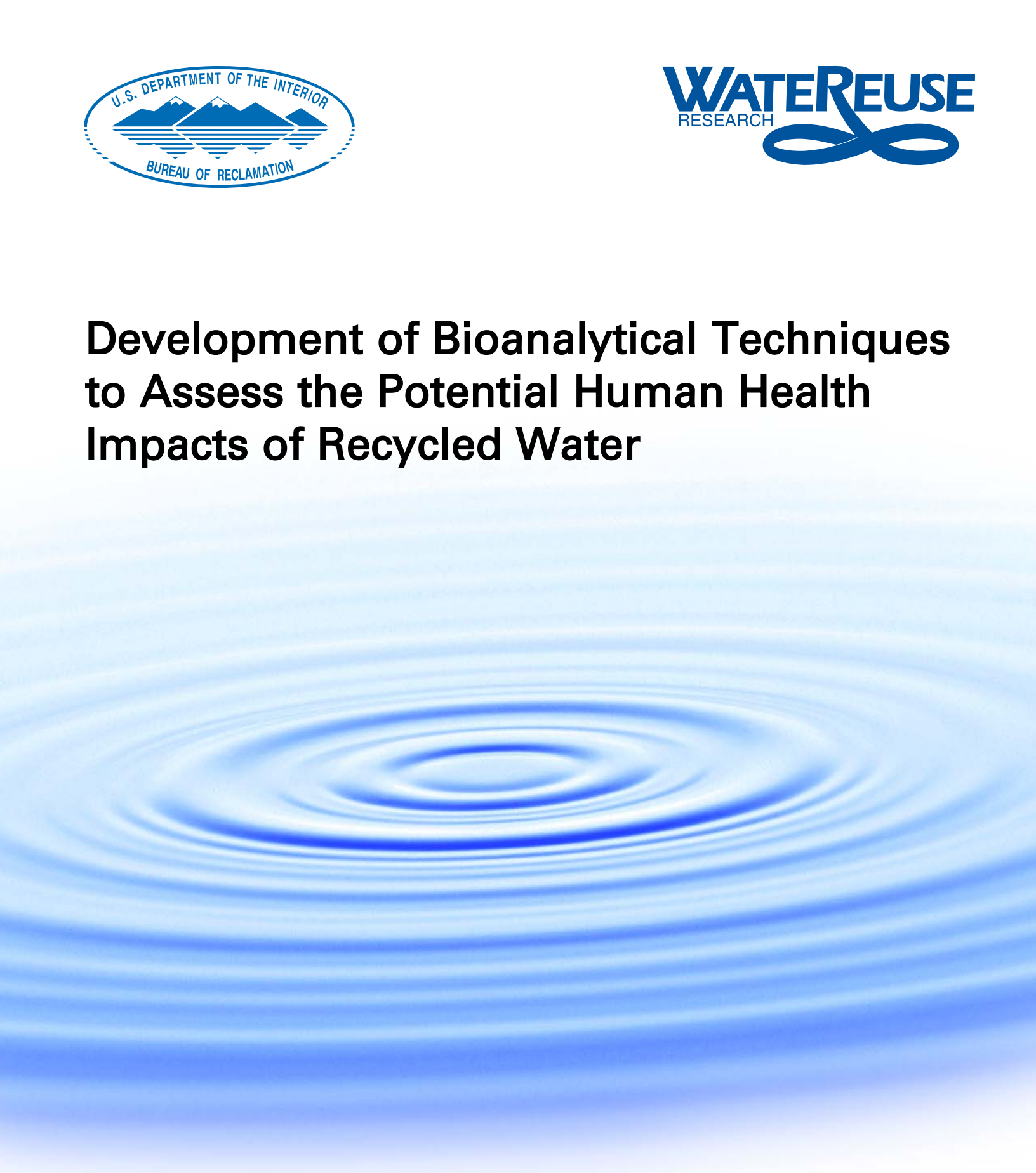
Development of Bioanalytical Techniques to Assess the Potential Human Health Impacts of Recycled Water



WateReuse Research Foundation

Development of Bioanalytical Techniques to Assess the Potential Human Health Impacts of Recycled Water

About the WateReuse Research Foundation

The mission of the WateReuse Research Foundation is to conduct and promote applied research on the reclamation, recycling, reuse, and desalination of water. The Foundation's research advances the science of water reuse and supports communities across the United States and abroad in their efforts to create new sources of high-quality water through reclamation, recycling, reuse, and desalination while protecting public health and the environment.

The Foundation sponsors research on all aspects of water reuse, including emerging chemical contaminants, microbiological agents, treatment technologies, salinity management and desalination, public perception and acceptance, economics, and marketing. The Foundation's research informs the public of the safety of reclaimed water and provides water professionals with the tools and knowledge to meet their commitment of increasing reliability and quality.

The Foundation's funding partners include the Bureau of Reclamation, the California State Water Resources Control Board, the California Energy Commission, and the California Department of Water Resources. Funding is also provided by the Foundation's subscribers, water and wastewater agencies, and other interested organizations.

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Acronyms

AO	advanced oxidation
AR	androgen receptor
ASR	adaptive stress response
ATG	Attagene
AWQC	Australian Water Quality Centre
BDS	BioDetection Systems
BEQ	bioanalytical equivalent concentration
CAPIM	Center for Aquatic Pollution Identification and Management
CAR	constitutive androstane receptor
CSIRO	Commonwealth Scientific and Industrial Research Organization
CT	cytotoxicity
CYP	cytochrome P450 mono-oxygenase enzyme
DART	embryo toxicity test with the zebrafish *Danio rerio*
DW	drinking water
EBT	effect-based trigger values
EC	effect concentration
EEQ	estradiol equivalent
Eff	effluent
ER	estrogen receptor
GR	glucocorticoid receptor
GU	Griffith University
HK	Hong Kong Baptist University
HTS	high-throughput screening
IC_{50}	50% inhibitory concentration
IR	induction ratio
IRCM	Cancer Research Institute of Montpellier
ISO	International Organization for Standardization
IWW	IWW Water Center
MF	microfiltration
MMC	mitomycin
MOA	mode of action
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF	nanofiltration
NIH	National Institutes of Health
NJU	Nanjing University
NRU	neutral red uptake
NWC	National Water Commission
OECD	Organiztion for Economic Cooperation and Development

PPAR	peroxisome proliferator-activated receptor
PR	progesterone receptor
PXR	pregnane X receptor
RAR	retinoic acid receptor
RCEES	Research Center for Eco-Environmental Sciences
RECETOX	Research Center for Toxic Compounds in the Environment
REF	relative enrichment factor
RFUs	relative fluorescence units
RLUs	relative light units
RXR	retinoid X receptor
RO	reverse osmosis
ROS	reactive oxygen species
RT-PCR	real-time PCR
RW	river water
SCCWRP	Southern California Coastal Water Research Project Authority
SPE	solid-phase extraction
SW	stormwater
SWISS	Center for Applied Ecotoxicology
TEQ	toxic equivalent
TR	thyroid receptor
UA	University of Arizona
UCR	University of California at Riverside
UF	University of Florida
UFZ	Helmholtz Center for Environmental Research
UQ	The University of Queensland
USF	University of South Florida
WRP	water reclamation plant
WWTP	wastewater treatment plant
XM	xenobiotic metabolism

Foreword

The WateReuse Research Foundation, a nonprofit corporation, sponsors research that advances the science of water reclamation, recycling, reuse, and desalination. The Foundation funds projects that meet the water reuse and desalination research needs of water and wastewater agencies and the public. The goal of the Foundation's research is to ensure that water reuse and desalination projects provide sustainable sources of high-quality water, protect public health, and improve the environment.

An Operating Plan guides the Foundation's research program. Under the plan, a research agenda of high-priority topics is maintained. The agenda is developed in cooperation with the water reuse and desalination communities including water professionals, academics, and Foundation subscribers. The Foundation's research focuses on a broad range of water reuse and desalination research topics including:

- Defining and addressing emerging contaminants, including chemicals and pathogens
- Determining effective and efficient treatment technologies to create 'fit for purpose' water
- Understanding public perceptions and increasing acceptance of water reuse
- Enhancing management practices related to direct and indirect potable reuse
- Managing concentrate resulting from desalination and potable reuse operations
- Demonstrating the feasibility and safety of direct potable reuse

The Operating Plan outlines the role of the Foundation's Research Advisory Committee (RAC), Project Advisory Committees (PACs), and Foundation staff. The RAC sets priorities, recommends projects for funding, and provides advice and recommendations on the Foundation's research agenda and other related efforts. PACs are convened for each project and provide technical review and oversight. The Foundation's RAC and PACs consist of experts in their fields and provide the Foundation with an independent review, which ensures the credibility of the Foundation's research results. The Foundation's Project Managers facilitate the efforts of the RAC and PACs and provide overall management of projects.

Bioanalytical tools are cell-based bioassays that give a measure of the toxicity and presence of known and unknown chemicals in complex environmental samples. Improved detection of bioactive chemicals in water will improve risk assessment methods and inform future water management options, particularly in the context of water reclaimed from impaired sources such as wastewater or stormwater. The overall goal of the project was to advance the science and application of bioanalytical tools for water quality assessment. The specific project objectives were (A) to review literature and give an overview on bioanalytical tools that can assess likely impacts on human health, (B) to validate novel endpoints and implement them together with existing bioassays for benchmarking water quality of recycled water, and (C) to develop interpretation guidelines.

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Executive Summary

A rising population and drier climate are leading to chronic water shortages in many cities, prompting exploration of alternate water sources and reuse of available waters. There is a need to thoroughly characterize the human risks associated with these new water sources. However, conventional chemical monitoring programs have been criticized because they cannot include the full range of chemical pollutants that could occur in water sources and recycled water and because they do not account for the combined effects of different chemicals or their transformation products. The proposed research involved review, validation, and interpretation of bioanalytical tools for cost-efficient water quality monitoring with a particular focus on water reuse matrices but also with attention to other water types for comparison, with particular reference to human health.

Bioanalytical tools are in vitro or low-complexity bioassays that target specific mechanisms of toxicity and give a measure of the toxicity of individual chemicals and of chemical mixtures, as well as the presence of unknown chemicals. These include, for example, pesticides, industrial chemicals, pharmaceuticals, personal care products and disinfectants, and their transformation products. Bioanalytical tools are complementary to chemical analysis. Although they cannot resolve single chemicals, they can provide measures of mixture effects and contribute to the assessment of the cumulative effects of chemicals that exhibit the same mode of toxic action and thus cause concentration-additive effects. Improved detection of the presence of groups of chemicals with common modes of action in water will improve risk assessment methods and inform future water management options, particularly in the context of water reclaimed from impaired sources such as wastewater or stormwater.

Furthermore, in addition to the identified chemicals, an unknown number and quantity of chemicals not yet identified are present in recycled water, including transformation products of chemicals known to occur therein that can be (1) introduced into wastewater after human metabolism and excretion of pharmaceuticals, (2) formed by biodegradation or advanced oxidation processes during wastewater and advanced water treatment, or (iii) formed from benign precursors during water disinfection processes. The potential of bioanalytical techniques for evaluation of "unknown unknowns" has been stressed by a Science Advisory Panel that was convened by the State of California to recommend monitoring strategies for chemicals of emerging concern in recycled water.

The overall goal of the project "Development of Bioanalytical Techniques to Assess the Potential Human Health Impacts of Recycled Water" was to advance the science and application of bioanalytical tools for water quality assessment, with a focus on recycled/reclaimed water, by achieving the following objectives:

- **Literature Review:** The objective was to give an overview on bioanalytical tools that can assess likely impacts on human health. To achieve this objective, we reviewed available in vitro or short-term in vivo bioassays, including those that have not previously been used for water quality assessment but that address endpoints of human health relevance. The result is a list of in vitro bioassays that are the most promising for assessing the potential for effects of recycled/reclaimed water exposure on human health.

- **Validation:** We selected nine bioassays on the basis of the outcomes of the literature review and validated them for application on water samples. The newly established bioassays together with bioassays already established in the laboratories of the core partners were used to assess 10 water samples collected in the United States at various sites including a pilot-scale facility equipped with ozone and UV that treated wastewater treatment plant effluent and a managed aquifer recharge facility. Furthermore, the team doing a larger interlaboratory study of 20 laboratories performed 103 bioassays on 10 Australian samples collected across the entire water cycle from treated wastewater to drinking water.

- **Development of Interpretation Guidelines:** This part included both recommendations for data evaluation and communication of data as well as the preliminary development of effect-based trigger (EBT) values for benchmarking water quality.

In Chapter 1, we reviewed the potential of bioanalytical tools for application in water quality assessment and evaluation of trace organic pollutants. For the purposes of this review, bioanalytical tools were defined as in vitro cell-based bioassays and were classified according to their mode of toxic action or toxicity pathway. A toxicity pathway is the chain of events that lead from the uptake of a chemical to the observed toxicity. Molecular and cellular effects are the main targets of in vitro assays, which can either assess toxicity or the activation of adaptive stress response pathways. Organ and system responses can be assessed only indirectly with cell-based bioassays.

For each human health-relevant toxicity endpoint, we reviewed assays that have previously been used in water quality assessment (Category 1 bioassays). In addition, we reviewed bioanalytical tools covering relevant modes of toxic action or toxicity pathways that have the potential for but are not being used for water quality assessment (Category 2 bioassays). Where available, examples of reference chemicals are listed; however, as most Category 2 bioassays were not originally developed for assessment of environmental samples, the test compounds often do not overlap with those expected to occur in water samples.

The nuclear xenobiotic receptors that are involved in the up-regulation of metabolism can provide early indicators of chemical exposure. The arylhydrocarbon receptor is the most widely assessed of the xenobiotic receptors because this receptor is activated by dioxin-like chemicals. The pregnane X receptor also has potential for application in water quality monitoring as it has been reported that it is induced by a large number of pesticides. The same holds for the peroxisome proliferator-activated receptor (PPAR), which is activated by a large number of trace organic chemicals but has so far not been applied for water quality assessment. It must be noted, however, that induction of xenobiotic metabolism is not directly related to toxicity but rather to detoxification or activation processes and must therefore be seen as an indicator of exposure and not necessarily of effect.

For reactive toxicity, genotoxicity has been the focus of most previous applications. As a wide variety of in vitro bioassays for genotoxicity have already been applied to water samples, there is no immediate need to implement further Category 2 bioassays for genotoxicity. It is advisable, however, to complement genotoxicity assays that quantify the actual damage done (direct genotoxicity) with assays that detect the activation of repair systems in response to DNA damage and possibly also with assays that detect epigenetic changes.

This last statement can be generalized: the induction of defense mechanisms to compensate for damage is a logical and more sensitive alternative because it assesses the potential to do harm rather than assessing damage. The general adaptive stress response pathways, particularly the ones responsible for management of inflammation, oxidative stress, and DNA damage, appear to be underutilized thus far in water quality assessment, even though extensive single-chemical testing has accumulated a large database and has demonstrated the relevance of these pathways for environmentally relevant chemicals.

Of the bioassays indicative of organ responses, hepatotoxicity and nephrotoxicity appear to be the most relevant for assessing trace organic contaminants in water. Although hepatotoxicity assays are widely applied for water quality testing, application gaps still need to be closed for nephrotoxicity.

With respect to system toxicology, neurotoxicity is highly relevant because insecticides are abundant trace organic pollutants. Several different assays are available, but many are cell free and should be replaced by cell-based bioassays in the future. Immunotoxicity is rarely assessed in water quality assessment, and many available assays are ex vivo rather than true in vitro assays. These applications clearly need further development, and the scope needs to be expanded to chemically induced immunosuppression.

In relation to reproductive and developmental effects, the pathways mediated by retinoid signaling appear to be most promising for applications in water quality testing. Yeast-based assays for retinoic acid receptor activity are most abundant in water quality testing, but there also are a number of mammalian-based systems with potential for future use. Last but not least, endocrine disruption, in particular estrogenic, androgenic, and progestagenic activity, is probably the most frequently tested aspect of system toxicology in water quality assessment and needs no further refinement or implementation of Category 2 bioassays. The disruption of glucocorticoid and thyroid function is, however, less well established and is recommended for further evaluation. Also, further assay development may be needed to reliably measure antagonistic activity.

In addition to individual bioassays, multiplex transcription reporter gene assays show great promise for applications in water quality testing by allowing the simultaneous profiling of the activity of a large number of nuclear receptors and transcription factors.

The second part of the literature review involved methods to evaluate and interpret data. In Chapter 2, we summarize established methods for the dose−response assessment and the derivation of effect concentrations from dose−response curves. In addition the method of using bioanalytical equivalent concentrations (BEQs) as an easy-to-communicate alternative to effect concentrations is discussed. We developed interpretation guidelines to help interpret the obtained data. Basic requirements for data quality including positive and negative controls were defined. There exist almost as many different ways to evaluate and report bioassay results as there are bioassays, but we were able to narrow data evaluation down to two major approaches:

1. If there is a clearly defined reference chemical that elicits a log-sigmoidal concentration effect curve, the bioanalytical equivalency concept is applicable and results can be expressed as BEQs and reported as the concentration of a reference chemical that induces the same effect as does a complex unresolved water sample.
2. If no maximum can be reached, if almost all chemicals in a sample contribute to the effect and/or if no reference compound can be defined, then the effects are typically

expressed as induction ratio, the quotient of the signal of the sample divided by the signal of the negative control. The limit of detection (defined as three times the standard deviation of the controls) was often about an induction ratio of 1.5, and therefore this threshold was chosen as the effect threshold for these types of effects.

After prioritization of Category 2 bioassays for further validation during the literature review, we selected nine bioassays for implementation and validation in Chapter 3. The PPARγ-GeneBLAzer assay was included as a representative for induction of the xenobiotic metabolism. This assay was successfully established with reference compounds, but none of the water samples evaluated here, some of which are highly treated, showed any effect in this bioassay even though environmentally relevant chemicals are known to induce this pathway.

Because of the lack of Category 1 bioassays indicative for induction of adaptive stress response pathways, we emphasized this class of bioassays and evaluated one bioassay for hypoxia (Switchgear), two for oxidative stress response (Nrf2-keap cell line and AREc32), and one for the inflammatory stress response (formation of an inhibitory protein involved in the NF-κB pathway in human lymphoblastoma cells). Whereas three bioassays did not show any or very small effects with water samples, AREc32 was highly responsive to reference compounds and water samples and a report on the successful implementation of this bioassay was published in a peer-reviewed journal.

With respect to endocrine disruption, we added bioassays for the glucocorticoid, thyroid, and retinoic acid receptors. These three endpoints have been previously applied for water quality testing but were newly established in the laboratories of the project team. The reporter gene assay for the glucocorticoid receptor from Switchgear was highly responsive to water samples, and this bioassay was then also used to fractionate the samples and search for the causative agents by using high-resolution mass spectrometry and powerful statistical tools. Dexamethasone and betamethasone were two of the compounds that were identified in more than 170 specific ions present during analysis of these samples.

The T-Screen assay measures cell proliferation in the presence of thyroid hormones or their xenobiotic mimics, but it was not sensitive enough to record reproducible results with the recycled water samples tested. There are other bioassays for thyroid activity, such as reporter gene assays, thyroid receptor binding assays, and transthyretin binding assays, but it is unclear which mode of action (and thus which assay) is the most relevant to detect thyroid disruption from environmental samples. A reporter gene assay (TR-CALUX) was used to test thyroid activity in the water samples, but no activity was detected in any of the water samples. It may be that thyroid active substances require metabolic activation (e.g., PBDE vs OH-PBDE) and that future work on the thyroid endpoint should include preincubation with a liver enzyme mix.

Finally the human neuroblastoma cell line SK-N-SH was used to detect cytotoxicity to neural cells by assessing the cell viability of these cells, but none of the samples displayed detectable "neurotoxic" activity.

Additional Category 2 bioassays were implemented by collaborating laboratories. The GeneBLAzer battery for estrogenicity and androgenicity and for progestagenic and glucocorticoid effects was implemented by laboratories at the University of California−Riverside, University of Florida, University of South Florida, and SCWWRP and was provided to this project through a collaboration agreement facilitated by WRRF.

Two application studies were then performed to apply the established Category 1 and 2 bioassays to water samples. Prior to sampling, the solid-phase extraction method was optimized to capture a wide range of chemicals typically occurring in wastewater, including hydrophilic and hydrophobic chemicals (Chapter 4). The best-performing extraction was accomplished by using a combination of Oasis HLB in sequence with coconut charcoal, which was used for the enrichment of all water samples in this project.

The goal of the application studies was to test the suitability of bioanalytical tools for benchmarking water quality and assessing treatment efficacy as well as to explore the potential application for future monitoring programs. Therefore, despite the overall limited number of samples investigated, it was ensured that they represent a wide variety of typical treatment technologies and water qualities. Thus, the focus on a limited number of facilities was appropriate to fulfill the objective of the project. In all cases the influents were secondary treated wastewater and the treated samples were compared directly with the influents to assess the treatment efficacy with respect to the different biological responses.

The first application study involved only the three core laboratories and evaluated 10 samples collected in Arizona in 39 bioassays including antagonist tests (Chapter 5). The 10 samples included a nitrified wastewater treatment plant (WWTP) effluent, a partially nitrified WWTP effluent, and a nitrified/denitrified WWTP effluent. A pilot-scale facility equipped with ozone and UV radiation was used to further treat the nitrified effluent. Additional samples also were collected from a managed aquifer recharge facility where water samples were collected from harvest wells at different travel times. Potential for genotoxicity and mutagenicity was evaluated by using *umu*C and Ames tests, both with and without metabolic activation. For screening of specific receptor-based mode of action, estrogen activity and glucocorticoid activity were important on the basis of observed effects. For induction of xenobiotic metabolism, the arylhydrocarbon receptor induction seemed to have the highest priority, and for adaptive stress response, AREc32 was the most responsive measure of oxidative stress response. The cytotoxicity test provided valuable information regarding the overall cell viability. The data showed that UV and ozone oxidation generally was quite efficacious for attenuating bioactivity.

The second application study involved a total of 20 laboratories, which applied 103 distinctly different bioassays in 137 experiments to 10 water samples collected in South East Queensland, Australia, across two water reuse schemes and also included surface water, stormwater, and drinking water for comparison (Chapter 6). The first treatment train consisted of microfiltration, reverse osmosis, and polishing with H_2O_2/UV, and the second treatment train used ozonation followed by biologically activated carbon filtration. Sixty-five out of 103 bioassays showed positive results in at least one sample. Each type of water had a characteristic bioanalytical profile with certain groups of toxicity pathways consistently being responsive and others not responding in any test system. The most sensitive and responsive endpoints were related to the xenobiotic metabolism (pregnane X receptor and arylhydrocarbon receptor), hormone-mediated modes of action (mainly related to estrogen, antiandrogen, and glucocorticoid receptors), reactive modes of action (genotoxicity), and adaptive stress response pathways (mainly oxidative stress response). The bioassays could be used to assess the treatment efficacy and benchmark water quality against positive controls (wastewater) and negative controls (ultrapure water). After reverse osmosis the water was not significantly different from the ultrapure water, and reverse osmosis and ozonation followed by biologically activated carbon filtration produced similar high-quality water.

In Chapter 7, a fractionation approach was developed on the example of the binding to the glucocorticoid receptor for the identification of bioactive fractions in the U.S. samples (Chapter 5).

In Chapter 8, EBT values were developed by translating existing water quality standards into preliminary EBT values for recycled and drinking water for four different bioassays, which were identified to be amongst the most relevant bioassays of the 100 evaluated ones (indicator bioassays). Mixture effects were considered in the derivation of these EBT values. The proposed values were compared with the data obtained in the application study of the Australian samples, assuming that the recycled water is targeted for direct and indirect potable reuse. All recycled water samples would be "compliant" with the proposed EBT values, but secondary treated effluent would be "noncompliant" with the EBT values for drinking water. This finding suggests that the EBT values are relevant to the real world and that they provide a clear benchmark separating water suitable for drinking (such as highly treated recycled water targeted for indirect potable reuse and drinking water) from that unsuitable for human consumption (such as treated wastewater).

Chapter 9 brings all parts of the project together and summarizes what we have learnt and how a typical test battery of bioassays should look and shows the way forward.

In conclusion, all goals of this project were achieved and the results of the study are a major leap toward the implementation and acceptance of bioanalytical tools to assess the quality of recycled water. The purpose of this study was not to investigate the occurrence of bioactivity at a large number of facilities but rather to demonstrate the proficiency and reproducibility of a battery of test assays that could be employed in reuse scenarios to screen complex mixtures. Thus, the most important aspect of the study was demonstrating that the assays were effective in screening both highly challenged waters (i.e., secondary effluents from WWTPs) as well as highly treated waters (i.e., post-reverse osmosis waters). Our team strongly endorses a follow-up study that would evaluate the bioactivity from hundreds, if not thousands, of water treatment systems around the globe.

Chapter 1

Literature Review of In Vitro Bioassays for Assessing Adverse Effects of Trace Organic Pollutants in Water

1.1 Introduction

This chapter provides a brief overview of the adverse effects of organic pollutants on human health.[1] The effects are categorized according to toxicity pathways. Toxicity pathways comprise the molecular events that take place from the moment a chemical is taken through all steps of distribution and binding to receptors, defense mechanisms up to its ultimate effect. This review considers only toxicity pathways linked to relevant adverse outcomes for human health via exposure to toxicants in recycled water. Then bioanalytical tools indicative of specific toxicity pathways are reviewed. The review focuses on cell-based bioassays, excluding cell-free assays, which often have difficulties with matrix effects by organic matter and other coextracted water constituents. For the purposes of this review, cell-based bioassays are subdivided into two categories.

- Category 1 bioassays are bioassays that have been fully or partially validated for application in water quality assessment on the basis of mode of action.

- Category 2 bioassays cover endpoints relevant to human health and associated bioassays that have the potential for but are not being used for water quality assessment.

In this introductory chapter, we provide a brief overview of the mechanistic background of toxicity pathways and the principles of cell-based bioassays. A more in-depth treatise of background information and terminology can be found in Escher and Leusch (2012). Section 1.2 summarizes the principles of in vitro toxicology with a focus on the Tox21 program. In Sections 1.3 to 1.7, we outline the different types of cell-based bioassays and explain how these can be classified according to toxicity pathways.

The Tox21 program (NRC, 2007; Shukla et al., 2010) aims to advance molecular toxicology, system biology, and computational toxicology to overcome shortcomings of traditional in vivo toxicity testing of chemicals. Tox21 brings a paradigm shift in toxicity testing as in vitro methods are used to elucidate mechanisms of toxicity and to prioritize chemicals for further testing. The ultimate goal is to develop predictive models in order to reduce or eliminate future in vivo testing. Tox21 is a joint initiative of the National Toxicology Program of the

[1] Sections of this chapter have been adapted with permission from Escher and Leusch (2012).

National Institutes of Health (NIH) (http://ntp.niehs.nih.gov/), NIH Chemical Genomics Center (NCGC), and the ToxCast Program of the U.S. EPA Office of Research and Development (http://www.epa.gov/ncct/toxcast/[2010]). These programs rely heavily on high-throughput cell-based and cell-free in vitro bioassays and are in the process of conducting high-throughput screening (HTS) of large numbers of chemicals for evaluation of their toxicity pathways. HTS allows testing large numbers of chemicals, but not all types of bioassays lend themselves to adoption of HTS. Practical issues that need to be addressed include sensitivity, reproducibility, accuracy, and economic feasibility (Inglese et al., 2007).

Although the programs mentioned earlier target risk assessment of single chemicals and are still in progress, the results are continuously made publicly available. Here, we reviewed the associated databases and literature for relevant information and toxicological endpoints that could be applied for water quality testing to ensure, that the tests selected in the proposed project are relevant for human health risk assessment. Chapters 3 to 7 give a more detailed account of individual toxicity pathways and associated bioassays, summarizing both relevant information from the Tox21 program and international peer-reviewed literature.

