, 39

Q

Quantitative real-time PCR, 69
Quantitative structure-activity relationship (QSAR), 74

R

Ranitidine, 261
Rawson sensor, *x*
Reactive oxygen species (ROS), 213
Real-world water analysis, 41
Receptor agonists, 85
Recombinant transthyretin (rTTR), 60
Recombinant yeast assay (RYA), 69, 74
 genetic setup, 77
 mathematical modelling, 81
Red tides, 220
Regulatory sequences, manipulation, 174
Reporter systems, 166
 colorimetric, 166
 electrochemical, 166
Reporting genes, 177
Response spectrum, expansion, 171
Reversion assays, 161, 177
Rhodamin B, 214
RIANA (River Analyzer), *xi*, 33, 57
 achievements, 35
 key features, 35
River Basin Management Plan (RBMP), 49
RNA quantification, 89, 93
RNases/RNA degradation, 91
RTG-2, 203

S

Salbutamol, 261
 SANDRINE, *xi*
 Saxitoxins, 220, 223
 Screen-printed carbon arrays (SPCAs), 132
 Screen-printed electrodes (SPEs), 52
 Selection markers, 177
Selenastrum capricornutum, 118, 123, 141
 Sensing elements, 163
 Sensitivity enhancement, 171
 Sensors, 2, 116
 electrical, 126
 mass-sensitive, 126
 thermometric, 11, 127
 SENSPOL network, *x*
 Simazine, 42, 232, 256
Skeletonema costatum, 118, 123
 Sodium diethyl dithiocarbamate (DDTC), 21
 Sodium dodecyl sulphate, 141
 SOS promoters, 163
 Spiggin, sticklebacks, 89
 Stanozolol, 61
 Statistical analyses, 211
 Steroid hormones, 39, 129
 receptors, 72, 129
 Sulfamethoxazole, 236
 Sulfapyridine (SPY), 56
 Sulfonamides, 42, 53, 56
 Sulphamethizole, 39
 Surface acoustic wave (SAW) devices, 63
 Surface plasmon resonance (SPR), 10, 124, 130
 Surface stress, 62
 Surface water, trace organics, *xi*

T

2,4,5-T, 232
 Target pollutants, 41
 Tebuconazole, 256
 Test strategies, 187
 Tetrabromobisphenol A, 60
 Tetrachlorobisphenol, 253
 Tetrachlorodibenzo-*p*-dioxin (TCDD), 63, 72, 100, 205
 Tetracycline repressor, 178
 Thiamphenicol (TAP), 60
 Thymine dimers, 203, 214, 217
 Thyroxine, 60
 Thyroxine-binding globulin (TBG), 60
 Titanium dioxide engineered nanoparticles (ENPs), 121
 Toluene, 140
 Total internal reflection fluorescence (TIRF), 9, 36, 57, 124
 ToxAlert, *xi*

Toxicity, *xi*, 116
 Toxicity identification evaluation (TIE), 118
 Trace organics, surface water, *xi*
 Triazines, 18, 39, 133, 232
 Triazoles, 83, 232
 Trichloroanisole, 16
 Tricyclic antidepressants, 235
 Triethyl tin, 246
 Trifluraline, 232
 Trihalogenemethanes, 222
 Trihydroxyisoflavone, 249
 Tyrosinase, 11, 51

U

Ultraviolet-B, pelagic fish embryos, 213
 umuC Test (ISO 13829), *x*
 Ureas, 232
 Uterotrophic test, 89
 UV-B radiation, 203

V

Validation, 33, 43
 VDR, coactivator RXR, 80
 Verapamil, 214
Vibrio fischeri, 118, 123
 VITELLO, *xi*
 Vitellogenin (VTG), *xii*, 89, 131

W

Wastewater, endocrine disruptors, 228
 Water Framework Directive (WFD), 2, 3, 117
 Water monitoring, 44
 Water quality, general parameters, *x*
 survey, 40
 Wavelength interrogated optical immunosensor (WIOS), 58
 Whole-cell biosensors, 21, 134
 WWTP (wastewater treatment plants), *x*

X

Xenoestrogens, 51

Y

Yeast biosensors, 74, 134, 142

Z

Zinc, 141
 Zona radiata proteins, 89