1.2 Principles of Cell-Based Bioassays

In vitro bioassays can employ primary or immortalized cell lines. Primary cells are taken from tissues of living organisms. Primary liver cells (hepatocytes), for example, are isolated from liver tissue. Compared to immortal cells that have been mutated, primary cells are generally considered more representative of normal cell function. Primary cells, however, have a limited life span, whereas immortal cell lines proliferate indefinitely. The longevity of immortal cells removes the need to exploit live animals, making this cell type both ethically and financially favourable. Using identical cells also reduces interindividual variability, which may exist when primary cells are derived from different individuals.

The choice of cell type will depend on the particular assay, the endpoint of interest, and its mode of action (Figure 1.1). Most organisms can be used, from simple life forms such as plants, yeasts, and bacteria to complex organisms, including humans and other mammals, such as rodents and monkeys. For this review, we mainly focus on human and mammalian cell-based assays because of their greater relevance as miniature models for assessing effects on human health.

Nonspecific cell toxicity (cytotoxicity) is typically measured as cell growth rate and survival. These endpoints can be evaluated by direct cell counting (using, for example, a flow cytometer or a hemocytometer) or indirect methods using dyes to measure membrane integrity and/or metabolic activity in cells. Indirect endpoints include mitochondrial dehydrogenase activity (e.g., tetrazolium salts and MTT and XTT assays), active transport and lysosomal function (e.g., neutral red uptake [NRU]), metabolic activity and energy metabolism (e.g., resazurin/Alamar Blue assay, adenosine triphosphate [ATP] chemiluminescence assay) and membrane integrity (e.g., lactate dehydrogenase [LDH] and 5-carboxyfluorescein diacetate acetoxymethyl methyl ester [CFDA-AM]). Methods that simply measure protein content (e.g., kenacid blue and sulforhodamine B [SRB] staining assays) are usually less reliable indicators of cell viability (Escher and Leusch, 2012).

Specific toxicity occurs when exposure to a chemical induces a specific response such as DNA damage or the activation of a nuclear receptor (NR). In some cases, when primary cells are exposed to a xenobiotic, the initiating event causes a specific detectable cellular response, such as the production of a protein, which is called a biomarker (Escher and Leusch, 2012). A

commonly measured biomarker in aquatic toxicity testing is the production of vitellogenin in fish liver cells as an indicator of exposure to estrogenic compounds. Natural biomarkers are often difficult to detect. To overcome this difficulty, recombinant cell lines are genetically engineered to produce a measurable product (e.g., enzyme or other protein) in response to the initiating event (Figure 1.1).

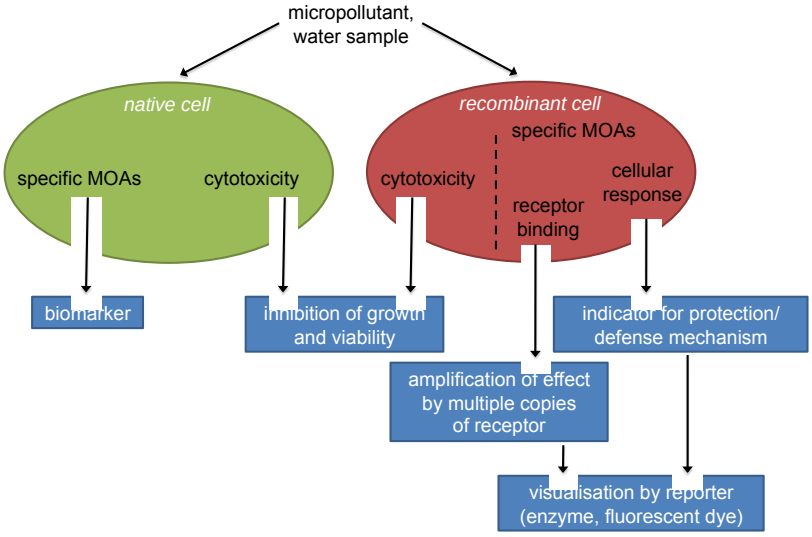

Figure 1.1. Types of cell-based assays and assessment endpoints.

Note: MOA = mode of (toxic) action.

Source: Reprinted with permission from Escher and Leusch (2012). Copyright 2012, IWA Publishing.

Reporter gene assays are popular genetically engineered tools in bioanalytical testing. Reporter cell lines are generated by transfecting cells with a gene that encodes an easily detectable product, a reporter, such as green fluorescent protein (GFP) or an enzyme (e.g., luciferase or β-galactosidase). The reporter is paired with a promoter region that is specific to a particular mode of action. Activation of this target mode of action then causes transcription of the reporter gene to messenger RNA (mRNA) followed by production of the fluorescent protein or enzyme, which can be quantified by fluorescence or enzymatic assays (Escher and Leusch, 2012) (Figure 1.2). The magnitude of the signal produced is proportional to the specific response of interest and is often quantified relative to the signal produced by exposure to a reference chemical.

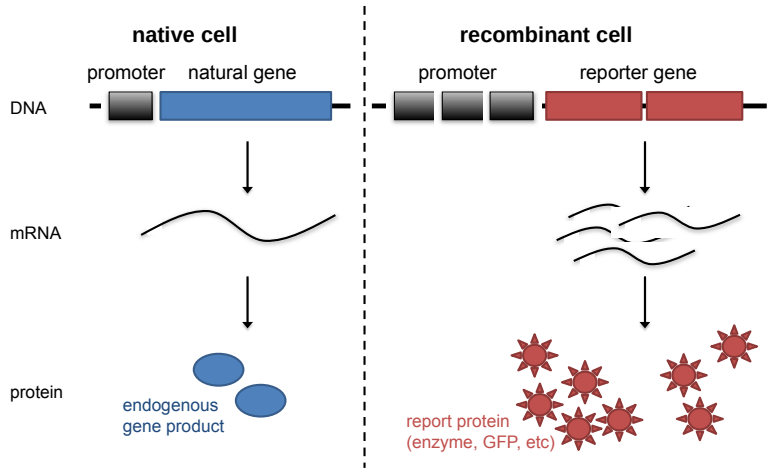

Figure 1.2. Principle of the reporter gene assay.

Note: A plasmid containing a reporter gene downstream of the natural gene promoter is inserted into a recombinant cell, and activation of the promoter results in production of a fluorescent reporter protein.

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1.3 Toxicity Pathways

The toxicokinetics describe all processes that link external exposure (e.g., via drinking water or food) to the biologically effective dose in the cell (Escher and Leusch, 2012). These processes comprise the absorption, excretion, and internal distribution and metabolism of a chemical within the body and cells.

The toxicodynamics describe the toxicity pathways that take place within the cell, starting with the initial molecular interaction of the chemical and its biological target through cellular defense mechanisms and other cell responses to observable toxic effect(s) or disease (Escher and Leusch, 2012). Toxicity pathways are defined as "the cellular response pathways after chemical exposure expected to ultimately result in adverse health effects" (Collins et al., 2008; Ankley et al., 2010) Figure 1.3). As cellular responses (e.g., gene activation, production/depletion of proteins, and changes in signaling) can occur via multiple steps, many overlaps and links exist between the many different toxicity pathways. Organic trace pollutants also can induce natural endogenous pathways, and toxicity pathways are sometimes referred to simply as biological pathways. Biological pathways may only modify the activity of a cellular response without causing a direct adverse effect but can be considered indicative of the presence of trace organic pollutants.

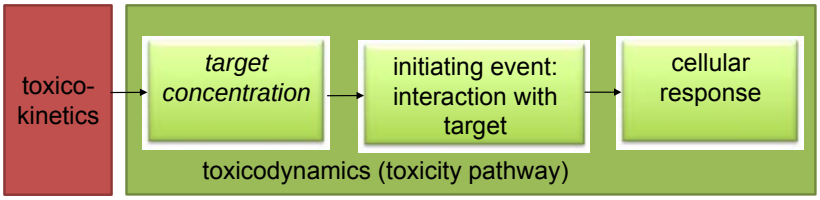

Figure 1.3. Principle of toxicity pathways.

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In vitro assays represent cellular level effects. Cell responses do not necessarily imply higher-level effects but are a prerequisite for whole-organism and -population responses (Escher and

Leusch, 2012). If the initiating event, the interaction of the organic pollutant with its biological target, is detected directly in a bioassay, we have a measure only of potential effect, as repair and defense mechanisms may still neutralize this effect. From a precautionary risk perspective, however, the potential to do harm is a crucial assessment endpoint (Escher and Leusch, 2012). As the initiating event often cannot be measured directly, steps in the cellular response pathway are often quantified by using a reporter gene assay, where a receptor or response element in the pathway of a cellular response is overexpressed. Many cellular responses are mediated via NRs, namely, proteins that trigger certain cellular processes. The activation of NRs is often directly or indirectly related to toxicity and also can be seen in reporter gene assays. The recombinant cells used in such assays are typically based on immortal cell lines (e.g., a rat hepatoma cell line or a human breast cancer cell line) that have been altered to include a reporter attached to a gene containing a responsive element for that receptor. The amount of response (enzyme activity or fluorescence intensity) quantified via the reporter is directly correlated to the amount of chemical bound to the receptor.

When the capacity of repair and defense mechanisms is exceeded, all pathways ultimately lead to cytotoxicity, namely, cell death (Escher and Leusch, 2012). Apoptosis is programmed cell death, which occurs as a final resort in the defense strategy and is initiated to remove damaged cells and plays an important role in the elimination of precancerous cells. Necrosis occurs following irreversible inhibition of vital cell function.

The concept of toxicity pathways can be expanded to so-called "adverse outcome pathways" (Ankley et al., 2010) (Figure 1.4). An adverse outcome pathway links the cellular toxicity pathway with the organ-level response, followed by the organism-level response and ultimately the population-level response. Organ-level effects are observed as altered physiology of the organ, disruption of homeostasis, altered tissue development, and/or disruption of organ function (Escher and Leusch, 2012). At the system or organism level, these effects translate to effects such as impaired development, reproductive failure, disability, and/or death. In ecotoxicology, individual effects may not be considered serious unless population levels are impacted, but in terms of human health assessment, we are concerned about individuals.

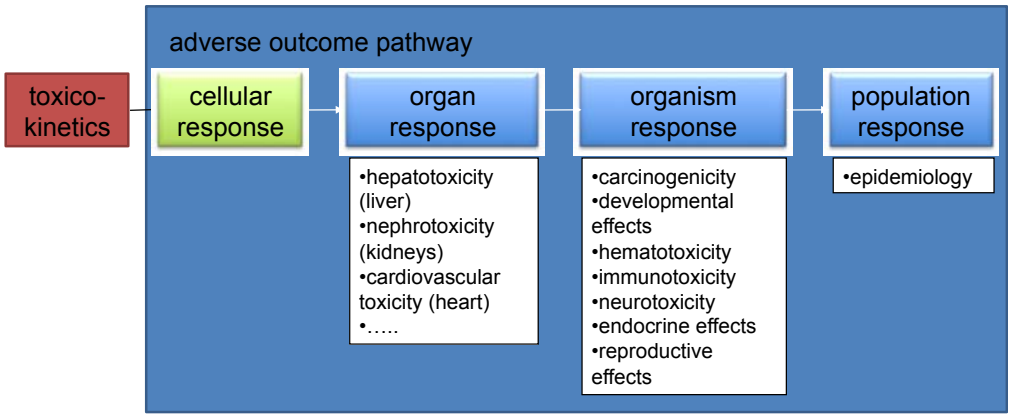

Figure 1.4. Adverse outcome pathways link the cellular response to the adverse health outcome.

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Cell-based bioassays can target all levels of toxicity and adverse outcome pathways:

1. Toxicokinetic indicators: The induction of metabolic pathways does not necessarily lead to cytotoxicity but is an indicator of the presence of trace organic pollutants.

2. Indicators of cellular effect: Some bioassays target the initiating event through sighting of primary events. The final response of the cell can be assessed by measurement of cell viability, growth, and/or proliferation. If the cell represents a specialized tissue, this gives an indication of impairment in this specific tissue. In addition, some general stress response pathways are triggered in cells in response to chemical insult and may act as early indicators of exposure to trace organic pollutants.

3. Organ response: Although cellular in vitro systems are not capable of mimicking the complex interactions occurring in organs, cytotoxicity measured in specific cell types provides a first-tier screening of the toxicity toward the associated organ (e.g., hepatocytes representative of the liver). In addition, there are ex vivo models, for example, 3D skin models and test systems involving multiple cell types cultured together that simulate more-complex interactions.

4. System response: Again a cell-based assay cannot model an entire system, but relevant cell types can be used as screening tools for toxicity toward a system. Often applied are tests for endocrine disruption, particularly estrogenicity.

The following bioassay review is structured according to the above four levels of effect. In Section 1.4, toxicokinetic indicators are reviewed. The bioassays indicative of cellular effects are subdivided in two chapters: Section 1.5 covers assays for detection of direct cellular effects caused by chemicals, and Section 1.6 reviews assays to detect the response of the cells to those insults, namely, adaptive stress response (ASR) pathways. In Section 1.7, bioassays that are related to organ response are reviewed, and in Section 1.8, we review bioassays indicative of system response.

In each main chapter, a brief background is given on the relevant response category and its underlying mechanisms, followed by a review of bioassays indicative of responses in this category. The authors of this report have recently reviewed applications of cell-based bioassays in water quality assessment (Escher and Leusch, 2012), and some content of the subsequent sections has been adapted from this book. This report expands the scope of the book by including not only bioassays that already have been used for water quality assessment (Category 1 bioassays) but also those that are promising for but have not yet been used for this application or cover a mode of toxic action that has not been linked to chemicals present in water (Category 2 bioassays). The bioassays are outlined with a brief description of the method, including measured endpoint, expression of results, and tested reference chemicals. Whereas listing of reference chemicals relevant for water quality screening is desirable, most Category 2 bioassays were not developed for environmental screening purposes and information on compounds likely to occur in water is often not available.

1.3.1 In Vitro Bioassays in Chemical Toxicity Assessment

The National Toxicology Program of NIH and the ToxCast Program of the U.S. EPA are in the process of conducting HTS of large numbers of chemicals to evaluate their toxicity pathways. Under Tox21, a large number of cell-based high-throughput assays were (or will be) tested on 1408 compounds (Shukla et al., 2010). The applied cells are of both human and mammalian origin, and the assays include cell viability, membrane integrity, mitochondrial

toxicity, DNA damage, cytokine production, and activation of NRs and toxicity pathways. Many are reporter gene assays, and some are single-cell imaging assays.

More than 600 endpoints of high-throughput tests have been tested on 309 chemicals in the first phase of ToxCast, and the findings have been made publicly available (Judson et al., 2010), (http://www.epa.gov/ncct/toxcast/). The results have been compared to in vivo animal study results, which are collected in the Toxicity Reference Database of the National Center for Computational Toxicology (http://epa.gov/ncct/toxrefdb/).

Phase I of ToxCast has been completed, and in addition to the database entries, the results were published in a series of papers in the peer-reviewed literature (Judson et al., 2009; Judson et al., 2010; Martin et al., 2010; Reif et al., 2010; Judson et al., 2011; Knudsen et al., 2011; Martin et al., 2011). In 2010, Phase II of ToxCast started with the goal of screening additional chemical compounds representing broader chemical structural classes to evaluate the predictive toxicity signatures developed in Phase I.

In Phase I of ToxCast, key transcription regulators were identified by profiling 309 environmental chemicals on 25 NRs and 48 transcription factor (TF) responses (Martin et al., 2010). Additionally 292 biochemical targets were profiled in cell-free assays with the same chemicals set using HTS of G-protein coupled receptors, cytochrome P450 mono-oxygenase enzymes (CYPs), kinases, proteases, phosphatases, ion channels, transporters, and various NRs to confirm known targets in signaling and metabolic pathways and to identify new targets (Knudsen et al., 2011). Overall, only 13 cell-based HTS tests were applied (Inglese et al., 2007), although a larger number of cell-based multiplex transcription reporter gene assays were included. From this large data set, 33 minimal pathways were extracted, showing that many of the tested 309 environmental pollutants were active in multiple pathways but rarely in more than 10 of the 33 minimal pathways (Judson et al., 2010). More-integrative assays such as the differentiation and cytotoxicity of mouse embryonic stem cells were also tested and compared to the other ToxCast endpoints (Chandler et al., 2011). The fact that a common set of test chemicals was evaluated in a large battery of assays facilitates direct comparison between different platforms. Such comparison was not previously possible, as different papers reported the results of different and much smaller sets of test chemicals.

Overall, the ToxCast chemical list includes 960 individual chemicals. Although Phase I included only pesticides, Phase II includes also the 386 chemicals evaluated by the National Toxicology Program, all 217 chemicals evaluated by the EPA Office of Water (CCl1 to 3 and PCCL lists), the 120 chemicals evaluated by the EPA endocrine disruptor-screening program, and various other environmentally relevant chemicals. As such, the chemical list is an excellent resource of toxicity profiles, and furthermore, if bioassays from the ToxCast program were to be used for water quality assessment, a link to the types of chemicals causing the observed effects appears to be within reach.

In this chapter, we review the cell-based bioassays of ToxCast separately, as these show great promise for water quality testing in having been and in being tested on large numbers of environmental pollutants. Most of these assays are clearly Category 2 bioassays, which have not yet been evaluated on water samples. We will refer to the individual assays in the following chapters but here give a general overview.

1.3.2 Cell-Based High-Throughput Screening of 1408 Chemicals

Xia et al. (2008) tested a total of 13 different cell lines from liver, blood, kidney, nerves, lung, and skin of humans and rodents against 1408 chemicals using a homogeneous, luminescent cell viability assay in HTS format that relies on the quantification of ATP as a measure of viable and metabolically active cells. About a third of the test chemicals showed cytotoxicity at concentrations of up to 92 μM in at least one of the cell types. Some chemicals were selectively active in one or few cell lines, whereas others were toxic to a wide range of cells. Although there was no single most sensitive cell type, the Jurkat cell line (derived from human blood) and a cell line derived from human neurons were amongst the most sensitive cell types. A negative point was the observation that the same cell types (e.g., liver hepatoma cells) often yielded substantially different responses depending on the species from which they were derived. Between humans and rodents, rodents often delivered more-sensitive cells. Even two very similar human neuronal cell lines showed differences in sensitivity, with the more differentiated daughter cell line showing the highest sensitivity (Xia et al., 2008). This extensive study demonstrates that a single cell line may not be sufficient to address all aspects of cytotoxicity but that a range of different cell types can deliver a sensitivity profile.

1.3.3 High-Throughput Screening of 3000 Chemicals on 10 Human Nuclear Receptors

Huang et al. (2011b) recently reported results from quantitative HTS of 3000 chemicals against a panel of 10 human NRs including the androgen receptor (AR), the estrogen receptor (ERα), the farnesoid X receptor (FXR), the glucocorticoid receptor (GR), the liver X receptor (LXRβ), the peroxisome proliferator-activated receptor (PPARγ and PPARδ), the retinoid X receptor (RXRα), the thyroid receptor (TRβ), and the vitamin D receptor (VDR). These receptors were expressed in β-lactamase reporter gene assays on the basis of HEK 293T cells developed by Invitrogen and named GeneBLAzer. To quantify the gene products, these assays use a fluorescent substrate, which was found to be unsuitable for about 26 of the tested chemicals because of their autofluorescence. All tests were run with a dilution series of chemicals in dimethyl sulfoxide (DMSO) in a 1536 well plate format and with a minimum of 14 different concentrations by using 10 mM DMSO stocks of all chemicals. All chemicals were tested in the agonistic mode (with only the test chemical present) and in the antagonistic mode (in the presence of a given concentration of a known agonist, tabulated in the Supplementary Information of Huang et al. [2011b]). Because the antagonist-mode assays would be compromised by cytotoxicity, parallel cytotoxicity tests were performed. More chemicals turned out to act as antagonists than as agonists. Most prominent were the antagonists of the AR (>10% of chemicals tested positive) and PPARγ (8% of chemicals). AR, ERα, and RXRα tested positive in 2−-3% of all test chemicals.

1.3.4 Cell-Based Multiplex Transcription Reporter Gene Assays Used in ToxCast

Although all individual receptors were tested individually in the GeneBLAzer reporter gene assays, the novel biosensor system “FACTORIAL” developed by Attagene, Inc. can simultaneously screen 25 NRs or 48 TF response elements in HepG2 human liver carcinoma cell lines (Martin et al., 2010). This technology was initially designed to overcome the problems of single-reporter gene assays that are limited to screening of one TF at a time. Further, single-reporter gene assays may yield variable responses if posttranscriptional mechanisms alter the expression of the reporter protein. This limitation has been overcome by

the construction of uniform reporter transcription units (RTUs), which are a common plasmid with individual TF-inducible promoters fused to a reporter sequence that varies only slightly between the different RTUs. The different reporters can be separated and quantified by capillary electrophoresis (Romanov et al., 2008). The same technology was also adopted for NRs, but only those that are expressed endogenously in the HepG2 cell line (25 NRs) could be implemented in this assay (Martin et al., 2010). Unfortunately, at this point in time the Attagene assays use transient transfections and can be carried out only in-house.

Both assays were tested with the 309 Phase I ToxCast chemicals by applying a series of concentrations that were demonstrated not to be cytotoxic. Through use of these tools, signaling pathways highly relevant for the pesticides in the ToxCast Phase I chemical list became evident. These pathways include the oxidative stress pathway Nrf2-ARE, the PPAR pathway, the VDR pathway, and the pregnane X receptor (PXR) pathway. This rich database is very useful for prioritizing endpoints and targets for water quality analysis.

1.4 Bioassays Indicative of Toxicokinetic Processes

Many foreign chemicals ("xenobiotics") trigger metabolic pathways activating and/or increasing the metabolic activity within a given cell (Escher and Leusch, 2012). Hepatocytes (liver cells) are particularly important for biotransformation; however, other cell types also exhibit various degrees of metabolic capacity.

Metabolic pathways can be used as indicators of the presence of chemicals. There are two ways to assess the activation of metabolic enzymes: (1) via direct measurement of enzyme activity or (2) via measurement of the induction of a xenobiotic receptor. Cellular pathways related to metabolism are regulated by xenobiotic receptors (Omiecinski et al., 2011). Binding of certain chemicals to these receptors induces the transcription of genes that encode metabolic enzymes. NR binding is not a toxic response in itself but an indicator of the presence of chemicals that interact with the receptor. A well-known example of a xenobiotic receptor is the arylhydrocarbon receptor (AhR), which responds to exposure to dioxin-like chemicals.

Xenobiotic NRs regulate metabolism of xenobiotics via similar key steps. Ligand binding of the chemical to the receptor causes dissociation of a bound protein from the receptor. The ligand−receptor complex translocates to the nucleus, where it binds to a receptor-specific response element on DNA, triggering the expression of the associated gene(s) that are involved in xenobiotic metabolism (Escher and Leusch, 2012).

Table 1.1 lists the nuclear xenobiotic receptors that are involved in up-regulation of metabolism. Each receptor has several functions taking part in various metabolic processes and in cell homeostasis.

Table 1.1. Nuclear Xenobiotic Receptors

Nuclear Receptor	Function	Inducing Chemicals
Pregnane X receptor (PXR)	Induction of various Phase I enzymes (CYPs), particularly CYP3A isoforms	Steroids
Constitutive androstane receptor (CAR)	Protective role against toxicity induced by bile acid, regulation of physiological functions	Indirectly activated by phenobarbital, various pharmaceuticals
Peroxisome proliferator-activated receptor (PPAR)	Glucose, lipid, and fatty acid metabolism	Phthalates, fibrate pharmaceuticals
Arylhydrocarbon receptor (AhR)	Induction of cytochrome P450 (CYP1A1)	PAHs, PCDDs, coplanar PCBs

Notes: CYP = cytochrome P450 mono-oxygenase enzymes, PAH = polycyclic aromatic hydrocarbon, PCB = polychlorinated biphenyl, PCDD = polychlorinated dibenzodioxin.

Source: Adapted with permission from Escher and Leusch (2012). Copyright 2012, IWA Publishing.

1.4.1 Activation of Metabolic Enzymes

1.4.1.1 Mechanistic Background and Relevance

As discussed earlier, the activation of metabolic enzymes is not necessarily connected to the development of toxicity, as metabolism generally detoxifies chemicals but the Phase I mono-oxygenases (i.e., CYPs) can activate chemicals such as polycyclic aromatic hydrocarbons (PAHs) to become carcinogens (Guengerich, 2008). Thus, the induction of metabolic enzymes can be used as an indicator of the presence of xenobiotic chemicals. Typically, all these assays are based on the provision of a substrate that is metabolized by some CYPs. The metabolite(s) are quantified and related quantitatively to the presence of the metabolic enzyme. Each cell type has a basal metabolic activity, and the difference compared to chemically induced activity is a measure of the activation of metabolic enzymes by chemicals.

1.4.1.2 Category 1 Bioassays

The most commonly applied assay indicative of metabolic activity is the ethoxyresorufin-*O*-deethylase (EROD) assay (Table 1.2). This assay quantifies the oxidation of ethoxyresorufin (7-ethoxyphenoxazone) to resorufin by the CYP isoforms CYP1A1 and CYP1A2, which are connected to the xenobiotic metabolism for dioxin-like chemicals. The reaction can easily be quantified because educt and product show a difference in the fluorescence excitation/emission pattern. The EROD assay was developed by Burke and Mayer (1974) by using microsomes (a fraction of liver homogenate) freshly isolated from rats fed with phenobarbital and was brought to the well-plate format by Kennedy and Jones (1994). Huuskonen et al. (1998), who assessed the toxicity of lake water receiving paper mill effluents in a fish hepatoma cell line, were among the first to apply the EROD well-plate assay for water quality assessment. Bols et al. (1999) optimized the EROD assay for application with a rainbow trout liver cell line, and Schirmer et al. (2001, 2004) quantified the EROD activity of water samples from petroleum refinery effluents and contaminated industrial sites.

The HepCYP1A2 assay measures the level of CYP1A2 in human hepatoma carcinoma (C3A) cells and also has been validated for water quality testing (NWC, 2011) (Table 1.2). Similar assays targeted specifically for the detection of hepatotoxicity are further detailed in Section 1.7.1.

Table 1.2. Category 1 In Vitro and Ex Vivo Assays for Detection of Activation of Cytochrome P450

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
EROD (ethoxyresorufin -*O*-deethylase) assay	Various liver cells from tissues of, e.g., rat, mouse, i.e., ex vivo	Induction of CYP1A, detected indirectly by fluorimetric measurement of ethoxyresorufin	Dioxins, dioxin-like chemicals, PAHs	BaP equivalents, TCDD-EQ (also referred to as BIO-TEQ (bioassay-derived TCDD equivalent concentration)	Huuskonen et al., 1998; Schirmer et al., 2004; Ma et al., 2005; Joung et al., 2007; Weston et al., 2010
EROD	Rainbow trout liver cells (ex vivo)	As above	PAHs	As above	Huuskonen et al., 1998; Ganassin et al., 2000; Schirmer et al., 2001; Schirmer et al., 2004
HepCYP1A2 assay	Human hepatoma carcinoma cells (C3A)	Induction of CYP1A2	PAHs including BaP, some pharmaceuticals	Fold induction compared to control	NWC, 2011

Notes: BaP = benzo[a]pyrene; CYP = cytochrome P450; TCDD = 2,3,7,8 –tetrachlorodibenzo-*p*-dioxin.

1.4.1.3 Category 2 Bioassays

A whole suite of assays indicative of metabolic enzyme activity exists, including CYP3A4, which plays a role in drug metabolism (Guengerich, 1999). Cheng et al. (2009) described a fluorescence-based, high-throughput assay for measuring the activity of CYP3A4 on the basis of the oxidative debenzylation of a substituted coumarin.

1.4.1.4 Conclusions

The activation of metabolism is a relatively generic indicator of both detoxification and toxification by metabolism. Particularly, the activation of the isoform CYP1A1 has been widely used as an indicator of the presence of dioxin-like chemicals. Well-validated experimental methods such as the DR-CALUX and the CAFLUX (see Section 1.4.5), which indicate the activation of CYP1A1 rather than the enzyme activity, are widely applied for water quality assessment. Alternative methods for the quantification of the activity of other isoforms of CYP exist but have not yet been applied for water quality testing. The link

between the activities of other isoforms of CYP and toxicity is weaker; most are involved mainly in detoxification reactions; therefore, the implementation of new Category 2 bioassays is of lower priority.

1.4.2 Pregnane X Receptor

1.4.2.1 Mechanistic Background and Relevance

The PXR is one of the hormone NRs identified as a potential candidate for endocrine-disrupting compounds found in contaminated aquatic environments (Creusot et al., 2010). The PXR is expressed in many different tissue types but is most highly expressed in the liver, kidney, and intestinal epithelia (Ihunnah et al., 2011). In its inactive state, the PXR is found within the cytoplasm of the cell bound to a number of corepressors. Upon ligand binding, the PXR dissociates from the corepressors and translocates to the nucleus, where it forms a heterodimer with the RXR before it binds to its DNA-binding site and initiates transcription (Ihunnah et al., 2011). The PXR controls the transcription of a large array of genes coding for Phase I metabolic enzymes, particularly the CYP3A family, which plays an important role in drug metabolism.

The PXR is named for its activation by pregnane steroids but also is activated by a wide range of compounds such as dietary compounds, pharmaceuticals, and environmental pollutants (Chai et al., 2013). The PXR can accommodate a wide range of ligands because its ligand-binding domain is large, about double the size of that of the CAR (Kortagere et al., 2010). In turn, this large number of possible ligands signifies lower specificity. In the ToxCast HTS, (i.e., testing of 25 NRs and 48 TFs), the PXR proved to be the most sensitive endpoint (Martin et al., 2010). Of the 309 ToxCast Phase I chemicals tested, 225 induced the PXR element (PXRE) and 102 activated the PXR. Furthermore, Kojima et al. (2011a) found 106 and 93 of a total of 200 pesticides to be able to activate human and mouse PXR, respectively.

1.4.2.2 Category 1 Bioassays

A number of in vitro bioassays designed to detect PXR activation are available, but mainly one has been adapted to assess sediment and water samples. The HG5LN-hPXR (or HG_5LNGal-4-PXR) cell line was constructed from cervical cancer (HeLa) cells in a two-step stable transfection with the intermediate cell line HG5LN (Seimandi et al., 2005) and expressed an hPXR ligand-binding domain fused to the GAL4 DNA-binding domain (Lemaire et al., 2006). This system is loosely based on the mammalian two-hybrid system and initiates a luciferase response when a PXR ligand binds to the hPXR ligand-binding domain. This event is followed by the binding of the GAL4 DNA-binding domain to GAL4RE5, which, in turn, initiates the expression of luciferase. This reporter gene assay has been applied widely in water quality monitoring, including for testing of wastewater, surface water, and reclaimed water (Table 1.3).

Kinani et al. (2010) used this stably transfected reporter cell system to identify potential PXR ligands among a number of organochlorine pesticides and to assess the presence of PXR xenobiotic chemicals in the sediments of several rivers in France. The same in vitro bioassay was also successfully utilized by Creusot et al. (2010) to assess the presence of PXR xenobiotic ligands in the wastewater effluent from a paper mill, an urban wastewater treatment plant, and surface water, although the compounds that contributed to PXR activity could not be identified. This reporter gene assay also has been applied for testing in

wastewater treatment plants (Mnif et al., 2010; Mnif et al., 2011) and reclaimed water (Mahjoub et al., 2009). With this assay and a bioassay-directed fractionation approach, it was also possible to identify di-iso-octylphthalate as PXR ligand (Creusot et al., 2013).

Table 1.3. Category 1 In Vitro Bioassays for Detecting Pregnane X Receptor (PXR) (Ant)agonists

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
PXR reporter gene assay	Human cervical cancer cells (HeLa)	Activated PXR quantified as luminescence (luciferase reporter gene)	Include POPs, pesticides, pharmaceuticals, plasticizers, rifampicine (Rf), SR12813, T0901317, pretilachlore	RfQ (or Rif EQ) and SR-EQ (Rf and SR12813 equivalent concentrations)	Mahjoub et al., 2009; Creusot et al., 2010; Kinani et al., 2010; Mnif et al., 2010; Mnif et al., 2011

Note: POP = persistent organic pollutant

1.4.2.3 Category 2 Bioassays

A myriad of approaches have been taken to (1) develop in vitro bioassays to determine the function of PXR and (2) to ascertain the most appropriate cell line to establish the in vitro bioassay (some of these are listed in Table 1.4). The development of these assays and a comparison of results of these assays established in different cell types, as well as species, have been extensively covered in a review by Stanley et al. (2006) and by Raucy et al. (2010, 2013). A common approach to developing an in vitro bioassay has been to clone the full-length PXR gene (or the ligand-binding domain of PXR) into an expression vector and to overexpress it in a mammalian cell line. At the same time a chimeric plasmid consisting of a PXR target (such as the response element of a *cyp* gene) fused to a reporter gene is transiently transfected into cells. The binding of a xenobiotic to PXR allows it to bind to its target and therefore drive the expression of the reporter gene (Casabar et al., 2010; Howe et al., 2011). A number of different reporter genes have been utilized for this purpose; the most common being the luciferase, β-lactamase, and CAT reporter vectors. There are a number of different cell lines proven adequate to support these cell-based in vitro assays as both transient and stable transfections, including HeLa, HuH7, CV-1, FLC-7, LS174T, LS180, MCF7, HEC1, LLC-PK1, HEK293, HepG2, and Caco-2 (Stanley et al., 2006; Raucy and Lasker, 2010).

In addition to the assay examples listed in Table 1.4, the research group behind the EcoScreen assays recently assessed the potential of 200 pesticides to activate the PXR by using transiently transfected monkey kidney cells (Kojima et al., 2011a). If a stable reporter cell line is developed to complement the EcoScreen NR assay battery, this may be a useful candidate for water quality screening. The EcoScreen assays are detailed in the sections for AhR, ER, AR, and TR.

Table 1.4. Category 2 In Vitro Bioassays for Detecting Pregnane X Receptor (PXR) (Ant)agonists

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
PXR reporter gene assay	Human liver carcinoma cells (Huh7)	Activated PXR quantified as luminescence (luciferase reporter gene)	Rifampicin	EC_{50} (μM)	Howe et al., 2011
PXR reporter gene assay	Human liver carcinoma cells (HepG2)	Activated PXR quantified as luminescence	Rifampicin	(Mean) fold induction over negative control	Casabar et al., 2010
Attagene multifactorial reporter gene assay (ATG_ PXRE_CIS)	HepG2 cells transfected with 48 RTUs and 25 NR elements including PXRE	PXRE binding quantified by fluorescence (via 6-carboxyfluorescein [6-FAM] labeling) and resolved from the remaining TFs by capillary electrophoresis	More than 200 chemicals including flufenacet	AC_{50} (50% of maximal response)	Romanov et al., 2008; Martin et al., 2010

Note: PXRE = pregnane X receptor response element

1.4.2.4 Conclusions

The PXR is a relevant xenobiotic receptor pathway related to endocrine disruption and codes for important detoxifying enzymes of the CYP3A3 family. There is much cross-talk between the PXR and CAR pathways, and if one of these pathways should be prioritized in future studies, we recommend including the PXR rather than the CAR because of its higher versatility and larger number of detected chemicals. Because the PXR binds, albeit, with low affinity to a wide range of ligands in the form of environmental pollutants and activates important detoxifying enzymes, its signaling pathway makes it a prime candidate for utilizing in an in vitro bioassay that screens for such chemicals.

The PXR reporter gene assay on the basis of HeLa cells (Lemaire et al., 2006) has been successfully adapted to water quality assessment but has been exclusively used by the group that developed the assay. Comparison with other PXR reporter gene assays would help ensure that the sensitivity of the HeLa-based variant is appropriate for water quality assessment.

1.4.3 Constitutive Androstane Receptor (CAR)

1.4.3.1 Mechanistic Background and Relevance

The constitutive androstane receptor (CAR) is similar to PXR in that it belongs to the superfamily of NRs and acts as a xenosensor to a wide range of both endogenous and exogenous compounds (Marino et al., 2011). The CAR regulates a number of Phase I metabolic enzymes of the CYP2 and CYP3 family and NADPH-CYP reductase but also

various Phase II enzymes (e.g., glutathione [GST]-S-transferase) and some xenobiotic receptors (Omiecinski et al., 2011). Furthermore, the receptor plays a role in regulating energy metabolism and lipid homeostasis.]

The mechanism by which CAR responds to xenobiotic exposure is not clearly understood (Raucy and Lasker, 2010). Similar to the PXR, the CAR is localized within the cytoplasm of the cell in its inactive state and is bound to a number of corepressors. Upon ligand binding, the CAR translocates to the nucleus, where it forms a heterodimer with RXR before binding to its DNA-binding site to initiate gene transcription. Attempts to fully characterize the activation and signaling pathways initiated by the CAR, however, have been difficult to perform because of the amount of overlap with PXR function and the lack of an in vitro bioassay system designed to specifically target CAR function (Raucy and Lasker, 2010).

1.4.3.2 Category 1 Bioassays

To our knowledge, no in vitro bioassays have yet been developed for assessing CAR activation in water samples.

1.4.3.3 Category 2 Bioassays

Many of the in vitro bioassays developed to determine CAR activation have been developed alongside those designed to determine the function of the PXR. One example is a set of in vitro assays developed to determine the ability of statins (a class of drugs commonly used to decrease cholesterol and thereby cardiovascular disease) to activate the CAR. The ligand-binding domain of CAR was cloned into a luciferase reporter plasmid (pBIND) and, by use of the mammalian two-hybrid system (adapted from the yeast two-hybrid system), was transfected into Huh7 cells (a human hepatocellular carcinoma cell line) (Howe et al., 2011) (Table 1.5). Using a different approach, Casabar et al. (2010) overexpressed human CAR in HepG2 cells alongside a number of *cyp* genes fused to the luciferase gene in a reporter plasmid, pGL4.10. *cyp* genes are downstream targets of activated CAR; therefore, when HepG2 cells are subjected to various chemicals (in this study, endosulfans), the overexpressed CAR becomes activated initiating the transcription of the *cyp* genes, which leads to the expression of luciferase (Casabar et al., 2010) (Table 1.5).

Table 1.5. Category 2 In Vitro Bioassays for Detecting Constitutive Androstane Receptor (CAR) (Ant)agonists

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
CAR reporter gene assay	Human hepatocarcinoma cells (Huh7)	Activated CAR quantified as luminescence (luciferase reporter gene)	CITCO	EC_{50} (μM)	Howe et al., 2011
CAR reporter gene assay	Human hepatocarcinoma cells (HepG2)	Activated CAR quantified as luminescence (luciferase reporter gene)	CITCO androstenol	(Mean) fold induction over negative control	Casabar et al., 2010
Attagene multifactorial reporter gene assay (ATG_ CAR_TRANS)	HepG2 cells transfected with 48 RTUs and 25 NRs including CAR	CAR-binding quantified by fluorescence (via 6-carboxyfluorescein (6-FAM) labeling) and resolved from the remaining TFs by capillary electrophoresis	Phosalone	AC_{50} (50% of maximal response)	Romanov et al., 2008; Martin et al., 2010

Notes: CITCO = 6-(4-chloropheny)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime.

These techniques and approaches have allowed for the identification of a vast array of activators of CAR (Marino et al., 2011). Of the 309 ToxCast environmental chemicals assessed in the multifactorial reporter gene assay (Table 1.5), however, only four induced the CAR (Martin et al., 2010).

1.4.3.4 Conclusions

There is a wide range of Category 2 bioassays available (Raucy and Lasker, 2013), but to our knowledge, these assays have yet to be adapted to identify CAR agonists, or antagonists, in water samples. We rank this a lower priority as the CAR is less sensitive to environmental chemicals than is the PXR (Martin et al., 2010) and as CAR is very similar to PXR, although it must be acknowledged that the CAR is linked to a number of very important xenobiotic metabolic enzymes from all three phases.

1.4.4 Peroxisome Proliferator-Activated Receptor (PPAR)

1.4.4.1 Mechanistic Background and Relevance

PPAR also is a TF that belongs to the superfamily of NRs. It performs a slightly different function from that of PXR and CAR in that it is more involved in the regulation of glucose and lipid metabolism than in xenobiotic metabolism (Scarsi et al., 2007). As the name indicates, the main function of PPAR is the delivery of peroxisomes, which are important for fatty acid oxidation and thus relevant for lipid metabolism. There are three isoforms of

PPAR: PPARα, PPARβ (also referred to as δ), and PPARγ, which different genes encode. They show different tissue expression and perform slightly different functions. PPARα is expressed predominantly in metabolically active tissues such as liver and kidney cells, where its ligands include fatty acids, hypolipidemic drugs, and xenobiotics (Seimandi et al., 2005). PPARγ is the key receptor in maintaining glucose and lipid homeostasis, and its activation increases the insulin resistance of the cell (Scarsi et al., 2007). As such, it has become a very attractive drug target for the treatment of type II diabetes. PPARβ, and the role it plays, is not as clearly understood. Upon ligand binding, PPAR forms a heterodimer with RXR followed by binding to specific DNA sequences to initiate transcription of various genes.

1.4.4.2 Category 1 Bioassays

To our knowledge, only one in vitro bioassay has been adapted for assessing PPAR activity in water samples. Liu et al. (2005) developed a reporter gene assay on the basis of rainbow trout gill cells (RT-W1) transiently transfected with a plasmid containing a PP response element (extracted from a rat gene most sensitive to PPARβ agonists) upstream of a luciferase gene. The assay was found to be particularly sensitive to PPARβ agonists such as bezafibrate. Further, the sensitivity of the assay could be greatly increased by coincubating with retinoic acid, which stimulated RXR. Treated Canadian domestic wastewater sample extracts were tested and produced a significant, but not quantified, response in the assay (Liu et al., 2005).

Table 1.6. Category 1 In Vitro Bioassays for Detecting Peroxisome Proliferator-Activated Receptor (PPAR) (Ant)agonists

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
Rainbow trout gill transient PPAR reporter gene assay	Rainbow trout gill cells (RT-W1)	PPARβ (empirically determined) activated luminescence (luciferase reporter gene)	Bezafibrate (+ retinoic acid)		Liu et al., 2005

1.4.4.3 Category 2 Bioassays

A couple of stable reporter cell lines have been designed to detect activated PPAR activity (Table 1.7), although these have been utilized to achieve quite different goals. The first reporter cell line was designed in 2005 by Seimandi et al. (2005) to characterize specific synthetic compounds that activated PPARs. HeLa cells stably expressed a chimeric protein containing the yeast transactivator GAL4 DNA-binding domain fused to the ligand-binding domain region of PPAR. This chimeric protein was used to coexpress the luciferase reporter gene driven by a pentamer of the GAL4 recognition sequence in front of the β-globulin promoter. Thus, ligand binding to the PPAR ligand-binding domain would allow the GAL4 DNA-binding domain to bind to the GAL4 recognition sequence, which then drives luciferase expression (Seimandi et al., 2005).

Table 1.7. Category 2 In Vitro Bioassays for Detecting Peroxisome Proliferator-Activated Receptor (PPAR) (Ant)agonists

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
PPAR reporter gene assay	Human hepatocarcinoma (HeLa) cells	Activated PPARα, PPARδ, PPAR quantified as luminescence (luciferase reporter gene)	Thioisobutyric acid derivative GW7647, phenoxyacetic acid derivative L-165041, 1,2-rosiglitazone (BRL49653)	EC_{50} (nM)	Seimandi et al., 2005
PPARγ-CALUX reporter gene assay	Human osteocarcinoma (U2OS) cells	Activated PPARγ quantified as luminescence (luciferase reporter gene)	Rosiglitazone	Relative potency of Rosigli-tazone	Gijsbers et al., 2011
PPARγ-GeneBLAzer	Human embryonic kidney cells (HEK293H)	Activated PPARγ quantified as β-lactamase activity (*bla* reporter gene)	Rosiglitazone		Huang et al., 2011b
Attagene multifactorial reporter gene assay (ATG_ PPARα_TRANS, ATG_ PPARδ_TRANS and ATG_ PPARγ_TRANS)	HepG2 cells transfected with 48 RTUs and 25 NRs including PPARα, -δ, and -γ	PPAR binding quantified by fluorescence (via 6-carboxyfluorescein [6-FAM] labeling) and resolved from the remaining TFs by capillary electrophoresis	Various (>100) chemicals including lactofen, flusilazole, resmethrin	AC_{50} (50% of maximal response)	Romanov et al., 2008; Martin et al., 2010

A second stable reporter cell line that detects activated PPARγ was designed to identify the regulation of downstream gene expression (Gijsbers et al., 2011). Briefly, an expression plasmid for PPARγ was generated by cloning full-length hPPARγ into pSG5-neo to create pSG5-neo-PPARγ. The Peroxisome Proliferator Response Element (PPRE) was cloned as three tandem repeats into the pGL3-luc plasmid, which contains an internal TATA box. Both plasmids were stably transfected into U2OS cells, a human osteoblastic osteosarcoma cell line, to generate PPARγ CALUX (Gijsbers et al., 2011).

The GeneBLAzer battery of β-lactamase assays includes a reporter cell line for PPAR and may be an attractive candidate for implementation in water quality assessment (Wilkinson et al., 2008). In a high-throughput study of 3000 environmentally relevant chemicals, roughly 1% were PPARγ agonists and 8% were PPARγ antagonists (Huang et al., 2011b).

In the "FACTORIAL" biosensor system that allows screening of 25 NRs and 48 TF response elements, the PPARγ was the most sensitive of the three PPAR subtypes with 146 out of 309 ToxCast Phase I chemicals being active (Martin et al., 2010). Conversely, only 3 of 200 pesticides tested positive for PPARα and none for PPARγ in a study applying transiently transfected monkey kidney cells (CV-1) (Takeuchi et al., 2006).

1.4.4.4 Conclusions

The PPAR covers a facet of xenobiotic metabolism that is highly relevant to human health and is not well covered by other xenobiotic metabolism pathways. Mice exposed to contaminated drinking water exhibited significantly induced PPAR expression (Shi et al., 2012), suggesting that this endpoint is likely to be relevant to drinking water. The only Category 1 assay used so far is unfortunately a transient transfection assay and as such is likely to produce somewhat variable results between tests. Some of the Category 2 reporter gene assays are based on stable platforms. We therefore recommend the implementation of one of the reviewed Category 2 bioassays.

1.4.5 Arylhydrocarbon Receptor (AhR)

1.4.5.1 Mechanistic Background and Relevance

The AhR is a ligand-dependent TF that is necessary for virtually all of the toxicity of halogenated aromatic hydrocarbons such as polychlorinated and brominated dibenzo-*p*-dioxins and biphenyls, as well as PAHs. The AhR also has some naturally occurring ligands such as flavonoids, carotinoids, and phenolics (Denison et al., 2002). After ligands have bound to the AhR, the ligand−receptor complex is translocated into the nucleus and binds to the dioxin-responsive element (DRE). Downstream of the DRE are the target genes coding for the metabolic enzymes CYP1A1, CYP1B1, and NADPH-quinone oxidoreductase (NQO1), but there also is cross-talk with Nrf2, the master regulator of antioxidant response, and the hypoxia-inducible factor HIF-1α, which are discussed in Section 1.6. Although the full physiological role of AhR remains unclear, its activation contributes to carcinogenicity because CYP can convert many of its ligands to reactive intermediates capable of causing DNA damage.

1.4.5.2 Category 1 Bioassays

Several reporter gene assays are available for testing AhR activity in water samples (Table 1.8), including the AhR-CALUX (chemical activated luciferase gene expression; Murk et al., 1996) and AhR-CAFLUX (chemically activated fluorescence expression; Nagy et al., 2002) assays. The AhR-CAFLUX assay applies a mouse hepatoma cell (Hepa1c1c7) stably transfected with a plasmid containing a reporter protein (the enhanced GFP, EGFP) downstream of a promoter consisting of four DREs. These recombinant cells produce EGFP upon exposure to dioxin-like compounds, and the amount of EGFP is directly correlated to the amount of AhR stimulation by the sample. The AhR-CAFLUX has been applied to test drinking water, surface water, and wastewater (Macova et al., 2010, 2011). In the AhR-CALUX, luciferase is used as the marker for AhR activity and is measured by luminescence. The AhR-CALUX also is called DR-CALUX, DRE-CALUX, and/or H4IIE bioassay (rat hepatoma cell line) and has been applied widely in water quality monitoring (e.g., Hurst et al., 2005; Gustavsson et al., 2007; Joung et al., 2007; Balaam et al., 2009; Rawson et al., 2009; Sato et al., 2010).

Table 1.8. Category 1 In Vitro Assays for Detection of AhR Activity

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
AhR-CALUX (also called DR-CALUX, H4IIE bioassay)	Rat hepatoma cell line (H4IIE)	Binding to AhR measured via luciferase	Dioxins, dioxin-like chemicals, PAHs	CALUX-TEQ (TCDD toxic equivalent concentration)	Murk et al., 1996
DRE-CALUX	Mouse hepatoma cell line (Hepa1c1c7)	Binding to AhR measured via luciferase	As above	As above	Joung et al., 2007
AhR-CAFLUX	Mouse hepatoma cell line (Hepa1c1c7)	Binding to AhR measured via GFP	As above	TCDDEQ (TCDD equivalent concentration)	Macova et al., 2010
HahLP reporter gene assay	HahLP (reporter cell line derived from human cervical cancer cells [HeLa])	AhR induction measured via luciferase	TCDD	Dioxin (TCDD) equivalents	Pillon et al., 2005
YCM3 yeast reporter gene assay	YMC3 reporter cell line derived by transfection of *Saccharomyces cerevisiae* strain w303a	AhR induction measured via β-galactosidase chemiluminescence	BaP, β-napthoflavone, hexachlorobenzene, mono-OH-PCBs, PCBs and PCB commercial mixtures, TCDD	βNF EQ (β−napthoflavone equivalents)	Allinson et al., 2011

Notes: BaP = benzo(a)pyrene, CYP1A = cytochrome P450 1A, mono-OH-PCB = mono-hydroxy-polychlorinated biphenyl, TCDD = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

Human cell-based reporter gene assays also exist for water testing. Pillon et al. (2005) developed the AhR-responsive HahLP cell line from HeLa cells and tested it with surface water samples. The HahLP cell line has since been applied for testing of wastewater (Dagnino et al., 2010; Mnif et al., 2010) and of reclaimed water (Mahjoub et al., 2009). Allinson et al. (2011) applied a yeast reporter cell line (YCM3; Miller, 1999; Kamata et al., 2009) with a human AhR-responsive element to assess AhR activity in wastewater.

1.4.5.3 Category 2 Bioassays

Given the toxicological importance of the induction of the AhR, there exists a wealth of cell-free bioassays on the basis of AhR ligand-binding and enzyme immunoassays (reviewed by Behnisch et al. [2001]). Few of these assays lend themselves to the investigation of water and environmental samples because of possible disturbances by matrix effects. The DR-EcoScreen™ applies a stably transfected mouse hepatoma (Hepa1c1c7) cell line with seven copies of the DRE (Takeuchi et al., 2008) (Table 1.9). The assay was first tested for 200 different pesticides, of which 11 demonstrated AhR activity (Takeuchi et al., 2008). Subsequently, Anezaki et al. (2009) employed the assay in 96 well plate format to assess air samples. Recently, the DR-EcoScreen was applied with biotic extracts and was compared with chemical analysis, which showed good correspondence (Kojima et al., 2011b). The DR-EcoScreen is commercially available and represents one NR reporter assay in an assay battery that further includes the ERα and -β, AR, and TR-EcoScreens. The Attagene multifactorial reporter gene assay AhR-responsive element tested positive for 54 of 320 chemicals (Martin et al., 2010) (Table 1.9).

Very recently, He et al. (2011a) developed the third generation of the CALUX system derived from Hepa1c1c7 cells stably transfected with 20 copies of DRE. This supersensitive CALUX variant pushes the detection limit significantly lower than do any other existing CALUX types, which is ideal for trace analysis in environmental water samples. Similar effort also has been made by Novotna et al. (2011) in HepG2 cells, which contains multiple copies of DREs and allows sensitive evaluation of AhR transcriptional activity triggered by xenobiotics.

Table 1.9. Category 2 In Vitro Bioassays for Detection of AhR (Ant)agonists[a]

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
DR-EcoScreen	Mouse hepatoma cells (hepa1c1c7) stably transfected with a plasmid containing a DRE	AhR activity measured via luciferase activity (luminescence)	Several pesticides (11) including chlorpyrifos and diuron	TCDD-EQ	Takeuchi et al., 2008; Anezaki et al., 2009
Attagene multifactorial reporter gene assay	Human hepatoma cells (HepG2) stably transfected with 48 RTUs and 25 NR response elements including AhRE	AhR binding quantified by fluorescence (via 6-carboxyfluorescein [6-FAM] labeling) and resolved from the remaining TFs by capillary electrophoresis	Various (54) chemicals including tetraconazole	AC_{50} (50% of maximal response)	Romanov et al., 2008; Martin et al., 2010
AZ-AHR	Human hepatoma HepG2 transfected stably with a construct containing several AhR-binding sites upstream of luciferase reporter gene	AhR transcriptional activity measured via luciferase activity (luminescence)	TCDD, omeprazole, 3-methylcholanthrene, resveratrol, indirubin, and SP600125	RLUs and fold induction	Novotna et al., 2011
AhR-CALUX	Mouse hepatoma cell line (Hepa1c1c7) stably transfected with a plasmid containing 20 DREs	AhR activity measured via luciferase activity (luminescence)	TCDD, PCDF, and 2 dioxin-like PCB congeners	RLUs and % maximum induction by TCDD	G. He et al., 2011

Notes: AhR = arylhydrocarbon receptor; AhRE = AhR-responsive element; DR = dioxin-responsive; PCB = polychlorinated biphenyl; PCDF = polychlorinated dibenzofuran; TCDD = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDD-EQ = TCDD equivalents; RLU = relative light unit.

1.4.5.4 Conclusions

The reporter gene assays AhR-CALUX and AhR-CAFLUX have been extensively validated with environmental samples, and there is presently no need to implement further bioassays for AhR activation. It would be desirable to work with human cell lines instead of with mouse or rat cell lines, and one such assay does exist (Pillon et al., 2005), although it appears not to be widely applied.

In addition to the assays mentioned earlier, the EROD assay (detailed in Section 1.4.1.2) in suitable liver cell lines is a good complement to the AhR activation assay because the EROD assay quantifies the CYP1A1 activity, which is mainly but not exclusively controlled by AhR. The EROD assay is thus a more integrative assay than any of the reporter gene assays for AhR.

1.5 Bioassays Indicative of Cellular Effects

1.5.1 Disturbance of Energy Production

1.5.1.1 Mechanistic Background and Relevance

Cells rely almost entirely on mitochondria for generation of energy. Interference with the mitochondrial electron transport chain and with oxidative phosphorylation lead to inhibition of ATP synthesis, thus resulting in depletion of energy (Nicholls and Ferguson, 1991). Energy depletion affects all cells. Disturbance of energy transduction can occur via nonspecific toxicity but mainly occurs through binding to proteins and disruption of ion gradients between membrane compartments (Escher and Leusch, 2012).

In plant cells, energy is produced by photosynthesis. Although this mechanism is evidently not relevant to human health, disturbances of photosynthesis are good predictors of the presence of herbicides and herbicide-like chemicals and should therefore also be included in a comprehensive bioassay battery.

A group of chemicals known as uncouplers can shuttle ions and protons across membranes without binding to specific receptors (Terada, 1990). Uncouplers are typically weak organic acids that form lipid-soluble conjugated bases that can shuttle protons back and forth across biological membranes (Spycher et al., 2008). Cyanide and rotenone are capable of binding to the quinone-binding sites in the mitochondrial electron transfer chain, whereas organotins (e.g., tributyl tin) and *N*,*N*'-dicyclohexylcarbodiimide are direct inhibitors of ATP synthase (Escher and Leusch, 2012).

1.5.1.2 Category 1 Bioassays

The most common assays for detecting cytotoxicity in water samples are bacterial bioluminescence inhibition assays (e.g., Microtox, ToxScreen). These assays are, however, more specific than general cytotoxicity assays in being indirectly indicative of ATP and in this way also indicative of the cell energy status. The principle of these assays is that the light emission in naturally bioluminescent bacteria (*Vibrio fischeri*, *Photobacterium phosphoreum*, or *P. leiognathi*) can be used as a measure of overall cellular energy status and health. A decreased light output is indicative of interference with energy metabolism and overall cellular health and reflects the combined baseline toxicity of all chemicals in the sample

(Escher and Leusch, 2012). Bacterial bioluminescence inhibition assays are therefore very suitable for screening of both overall nonspecific toxicity and specific inhibition of energy production. These assays are simple and cost efficient, and their wide applications are well covered in the literature, allowing plenty of data for comparative purposes. Examples of the use of Microtox include assessment of effluents of coal gasification (Timourian et al., 1982), oil refineries (Chang et al., 1981), pulp mills (Rosa et al., 2010), and sewage treatment plants (Farré et al., 2002) as well as of environmental waters (Dizer et al., 2002) and drinking water (Guzzella et al., 2004). The assay also has been adapted to 96 well plate format (Macova et al., 2010).

Photosynthesis inhibition, albeit not of direct relevance to human health, is an important assessment endpoint for water quality monitoring, as protection of the photosynthesizing primary producers at the bottom of the food chain is crucial to ensure a healthy ecosystem (Escher and Leusch, 2012). The imaging-PAM (IPAM) assay measures inhibition of Photosystem II (PSII)-derived photosynthesis in green algae exposed to water samples (Bengtson Nash et al., 2006). The IPAM was optimized to measure inhibition of photosynthesis and growth together in the combined algae assay, which was applied for testing of surface water and wastewater (Escher et al., 2008b) (Table 1.10).

Table 1.10. Category 1 In Vitro Assays To Detect Disturbance of Energy Production

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
Bioluminescence inhibition test (also called Microtox, Biotox, MicroLumo, Lumistox, Luminotox, ToxAlert, and ToxScreen)	*V. fischeri*, *P. phosphoreum*, or *P. leiognathi*	Reduction in luminescence of naturally bioluminescent marine bacteria	Phenol	EC_{50}	ISO, 1998; Ulitzur et al., 2002; Johnson, 2005; Farre et al., 2006; Escher et al., 2008b
IPAM and combined algal assay	Various green algae including *Pseudokirchneriella subcapitata*	PSII-derived photosynthesis inhibition (via Chl A fluorescence, which is inversely proportional to PSII photosynthetic yield) and growth inhibition (via optical density)	Herbicides, diuron	DEQ (diuron equivalent concentration)	Bengtson Nash et al., 2006; Escher et al., 2008b

1.5.1.3 Category 2 Bioassays

ATP is generated as an end product of energy production. General cytotoxicity assays often rely on the determination of metabolic activity, which is often accomplished by directly measuring ATP. In the compound profiling by Xia et al. (2008) (Section 1.3.2), a reporter system with luciferase coupled to ATP was applied to a range of cell types for profiling of 1400 compounds. This method is not only suitable for the determination of general

cytotoxicity but also may have potential for detecting specific inhibition of energy production. Other researchers have used mitochondrial membrane potential ($\Delta\psi m$) by fluorescent probes (Bensassi et al., 2011; Padmini and Usha Rani, 2011; Vongs et al., 2011) and mitochondrial morphology by autofluorescence (Rodrigues et al., 2011) as indicators of mitochondrial toxicity; however, these responses also can follow nonspecific toxicity.

Table 1.11. Category 2 In Vitro Assays To Detect Disturbance of Energy Production

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
Cytotoxicity on the basis of metabolic activity	13 different human and rodent cell lines	Luciferase-coupled ATP quantitation assay	1408 chemicals from the National Toxicology Program compound library	50% inhibitory concn (IC_{50})	Xia et al., 2008

1.5.1.4 Conclusions

Although energy production is a vital process for sustenance of cells, most assays rely on the quantification of the final currency of energy, ATP. As such these assays overlap with general cytotoxicity. More-specific inhibition of energy production, such as the inhibition of photosynthesis, does not affect human health directly but is nevertheless relevant in the context of water quality assessment to ensure that treated water is clear of herbicides. Therefore, we recommend the inclusion of the combined algae assay in any test battery of water quality assessment.

1.5.2 Direct Genotoxicity

1.5.2.1 Mechanistic Background and Relevance

DNA can be damaged by direct electrophilic attack by exogenous chemicals, reactive oxygen species (ROS) generated in the cell, or nonchemical stressors such as UV light. Alkylating agents, e.g., fluorouracil or methyl iodide, can covalently bind to DNA, creating methyl adducts, particularly via the nitrogen atoms of the guanine and adenine bases in DNA (Escher and Leusch, 2012). Larger multifunctional molecules can react with DNA to produce cross-links within or between strands, and large adducts can create errors in translation or replication. Structural alterations of DNA (potentially leading to mutations and other errors) also can occur via intercalation of large planar molecules into DNA. DNA damage may result in loss of bases or strand breaks. During repair, incorrect bases may be inserted, resulting in irreversible mutations, which can cause errors in protein synthesis and are a major cause of cancer.

1.5.2.2 Category 1 Bioassays

Assays for direct genotoxicity include those that detect mutagenic (i.e., introduction of mutations such as the Ames test) and cytogenetic (i.e., structural DNA damage such as the Comet assay) damage (Table 1.12). A multitude of assays are available, and many of these are utilized for water quality assessment.

The Ames test detects mutagens and has been employed for water quality testing since early after its publication in 1975 (Ames et al., 1975). Various water matrices have been tested in the assay, including surface water (Pelon et al., 1977; Vankreijl et al., 1980), ozonated recycled water (Gruener, 1978), coal gasification process water (Epler et al., 1978), drinking water (Simmon and Tardiff, 1976; Nestmann et al., 1979; Cheh et al., 1980), marine water (Kurelec et al., 1979), pulp and paper mill effluents (Bjorseth et al., 1979; Carlberg et al., 1980), and different wastewaters (Rappaport et al., 1979; Saxena and Schwartz, 1979). The Ames test is still applied for water quality monitoring, and, according to Claxton et al. (2010), will play an "indispensable role in the foreseeable future of 21st century toxicology."

Modern applications include the assessment of mutagenicity of pool water (Richardson et al., 2010). For further applications refer to Poulsen et al. (2011). The Ames test uses different strains of *Salmonella typhimurium*, which are capable of detecting specific types of mutations (e.g., caused by DNA frameshifts or base-pair substitutions [OECD, 1997]) or even specifically acting mutagens (e.g., alkylating agents (Emmert et al., 2006]).

Mutagenicity assays utilizing marine bacterial cells have also been developed for marine water quality testing and include the Mutatox (Ulitzur et al., 1980; Guzzella et al., 2004; Zani et al., 2005), the *Vibrio harveyi* mutagenicity assay (Czyz et al., 2000, 2003), and the *V. harveyi* luminescence mutagenicity assay (Podgorska and Wegrzyn, 2006; Podgorska et al., 2007) (Table 1.12).

The Comet assay, which also is known as the single-cell gel electrophoresis (SCGE) assay, is a popular technique used for detection of reactive toxicity in polluted waters (Table 1.12). The Comet assay utilizes the differences in migration behavior between intact DNA and damaged DNA in an electric field (Rydberg and Johanson, 1978; Ostling and Johanson, 1984). Singh et al. (1988) optimized the assay from being able to detect double-strand breaks also to detect single-strand breaks. The Comet assay has been used widely for water quality assessment since its first water applications were carried out in 2001 to assess the genotoxicity of rivers in Germany and China (Schnurstein and Braunbeck, 2001; Zhong et al., 2001).

Surface water (i.e., rivers and lakes) and wastewater have been most frequently tested by means of in vitro bioassays. Disinfection byproducts (DBPs) formed during chemical disinfection of drinking water (with, e.g., chlorine, chlorine dioxide, and ozone) are, however, receiving increased attention. Bioanalytical techniques are very promising tools for detection of DBPs, as many of these remain unidentified and would go undetected by chemical analysis (Richardson et al., 2007). Plewa and coworkers adapted the SCGE assay for assessment of genotoxicity by DBPs (Table 1.12). The modified SCGE assay applies a Chinese hamster ovarian (CHO) cell line and is run in conjunction with the CHO microplate cytotoxicity assay (Plewa et al., 2002, 2004a). This assay has been utilized for assessment of recreational waters (Liviac et al., 2010; Plewa et al., 2011).

The micronucleus (MN) assay detects MN formation as an indicator of chromosome breaks or loss. Various adaptations of the original MN assay (Countryman and Heddle, 1976) have been applied for water quality monitoring, including the cytokinesis block MN (CBMN) assay (Fenech and Morley, 1985), which has been utilized for testing of, for example, drinking water (Buschini et al., 2004), surface water (Lemos et al., 2011), and wastewater (Reifferscheid et al., 2008) (Table 1.12). The ability to detect MN by flow cytometry (Nusse et al., 1994; Laingam et al., 2008) also has been exploited to detect genotoxicity in human

lymphocytes (WIL2-NS) exposed to a variety of water matrices, including treated sewage, reclaimed water, and drinking water (NWC, 2011) (Table 1.12).

Krishnamurti et al. (2008) measured several endpoints for genotoxicity in human peripheral mononuclear blood (PBMC) cells exposed to extracts of industrial wastewater. The endpoints targeting direct genotoxicity were DNA strand breaks measured in the fluorometric analysis of DNA unwinding (FADU) assay (Birnboim and Jevcak, 1981), chromosome aberrations (Evans, 1976; Api and San, 1999), and DNA fragmentation (Martikainen et al., 1991; Brulport et al., 2007) (Table 1.12). Chromosome aberrations also are a common endpoint applied in plant cell-based assays for water testing using, for example, *Allium cepa* (brown onion) (Evandri et al., 2000). Sister chromatid exchange (SCE) (Perry and Wolff, 1974) is another endpoint for direct genotoxicity used in monitoring of, for example, drinking water (Ergene et al., 2008) and surface water (Ohe et al., 2009) (Table 1.12).

Table 1.12. Category 1 In Vitro Assays To Detect Direct Genotoxicity in Water Samples

Target Mode of Action	Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
Mutagenicity	Ames test (and modified Ames test)	Bacterial cells *S. typhimurium* (many strains including TA98, TA100, and 98NR)	Number of histidine revertants	Various including aflatoxin B1, nitrofuran carcinogens, acetylenic diarylcarbamates, methyl methanesulfonate (MMS), PAHs including benzo(a)pyrene (BaP), trichlorfon, sodium azide (SA), 9-aminoacridine, 4-nitroquinoline-N-oxide (4QNO), methylglioxal	Revertants per µmol of reagent (revertants per L-equivalent for water samples)	Richardson et al., 2010
Mutagenicity	Mutatox assay	Dark strains of luminescent bacterial cells *P. leiognathi*, *V. fischeri*	Genotoxic damage such as frameshift or base-substitution point mutations and more. All endpoints induce a dark strain to regain luminescence	Various including PAHs (e.g., BaP), phenol, *N*-methyl-*N*'-nitrosoguanidine, hydroxylamine, ethyl methanesulfonate (EMS), ethidium bromide, acriflavine sulfate, proflavine, acridine orange, 9-aminoacridine, alidixic acid, mitomycin C, arabinosyl-cytosine	Mutagenic ratio (light emission sample/light emission negative control)	Guzzella et al., 2004
Mutagenicity	*V. harveyi* luminescence mutagenicity assays	Genetically modified *V. harveyi* that produces neomycin (antibiotic)-resistant mutants upon mutagen exposure	Formation of neomycin-resistant colonies following exposure to mutagens	2-aminofluorene (2-AF), 2-methoxy-6-chloro-9-(3-(2-chloroethyl)amino propylamino)acridine x2HCl (ICR-191), 4-nitro-o-phenylene diamine (NPD), MMS, 4QNO	Mutagenicity (% mutagens in sample/% mutagens in control)	Czyz et al., 2003

Mutagenicity	*V. harveyi* luminescence mutagenicity assays	Genetically modified strains of *V. harveyi*	Exposure to mutagens reverts mutated dark variants to luminescent variant (measured by luminescence)	BaP, ICR-191, NPD, NQNO, SA, 2-AF	RLUs	Podgorska et al., 2007
DNA damage (strand breaks)	Comet, also called SCGE	A variety of mammalian (including human) cells such as CHO cells and fish liver cells (zebrafish *Danio rerio* and rainbow trout *Oncorhynchus mykiss* RTL-W1, RTH-149)	Measures DNA double-strand breaks in single cells (single-strand break in some variants). Staining technique, fluorescence. Image analysis results in an output image resembling a comet. The body of the comet represents undamaged cells, and the tail, the damaged cells	Hydrogen peroxide, DBPs including haloacetic acids and halonitromethanes	Concentration−response with, e.g., hydrogen peroxide or EMS as positive control	Schnurstein and Braunbeck, 2001; Plewa et al., 2002; Wagner and Plewa, 2008
DNA damage (strand breaks)	Alkaline yeast comet/SCGE	Yeast *S. cerevisiae* DLH3	Same as normal Comet but appears to be more sensitive than with mammalian cell lines	Hydrogen peroxide, MMS	Tail length, % DNA in tail	Miloshev et al., 2002
DNA damage (MN formation, i.e., chromosome breakage or loss)	MN test, cytokinesis block MN (CBMN)	Human lymphocytes, HepG2 cells	MN formation by staining techniques	Bleomycin	E.g., nucleation index (NI) or MN/1000 cells (positive if > twice the spontaneous MN formation)	Lemos and Erdtmann, 2000; Buschini et al., 2004; Reifferscheid et al., 2008; Lemos et al., 2011

DNA damage (MN formation)	FCMN or FCMNN (flow cytometry MN formation)	Nonsecreting human lymphoblast (WIL2-NS)	MN formation measured by flow cytometry	BaP, MMS, mitomycin C, etoposide, vinblastine	Statistical significant or not, genotoxic or not (positive if > 3 × SD over control)	NWC, 2011
DNA damage (strand breaks)	FADU assay	Human PBMC cells	(Undamaged) double strands are preferentially detected via fluorescent dye	BaP	Relative light units with BaP as positive control	Krishnamurthi et al., 2003, 2008
DNA damage (chromosome aberrations)	Chromosome aberration assay	Human PBMC cells	Chromosome aberrations, determined via staining (Giemsa)	BaP, *N*-methyl-*N*-nitro-*N*-nitroso-guanidine and fragrance ingredients 6-acetyl-1,1,2,4,4,7-hexamethyltetraline and 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta-γ-2-benzopyran	% aberrant cells	Krishnamurthi et al., 2008
DNA damage (DNA laddering/fragmentation)	DNA fragmentation assay	Human PBMC cells	DNA fragmentation. DNA is extracted and quantified by immunostaining	BaP, 5-fluorodeoxy-uridine, trifluoro-thymidine	Significant or not significant	Krishnamurthi et al., 2008
DNA damage (induction of SCE)	SCE assay	Chinese hamster lung (CHL) cells, human lymphocytes	SCE is measured by a fluorescence staining technique (Giemsa)	Mitomycin C, dichlorobiphenyl derivatives	SCE frequency	Ergene et al., 2008; Ohe et al., 2009

1.5.2.3 Category 2 Bioassays

With the vast range of Category 1 in vitro assays available for detection of direct genotoxicity in water, it is of less urgency to establish further assays for this endpoint. We note one alternative bacterial assay here for detection of genotoxicity by DBPs that was developed by the Plewa group in addition to the mammalian SCGE microplate assay discussed in the previous section. The Salmonella preincubation assay (Table 1.13) is a modified version of the Ames mutagenicity assay (Kargalioglu et al., 2002; Plewa et al., 2004b) that is run alongside the *S. typhimurium* microplate cytotoxicity assay. The Salmonella assay has, however, not yet been tested with water samples and is probably of less relevance for drinking water testing than is the mammalian SCGE microplate assay.

Generally, the Ames assay is increasingly applied as a fluctuation assay in microplates (also referred to as Ames II) instead of the conventional setup with agar plates (Reifferscheid et al., 2012; Smith et al., 2013). This modification was developed by Green et al. (1977) and was further modified according to Reifferscheid et al. (2012), which simplifies the procedure of the original Ames assay and delivers comparable results (Umbuzeiro et al., 2010). Recently, the Ames fluctuation assay has been adapted to a headspace-free setup to allow the testing of volatile compounds (Stalter et al., 2013). This setup allows the direct exposure of volatile compounds in liquid media. Additionally, different tester strains can be chosen, depending on the target compounds. For instance, if alkylating agents or nitrosamines are expected to be present in water samples, YG71XX tester strains should be selected, for example, YG7108 (Emmert et al., 2006). Further strains have been developed, which express mammalian enzymes that are involved in the activation of mutagens in vivo; e.g., several strains express different sulfotransferases, which leads to a higher sensitivity toward furans than shown by the parent strain (Glatt et al., 2012). Another strain expresses a mammalian GST-S-transferase, which increases the sensitivity toward dihalomethanes (Thier et al., 1993) and trihalomethanes (Zwiener et al., 2007) and may therefore be of particular importance for the evaluation of DBPs in drinking or swimming pool water.

Recently, Smart et al. (2011) developed a genotoxicity screening via the γH2AX by flow assay, which utilizes the measurement of serine 139-phosphorylated histone H2AX (γH2AX) as the biomarker of DNA double-strand breaks. In this assay, H2AX is evaluated by flow cytometry (Smart et al., 2011).

It is important that cancer also can be caused by nongenotoxic mechanisms. Epigenetic carcinogens including pesticides (Rakitsky et al., 2000) and benzene (Gao et al., 2011) do not react directly with DNA but are capable of altering gene expression via, for example, silencing of tumor suppression genes and/or activating proto-oncogenes (Ziech et al., 2010). Additional steps of epigenetic carcinogenesis include proliferation and apoptosis in transformed cells (Combes et al., 1999; Gao et al., 2011). Endpoints for epigenetic carcinogenesis are thus typically assessed in gene expression (Gao et al., 2011) and in cell transformation assays including the Balb/c 3T3 assay (Combes et al., 1999).

Table 1.13. Category 2 In Vitro Assays for Detection of Direct Genotoxicity

Target Mode of Action	Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
Mutagenicity	Salmonella preincubation assay	*S. typhimurium* strains TA98 and TA100	No. of histidine revertants	Haloacetic acid (drinking water DBPs)	Induced revertants per µmol of reagent	Kargalioglu et al., 2002; Plewa et al., 2004b
DNA damage (strand breaks)	γH2AX by flow assay	Mouse lymphoma L5178Y cells	γH2AX measurement	MMS	Relative γH2AX change	Smart et al., 2011

1.5.2.4 Conclusions

The Ames test was one of the earliest cell-based bioassays applied in water quality assessment and still plays a dominant role in detecting direct genotoxicity. The large number of available Category 1 bioassays for direct genotoxicity underlines the relevance of this mode of toxic action. It is therefore not necessary to implement any further bioassays targeting genotoxicity. However, simplified test setups should be selected if a direct genotoxicity test system is to be established in the lab (e.g., the γH2AX by flow assay instead of the Comet assay or the Ames fluctuation assay instead of the conventional Ames test). Furthermore, it is advisable also to include assays indicative of the repair mechanisms for DNA damage, such as the p53 pathway (Section 1.6.6), as assessment of repair may be more sensitive than assessment of the actual damage done

1.5.3 Nonspecific Reactivity Toward Proteins

1.5.3.1 Mechanistic Background and Relevance

Biocides such as the antifoulant Sea-Nine, electrophilic chemicals (e.g., acrylates), and dithiocarbamate pesticides can react directly with the thiol group in the amino acid cysteine (Timbrell, 2009). Heavy metals such as mercury (Hg^{2+}) and cadmium (Cd^{2+}) also can form complexes with thiol groups. These complexes can cause structural damage to proteins, and if this damage affects an enzymatic site of a protein, nonspecific enzyme inhibition also may occur.

GSH is a small cysteine-containing peptide that plays an important role in the defense against electrophilic chemicals and internal ROS. Exposure to trace organic pollutants and subsequent defense mechanisms can lead to GSH depletion, which can cause proteins to lose their protection resulting in direct protein damage.

Reactive toxicity resulting from covalent binding to proteins or endogenous biochemical substrates has the potential to initiate severe adverse biological effects (Chan and O'Brien, 2008; Bohme et al., 2009, 2010). Electrophilic compounds can react with nucleophilic groups on proteins by several mechanisms of chemical reaction, including the Michael-type reaction, Schiff base formation, acylation, aromatic nucleophilic substitution (S_NAr), and aliphatic nucleophilic substitution (S_N2) (Schwobel et al., 2011). Different electrophilic xenobiotics react at different nucleophilic sites within the cell, and these patterns are governed by the hard/soft acid base principle (Chan et al., 2008). Hard species have small atomic radii, a high electronegativity and low polarizability, whereas soft species are larger and highly polarizable in comparison (Carlson, 1990). Hard electrophiles have affinity for hard nucleophiles such as amino groups of lysine and DNA, whereas soft electrophiles have affinity for soft nucleophiles such as the sulfhydryl group of cysteine or GSH (T. W. Schultz et al., 2006). Electrophilic reactivity can cause various adverse outcomes including skin sensitization, mutagenicity, carcinogenicity, respiratory allergy, organ toxicity, and necrosis (Aptula et al., 2006; Chan et al., 2008). Electrophilic reactivity is therefore a key step in determining the toxic potential of a substance (Aptula et al., 2006). GSH is one of the most widely utilized nucleophilic reference molecules in reactivity assays (Schwobel et al., 2011) because it is the most prevalent cellular thiol and the most abundant low-molecular-weight peptide in cells (Aptula et al., 2006). GSH protects cells by detoxifying electrophilic compounds and by serving as an antioxidant; it is a soft nucleophile that can be used as an

indicator of the reactivity of electrophilic agents toward the thiol group and other electron-rich sites of molecular structures (Bohme et al., 2010).

1.5.3.2 Category 1 Bioassays

Currently no Category 1 bioassays for soft electrophiles are available for water quality assessment. GSH is present in high concentrations in cells and its depletion can be quantified after extraction of cells with chemical analytical methods or by an enzymatic assay. Depletion of cellular GSH has been assessed in water samples by using various cell types (e.g., HepG2 cells and rainbow trout primary cells) (Marabini et al., 2006; Farmen et al., 2010). The significance of the obtained results as a measure of direct electrophilic reactivity is, however, difficult to interpret as additional mechanisms also can cause GSH depletion.

1.5.3.3 Category 2 Bioassays

To our knowledge, only one Category 2 in vitro cell-based assay has been developed to measure the reactivity of soft electrophiles. This assay quantifies the cytoprotective effect of GSH by measuring the bacterial growth inhibition in normal and genetically modified *Escherichia coli* cells exposed to chemicals (Harder et al., 2003) (Table 1.14). The parent strain MJF276 (GSH^+) is capable of synthesizing GSH, whereas the mutant strain MJF335 (GSH^-) lacks both γ-glutamylcysteine synthase and GSH synthase. The comparison of growth inhibition by reactive chemicals in a strain deficient of GSH and by reactive chemicals in its fully functional parent strain can be used for indication of detoxification via GSH conjugation and of related toxic effects as well as direct reactivity with cysteine-containing proteins.

A number of in chemico bioassays targeting soft electrophiles have also been reported in the literature (Gerberick et al., 2007; Bohme et al., 2009, 2010) (Table 1.14). Schultz et al. (2005) developed an in chemico assay using GSH as a model nucleophile. Test compound and GSH are dissolved in an aqueous phosphate buffer solution. After 2 h of exposure, the concentrations of free thiol groups are quantified spectrophotometrically at 412 nm through their reaction with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB). Bohme et al. (2009) modified the Schultz assay and developed a kinetic GSH assay that quantifies GSH loss and measures the second-order rate constants of the reaction between electrophilic substances and GSH. Moreover, some study teams conducted the *Tetrahymena pyriformis ciliate* bioassay alongside the Schultz assay and found significant correlations between 48 h ciliate toxicity and thiol reactivity for α,β-unsaturated ketones, esters, aldehydes, and halogenated carbonyls (Yarbrough and Schultz, 2007; Bohme et al., 2009; Roberts et al., 2010).

Gerberick et al. (2004) introduced a combined GSH and pentapeptide depletion assay for assessing the skin sensitization potential of chemicals. The authors suggested using a battery of peptide assays and high-performance liquid chromatography (HPLC) analysis. UV detection was used to monitor the depletion of GSH or the peptide.

Table 1.14. Summary of Category 2 In Vitro and In Chemico Bioassays for Reactive Toxicity

Target Mode of Action	Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
Cytotoxicity in GSH-deficient cells	In vitro *E. coli* expression	Genetically modified *E. coli* strains: MJF276 (GSH^+) and MJF335 (GSH^-)	Growth inhibition of GSH-deficient and parent strain measured by absorbance OD_{600}	Ratio between EC_{50} values for growth inhibition between GSH^+ and GSH^- strains	Organochlorines, acrylates, acrylic compounds	Harder et al., 2003; Richter and Escher, 2005
GSH depletion	In chemico thiol reactivity	Pure GSH (nonenzymatic chemical reactivity with free thiol group)	Free GSH concn measured spectrophotometrically (OD_{412}) via reaction with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB)	GSH concn	Esters, ketones, aldehydes, amides, halogens, acetonitriles, heterocyclic organics, aliphatic isothiocyanates	Schultz et al., 2005; Aptula et al., 2006; T. Schultz et al., 2006; T. W. Schultz et al., 2006; Gagan et al., 2007; Schultz et al., 2007a, 2007b; Schultz et al., 2007c; Yarbrough and Schultz, 2007; Dawson et al., 2008, 2010; Roberts et al., 2010; Schultz et al., 2010; Bajot et al., 2011

1.5.3.4 Conclusion

Both the in vitro *E. coli* assay and the in chemico thiol reactivity assay show potential for quantification of reactive toxicity for water quality assessment. Only the *E. coli* assay is, however, capable of demonstrating the relevance of GSH conjugation as an in vivo detoxification step.

1.5.4 Reactive Toxicity Toward Membranes

1.5.4.1 Mechanistic Background and Relevance

ROS can induce cellular oxidative stress, not only by damaging DNA and proteins but also by causing lipid peroxidation. Polyunsaturated phospholipids are very vulnerable to this attack, and saturation of double bonds causes alteration in fluidity of membranes. Ultimately, lipid peroxidation leads to a chain reaction breakdown of fatty acids, which causes structural damage of membranes. Lysosomes are at risk of losing their hydrolytic content, and the function of membrane-bound enzymes in the mitochondria and endoplasmic reticulum (EpR) can be disturbed, ultimately causing adverse outcomes for the cell.

Modern methods of disinfecting urban drinking water form DBPs, which may, in turn, adversely affect health. Cell exposure to these DBPs appears to coincide with an increase in cellular oxidative stress, genotoxicity, and general toxicity (Buschini et al., 2004; Zani et al., 2005; Y. Shi et al., 2009; Xie et al., 2010). It must be noted, however, that in a review of the literature, lipid peroxidation assays did not appear to be as sensitive at measuring oxidative stress as did genotoxicity assays (Y. Shi et al., 2009). Within the context of a battery of assays, however, lipid peroxidation assays may contribute to our understanding of xenobiotics in water (Damasio et al., 2011), cellular stress, and the potential adverse effects oxidative stress may have on health.

1.5.4.2 Category 1 Bioassays

Malonaldehyde (MDA) is a lipid peroxidation product that is used as biomarker for this endpoint (Yagi, 1998). Studies have, for example, determined the MDA concentration in human liver cells exposed to drinking water by using a commercially available kit (Y. Shi et al., 2009; Xie et al., 2010). MDA also has been used as endpoint for water quality tests with duckweed (*Lemna minor*) (Radić et al., 2010) or different algal species (Kumari et al., 2012) showing that it is a useful biomarker for lipid peroxidation in various species. However, species or cell lines should be carefully selected as some exhibit a significantly higher sensitivity than others do. For example, the mammalian neuro 2A cell line exhibited a high sensitivity toward saxitoxin, whereas *Chlamydomonas reinhardtii* alga did not show any MDA increase (Melegari et al., 2012).

Table 1.15. Category 1 In Vitro Assays To Detect Reactive Toxicity Toward Membranes

Target Mode of Action	Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
Lipid peroxidation	MDA assay	Human liver cells (Hep-G2, L-02)	Cellular concn of MDA, which is a product of lipid peroxidation	H_2O_2	MDA concn (MDA per mass protein), significant or not significant	Y. Shi et al., 2009; Yie et al., 2010

1.5.4.3 Category 2 Bioassays

Another method for detecting lipid peroxidation in a cell-based in vitro assay is to measure the concentration of thiobarbituric acid-reacting substances. This method is an indirect way of measuring MDA (discussed earlier), whereby MDA reacts in a [1:2] ratio with 2-thiobarbituric acid to produce the compound tetramethoxypropane. The absorbance of tetramethoxypropane can be read at 540 nm, thereby indirectly determining the concentration of cellular MDA. This method has been used to observe the effect that mycotoxins have on lipid peroxidation in porcine kidney cells (Klaric et al., 2007) and to measure the effect of xenobiotics on the lugworm (*Arenicola marina*) in sediment samples (Ramos-Gomez et al., 2011). Although the latter example is not a cell-based in vitro assay, it still indicates that the method may be sensitive enough to detect such xenobiotics in environmental samples and therefore should be considered for review. To our knowledge, this method of lipid peroxidation detection has not yet been applied to cells exposed to environmental water samples.

Table 1.16. Category 2 In Vitro Assays To Detect Reactive Toxicity Toward Membranes

Target Mode of Action	Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference
Lipid peroxi-dation	Thiobarbit uric acid-reacting substances (TBARS) assay	Porcine kidney epithelial cells (PK15)	Increase in TBARS concn as a measure of lipid peroxidation and determined by absorbance (532 nm)	Mycotoxins monisin B1, beauvericin, and ochratoxin A. 1,1,3,3-Tetramethoxypro pane as reference compound for developing the standard curve	% concn of TBARS relative to control	Klaric et al., 2007

1.5.4.4 Conclusions

Lipid peroxidation is one of the measures indicating that the cell is experiencing oxidative stress. Although more focus is typically on oxidative damage of DNA and proteins, oxidative damage of lipids cannot be neglected as loss of the structure and of the functionality of membranes leads to a loss of many vital cellular functions. All bioassays indicative of lipid peroxidation quantify the ultimate product of fatty acid degradation, MDA. As such, these MDA bioassays are very selective of the lipid peroxidation process but may not be very sensitive, as mild lipid peroxidation (e.g., the formation of saturated fatty acids) will not be detected. More-sensitive indicators of oxidative stress, such as the ASR pathway Keap-Nrf2-ARE, are possibly more suitable early indicators of oxidative stress, and implementation of assays that can detect these pathways should be given higher priority than that of assays detecting lipid peroxidation.

1.6 Bioassays Indicative of General Adaptive Stress Response Pathways

Cells function and remain viable under very defined external conditions, which are dictated by temperature, concentration, pH, ionic strength, etc. If these conditions are changed, the cell becomes stressed and ceases to function at its optimal capacity. When external changes do occur, cells can respond via a number of adaptive stress pathways to mitigate any detrimental effects potentially induced and to restore the cell to homeostasis. Many ASR pathways are triggered by the activation of TFs. TF activation initiates the transcription of cytoprotective genes (and, thus, the expression of cytoprotective proteins), which repair cellular damage caused by any changes in condition that the cell has endured (Simmons et al., 2009). As stress response pathways are induced only by chemicals or other external stressors, these are referred to as adaptive.

Activation and detection of ASR pathways are much more sensitive endpoints than is general cellular cytotoxicity and thus provide the body with early warning signals of exposure to chemicals (Escher and Leusch, 2012).

The common ASR pathway is sketched in Figure 1.5. Under normal conditions, the TF, which is the protein responsible for triggering the adaptive response, is bound by a sensor molecule. The sensor−TF complex cannot enter the nucleus and keeps the TF dormant until the cell is exposed to stress. During stress, a family of enzymes, called transducers, activates the TF, which releases it from the sensor. The activated TF is then free to enter the nucleus, where it binds to specific sites on the DNA, triggering the expression of the associated, protective genes (Escher and Leusch, 2012).

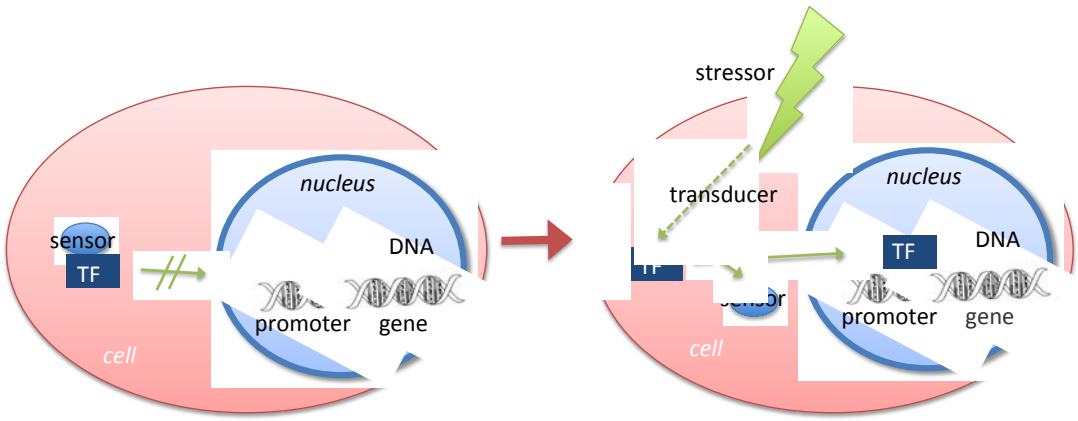

Figure 1.5. Activation of an adaptive stress response pathway.

Note: TF = transcription factor.

Source: Reprinted with permission from Escher and Leusch (2012). Copyright 2012, IWA Publishing.

The general pathway for adaptive stress responses is similar to those for xenobiotic metabolism and hormones with one important exception; adaptive stress pathways occur in all cells, whereas other toxicity pathways are specific to certain tissues and organs (Escher and Leusch, 2012). Furthermore, the chemical stressor in the ASR pathway is not bound to the TF and is not collocated into the nucleus.

An overview on the cellular response pathways is given in Table 1.17, and details are discussed in the subsequent chapters of this review.

Table 1.17. General Cellular Stress Response Pathways

Pathway	Sensor	TF	Inducing Chemicals
Heat shock response	Hsp90, Hsp70	HSF-1	Temperature, metals
Hypoxia	VHL	HIF-1	Oxygen depletion (can be caused by metals)
Metal stress	None	MTF-1	Heavy metals
Endoplasmic reticulum stress	BiP	XBP-1, ATF6, ATF4	Tunicamycin, thapsigargin, caplain, brefeldin A
Osmotic stress	None	NFAT5	High salt, glycol
Inflammation	IkB	NF-κB	Metals, PCBs, smoke, particles
Oxidative stress	Keap1	Nrf2	Chemicals that produce ROS
DNA damage	MDM2	p53	Electrophilic chemicals, UV radiation

Source: Adapted with permission from Simmons et al. (2009). Copyright 2009, Oxford University Press.

1.6.1 Heat Shock Response

1.6.1.1 Mechanistic Background and Relevance

The heat shock response (HSR) was one of the first stress response pathways to be discovered and described for its importance for cellular adaptation to hyperthermia, although it was later found also to be induced in response to protein-denaturing chemicals (Simmons et al., 2009). The HSR is regulated by heat shock proteins (Hsps), which have a cytoprotective role following heat and oxidative and chemical stress (El Golli-Bennour and Bacha, 2011). The Hsps are classed according to molecular size from small Hsps (also referred to as sHsps) to Hsp104, and their specific functions were summarized by Morimoto and Santoro (1998). Hsp70 and Hsp90, which are the main sensors of protein misfolding (following, e.g., oxidative stress), regulate the HSR by releasing the heat shock TF 1 (HSF-1) during stress (El Golli-Bennour and Bacha, 2011). HSF-1 induction also has been linked to nuclear factor kappa B (NF-κB, discussed in Section 1.6.5) (Morimoto and Santoro, 1998). Because of the key role of the HSR in cellular defense following chemical stress, HSF-1 induction may be useful as an indicator of the presence of xenobiotics in water samples.

1.6.1.2 Category 1 Bioassays

Whereas researchers have measured the levels of Hsps in biota sampled from polluted waters as biomarkers for the HSR, to our knowledge, no studies have used in vitro assays to measure the HSR in water samples.

1.6.1.3 Category 2 Bioassays

Immunoassays can be used to measure the levels of Hsps as indicator of Hsp induction after in vitro exposure to toxicants and other stressors. El Golli et al. (2006), for example, measured Hsp70 induction and cytotoxicity in green monkey kidney cells in response to mycotoxin exposure with and without Vitamin E and prior heat shock (Table 1.18).

Table 1.18. Category 2 Bioassays for Detection of Heat Shock Response in Water Samples

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
Hsp70 induction via immunoassay	Green monkey kidney cells (Vero)	Hsp70 induction measured as Hsp70 concn via immunoassay	Mycotoxins citrinin, zearalenone, and T2 toxin	% Hsp concn compared to control, IC_{50}	El Golli et al., 2006
HSE luciferase assay	Transfected human prostate adenocarcinoma cells (LNCaP), human lymphoblast cells	HSE binding measured as luciferase and/or galactosidase activity via luminescence	Hsp90 inhibitors PF-04928473 and (17-AAG), anti-inflammatory drug sodium salicylate	% or fold induction luciferase activity compared to control	X. Z. Wang et al., 2000; Lamoureux et al., 2011
HSE β-lactamase (HSE-*bla*) reporter gene assay	Transfected HeLa (human cervical cancer) cells	HSE binding measured as β-lactamase activity via fluorescence	Heat (42 ºC), 17-AAG, celastrol, bortezomib	Response ratio (460 nm/530 nm emission ratio) relative to control, EC_{50}	Hancock et al., 2009
Attagene multifactorial reporter gene assay (ATG_CIS_HSE)	Human liver hepatoma cells (Hep-G2) transfected with 48 RTUs including one for HSE	HSE binding quantified by fluorescence (via 6-carboxyfluorescein [6-FAM] labeling) and resolved from the remaining TFs by capillary electrophoresis	Various pesticides including prallethin, allethrin (d-*cis*,*trans*), quintozene, fenarimol, fenoxycarb, fipronil, tetramethrin, thidiazuron	AC_{50} (50% of maximal response)	Romanov et al., 2008; Martin et al., 2010

Notes: HSE = heat shock element; HSP = heat shock protein; 17-AAG = 17-allylamino-17-demethoxy-geldanamycin; PF-04928473 = 4-(6,6-dimethyl-4-oxo-3-trifluoromethyl-4,5,6,7-tetrahydroindazol-1-yl)-2-(4-hydroxy-cyclohexylamino)-benzamide; IC_{50}, 50% inhibitory concentration.

Assays specifically targeting HSF-1 also are available. After release, HSF-1 binds to heat shock elements (HSEs) in the nucleus, and this binding has been utilized as an indicator of HSF-1 activation (Table 1.18). In one example, transfected human prostate adenocarcinoma cells (LNCaP) were exposed to Hsp90 inhibitors and HSE binding was measured in a luciferase assay alongside immunoassay for HSF-1 quantification (Lamoureux et al., 2011).

The HSE β-lactamase, or HSE-*bla* reporter gene assay, was developed under the Tox21 program by Hancock et al. (2009) (Table 1.18). This assay measures β-lactamase activity fluorometrically via CCF4-AM substrate as a marker for HSE binding and has been validated for HTS. Further, the ToxCast program tested 309 compounds in the Attagene multifactorial reporter gene assay for the HSE response and found 22 of these compounds responsive (Martin et al., 2010; Table 1.18).

Using a rather different approach, Garces-Sanchez et al. (2009) used quantitative reverse transcriptase PCR (RT-qPCR) to detect the level of *Cryptosporidia* present in various water samples. The assay was designed by using oligonucleotides directed specifically against *Cryptosporidia*'s heat shock *hsp70* gene. The amount of *hsp70* mRNA detected in the assay was directly proportional to the levels of viable protozoa in the water samples. The authors found that RT-qPCR was rapid and highly specific and easily integrated into their high-throughput analysis of water samples (Garces-Sanchez et al., 2009). Such an approach might be readily adapted to detect *hsp70* mRNA produced in human cell lines when they are exposed to water samples containing chemical inducers of the HSR pathway.

1.6.1.4 Conclusions

The HSR can be induced by heat shock as well as by a range of inorganic and organic chemicals and therefore may be an important marker of xenobiotics and other stressors in water. This response is mainly measured via immunoassays, which are less practical for routine screening. Commercially available reporter gene assay kits are, however, also emerging and may be useful for water quality testing. The HSE β-lactamase reporter gene assay appears particularly promising because it is already optimized for high-throughput testing of chemicals in DMSO and would need only adaptation to water samples.

1.6.2 Hypoxia Response

1.6.2.1 Mechanistic Background and Relevance

All cells require oxygen for normal function. The hypoxia response pathway protects cells during oxygen depletion (hypoxia) by activating genes that trigger, for example, increased oxygen transport and glucose uptake (Simmons, 2009). This ASR pathway is regulated by the hypoxia inducible factor 1 (HIF-1), which is comprised of two subunits, HIF-1α and HIF-1β (Xia et al., 2009). Prolyl hydroxylase enzymes, which are important for degradation of HIF-1α, are impaired during hypoxia allowing the buildup of HIF-1α, which then forms a heterodimer with HIF-1β (Xia et al., 2009). The heterodimer translocates to the nucleus and binds to hypoxia response elements (HREs), ultimately leading to target gene expression (Xia et al., 2009). Hypoxia can be induced by metals and carbon monoxide, and additional chemicals have been demonstrated to activate HIF-1α (Xia et al., 2009; Martin et al., 2010).

1.6.2.2 Category 1 Bioassays

To our knowledge, no studies have measured hypoxia response following in vitro exposure to water samples.

1.6.2.3 Category 2 Bioassays

Activation of the hypoxia response pathway can be measured through quantification of hypoxia target genes or reporter gene assays with amplified HIF-1 response. Xia et al. (2009) tested a chemical library of 1408 compounds for their ability to induce HIF-1α. This example provides a good case study for this review as several assays were employed to support each other. First, all test compounds were screened in the HRE-*bla* reporter gene assay in which 12 compounds tested positive (Table 1.19). These 12 positive compounds were subjected to additional tests including an immunoassay for measuring secretion of the vascular endothelial growth factor (VEGF), which is one of 70 known hypoxia target genes (Xia et al., 2009). Five compounds tested positive in the VEGF assay, and these were tested further in a reporter gene assay targeting multiple hypoxia-responsive promoter genes (Table 1.19). Altogether, only 3 (o-phenanthroline, iodochloro-hydroxyquinoline, and cobalt sulfate heptahydrate) of the 12 HIF-1α-inducing compounds were confirmed as hypoxia mimetics, whereas two compounds (7-diethylamino-4-methyl-coumarin and 7,12-dimethylbenz[a] anthracene) were found to induce HIF-1α via a nonhypoxia pathway (Xia et al., 2009).

As noted in the previous section, the ToxCast program tested 320 compounds in the Attagene multifactorial reporter gene assay (Martin et al., 2010). When it was used, 25 compounds tested positive for HIF-1α induction, and interestingly, these are all different from the 12 compounds that tested positive in the HRE-*bla* assay (Table 1.19).

Table 1.19. Category 2 Bioassays for Detection of Hypoxia Response in Water Samples

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
HRE β-lactamase (HRE-*bla*) reporter gene assay	Transfected human cervical cancer (ME-180) cells	HSE binding measured as β-lactamase activity via fluorescence	2-Aminoanthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, $CoSO_4$, dibenz(a,h)anthracene, 7-diethylamino-4-methylcoumarin, 7,12-dimethylbenz(a)anthracene, iodochlorohydroxyquinoline, o-phenanthroline, prednisone, salicylazosulfapyridine, triamterene, $CoCl_2$	EC_{50}	Xia et al., 2009
VEGF secretion by immunoassay	Human cervical cancer (ME-180) cells	VEGF secretion measured in immunoassay	7-Diethylamino-4-methylcoumarin, 7,12-dimethylbenz(a)-anthracene, iodochlorohydroxy-quinoline, $CoSO_4$, o-phenanthroline, $CoCl_2$	EC_{50}	Xia et al., 2009
Multipromoter/long-range transcriptional regulatory element reporter gene assay	Human colon cancer cells (HCT116) transfected with 36 human hypoxia-regulated promoters and long-range transcriptional regulatory elements	Promoter/response element binding measured via luciferase activity (luminescence)	o-Phenanthroline, iodochlorohydroxy-quinoline, $CoSO_4$, $CoCl_2$	log 2 ratio of activity	Xia et al., 2009
Attagene multifactorial reporter gene assay (ATG_HIF1a_CIS)	Human liver hepatoma cells (Hep-G2) transfected with 48 RTUs including one for HIF-1	HIF-1α binding quantified by fluorescence (via 6-carboxyfluorescein [6-FAM] labeling) and resolved from the remaining TFs by capillary electrophoresis	2-Phenylphenol, bifenazate, cyprodinil, allethrin (d-*cis*,*trans*), dibutyl phthalate, dimethomorph, fenoxycarb, fluoxastrobin, flusilazole, imazalil, isoxaben, MGK, phosalone, pirimiphos-methyl, prallethrin, prodiamine, propargite, propiconazole, propoxycarbazone-sodium, pyridaben, quintozene, spiroxamine, tetraconazole, tetramethrin, thiobencarb	AC_{50} (50% of maximal response)	Romanov et al., 2008; Martin et al., 2010

1.6.2.4 Conclusions

The importance of the hypoxia pathway in living cells is clear. Relatively few chemicals tested in the HRE β-lactamase reporter gene assay were, however, identified as hypoxia-inducing compounds (i.e., 12 of 1408 compounds). A more considerable proportion of chemicals (25 of 320 compounds) responded to HIF-1α in the Attagene multifactorial assay. Similarly, a small proportion of chemicals (25 of 320 compounds) responded to HIF-1α in the Attagene multifactorial assay. However, both of these approaches effectively identified chemicals able to induce the hypoxia pathway and, therefore, may be adapted to assessing environmental water samples.

1.6.3 Endoplasmic Reticulum Stress Response

1.6.3.1 Mechanistic Background and Relevance

The EpR is important for lipid synthesis, folding and maturation of proteins, and maintenance of overall cellular homeostasis. The protein folding capacity of the EpR is continuously adjusted according to cell demands, but when the load of unfolded proteins exceeds this folding capacity, so-called EpR stress occurs (Ron and Walter, 2007).

The EpR responds to stress via the EpR stress response pathway, which also is termed the unfolded protein response (UPR) pathway and induces expression of genes that help refold proteins and remove those that are damaged. The UPR pathway entails three main response pathways: (1) a transient adaptation that helps reduce the unfolded protein load, (2) a longer-term adaptation that increases the protein folding capacity of the EpR, and finally, if homeostasis cannot be maintained through the UPR pathway, (3) apoptosis (Ron and Walter, 2007). Although EpR stress does not necessarily lead to toxicity, detection of this stress response can be used as an indicator of the presence of EpR stress-inducing compounds that have potential to cause an adverse outcome.

The UPR pathway of mammalian cells involves at least three parallel intracellular signaling pathways, which are regulated by the proteins inositol-requiring protein-1 (IRE1), activating TF-6 (ATF6), and protein kinase RNA (PKR)-like ER kinase (PERK) (reviewed by Ron and Walter, 2007; Samali et al., 2010; Hiramatsu et al., 2011). Common to each of these signaling pathways is the interaction with the immunoglobulin-binding protein (BiP; also referred to as glucose-regulated protein (GRP78 or BiP/GRP78) (Simmons et al., 2009). Three possible models have been described for sensing of unfolded proteins (as illustrated by Ron and Walter, 2007). One model suggests that unfolded proteins bind directly to IRE1 and PERK. A second model suggests that under normal conditions, IRE1, ATF6, and PERK form complexes with BiP, which later dissociates from the complex during EpR stress to bind to unfolded protein (Samali et al., 2010). Finally, a third model suggests that both former models are involved with activating IRE1 and PERK (Ron and Walter, 2007). None of these models has been fully characterized.

It is not known which of these signaling pathways, if not all of them, is favored during EpR stress. It may thus be more practical to use downstream protein targets of EpR stress as molecular markers for the UPR (Samali et al., 2010). Relevant markers include BiP, X-box binding protein 1 (XBP-1), the TF C/EBP-homologous protein (CHOP), eukaryotic translation initiation factor-2 (eIF2α), activating TF 4 (ATF4), and homocysteine-induced EpR protein (HERP). XBP-1 is an important regulator of UPR (Lee et al., 2003) but also has been linked to regulating insulin sensitivity through this pathway, as well as regulating glucose tolerance independently of its role in UPR (Ueki and Kadowaki, 2011; Zhou et al., 2011). XBP-1 is specific for UPR induction of IRE1, during which IRE1 cleaves unspliced X-box binding protein 1 (X*bp*-1) mRNA to its active (spliced) form, the TF

XBP-1 (Yoshida et al., 2001). BiP is a marker for all UPR pathways and is the typical marker used for ATF6 (Hiramatsu et al., 2011). CHOP, eIF2α, and ATF4 are all molecular markers for PERK activity, and HERP is an EpR membrane protein, expression of which is increased in response to accumulation of unfolded protein (Kokame et al., 2001).

1.6.3.2 Category 1 Bioassays

To our knowledge, no studies have included efforts to apply in vitro assays to detect EpR stress response in water samples.

1.6.3.3 Category 2 Bioassays

The available methods for monitoring of UPR in mammalian cells were recently reviewed (Samali et al., 2010; Hiramatsu et al., 2011). Although research into EpR stress is increasing, available techniques for assessing the related pathways are still limited (Samali et al., 2010). X*bp*-1 mRNA splicing to XBP-1 and HERP is typically measured by real-time PCR (RT-PCR). qPCR is a common tool applied for quantifying ATF4, eIF2α, and BiP, whereas Western blotting is often used to measure protein levels of CHOP. ATF6 can be measured directly by use of a recombinant FLAG-tagged construct (Shen and Prywes, 2005) for Western blot analysis or indirectly by quantification of BiP. These most commonly used techniques for UPR detection are not suitable for HTS.

Reporter gene assays have more potential for water quality screening. Reporter systems that contain EpR stress response elements (ERSE or ESRE) and UPR elements (UPRE) have been developed (reviewed by Samali et al., 2010; Hiramatsu et al., 2011). Some examples of available reporter systems are listed in Table 1.20. Lee et al. (2003) used transiently transfected mouse embryo fibroblast (MEF) cells to develop a luciferase reporter system (4xXBPGL3) responsive to XBP-1 binding. Additional luciferase reporter systems with promoter regions for HERP (Kokame et al., 2001) and ATF6 and BiP (Y. Wang et al., 2000) have also been developed. A GFP reporter system also has been used to assess XBP-1 activation (Iwawaki et al., 2004). These reporters provide sensitive techniques for detection of UPR and are useful for transfection with many mammalian cell lines, but common to all is the uncertainty of detecting activation of XBP-1, ATF6, or a combination of the two (Samali et al., 2010). Specific binding can be elucidated only in combination with further assays such as immunoblotting. Furthermore, most assays use transient transfection and are not validated for high-throughput chemical assessment.

As with the hypoxia and heat shock stress responses, a β-lactamase assay also is available for ESRE (ESRE-*bla*, Table 1.20), but this assay responded to only a few of a total 1408 tested chemicals (Houck, 2009). The Attagene multifactorial assay also includes a RTU for X*bp*-1, but it tested positive for only 12 of the 320 chemicals subjected to this assay by the ToxCast program (Martin et al., 2010).

Table 1.20. Category 2 Bioassays with Potential for Water Quality Screening for Endoplasmic Reticulum Stress Response

Assay	Cell Line	Endpoint	Reference Chemical	Expression of Results	Method Reference(s)
Luciferase reporter gene (4xXBPGL3) assay for XBP-1	Mouse embryo fibroblast (MEF) cells transfected with promoter for an XBP-1 binding site	Induction of XBP-1 measured via luciferase activity	Tunicamycin	Fold induction relative to the control	Lee et al., 2003
Luciferase reporter gene assay for ATF6 and BiP	Human cervical cancer (HeLa) cells transfected with an ATF6 site reporter and a promoter for BiP	Binding to ATF6 binding site measured as luciferase activity	Tunicamycin, ATF6	Relative activity compared with the control	Y. Wang et al., 2000
Luciferase reporter gene assay for HERP	Human umbilical vein endothelial cells (HUVECs) transfected with a plasmid containing a promoter for HERP	HERP activation measured via luciferase activity	Tunicamycin, thapsigargin	Relative activity compared with the control	Kokame et al., 2001
GFP reporter gene assay for XBP-1	Human embryonic kidney (HEK293T) and HeLa cells transfected with XBP-1 DNA fragments fused to Venus (a GFP variant)	XBP-1 activation measured via fluorescence	Tunicamycin, thapsigargin, etoposide, thenoyltrifluoroacetone	Fluorescence intensity	Iwawaki et al., 2004
ESRE β-lactamase (ESRE-*bla*) reporter gene assay	Transfected human cervical cancer (HeLa) cells	ESRE-activation measured as β-lactamase activity via fluorescence	Tunicamycin	EC_{50}	Invitrogen
Attagene multifactorial reporter gene assay (ATG_Xbp1_CIS)	Human liver hepatoma cells (Hep-G2) transfected with 48 RTUs including one for XBP-1	XBP-1 activation quantified by fluorescence (via 6-carboxyfluorescein [6-FAM] labeling) and resolved from the remaining TFs by capillary electrophoresis	Prallethrin and 11 other compounds (of a total 320 chemicals) tested positive	AC_{50} (50% of maximal response)	Romanov et al., 2008; Martin et al., 2010

Notes: ATF = activating TF; BiP = immunoglobulin-binding protein (also called GRP78); EpR = endoplasmic reticulum; HERP = homocysteine-induced EpR protein; XBP-1 = X-box binding protein 1 (TF).

1.6.3.4 Conclusions

The field of research into the EpR stress response is growing, and researchers acknowledge the need to develop simple assays targeting specific pathways. It is likely that reporter gene techniques with potential for water quality screening will emerge in coming years; however, current techniques need considerable optimization before they can be applied for routine water testing. Another reason for assigning a low priority to the EpR stress response is that it was not induced by many environmentally relevant chemicals.

1.6.4 Osmotic Stress Response

1.6.4.1 Mechanistic Background and Relevance

Maintaining osmotic homeostasis is important for all living organisms as both hypotonic and hypertonic situations cause cellular stress. Thus, all living organisms have systems that sense, and respond, to changes in osmolality that exist at the cellular level (Naguro et al., 2012). Cells need to determine not only *how much* the ionic strength of their surrounding is changing but in what direction that change is occurring, namely, are the surroundings becoming more hypotonic or hypertonic? Cells must then trigger the appropriate signaling pathways to protect the cell from damage and to restore homeostasis (reviewed by Go et al., 2004; Ho, 2006). Although these signaling pathways have been well characterized in both yeast (Hohmann, 2002) and bacteria (Wood, 2011), they remain less understood in mammalian cells (Simmons et al., 2009).

In general, mammalian cells are not considered to be as vulnerable as unicellular organisms to the external pressures of hypertonic stress because these cells exist within tissues and organs, which come equipped with sensitive and dynamic regulatory mechanisms that help to maintain the body's fluid homeostasis. The only known mammalian TF that has been linked to the osmotic stress response pathway is NFAT5 (nuclear factor of activated T cells 5), also known as TonEBP (tonicity enhancer binding protein) (Miyakawa et al., 1999) and NFATL1 (Trama et al., 2000). NFAT5 is in the same family as NF-κB (Simmons et al., 2009), which is discussed in Section 1.6.5. NFAT5 acts by inducing the expression of genes that are responsible for increasing osmolytes within cells in order to maintain tonicity under osmotic stress (Berga-Bolanos et al., 2010). For mammals, osmotic stress was previously believed to be relevant exclusively to renal function, but NFAT5 is now known to be expressed in, and involved with, osmotic adaptive functions in mammalian tissues other than those of the kidney (Go et al., 2004; Ho, 2006). NFAT5 has been found to play a role, for example, in the adaptation of T lymphocytes to hypertonic stress (Ho, 2006; Berga-Bolanos et al., 2010).

The mitogen-activated protein kinase (MAPK) signaling pathway is highly conserved between yeast and humans, and this signaling pathway is a known response in yeast exposed to osmotic pressures. It also has been suggested to be triggered in mammalian cells that have been exposed to hypertonic stress. However, mammalian signaling pathways, in response to osmotic stress, are only now being characterized (Naguro et al., 2012). As such, few in vitro assays or reporter gene assays exist to assess water samples.

1.6.4.2 Category 1 Bioassays

To our knowledge, no existing in vitro assay for the osmotic stress response has been applied for water quality screening.

1.6.4.3 Category 2 Bioassays

Reporter gene constructs have been developed for assessing the activity of NFAT5 in response to osmotic stress (Table 1.21). These reporter systems utilize a luciferase reporter gene, which comprises a dimer of the human tonicity-responsive enhancer (hTonE) site inserted within a minimal promoter from the human interleukin-2 (IL-2) gene. The original assay was performed in Madin-Darby canine kidney (MDCK) cells (Miyakawa et al., 1998, 1999), but human Jurkat cells (Trama et al., 2000) and mouse embryonic fibroblast cells (Go et al., 2004) have been used for transfection (Table 1.21). To our knowledge, these assays are yet to be adapted for high-throughput testing and have been tested only with a few compounds.

Additional reporter gene assays have been developed that target NFAT5 activity indirectly. These assays have utilized both immortalized (Jurkat T and HEK293) and primary (mouse embryonic fibroblast) cells to assess the ability of NFAT5 to activate other endogenous biomolecules, including human tumor necrosis factor α (TNF-α) (López-Rodríguez et al., 1999) and aldose reductase (Lopez-Rodriguez et al., 2004). These reporter systems were not constructed to specifically measure NFAT5 activity but rather its activation of response elements for other molecules and thus in terms of specificity hold little promise for screening purposes.

Table 1.21. Category 2 Bioassays with Potential for Water Quality Screening for Osmotic Stress Response

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
NFAT5 reporter gene assay (luciferase)	Human lymphoblast (Jurkat) cells, MDCK cells	Activation of NFAT5, which is measured through binding to the hTonE site (quantified as luciferase activity)	Ionomycin, cyclosporine A, phorbol 12-myristate 13-acetate, raffinose	RLUs, relative luciferase activity relative to control	Miyakawa et al., 1998, 1999; Trama et al., 2000
As above	Mouse embryonic fibroblast (MEF) immortalized from primary MEF cells	As above	NaCl, raffinose	As above	Go et al., 2004

Notes: hTonE = human tonicity-responsive enhancer; NFAT5 = nuclear factor of activated T cells 5.

1.6.4.4 Conclusions

Reporter gene constructs are available for measuring the activity of NFAT5. Similar to research into the EpR stress response, however, research into the osmotic stress response is in its infancy in terms of chemical assessment. To our knowledge, no assay is available that has been subjected to trial for high-throughput testing, let alone for its compatibility with water samples.

1.6.5 Response to Inflammation

1.6.5.1 Mechanistic Background and Relevance

Inflammation is a protective measure of an organism against a variety of physical and chemical insults and also initiates the healing process. On the cellular level, the NF-κB TF represents a key regulator mediating the inflammatory stress response. In fact, NF-κB is a collective term of the homo- or heterodimers assembled from members of the NF-κB and Rel protein families, among which the p50 (NF-κB)/p65 (RelA) heterodimer has been by far the best characterized (Gilmore, 2006). In most cells, NF-κB is inactivated in the cytoplasm by complexing with its sensor, inhibitor kappa B (IκB) (Hayden and Ghosh, 2008). Extracellular stimuli can activate the pathway transducer called IκB kinase (IKK). IKK phosphorylates IκB proteins, which results in the dissociation of IκB from NF-κB and eventual degradation of IκB by proteosome. The released NF-κB is rapidly shuttled into the nucleus, where it activates expression of genes regulating the inflammatory stress response. Almost 400 target genes have been identified for NF-κB (Miller et al., 2010), which is closely related to immune responses and leads to induction of cytokines (Section 1.8.3 on immunotoxicity), CYPs (Sections 1.4.1 and 1.7.1), Hsp90 (Section 1.6.1), and regulators of apoptosis (Simmons et al., 2009). Furthermore, NF-κB plays a crucial role in embryonal and neuronal development, and impairment of NF-κB signaling has been linked with some cancers and inflammatory diseases (Miller et al., 2010).

1.6.5.2 Category 1 Bioassays

To our knowledge, no in vitro assay is available that has been adapted and/or applied for water quality screening.

1.6.5.3 Category 2 Bioassays

Several reporter gene assays are available for assessment of NF-κB-active/inhibitory compounds (Table 1.22). Miller et al. (2010) employed a battery of commercially available assays to screen 2800 clinically approved drugs and bioactive compounds for their ability to inhibit NF-κB (i.e., for their potential use as anticancer drugs, as NF-κB has been found to be constitutively expressed in cancer cells). The assay battery included the NF-κB β-lactamase assay (CellSensor® NF-κB-*bla* ME180 assay) and an NF-κB luciferase reporter gene assay (NF-κB *luc* assay), which were applied first to identify the NF-κB inhibitors. The assay battery was complemented by assays to assess the ability of these compounds to affect IκBα phosphorylation (the GFP reporter gene assay LanthaScreen™ IκBα GripTite assay) and caspase 3/7 activity (as an indicator of apoptosis) and to further establish the mechanism behind NF-κB-inhibition (Miller et al., 2010). All the assays mentioned earlier are human cell based and all were conducted in 1536 well plate format.

Oostingh et al. (2008) developed a series of luciferase reporter gene assays for assessment of immune responses. One stable reporter cell line was also established for NF-κB. This reporter assay in combination with the battery of stable cell lines for cytokine release (Table 1.22) appears to be an attractive set of assays capable of assessing various aspects of immunotoxicity, while also specifically targeting the inflammation stress response pathway.

A β-lactamase assay also is available for IκBα (IκBα-*bla* assay) (Robers et al., 2008). Recently, Kim et al. (2011) used a mouse-based reporter cell line to assess the effect of a phytotoxin on NF-κB; however, this reporter system used transient transfection (Table 1.22).

1.6.5.4 Conclusion

The inflammatory stress response pathway is a multifaceted stress response pathway. The association of NF-κB to immunotoxic responses makes this response pathway interesting and important from more than one toxicological perspective. Several suitable reporter gene assays have been developed and optimized for high-throughput testing of chemical compounds. The commercial NF-κB-*bla* ME180 assay appears most appropriate for water quality assessment in that it has been tested on hundreds of compounds.

Table 1.22. Category 2 Bioassays To Detect the Inflammatory Stress Response

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
NF-κB luciferase reporter gene assay (NF-κB *luc* assay)	Human embryonic kidney cells stably transfected with a plasmid containing a luciferase gene (*luc2P*) and multiple response elements for NF-κB response (NF-κB−*luc2P* Hek293 cell line)	NF-κB inhibition/activation measured via luciferase activity (luminescence)	18 compounds tested positive (inhibitory) including ectinascidin and the cardiac glycoside drugs digitoxin and ouabain	IC_{50} (with TNF-α as reference compound)	Miller et al., 2010
Luciferase reporter gene assay for NF-κB	Human lymphoma cell line (Jurkat T) stably transfected with a binding domain for NF-κB	NF-κB expression measured via luciferase activity (luminescence)	Fluoranthene, phenanthracene, pyrene, and anthracene	Induction fold	Oostingh et al., 2008
Luciferase reporter gene assay for NF-κB	Murine macrophages (RAW 264.7 cell line) transiently transfected with luciferase reporter gene containing an NF-κB promoter	NF-κB activation measured via luciferase activity	Phlorofucofuroeckol A	NF-κB promoter activity normalized to control	Kim et al., 2011
CellSensor® NF-κB-*bla* ME180 assay (β-lactamase (*bla*) reporter gene assay)	Human cervical cancer (ME180) cells that stably express a *bla* reporter gene under the regulation of an NF-κB response element	NF-κB activation/inhibition measured as *bla* activity via fluorescence	55 compounds tested positive (inhibitory) including ectinascidin and digitoxin and ouabain	IC_{50} (with TNF-α and IL-1β as reference compounds and MG-132 as positive control)	Miller et al., 2010
IκBα-*bla* assay with FRET quantification	Human embryonic kidney (HEK293) cells stably transfected with a plasmid expressing IκBα-*bla*	Degradation of IκBα-*bla* measured by fluorescence (FRET)	5-(*p*-fluorophenyl)-2-ureido]thiophene-3-carboxamide (IκBα inhibitor)	FRET response ratio (with TNF-α as reference compound)	Robers et al., 2008
LanthaScreen™ IκBα GripTite assay with TR-FRET	Human embryonic kidney (HEK293) cells stably transfected with IκBα fused to GFP	Phosphorylation and ubiquitination of IκBα measured by TR-FRET via terbium labeling	12 compounds tested positive (inhibitory) including ectinascidin	IC_{50} (with TNF-α as reference compound)	Robers et al., 2008; Miller et al., 2010

Notes: IκBα = inhibitor κBα; MG-132 = a known inhibitor of the NF-κB pathway; NF-κB = nuclear factor kappa B; TNF-α = tumor necrosis factor α (a known activator of the NF-κB pathway); TR-FRET = time-resolved Förster resonance energy transfer; IC_{50}, 50% inhibitory concentration.

1.6.6 Response to DNA Damage

1.6.6.1 Mechanistic Background and Relevance

Repair mechanisms in place for DNA involve enzymes that are able to recognize damaged DNA (Timbrell, 2009). Strand breaks are detected by p53, which triggers DNA repair (Simmons et al., 2009). Further repair mechanisms are in place, however, as these are all prone to error and as failure to repair DNA generally triggers cell death via apoptosis (Escher and Leusch, 2012).

The p53 family of TFs is important for DNA repair. p53 is negatively regulated by the sensor MDM2 under normal conditions but is activated in response to DNA damage. p53 then initiates a series of DNA repair mechanisms, among them regulators of the cell cycles and genes for repair enzymes. p53 also regulates apoptosis and is thus referred to as the tumor suppressor gene. Accordingly, p53 induction is regarded as an excellent predictor of genotoxic carcinogens (Duerksen-Hughes et al., 1999). p53 also regulates apoptosis and is thus referred to as a tumor suppressor gene. However, nongenotoxic mechanisms too can increase the cellular p53 concentration, such as hypoxia, ribonucleotidyl depletion, or p53-stabilizing mechanisms (Stenius and Hogberg, 1999). Another DNA-damaging response mechanism involves ATAD5, which supports the suppression of genomic instability and tumorigenesis (Fox et al., 2012). As ATAD5 is stabilized in response to various types of DNA damage, it was shown that it is possible to identify genotoxic compounds by monitoring ATAD5 protein levels (Fox et al., 2012).

1.6.6.2 Category 1 Bioassays

A range of assays is available to detect cellular responses to DNA damage (Table 1.23). The SOS response is one indicator that DNA damage has taken place. A range of bacterial cell-based assays is available for detection of the SOS response in water samples, including the *umu*C (also called SOS/*umu* and *umu*) assay (Oda et al., 1985; Reifferscheid et al., 1991), SOS chromotest (Quillardet et al., 1982), *umu* microtest, and the Vitotox assay (van der Lelie et al., 1997; Verschaeve et al., 1999), which have all been widely used in water quality monitoring (Langevin et al., 1992; Dizer et al., 2002). Aleem and Malik (2003, 2005) utilized SOS-defective bacterial cells to detect this response indirectly by comparing cytotoxicity in defective and wild-type bacterial cells exposed to surface water extracts. Alternatively, the GreenScreen reporter yeast-based assay measures DNA repair via the RAD54 repair gene (Cahill et al., 2004) and also has been found useful for water testing (Keenan et al., 2007). Krishnamurti et al. (2008) determined p53 accumulation in human PBMC cells (Trost et al., 2005; Hermes et al., 2008) following exposure to industrial effluents as an indicator of DNA repair.

Table 1.23. Category 1 In Vitro Assays To Detect Response to DNA Damage

Target Mode of Action	Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
SOS response	SOS response assays: *umu*C assay (also called *umu* and SOS/*umu*), *umu* microtest and SOS chromotest	Bacterial cells *S. typhimurium* TA 1535/pSK1002	Induction of the *umu* operon (SOS response), measured via activation of β-galactosidase, which can metabolize the substrate to a colored product for colorimetric measurement	Various including benzo(a)pyrene (BaP), 4-nitroquinoline-1-oxide (4NQO), 2-amino-anthracene, DDT, ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS)	EC_{10} in units of relative enrichment factor (REF). Genotoxic if induction factor (response of sample/response of control) is above 1.5	Reifferscheid et al., 1991; Langevin et al., 1992; White et al., 1996; ISO, 2000; Hu et al., 2007; Escher et al., 2008b; Cao et al., 2009; Macova et al., 2010
SOS response	Cytotoxicity in SOS defective *E. coli*	Bacterial *E. coli* cells (several K12 AB and KL strains)	Colony formation	None	% survival	Aleem and Malik, 2003, 2005
SOS response	Vitotox assay (kit for detection of SOS response)	Bacteria, genetically modified *S. typhimurium* (TA 104 *rec*N2-4 strain)	SOS response, measured via luminescence	4NQO, 2-aminofluorene, BaP, chrysene, acridine, $K_2Cr_2O_7$, MMS, novobiocine, 2,4,5,7-tetra-nitro-9-fluorenone	Genotoxic when genotoxin-induced luminescence is 1.5 times higher than luminescence in the positive control (TA 104 *pr1* strain, which constantly expresses lux)	Pessala et al., 2004
DNA repair	GreenScreen EM (yeast DNA repair reporter gene assay formerly known as RAD54-GFP)	Yeast *S. cerevisiae* transfected with a plasmid incorporating γEGFP3	Induction of the DNA repair gene, the RAD54 promoter, measured via induction of GFP	Various including BaP, MMS, 4NQO, chloramphenicol, benzaldehyde, benzoyl chloride	LOEC and induction ratio (relative to control); a ratio of ≥ 1.3 is considered genotoxic	Keenan et al., 2007
p53 accumulation	Immunoblot analysis	Human PBMC cells	p53 accumulation, determined by Western blotting	BaP	Semiquantitative	Krishnamurthi et al., 2008

1.6.6.3 Category 2 Bioassays

Although there is an abundance of yeast- and bacterium-based assays for DNA repair applied in water quality assessment, corresponding mammalian cell-based assays are lacking. The GreenScreen HC GADD45a-GFP genotoxicity assay is based on the pathway MDM2-p53-GADD45a (GADD = growth arrest and DNA damage; gene for repair enzymes). This assay not only was derived from a human cell line but also proved to be highly specific and sensitive in a study with 75 chemicals, about half of which were known genotoxicants and the other half not having tested positive in other assays (Hastwell et al., 2006) (Table 1.24). Walmsley (2008) scrutinized the GreenScreen HC assay and concluded that it performed favorably compared to other assays and would be a good complement to the Ames test. However, recently it was demonstrated that GADD45α induction in the GreenScreen HC assay did not occur independently of cytotoxicity, which is a known phenomenon for most genotoxicity assays (Olaharski et al., 2011). A major disadvantage of the GreenScreen HC GADD45a-GFP genotoxicity assay is that the cell line is not available but that testing is performed exclusively by a commercial laboratory (Gentronix).

Table 1.24. Category 2 In Vitro Assays To Detect Response to DNA Damage

Target Mode of Action	Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
Activation of MDM2-p53-GADD45a pathway	GreenScreen HC GADD45a-GFP genotoxicity	Human lymphoblastoid cell line TK6	GFP reporter fluorescence (and control for cell proliferation)	Carboplatin, etoposide, 2,4-dichlorophenol, and methyl nitrosourea	Lowest effective concn for GFP induction >1.5-fold (and for growth inhibition)	Hastwell et al., 2006
ATAD5 induction	ATAD5-luciferase assay	Human embryonic kidney cells HEK293T	Luciferase expression	Methyl methanesulfonate	Fold induction of luciferase	Fox et al., 2012
p53 production	CellCiphr p53	Human hepatocellular cell line HepG2	Via a fluorescent anti-p53 antibody	Mitomycin, Nutlin 3	Induction ratio (relative to control)	Knight et al., 2009
p53 induction	CellSensor® p53RE	Human colon carcinoma cell line HCT-116	Via expression of a β-lactamase reporter gene	Mitomycin, Nutlin 3	Induction ratio (relative to control)	Knight et al., 2009

The Attagene multifactorial assay includes the *cis*-regulated transcriptional factor for p53. Only 6 of the 309 ToxCast chemicals tested positive in this assay (Martin et al., 2010). Fox et al. (2012) recently developed the ATAD5-luciferase assay for the identification of genotoxic compounds.

A number of recently developed genotoxicity test systems are based on the detection of p53 induction, either directly via Western blotting or enzyme-linked immunosorbent assays (ELISAs)

(e.g., Boehme et al., 2010; Zwart et al., 2012) or indirectly via reporter genes (Westerink et al., 2010; Mizota et al., 2011). Hendriks et al. (2011) identified potential biomarkers for mammalian genotoxicity by identifying genes that were strongly induced upon exposure to specific carcinogens in mouse embryonic stem cells and subsequently transfected the cells with a DsRed fluorescent reporter gene to assess the gene induction. However, p53 detection via ELISA and Western blotting as well as culturing of embryonic stem cells is very tedious and p53 reporter gene assays are either only transiently transfected (Mizota et al., 2011) or not accessible for external research groups (Westerink et al., 2010). Commercially available test systems are the GreenScreen, CellCiphr p53, and CellSensor® p53RE (Table 1.24). These three bioassays were compared with the 320 ToxCast I chemicals (Knight et al., 2009), and the assays were of relatively low sensitivity, presumably because of lack of metabolic activation but high specificity.

1.6.6.4 Conclusions

A wide range of in vitro bioassays is available to detect the response of cells to DNA damage, including the induction of repair mechanisms, but most of these assays were derived from yeast and bacterial cells. It is therefore recommended to attempt the validation of a human cell line-based assay for testing of water samples, e.g., the CellCiphr p53 assay (Cellumen Inc.), the CellSensor® p53RE assay (Invitrogen), or the ATAD5-luciferase assay (Table 1.24).

1.6.7 Response to Oxidative Stress

1.6.7.1 Mechanistic Background and Relevance

ROS such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\bullet}$) can be formed by certain radical chemicals (e.g., paraquat) and redox cyclers (e.g., quinones) (Figure 1.6). Inhibition of the mitochondrial electron transfer chain will also lead to the formation of ROS (Escher and Leusch, 2012). Particularly reactive hydroxyl radicals are formed in the presence of divalent iron (Fe^{2+}). The potential adverse effects of ROS include lipid peroxidation, DNA damage, and oxidation of proteins followed by loss of enzymatic activity.

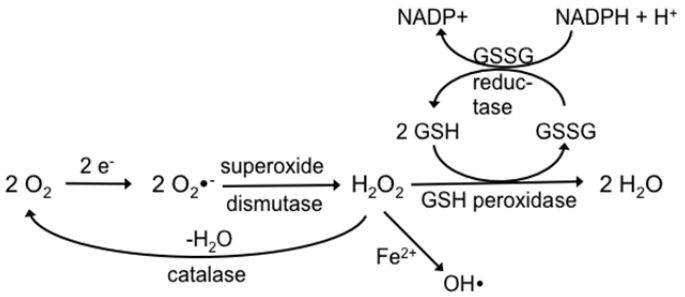

Figure 1.6. Formation and deactivation of reactive oxygen species.

Notes: GSH = glutathione, GSSG = glutathione disulfide, O2 = molecular oxygen, O2•- = superoxide, H2O2 = hydrogen peroxide (H2O2), OH• = hydroxyl radicals, NADP+ = nicotinamide dinucleotide phosphate, NADPH = reduced NADP+.

Source: Reprinted with permission from Escher and Leusch (2012). Copyright 2012, IWA Publishing.

GSH and catalase are important for detoxification of ROS, during which GSH is oxidized to GSH disulfide (GSSG) (Figure 1.6). Oxidative stress thus causes a change in the ratio of GSH to GSSG that ultimately leads to a disturbance of the cellular redox homeostasis and of other cellular redox systems. As NADPH is involved in the reduction of GSSG to GSH, the $NADP^+$/NADPH will, for example, also be affected by a change in GSSG/GSH. A reduction of the amount of NADPH can

have implications for other vital functions, such as acting as coenzyme for the Phase I metabolic enzyme CYP (Escher and Leusch, 2012).

In mammals, the NF-E2-related factor 2 (Nrf2; Figure 1.7) regulates the cellular defense mechanism against oxidative stress through activation of detoxification and antioxidant genes (Nguyen et al., 2009; Giudice et al., 2010; Q. Zhang et al., 2010). Nrf2 activates the transcription of sequences containing the antioxidant response element (ARE), which is a *cis* element found in the promoter region of genes encoding proteins that protect the cell from damage by counteracting the harmful effects of ROS and environmental carcinogens.

Induction of ARE activates the major detoxification enzymes, including the two major contributors to cellular protection: GSTA2 (GSH S-transferase A2) and NQO1 (NADPH:quinone oxidoreductase (Uda et al., 1997). The important role of Nrf2 in cytoprotection and health is clearly demonstrated in Nrf2 knockout mice, which display an increased sensitivity to chemical toxicants and carcinogens and are resistant to the protective actions of chemopreventive compounds (Ramos-Gomez et al., 2001).

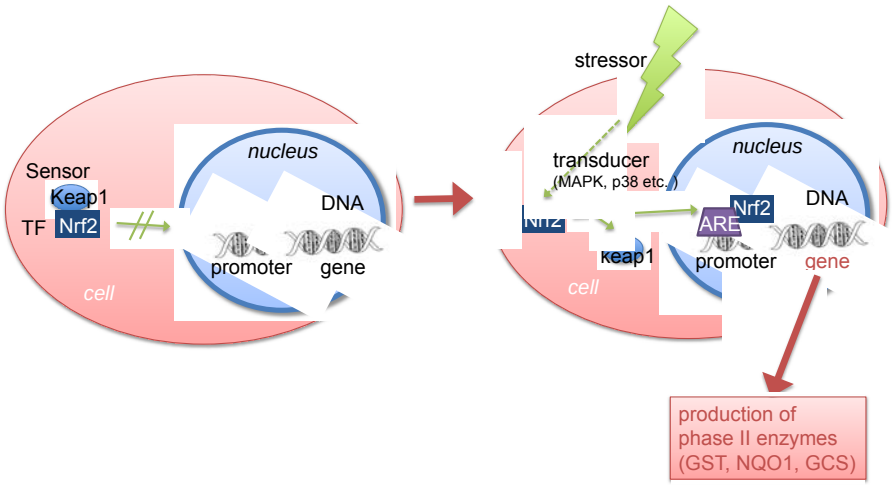

Figure 1.7. Schematic model of the Nrf2 signal pathway.

Notes: In basal conditions, Nrf2 is bound to Keap1 and targeted for degradation. In response to oxidative stress, Nrf2 translocates to the nucleus and initiates transcription of ARE-containing genes. The enzymes transcribed from these Phase II genes work together to maintain cellular integrity.

1.6.7.2 Category 1 Bioassays

A reporter cell line allowing the quantification of luciferase expression in response to various chemicals is the AREc32 cell line generated by Wang et al. (2006). These cells are derived from the human breast cancer cell line MCF7, with the addition of a luciferase gene construct attached to the ARE *cis* element. The antioxidant response of the AREc32 cells can be measured by luciferase expression. On the basis of the induction of reporter genes by the reference compound *tert*-butylhydroquinone (*t*-BHQ), AREc32 cells gave substantially greater levels of induction than do HepG2 cells (Wang et al., 2006). The Nrf2-Keap1-ARE toxicity pathway also is relevant for skin sensitization (Natsch, 2010), and the AREc32 cell line has been used as an in vitro screen for 116 reference chemicals and potential skin sensitizers. The results from AREc32 correlated well with the in vivo local lymph node assay in mice (Natsch et al., 2009). Most recently AREc32 also has been applied successfully for water quality assessment (Escher et al., 2012, 2013b), and another cell construct based on HepG2 cells also showed induction of the Nrf2 pathway by extracts from drinking water (Wang et al., 2013).

In addition, there are bioassays that attempt to quantify the ROS or the redox status of the cells by determination of GSH (Table 1.25). The presence of ROS is used as an indicator of oxidative stress in water samples (Marabini et al., 2006). ROS can be quantified colorimetrically by adding a substrate that fluoresces in the presence of ROS (Wang and Joseph, 1999). The presence or lack of ROS in water samples should, however, be interpreted with caution. One study detected indicators of oxidative stress in samples of disinfected drinking water in which no ROS could be detected (Y. Shi et al., 2009). ROS also can be measured as the production of free radicals (superoxide and hydroxyl radical) (Xie et al., 2010).

Table 1.25. Category 1 Bioassays Indicative of Oxidative Stress

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
Binding to ARE	Luciferase	HepG2	*t*-BHQ	Fold induction of luciferase	Wang et al., 2013
Binding to ARE	Luciferase	1.1 Human mammary reporter cell line (AREc32 derived from MCF7)	*t*-BHQ	$EC_{IR1.5}$ (effect concn causing an induction ratio of 1.5)	Wang et al., 2006; Natsch et al., 2009; Escher et al., 2012, 2013
ROS assay (indirect detection of ROS)	Human liver cells (Hep-G2)	Oxidation of a substrate (DCFH-DA) leads to a fluorescent product			Marabini et al., 2006; Y. Shi et al., 2009
ROS assay (indirect detection of ROS)	Rainbow trout primary hepatocytes	Oxidation of a substrate (H_2-DCFA) leads to a fluorescent product, total GSH by the GSH reductase enzymatic recycling assay	PAHs	LOEC for relative ROS, total GSH	Farmen et al., 2010
Oxidative status in cells via GSH measurement	Human normal liver cell line L-02	Quantification of reduced GSH	Concentration of GSH in mg per g of protein	Hydrogen peroxide	Xie et al., 2010

Note: ARE = antioxidant response element.

1.6.7.3 Category 2 Bioassays

A number of Category 2 bioassays have been developed to measure cellular oxidative stress. These assays are discussed in further detail later and are summarized in Table 1.26.

Boerboom et al. (2006) developed the EpRE(hNQO1)-LUX luciferase reporter cell line, derived from the human hepatoblastoma cell line HepG2. These cells can be cultured in the presence of various toxicants that induce oxidative stress via Nrf2 activation, and the resulting luciferase expression may be quantified. This group also has developed a similar system using the murine EpRE(mGST-Ya)-LUX cells, which are derived from the mouse liver hepatoma cell line Hepa-1c1c7.

Villeneuve et al. (2008) developed the ARE-Luc cell line, in which the MDA-MB-231 mammary gland cell is transfected with an ARE luciferase plasmid. This work allows the quantification of induction of the Nrf2 signaling pathway in response to oxidative stress via the luciferase gene assay, similar to the AREc32 cell line, which had already been applied for water quality assessment. The MDA-MB-231 ARE-Luc cell line also has been validated by using Western blotting and RT-PCR to measure the levels of Nrf2 protein and mRNA expression, respectively, in response to oxidative stress. ARE-Luc cells are therefore another robust and well-validated cell line that would be a good candidate for measuring the effects of recycled or reclaimed water on human health.

Keenan et al. (2009) validated the use of the human bronchial epithelial cell line 16HBE14o to quantify the levels of lactate dehydrogenase and ROS as markers of oxidative stress. The drawback of this cell line is that it has been validated only in response to nZVI (nanoparticulate zerovalent iron) and Fe(II) (ferrous iron) with no other inducers of oxidative stress tested. Additionally, the other Category 2 bioassays discussed quantify Nrf2 activation in response to oxidative stress rather than the end products of oxidative stress (production of ROS). Measuring the cellular oxidative stress response is arguably the most reliable method for quantifying cellular oxidative stress, rather than merely using the end products that are generated when the oxidative stress response is insufficient to protect the cell.

Table 1.26. Category 2 Bioassays Indicative of Oxidative Stress

Target Mechanism	Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
Binding to ARE	Luciferase	Human mammary reporter cell line (AREc32 derived from MCF7)	Reduction in luminescence from the GFP-tagged ARE	Fold induction of luciferase	All chemicals, skin sensitizers	Wang et al., 2006; Natsch et al., 2009
Binding to ARE (called EpRE)	Reporter gene assay/luciferase	Mouse EpRE(mGST-Ya)-LUX cells (derived from the mouse liver hepatoma cell line Hepa-1c1c7)	Luciferase expression	Fold induction of luciferase	Benzyl isothiocyanate, tBHQ, flavonoid antioxidants	Boerboom et al., 2006
Binding to ARE	Reporter gene assay/luciferase	Human EpRE(hNQO1)-LUX (derived from the human hepatoblastoma cell line HepG2)	Luciferase expression	Fold induction of luciferase	As above	Boerboom et al., 2006
LDH leakage and ROS formation	Cell viability and HDCF-DA	Human bronchial epithelial cells (16HBE14o)	Cell viability measured by quantifying LDH released (proportional to membrane damage). The HDCF-DA assay can be used to measure ROS production.	% cell viability	nZVI; Fe0(s) and Fe(II)	Keenan et al., 2009
Activation of Nrf2 and binding to ARE	RT-PCR and Western blotting; luciferase reporter gene assay	Human MDA-MB-231 mammary gland cells transfected with an ARE luciferase plasmid	Nrf2 protein measured in the Western blot, or mRNA expression measured by RT-PCR	Fold induction of luciferase	tBHQ	Villeneuve et al., 2008
Activation of Nrf2	Multiplexed reporter gene transcription units	Human liver hepatoma cell line (HepG2)	Activity of endogenous TFs	Changes in TF activity	Dichlorvos	Martin et al., 2010

Notes: ARE = antioxidant response element; EpRE = electrophile-responsive element; HDCF-DA = 2′7′-dichlorodihydoofluorescein diacetate; LDH = lactate dehydrogenase; Nrf2 = NF-E2-related factor 2; nZVI; Fe0(s) = nanoparticulate zerovalent iron; Fe(II) = ferrous iron; tBHQ = *t*-butylhydroquinone.

1.6.7.4 Conclusion

In the evaluation by Martin et al. (2010) of the impact of 309 chemicals on 25 NRs and 48 TF response elements, it was concluded that Nrf2 activity was one of the outstanding stress response pathways. Nrf2 activity was considered to be highly correlated with the overall effect, as 165 out of the 309 environmental pollutants had a positive response in this endpoint. Defense against oxidative stress should therefore be included in any test battery for water quality.

Both ARE-Luc and AREc32 represent good candidates to quantify cellular oxidative stress. Both cell lines show sensitivity in response to a variety of toxicants and have demonstrated reliability and robustness. In conclusion, these Category 2 bioassays appear to be a more promising tool than the attempt to measure ROS directly. Therefore, we propose the implementation of one of the reporter gene assays indicative of the Nrf2-Keap1-ARE toxicity pathway for water quality assessment.

1.7 Bioassays Indicative of Organ Response

1.7.1 Hepatotoxicity

1.7.1.1 Mechanistic Background and Relevance

All circulating xenobiotics must pass the liver to be metabolized. Although many xenobiotics are metabolized into stable metabolites, others are transformed into potentially reactive metabolites. Reactive metabolites are often capable of inducing hepatotoxicity (Tolosa et al., 2012). Xenobiotics may induce hepatotoxicity via a number of different signaling mechanisms e.g., apoptosis, DNA synthesis/genotoxicity, oxidative stress, mitochondrial damage, and bioactivation (Tolosa et al., 2012). Many of these mechanisms are highly related, and indeed, one event may act as the trigger for other events, all of which result in cell damage. The damage that occurs at the molecular level may manifest itself as direct cytotoxicity to hepatocytes, damage to the epithelial cells of liver capillaries, impaired bile excretion (usually from interference of bile salt export pumps), and excessive cell proliferation to replace dead cells (hyperplasia). Thus, cytotoxicity and hepatocyte viability are commonly used as measurable endpoints of hepatotoxicity

Xenobiotic exposure induces metabolic activity, which is particularly high in liver cells. Liver enzymes are, therefore, commonly used biomarkers for contaminant exposure. Phase I (e.g., enzymes in the CYP family) and Phase II (e.g., transferases) enzymes can be measured both in vivo and in vitro. Often, primary hepatocytes are coincubated with other cells to simulate metabolism in bioassays targeting other endpoints (Coecke et al., 1999). The vulnerability of the liver to xenobiotics and the role it plays in their metabolism means that there is a clear requirement for an in vitro assay that can accurately determine the presence of hepatotoxicity-inducing chemicals in water samples.

1.7.1.2 Category 1 Bioassays

Most bioassays targeting hepatotoxicity quantify the activation of metabolic enzymes. The EROD and HepCYP1A2 assays target CYP1A1 and CYP1A2 enzymes, respectively, and are detailed in Section 1.4.1.

Additional assays for liver toxicity have been applied for water quality monitoring (Table 1.27). Various studies have measured cytotoxicity in liver cells such as rainbow trout hepatocytes (e.g., Gagné and Blaise, 1998; Klee et al., 2004; Farmen et al., 2010) and human liver carcinoma

(HepG2) cells (Marabini et al., 2007; Maffei et al., 2009) following exposure to water samples. The HepaTOX assay is an example of an assay that targets liver toxicity by measuring nonspecific toxicity in human hepatocellular carcinoma (C3A) cells using the resazurin assay (NWC, 2011).

The PP2A assay is specific for cyanobacterial hepatotoxins such as microcystins and nodularins, which act by inhibiting the protein phosphatases PP1 and PP2A. The PP2A assay was validated for detection of cyanobacterial hepatotoxins below the Australian drinking water guideline (1 µg/L) with no preconcentration step necessary (Heresztyn and Nicholson, 2001).

Table 1.27. Selection of In Vitro Bioassays To Measure Hepatotoxicity in Water Samples

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
HepaTOX	Human C3A liver cell line, a subclone of the hepatoma-derived HepG2 cell line	Hepatocyte-specific cytotoxicity by resazurin reduction assay	Several including chloroform, diuron, bisphenol A, methyl methanesulfonate	% viable cells compared to control. Cytotoxic if the response is at least 3 times greater than that of the control	Page et al., 1993; NWC, 2011
PP2A assay for cyanobacterial hepatotoxins	Rabbit skeletal muscle (commercially available)	Inhibition of protein phosphatase (PP2A). The substrate (*p*-nitrophenyl phosphate) releases *p*-nitrophenol, which is measured colorimetrically	Microcystins, nodularins	% PP2A activity relative to control. IC_{50}	An and Carmichael, 1994; Heresztyn and Nicholson, 2001

Note: IC_{50} = 50% inhibitory concentration.

1.7.1.3 Category 2 Bioassays

Several immortalized liver cancer cell lines are available as in vitro models for hepatotoxicity (e.g., HepG2, human hepatoma BC2). It should be noted, however, that cancer cell lines exhibit differences in gene and protein expression and have reduced metabolizing capacity compared with primary hepatocytes (Donato et al., 2008). Noncancerous immortalized human hepatocytes (Fa2N-4 cells), which closely resemble primary hepatocytes, have recently become commercially available (Steen, 2004) and may be useful in in vitro models to monitor nonspecific and specific effects of xenobiotics (Escher and Leusch, 2012).

Although hepatotoxicity can be induced via a number of different signaling mechanisms, most in vitro assays measure only one endpoint of a particular signaling pathway. The assays, therefore, can appear to have a very low level of sensitivity to measure hepatotoxicity. On that basis, Tolosa et al. (2012) suggested that hepatotoxicity should be assessed upon multiple parameters. To that end, this group developed a multiparametric cell-based protocol to screen the hepatotoxicity potential of new drugs, where data on nuclear morphology, mitochondrial function, cell viability,

intracellular calcium concentration, and oxidative stress were obtained simultaneously and integrated. These in vitro assays were conducted by using human liver HepG2 cells. The authors acknowledged that, though HepG2 cells had been used extensively to predict hepatotoxicity, these cells expressed very low levels of the P450 enzymes. A subsequent study used adenoviruses to overexpress a range of CYP genes to elevate the sensitivity of HepG2 cells to hepatotoxicity-inducing drugs (Tolosa et al., 2013). These later cells, combined with a high-content screening protocol, may be adapted to assess the hepatotoxicity potential of environmental water samples.

Using a radically different approach in the same domain of drug development, companies employ mathematical modeling to determine the toxicity of various compounds to specific organs or biological systems. Howell et al. (2012) examined the possibility of extrapolating in vivo data from in vitro data by using the Drug-Inducing Liver Injury (DILIsym). The DILIsym is a predictive model that determines the hepatotoxicity of drugs and chemicals. It is to be used early in research and development to identify those drugs that show a potential for hepatotoxicity. In this paper the authors used known hepatotoxins in in vitro assays to validate the predictive modeling of DILIsym.

1.7.1.4 Conclusions

Hepatoxicity is clearly a relevant endpoint for water quality testing. The HepaTOX assay has been validated for application with a wide range of water samples and is probably the most promising candidate for implementation in a test battery for water quality testing.

1.7.2 Nephrotoxicity

1.7.2.1 Mechanistic Background and Relevance

The kidneys are important for filtering blood and, therefore, for maintaining whole body homeostasis. Hence, the kidneys are susceptible to receiving a considerable load of blood-borne toxicants. As passive and active (ATP-driven) transport mechanisms are crucial for kidney function, the kidneys can be seriously impacted by toxicant-induced interruptions of the energy production that sustains active transport mechanisms or by interference with critical membrane-bound enzymes and/or transporters. Tight control of capillary pressure also is important for kidney function, which is particularly sensitive to vasoactive substances that modulate blood pressure. Kidney toxicity can lead to impaired detoxification, hyperplasia, and tumors and eventually to kidney failure and loss of homeostasis.

1.7.2.2 Category 1 Bioassays

Kidney cells such as the green monkey kidney cell line CV-1 and the human embryonic kidney cell line HEK293 are commonly used in reporter gene assays applied with water samples for other specific endpoints. To our knowledge, however, few studies have used in vitro bioassays specifically targeting nephrotoxicity for water quality testing.

One study was identified that measured cell viability in human kidney cells (HK-2) using colorimetric assays (Alamar Blue and MTT) following exposure to drinking water; however, no clear results were obtained (Bunnell et al., 2007).

1.7.2.3 Category 2 Bioassays

Several models including both primary cultures and immortalized cell lines are available to assess in vitro kidney toxicity. Nephrotoxic endpoints such as kidney cell-specific cytotoxicity, cell proliferation, and glucose uptake can be measured in vitro (Hawksworth et al., 1995; Morin et al., 1997; Pfaller and Gstraunthaler, 1998). One challenge in selecting assays for nephrotoxicity is the

selection of appropriate cell lines. A common shortcoming of immortal cell lines is that they generally lack many of the endogenous functions specific to kidney cells (Beeson et al., 2010). Even primary kidney cells lose these functions with time (Pfaller and Gstraunthaler, 1998). For this reason, the most practical nephrotoxic endpoint for water quality screening may be general cytotoxicity to kidney cells. As nephrotoxicants often cause mitochondrial damage (Beeson et al., 2010; Johnson-Lyles et al., 2010), the assessment can potentially be made somewhat more comprehensive by comeasurement of mitochondrial toxicity. Examples of such studies include that by Johnson-Lyles et al. (2010), who assessed cell viability, ATP content, and other endpoints in an immortal kidney cell line (LLC-PK1) exposed to fullerenol, and Beeson et al. (2010), who used primary kidney cells to assess mitochondrial function and cytotoxicity in response to nephrotoxicants.

More recently Astashkina et al. (2012) developed a 3D organoid cell culture system derived from murine kidney proximal tubules. These cultures were able to maintain the phenotypical stability of primary cells over a 6 week period and were chosen because of their ability to grow in a matrix and their large epithelial surface area, which potentially made them more susceptible to environmental assault. As a 3D structure, they were considered more physiologically relevant than cells growing in a 2D culture or immortalized cells. Although these assays were designed to assess drugs with the potential to induce nephrotoxicity, they may be adapted also to assess environmental water samples (Astashkina et al., 2012).

1.7.2.4 Conclusions

Neprotoxicity is clearly relevant from a human health perspective. The most suitable assays for assessing this endpoint in water samples are assays that detect nonspecific cytotoxicity in various kidney cell lines. The specificity of evaluation of nephrotoxicity may be improved by coassessment of mitochondrial toxicity/disturbance of energy production, which is often associated with nephrotoxicity.

1.7.3 Cardiovascular Toxicity

1.7.3.1 Mechanistic Background and Relevance

All absorbed xenobiotics will inevitably come into contact with the cardiovascular system via the blood vessel epithelium. The heart (cardio) system can be affected by toxins that disturb energy production (Section 1.5.1) and ion channels and pumps (Section 1.6.4). Vascular function can be affected via neurotoxicity (Section 1.8.2) and/or endocrine disruption (Section 1.10) because dilation and constriction of blood vessels are controlled remotely by neurons and hormones (e.g., epinephrine, norepinephrine, and angiotensin). By disrupting the heart and blood vessels, cardiovascular toxicity can ultimately cause heart failure and irregular blood pressure.

1.7.3.2 Category 1 Bioassays

To our knowledge, no in vitro bioassay specific for cardiovascular toxicity has yet been applied to water quality testing.

1.7.3.3 Category 2 Bioassays

The cardiac muscle cell line HL-1 (Claycomb et al., 1998) can be used to test cytotoxicity through cardiomyocyte viability (using the neutral red uptake assay) and electrophysiology (in

particular membrane potential). Additional in vitro tests (reviewed by Netzer et al., 2001) also may be compatible with HL-1 cells.

Recent progress in human-induced pluripotent stem cells means that large quantities of undifferentiated cells can be generated and then forced to differentiate into any number of different cell types to be used in drug toxicity studies (Scott et al., 2013). Thus, drug-induced toxicity can be assessed by using these cells in high-throughput in vitro assays, which target and quantify very tissue-specific biological properties. Although further development is required before cell-based in vitro toxicity assays accurately reflect the toxicity observed in mature tissue in vivo, these techniques may potentially be adapted to test environmental water samples in the future.

1.7.3.4 Conclusions

The endpoint of cardiovascular toxicity has no high priority for water quality assessment at this stage.

1.8 Bioassays Indicative of System Response

Some biological functions are dependent on systems composed of multiple organs. For such systems, toxicity to any one organ involved may result in failure of the whole system. System toxicity includes toxicity to the blood (hematotoxicity), immune, nervous, hormone (endocrine disruption), reproductive, sensory organ (e.g., ocular toxicity), respiratory, cutaneous, and musculoskeletal (e.g., myotoxicity) systems. For this review, we focus on hematotoxicity, immunotoxicity, neurotoxicity, endocrine disruption, and reproductive/developmental effects, which are more pertinent in regard to water consumption.

1.8.1 Hematotoxicity

1.8.1.1 Mechanistic Background and Relevance

Hematopoiesis (the production of blood cells) is important for oxygen transport, host defense and repair, and blood homeostasis. The major organs involved in this process are bone marrow and the spleen. Hematotoxicity is defined as a significant decrease in the overall number of blood cells (L. Zhang et al., 2010) and often occurs when toxicants interfere with hematopoiesis, or affect the viability of red blood cells, which can cause anemia and/or hypoxia (lack of blood and/or oxygen, respectively). Red blood cell viability can be affected by oxidative damage, which compromises the oxygen-carrying capacity of hemoglobin. Also, interference with cell surface proteins can result in the loss of "self"-antigens (antigens recognized as noninvasive by the immune system) and in their subsequent destruction by white blood cells.

1.8.1.2 Category 1 Bioassays

Some studies use blood cells as in vitro model for screening of water samples. To our knowledge, however, the applied techniques do not include in vitro assays specifically detecting hematotoxicity.

1.8.1.3 Category 2 Bioassays

Current in vitro methods in hematotoxicology mainly focus on the development of progenitor cells and stromal cells that support hematopoiesis in response to drug therapies that target cancer

(Haglund et al., 2010). This is typically measured by using colony-forming unit (CFU) assays in hematopoietic progenitor cells and fibroblasts (reviewed by Gribaldo et al., 1996; Parent-Massin, 2001; Rich, 2003; Wang et al., 2012). Although CFU assays provide clear evidence of a particular drug's toxic effect on hematopoietic progenitor cells, they have a very low throughput capacity. Haglund et al. (2010) recently developed a nonclonogenic fluorometric microculture cytotoxicity assay (FMCA) using human $CD34^+$ progenitor cells, which has been validated for high-throughput toxicity in drug discovery. It is based on the measurement of fluorescence generated from the hydrolysis of fluorescein diacetate (FDA) to fluorescein by cells with an intact plasma membrane. This type of cell-based assay may be adapted for testing hematotoxicity in water samples.

1.8.1.4 Conclusions

In general, hematotoxicity may be of minor importance for water quality testing as it can be indirectly assessed by using cytotoxicity- and immunotoxicity-related assays. There are, however, some industrial chemicals that cause toxicity to the hematopoietic system. The biological effect of benzene on the hematopoietic system and its links to leukemia have been known for a long time (Wang et al., 2012). The exact mechanism by which benzene affects blood cells remains unknown, but chronic exposure has been shown to lead to a number of blood-based diseases. Although benzene is highly volatile, there is some concern that it may contaminate groundwater as a result of fracking in coal seam gas mining and other industrial processes. It may therefore be worth developing a cell-based assay to measure hematotoxicity in water samples.

1.8.2 Neurotoxicity

1.8.2.1 Mechanistic Background and Relevance

A neurotoxin is a toxic compound that interferes with the development and function of the nervous system, which comprises two types of cells, neurons and glial cells. Neurons are important for generation, reception, and transfer of information, which take place via neurotransmitters (e.g., acetylcholine (ACh) and epinephrine). Glial cells are responsible for homeostasis and for physical and nutritional support of neurons. The nervous system can be disrupted via, for example, the neuron, the axon (the projection of a neuron toward other neurons by which electrical impulses are transduced), the myelinating cells (including glial cells), and the neurotransmitter system.

Many insecticides act via disruption of electrical signal transduction or by inhibition of chemical signal transduction at the synapses. The opening and closing of sodium channels allow the transmission of action potentials, and thus electrical signals, along the neuron. By inhibiting the reclosure of these ion channels, natural and synthetic pyrethroids (e.g., pyrethrin and permethrin) can cause overexcitation of the nervous system. Many pesticides and pharmaceuticals as well as naturally occurring toxicants are capable of inhibiting neurotransmitters. Organophosphate and carbamate pesticides, for example, inhibit the neurotransmitter ACh through inhibition of the enzyme acetylcholinesterase (AChE), which is responsible for its recycling via breakdown. Similarly the neonicotinoid imidacloprid has an antagonistic effect on the nicotinic ACh receptor. The GABA (γ aminobutyric acid) receptor is another target of the nervous system. The GABA receptor acts as a gate for chloride channels, thereby controlling the flow of chloride ions across the plasma membrane of the neuron. Thus, the GABA receptor functions as an inhibitory neurotransmitter. Pesticides such as dieldrin, lindane (γ-hexachlorocyclohexane), and antiparasitic avermectins are agonists of the GABA receptor, again disrupting the electrical signals of the nervous system.

From a human risk assessment perspective, neurotoxic insecticides are generally more toxic to insects than to humans, who have higher metabolizing capacity for, e.g., the organophosphates. The relevant receptors of some insecticides often also play a different role in mammals and insects. Further, the central nervous system is surrounded by the blood-brain barrier (BBB), which provides additional protection against xenobiotics. This consideration is crucial for human risk assessment because some otherwise neurotoxic chemicals simply cannot cross the BBB. The GABA receptor, for example, is important for the peripheral nervous system in invertebrates, where agonistic activity will lead to paralysis. Conversely in mammals, the GABA receptors are of importance only to the central nervous system, which, because of the BBB, is impenetrable to many of the GABA agonistic insecticides, including the macrocyclic lactones.

Finally, as neuronal function is dependent on energy, neurons are extremely sensitive to interruptions in the oxygen and glucose supply.

1.8.2.2 Category 1 Bioassays

AChE inhibition is a commonly applied in vitro endpoint for detection of specific neurotoxicity in water samples (Table 1.28). Ellman et al. (1961) first developed the AChE inhibition assay, which was later optimized for use with environmental samples by Hamers et al. (2000), who validated the technique with rainwater samples. Various studies investigating wastewater and recycled water as well as surface waters have included this test (Escher et al., 2009; Macova et al., 2010, 2011). A drawback of the AChE inhibition assay is that it is impossible to differentiate between the specific inhibition and nonspecific denaturation of AChE. This limitation can lead to false-positive results in highly contaminated samples such as wastewater. This disadvantage is common to all cell-free bioassays. We have therefore generally omitted these types of assays in this review but have made an exception for the AChE inhibition assay, as it is so commonly used and is established in the laboratory of the report authors.

In addition to anthropogenic chemicals, natural toxins also are important for water quality assessment as, for example, neurotoxic cyanotoxins can occur in harmful concentrations in recreational waters such as lakes following algal blooms. Natural toxins also can be important for assessment of drinking water, where it is derived from surface water. Various analytical and bioanalytical tools have been reviewed by Humpage et al. (2010). The neuroblastoma assay is useful for detection of cyanotoxins and paralytic shellfish toxins (Kogure et al., 1988; Jellett et al., 1992; Manger et al., 1993, 1995). This assay detects agents that block the sodium channels during signal transmission and has been validated thoroughly through interlaboratory comparison (Humpage et al., 2007). Although this assay has been mostly tested with mat and/or freeze-dried samples, it is seeing increased use in testing of water quality (Wood et al., 2006; Cetojevic-Simin et al., 2009; Campora et al., 2010; Kerbrat et al., 2010) (Table 1.28).

Some studies have also used ex vivo and receptor binding assays (RBAs) to detect the presence of neurotoxins in water samples. Although these assay types are less suitable for routine screening purposes, we name a couple in the following. Kerbrat et al. (2010) applied an RBA with rat brain synaptosomes to detect the presence of natural neurotoxins such as brevetoxins that have a differential affinity to bind to a specific site (5) of the voltage-sensitive sodium channel (Table 1.28). Basu et al. (2009b) evaluated solvent extracts from pulp and paper mill effluents for their ability to bind to a number of receptors and enzymes involved in neurotransmission, which were extracted from the brain tissue of goldfish (Table 1.28).

Table 1.28. Category 1 In Vitro Bioassays for Detection of Neurotoxicity

Target Mode of Action	Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
Enzyme inhibition	AChE inhibition assay	Purified AChE from electrical eel (*Electrophorus electricus*) or honeybee head (*Apis mellifera*)	AChE inhibition. AChE hydrolyzes the added substrate to yield a product that can be measured fluorimetrically	Organophosphates and carbamate insecticides	Parathion toxic equivalent concns (PT-EQ)	*Hamers et al., 2000; Escher et al., 2009; Macova et al., 2011*
Sodium channel blocking	Neuro-blastoma assay	Mouse neuroblastoma cells (neuro-2A), human neuroblastoma cells (SK-N-SH)	Inhibition of the channel-opening effect of veratridine. In this assay, the inhibitory effect protects cells from swelling and lysis	Tetrodotoxin (pufferfish toxin), saxi-, gonyau-, and C-toxins (paralytic shellfish toxins produced by dinoflagellates and cyanobacteria)	% cell survival relative to control, EC_{50}, significant or not significant	Wood et al., 2006; Cetojevic-Simin et al., 2009; Campora et al., 2010; Kerbrat et al., 2010
Receptor binding (site 5 of the voltage-sensitive sodium channel [VSSC])	RBA	Rat brain synaptosomes (ex vivo)	Binding to site 5 of VSSC quantified via competitive binding against a radio-labeled reference compound (^{3}H-PbTx-3)	Brevetoxins (PbTx-1 to PbTx-8) and ciguatoxins (CTX-1B, CTX-2A1, CTX-2A2, CTX-3C, and CTX-4B)	IC_{50}, the concn of extract required to induce 50% inhibition of binding of ^{3}H-PbTx-3	Poli et al., 1986; Dechraoui et al., 1999; Darius et al., 2007; Kerbrat et al., 2010
Receptor binding (D2R, $GABA_AR$, NMDAR, mAChR)	RBA	Goldfish brain (ex vivo)	Binding to receptor quantified via competitive binding against a radiolabeled reference compound	^{3}H-spiperone, ^{3}H-muscimol, ^{3}H-MK801, ^{3}H-QNB for D2R, $GABA_AR$, NMDAR, mAChR, respectively	% inhibition of binding of reference compound	Basu et al., 2005a, 2005b, 2009a, 2009b
Neurotransmitter enzyme activity (AChE, $GABA_T$, GAD, MAO)	Inhibition of various neurotransmitter-related enzymes: monoamine oxidase, GABA-trans-aminase, glutamic acid decarboxylase, AChE	Goldfish brain (ex vivo)	Enzyme activity (concn) measured by colorimetric labeling; competitive radioligand binding	None	% of activity measured in the control	Awad et al., 2007; Basu et al., 2007, 2009a, 2009b

Notes: AChE = acetylcholinesterase; D2 = dopamine 2 receptor; $GABA_AR$ = γ aminobutyric acid (A) receptor; $GABA_T$ = γ aminobutyric acid (T); GAD = glutamic acid decarboxylase; mAChR = muscarinic ACh receptor; MAO = monoamine oxidase; NMDAR = *N*-methyl-D-aspartate receptor; IC_{50} = 50% inhibitory concentration.

1.8.2.3 Category 2 Bioassays

Additional in vitro neurotoxicity assays that have not been validated for water quality assessment are available (Table 1.29) and include neuronal and glial cell viability assays using the human neuroblastoma cell line SK-N-SH (and derivatives, such as SH-SY5Y cells) and C6 glial cells (Tang et al., 2003; Xia et al., 2008). Xia et al. (2008) tested a large range of different cell types on more than 1000 chemicals and found the cell lines derived from neurons to be among the most sensitive.

Precursor cell differentiation and apoptosis assays with neuroblastoma cells, glial maturation (myelination) in U-373MG human astrocytoma cells, neurotransmitter receptor profiles in neuroblastoma cells, and interference with neurotransmitter enzymes or postsynaptic receptors also are available (reviewed by Atterwill et al., 1994; Costa, 1998; Tiffany-Castiglioni et al., 2006; Coecke et al., 2007).

Because of the partial protection of the central nervous system by the BBB, it is important to first establish a chemical's potential to cross the BBB before assessing its potential to cause adverse effects on the central nervous system. The BBB can be simulated in vitro, and a number of immortalized brain endothelial cell lines are available for this purpose (e.g., SV-HCEC, HBEC-51, or BB19 cells) (Prieto et al., 2004). As immortalized cells often lose the BBB properties of primary cells, cocultures with astrocytes or other glial cells (e.g., the C6 rat glioma cell line) are often used to partly regain these characteristics (Neuhaus et al., 2008; Culot et al., 2009; Helms et al., 2010; G. L. Li et al., 2010; Mabondzo et al., 2010; Wilhelm et al., 2011). Two-component in vitro BBB models consist of an upper (blood, i.e., endothelial cells) and lower (brain, i.e., astrocytes) compartment separated by a semipermeable filter (commercially available insert) (Culot et al., 2008; Mabondzo et al., 2010; Wilhelm et al., 2011). The permeability of the BBB is then measured by tracing transport across the "membrane" from the upper to the lower compartment by using radioactive, fluorescent, or luminescent labeling. In three-component in vitro BBB systems, target cells (e.g., the neuroblastoma cell line SH-SY5Y) are inserted at the bottom of the lower compartment for assessment of toxicity of the chemical proportion reaching these cells (Hallier-Vanuxeem et al., 2009; Balbuena et al., 2010). Although BBB penetration is a relevant endpoint for assessment of water intended for human consumption, these assays are too complex for the high-throughput requirements of water quality screening.

Neurotoxins that are incapable of crossing the BBB may still be of risk to the developing brain in utero and during early postnatal exposure (Harrill et al., 2011), when the BBB has not yet fully differentiated (Bal-Price et al., 2010). Developmental neurotoxicity (DNT) is thus of high relevance when assessing the quality of water intended for human consumption and use. As reviewed by Breier et al. (2010), DNT can be assessed by using neurosphere cultures of human neural progenitor cells (hNPCs). Schreiber et al. (2010) used primary fetal hNPCs to determine cell viability, proliferation, differentiation, and migration when exposed to brominated flame retardants (BFRs), which were found to reduce cell migration and differentiation. These hNCPs were then coexposed with a TR agonist, which appeared to protect the neurospheres from the observed effects of BFRs on neurodevelopment. A similar study found PAHs to cause no DNT in hNPCs and an AhR-deficient mouse cell line, whereas cell proliferation and migration were affected in wild-type mouse NPCs (Gassmann et al., 2010). Culturing and plating out single neurospheres are a very time-consuming process, which means that this technique, as it stands, cannot be utilized for HTS for neurotoxins. Gassmann et al. (2012) have recently shown, however, that a complex object parametric analyzer and sorter (COPAS) effectively sorted and plated neurospheres without detrimentally affecting their performance. A COPAS may thus help to automate this type of assay, thus lending it to HTS of water samples for neurotoxins.

Developmental toxicity is usually assessed by observing changes in behavior in animal studies. A review by van Thriel et al. (2012) targeted studies attempting to bridge the gap between behavioral and ex vivo studies and in vitro assays that attempt to explain cellular mechanisms behind the observed behavioral changes. To this end, Dingemans et al. (2007) used rat PC12 cells to study changes in intracellular Ca^{2+} as a measure of toxicity caused by polybrominated diphenyl ethers.

Table 1.29. Category 2 In Vitro Bioassays with Potential for Water Quality Assessment of Neurotoxins

Target Mode of Action	Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
Cytotoxicity to neuronal and glial cells	Cytotoxicity assays	Human neuroblastoma cell lines (e.g., SK-N-SH, SH-SY5Y) and C6 glial cells	Cell viability (cell count, colorimetric assessment, metabolic activity as ATP)	PAHs, organochlorines	% viable cells compared to control	Tang et al., 2003; Xia et al., 2008
Permeability of the BBB	2-Component in vitro BBB model (also known as 4d/24w (4 day/24 well) in vitro BBB model)	Coculture of brain endothelial cells (e.g., mouse brain microvascular bEnd.3) and astrocytes (e.g., rat glioma cell line C6)	BBB permeability measured via fluorescence (e.g., 8-aminopyrene-1,3,6-trisulfonate (APTS), sodium fluorescein, Fluo-3AM) or luminescence (e.g., Lucifer Yellow (LY) labeling	Various including 1,2,3,4-tetrachlorobenzene, malathion, mercury II chloride, pentachlorophenol, phenanthrene, phenobarbital, pyrene	Permeability coefficient Pe (cm/min)	Neuhaus et al., 2006; Culot et al., 2008; Wang et al., 2011; Wilhelm et al., 2011
Permeability of the BBB followed by neurotoxicity	3-Component in vitro BBB model	As above but further including target neuronal cells such as the human neuroblastoma cell line SH-SY5Y	BBB permeability followed by assessment of cytotoxicity and, e.g., caspase activation or AChE inhibition in target cells	Various including lindane, methylmercury(II) chloride, triethyltin chloride, malathion	Permeability coefficient Pe, (cm/min) and % mortality and enzyme activation/inhibition compared to control	Hallier-Vanuxeem et al., 2009; Balbuena et al., 2010
DNT	Cell viability assays (alamarBlue and CytoTox ONE)	Primary fetal human neural progenitor cells (hNPCs, commercially available)	Reduction in cell viability	Polybrominated diphenyl ethers BDE-47 and BDE-99	% viable cells relative to control	Schreiber et al., 2010
DNT	Cell proliferation assay	As above	Reduced proliferation (sphere size)	BDE-47, BDE-99, mercury chloride, H_2O_2	Sphere diameter (mm)	Moors et al., 2009; Schreiber et

						al., 2010
DNT	Cell migration assay	As above	Reduced cell migration determined by microscope analysis	BDE-47, BDE-99, phorbol-12-myristate-13-acetate, ethanol	% migration distance relative to control	Moors et al., 2007; Schreiber et al., 2010
DNT	Cell differentiation (immunostaining and RT-PCR)	As above	Reduced differentiation to neurons and oligodendrocytes by immunostaining. Nestin (marker of undifferentiated progenitor cells) expression by RT-PCR	BDE-47, BDE-99	% differentiated cells relative to control	Schreiber et al., 2010
DNT	Attagene multifactorial reporter gene assay (ATG_Pax6_CIS)	Human liver hepatoma cells (Hep-G2) transfected with 48 RTUs including one for Pax6	Pax6 binding quantified by fluorescence (via 6-carboxyfluorescein [6-FAM] labeling) and resolved from the remaining TFs by capillary electrophoresis	Various pesticides including prallethrin, spiroxamine, tetraconazole, tetramethrin, thidiazuron	AC_{50} (50% of maximal response)	Romanov et al., 2008; Martin et al., 2010
DNT	Intracellular Ca^{2+}	Rat PC12 cells	Cellular fluorescence: excitation at 340 nm and 380 nm, emission at 510 nm	BDE-47	Ratio of wavelengths F340/F380	Dingemans et al., 2007

1.8.2.4 Conclusions

Over the last 3 decades there appears to have been an increase in learning and neurodevelopmental disorders in children, which is potentially caused by early exposure to a range of chemicals (de Groot et al., 2013). A recent review of DNT, and the in vitro assays that have been validated to measure this endpoint, highlighted the importance of these assays, not only to determine the dose and the DNT of a chemical but because of the sensitivity of a developing brain to chemicals during different stages of its development. Despite this, however, DNT testing is not usually a primary objective in the testing regimen of new chemicals (de Groot et al., 2013). With this in mind, the neuroblastoma assay has been successfully applied for water quality assessment and is therefore recommended for future use. Its derivative, the SH-SY5Y cell, appears to be more sensitive than the parent SK-N-SH, and although this cell line has not yet been applied for water quality assessment, it should be evaluated for possible use. The neuroblastoma assay covers only one aspect of neurotoxicity, the inhibition of sodium channels, and should therefore be complemented by additional assays. The AChE inhibition assay may be a good complementary assay, as many insecticides act via the AChE signaling mechanism. Unfortunately, the AChE assay is a cell-free assay, which in itself creates its own major limitations (cell-free assays being unable to differentiate between specific inhibition and nonspecific deactivation of enzymes). Thus, we find that established in vitro assays for assessing water quality in relation to the DNT of chemicals are underrepresented in the literature.

1.8.3 Immunotoxicity

1.8.3.1 Mechanistic Background and Relevance

Immunotoxicity refers to the deleterious effects of xenobiotics on the immune system. Compounds can elicit immunotoxicity via direct and indirect modes of action. Direct immunotoxicity occurs when the immune system is the direct target for chemicals, resulting in compromised immune function or immunosuppression and subsequently reduced resistance to infection upon external challenge (de Jong and van Loveren, 2007). Indirect immunotoxicity is the specific immune response to the chemical itself or to a chemical-altered self-antigen, leading to allergy or autoimmunity (Lankveld et al., 2009). To date, no validated in vitro bioassay for allergy and autoimmunity is available. Driven by the cosmetic industry, efforts to develop in vitro methods to screen the sensitizing potential of chemicals have had greater success in recent years than those to screen for immunosuppression (Luebke, 2012). Some skin sensitization tests show potential for drinking water quality assessment, but the modes of action are by definition reactive toxicity (e.g., the AREc32 reporter gene assay, Table 1.25). The following text thus focuses on direct immunotoxicity.

General cytotoxicity and specific cytokine production are the common endpoints investigated in current in vitro bioassays for direct immunotoxicity. Cytokines are produced in the first steps of the immune response, and quantitative alterations in their levels can provide insights into immunomodulation by certain chemicals. Of all cytokines, interleukins (ILs) represent a group for which the function of the immune system is largely dependent on IL-4, which serves, for instance, as a key regulator in humoral and adaptive immunity, inducing native helper T cells (Th0 cells) to Th2 cells. Besides ILs, gamma interferon (IFN-γ) is another cytokine critical in innate and adaptive immunity to viral and intracellular bacterial infections. For tumor control, TNF-α also is a critical cytokine involved in systemic inflammation and acute-phase reaction with the primary role of regulating immune cells. In addition to cytokines, NF-κB (Section 1.6.5) is an important protein complex involved in cellular responses to a variety of stimuli, both specifically

to infection and nonspecifically to physical and chemical insult, such as stress, UV radiation, and free radicals.

1.8.3.2 Category 1 Bioassays

Only few immunotoxicity bioassays have been applied to assess water quality (Table 1.30). One of these assays is based on mouse splenocytes and was used to test wastewater. Lymphocyte proliferation was measured by using the tritiated thymidine incorporation assay, and the activity of IL-1 or -2 was measured by using the proliferation assay for the associated target immune cells. Levels of several cytokines (IFN-γ and IL-6 and -10) were also examined by using ELISA (Kontana et al., 2008, 2009). The second application is based on the human whole blood cytokine release assay. The LDH leakage assay and ELISAs were applied to investigate the potential effects of surface water extracts on cell viability and cytokine production (IL-10, IFN-γ, and TNF-α), respectively (Pool and Magcwebeba, 2009). More recently, a study measured both cytotoxicity to lymphocyte precursor cells (WIL2NS TOX assay) and modulation of the cytokine IL-1β in macrophage precursor cells (THP1 cytokine production assay [THP1-CPA]) in a variety of water matrices, including treated sewage, reclaimed water, and drinking water (NWC, 2011).

Generally, these assays have provided meaningful information about the potential of the water extracts to affect different aspects of the immune system on the basis of the functionality of cytokines. The radioactive method used to determine lymphocyte proliferation is, however, not suitable for routine application. The ex vivo nature of the human whole blood cytokine release assay suggests no easy access to this tissue type, limiting its future use in screening a large number of water samples. Also, the use of ELISAs in the context of environmental matrices is a common drawback in the two cases mentioned earlier. In addition to the labor intensiveness and high cost of the ELISA, the sensitivity of this assay can be compromised by matrix components of environmental water, such as dissolved organic carbon, which may interfere with specific binding of the cytokine to its corresponding antibody. Yet cytotoxicity measurement using specific cell types is of value.

Table 1.30. Category 1 Bioassays Indicative of Adverse Effects on the Immune System

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
Lymphoproliferation bioassay and IL-1/IL-2, characterization	Mouse spleen cells	Cell proliferation using ^{3}H-labeling	None reported	^{3}H-thymidine incorporation	Kontana et al., 2008, 2009
Cytokine expression by ELISA	Mouse spleen cells, whole human blood	Cytokine expression (IL-10, IFN-γ, and TNF-α) measured by ELISA	None reported	Concn of IL-10, IFN-γ, and TNF-α (pg/mL)	Kontana et al., 2008; Pool and Magcwebeba, 2009
THP1-CPA	Human acute monocytic leukemia cells (THP1)	Stimulation or suppression of IL-1β production, determined by ELISA	Phorbol-12-myristate-13-acetate and dexamethasone for the agonist and antagonist modes, respectively	EC_{50}	NWC, 2011
WIL2NS TOX	Human lymphoblastoma cells (WIL2-NS is a nonsecreting variant of WIL2)	Lymphocyte-specific cytotoxicity, determined by resazurin assay	Methyl methane sulfonate	% reduction in cell viability relative to solvent control	NWC, 2011

Notes: ELISA = enzyme-linked immunosorbent assay; IFN-γ = gamma interferon; IL = interleukin; TNF-α = tumor necrosis factor α.

1.8.3.3 Category 2 Bioassays

There are a number of Category 2 bioassays available for immunotoxicity (Table 1.31). In direct immunotoxicity testing, all immune cells originate from hematopoietic stem cells in the bone marrow and the potential toxic effect of a chemical on these cells (myelotoxicity) can be prioritized for immunotoxicity screening. If a chemical possesses myelotoxic potential, it is intrinsically immunotoxic, and there is thus no need for further immunotoxicity testing. The CFU−granulocyte/macrophage (CFU-GM) assay, using human umbilical cord blood cells (Pessina et al., 2003) or murine bone marrow progenitors (Pessina et al., 2001), is the common tool for this type of immunotoxicity. The CFU-GM assay is a cell viability assay in nature, counting the surviving cells following chemical exposure. The assay has been applied to screen various drugs and a pesticide, lindane (Pessina et al., 2003). Toxic potencies are expressed as the 50% or 90% inhibitory concentration (IC_{50} or IC_{90}), which can be translated to the TEQ concept. The cost of the cell/tissue culture involved in the CFU-GM assay and the lack of a specific endpoint for meaningful indication of immunotoxicity, however, make this assay less attractive for screening of water quality.

Should a chemical not be myelotoxic, it may be lymphotoxic. Similar to the human whole blood cytokine release assay, several currently used “in vitro” lymphotoxicity bioassays are ex vivo in

nature. Such assays include the dendritic cell maturation assay (Toebak et al., 2008), the T-cell-dependent antibody response assay (Gennari et al., 2005) and the natural killer cell activity assay (Marcusson-Stahl and Cederbrant, 2003), which involve cell/tissue collection from human or animal subjects for subsequent chemical exposure and endpoint examination in cells transferred in vitro. As noted, these factors hamper practical application of these techniques in HTS of environmental samples and these assays were excluded from Table 1.31.

Recent efforts have incorporated reporter gene assays in the development of an HTS system for direct immunotoxicity. The "fluorescent cell chip" (Ringerike et al., 2005; Trzaska et al., 2005; Wagner et al., 2006) and the luciferase expression assay (Oostingh et al., 2008) are examples of such reporter gene assays. In the "fluorescent cell chip," the genetically modified EL-4 murine thymoma cell line harbors an enhanced GFP (EGFP) gene under the control of the promoter of a cytokine. The applied endpoints are IL-2, -4, and -10 and IFN-γ expression as measured by EGFP intensity (Ringerike et al., 2005; Wagner et al., 2006). A similar system is tagging BW5147.3 murine thymoma cells with an enhanced cyan fluorescent protein (ECFP) downstream of the promoter region of the *c-fos* gene, indicating pro-oncogene activation (Trzaska et al., 2005). Various immunosuppressants, allergens, and autoimmunity-inducing agents have been tested in these fluorescent protein-based assays. Sakamoto et al. (2011) also transfected RAW264 macrophages with GFP reporters for two proinflammatory cytokines (TNF-α and monocyte chemoattractant protein-1 [MCP-1]) in search of anti-inflammatory compounds.

A set of luciferase expression assays have been developed to monitor expression of a series of cytokines after xenobiotic challenge. These luciferase assays are based on the Jurkat T-cell lymphoma cell line (transfected with plasmids containing the binding domain for NF-κB or the promoter region for IFN-γ, TNF-α, TGF-β, or IL-4) and the A549 human lung carcinoma epithelial cell line (transfected with promoter regions for TNF-α and IL-6 or -8). These systems have been applied to test the potential of several PAHs, including fluoranthene, phenanthracene, pyrene and anthracene, to induce the expression of the above cytokines and NF-κB (Oostingh et al., 2008).

A dual luciferase reporter gene system can simultaneously monitor IL-8 expression and cell viability in response to contact and respiratory allergens (Takahashi et al., 2011). Although designed to screen skin sensitzers, this assay can be applied to chemicals and environmental samples for immunotoxicity screening in general, as IL-8 is a representative proinflammatory cytokine.

Table 1.31. Category 2 Bioassays Indicative of Adverse Effects on the Immune System

Target Mode of Action	Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference
Cytotoxicity	CFU-GM	Human umbilical cord blood cells/murine bone marrow progenitors	Cytotoxicity	Various drugs and lindane (pesticide)	IC_{50} or IC_{90}	Pessina et al., 2001, 2003
Cell-mediated/humoral immunity	Fluorescent cell chip	Murine thymoma cell line (EL-4) transfected with EGFP under control of a promoter for IL-2, -4, or -10 or IFN-γ	IL-2, -4, -10, IFN-γ expression measured via EGFP expression	Various immunosuppressants, allergens, and autoimmunity-inducing agents	Induction fold	Ringerike et al., 2005; Wagner et al., 2006
Pro-oncogene activation (via *c-fos* gene expression)	Fluorescent cell chip	Murine thymoma cells (BW5147.3) tagged by an enhanced cyan fluorescent protein (ECFP) downstream of the promoter region of the *c-fos* gene	Expression of *c-fos* gene measured via ECFP intensity	$NaNO_3$, RbCl, K_2PtCl_4, $CdCl_2$, $Pb(NO_3)_2$, $BaCl_2$, $CoCl_2$, $NiSO_4$	Induction fold	Trzaska et al., 2005
Cell-mediated/humoral immunity	Luciferase expression	Human lymphoma cell line (Jurkat T) stably transfected with promoter regions for TNF-α, TGF-β, IL-4, or IFN-γ or a binding domain for NF-κB	NF-κB, TNF-α, TGF-β, IL-4, and IFN-γ expression measured via luciferase activity (luminescence)	Fluoranthene, phenanthracene, pyrene, and anthracene	Induction fold	Oostingh et al., 2008
Cell-mediated/humoral immunity	Luciferase expression	Human lung carcinoma epithelial cell line (A549) stably transfected with promoter region for TNF-α, IL-6, or IL -8	TNF-α, IL-6, and IL-8 expression measured via luciferase activity (luminescence)	As above	Induction fold	Oostingh et al., 2008
Allergic responses	Human Cell	Human monocytic	Expression of cluster of	Dinitrochlorobenzene, *p*-	Relative	Ashikaga et al.,

Target Mode of Action	Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference
(Indirect immunotoxicity)	Line Activation Test (h-CLAT)	leukemia cell line (THP-1) and human histiocytic lymphoma cell line (U937)	differentiation (CD)—CD86 and CD54	phenylenediamine, 2-mercaptobenzothiazole, $NiSO_4 \cdot 6H_2O$, $CoSO_4 \cdot 6H_2O$, $(NH_4)2PtCl_4$	fluorescence intensity	2006; Sakaguchi et al., 2006
Cell-mediated/humoral immunity	Luciferase expression	The human macrophage-like cell line (THP-1) stably transfected with the promoter region for IL-8	IL-8 expression measured via luciferase activity	Various contact or respiratory sensitizers	Induction fold	Takahashi et al., 2011
Cell-mediated/humoral immunity	GFP expression	RAW264 macrophages transfected with the promoter region for TNF-α and MIP-1	TNF-α and MIP-1 expression quantified by fluorescence	LPS	% of control	Sakamoto et al., 2011
Cell-mediated immunity	Flow cytometric	Tall-104 human leukemic CTLs	Blocked binding of an antibody to a luminal epitope of a lysosomal membrane protein (LAMP-1) measured via fluorescence by flow cytometer	A library of 91 compounds	IC_{50}	Florian et al., 2013
Cytotoxicity and cell-mediated/humoral immunity	ATP-based cell viability and HTRF-based TNF-α	Human monocytic leukemia cell line (THP-1)	Inhibition of LPS-induced TNF-α production quantified by FRET	A library of 1280 pharmacologically active compounds	IC_{50} for both cytotoxicity and TNF-α inhibition	Leister et al., 2011
Cytotoxicity and cell-mediated/humoral immunity	ATP-based cell viability and AlphaLISA-based TNF-α	Human monocytic leukemia cell line (THP-1)	Inhibition of LPS-induced TNF-α production quantified by fluorescence	As above	IC_{50} for both cytotoxicity and TNF-α inhibition	Leister et al., 2011

Notes: CTL = cytotoxic T-lymphocyte; FRET = Förster resonance energy transfer; HTRF = homogeneous time resolved fluorescence; IFN-γ = gamma interferon; IL = interleukin; LPS = lipopolysaccharide; MIP-1 = macrophage inflammatory protein 1; NF-κB = nuclear factor kappa B; TGF-β = transforming growth factor beta; TNF-α = tumor necrosis factor α.

Florian et al. (2013) developed a homogeneous phenotypic fluorescence endpoint assay for cytotoxic T-lymphocyte lytic granule exocytosis. The flow cytometric assay measured binding of an antibody to a luminal epitope of a lysosomal membrane protein (LAMP-1) that is exposed by exocytosis to the compound solution. The assay was targeted for a library of 91 compounds in 96 well plate format, which shows its promise in HTS of immunosuppressants and potential in water quality assessment.

Besides reporter gene-based assays, technologies for measuring native cytokine production beyond traditional ELISA methods have been advancing. Leister et al. (2011) evaluated two commercially available homogeneous time resolved fluorescence (HTRF)-based TNF-α assays. A library of 1280 compounds with pharmacological activity was screened for the potential to suppress lipopolysaccharide (LPS)-induced TNF-α production and cytotoxicity in THP1 cells in 1536 well plate format. The two assays demonstrated their application potential in HTS of immunosuppressive chemicals and environmental samples. It should be noted that these assays involve specific bindings of antibodies to TNF-α such as in ELISA and may be hampered by complex matrices including dissolved organic carbon, whereas fluorescence reading favors measurement specificity.

Although comparatively simple, the assays discussed earlier remain to be validated and adapted for water quality assessment. In addition to induction of endpoints, suppression, and potentiation of endpoints by pathogenic compounds (e.g., *E. coli* LPS), mimicking immunosuppressive and immunostimulating effects should also be included. Ideally, the candidate assays would allow simultaneous measurement of multiple specific endpoints as well as cytotoxicity. Prior to the application of these assays, potent reference compounds need to be sought and dose−response curves must be established. Similar to work on other Category 2 bioassays, the matrix effect due to recycled water should also be evaluated. Finally, the human Cell Line Activation Test (h-CLAT) is widely used for skin sensitization testing of allergenic chemicals (Ashikaga et al., 2006; Sakaguchi et al., 2006) and has potential for application in water quality testing.

1.8.3.4 Conclusions

The available reporter gene assays as well as HTRF- and AlphaLISA-based assays for immunotoxicity represent promising high-throughput in vitro screening systems for large quantities of water samples and future routine monitoring tasks. The practical value of these assays remains to be evaluated. Firstly, assay robustness needs to be evaluated through establishment of the dose−response relationship of potent reference chemicals and investigation of potential matrix effects of water samples on the bioassays. Immunotoxicity is system toxicity in nature and involves complex interactions between effectors and receptors. In order for simplified in vitro immunotoxicity assays to be meaningful, the relevance of various endpoints to immune function should be well explored.

1.9 Reproductive and Developmental Effects

Reproduction is a complex metacellular process relying on the successful completion of multiple individual and organism-level events. Reproductive and developmental defects are typically reported as macro-level observations, e.g., fertility, gonadal histopathology, and gonadal-somatic index (Wolf et al., 2010; Colman et al., 2011). These observations, however, require whole animals and only imply an association of exposure to environmental xenobiotics and reproductive defects rather than addressing molecular-level causality.

Because of the complexity and the many different processes involved in reproduction and embryonic development, no single in vitro assay can be used as a model to assess all reproductive and developmental toxicity occurring in a system. In view of this complexity, the design of in vitro bioassay alternatives that predict whole-organism effects is challenging (Piersma, 2006). This entire process may, however, be broken down to individual stages that may be assessed by specific in vitro assays (reviewed by Brown et al., 1995; Bremer et al., 2005). In the following, we outline some examples of cellular processes and/or tissue-specific cells that can be used to indicate and/or detect reproductive (Section 1.9.1) and developmental (Section 1.9.2) effects.

1.9.1 Reproductive Toxicity

1.9.1.1 Mechanistic Background and Relevance

Interest in the ability of environmental pollutants to disrupt reproductive homeostasis has grown over the last few decades as more reports show that xenobiotics may cause reproductive disorders. The sensitivity of male reproduction to xenobiotics has been demonstrated in a range of studies identifying three susceptible cell types: spermatogenic cells, Sertoli cells, and Leydig cells. Similarly, xenobiotics may harm female reproduction at three different levels: (1) the ovaries and germ cells, (2) the uterine lining and implantation, and (3) placental integrity and fetal development (Bremer et al., 2005; Hareng et al., 2005). Although the concentration of many industrial chemicals found in surface water may fall below the recommended guidelines of the particular countries in which these are measured, there is evidence to suggest that bioaccumulation of these chemicals may have long-term effects on fertility (Wang et al., 2010).

1.9.1.2 Category 1 Bioassays

Cytotoxicity in reproductive cells can be used as a semispecific indicator of reproductive effects. A number of in vitro assays have been developed to identify the sensitivity of male reproductive cells to xenobiotics. Wang et al. (2010) evaluated reproductive toxicity in water through a range of cytotoxicity assays using Sprague−Dawley rat spermatogenic, Sertoli, and Leydig cells (Table 1.32). One approach was to assess the integrity of the plasma membrane by using two different fluorescent dyes: fluorescein diacetate (FDA), which is a lipophilic compound that is cleaved to its fluorescent form in the cytoplasm of an intact cell, and propidium iodide (PI), which binds to DNA and can enter the cell only if the cell membranes are compromised. This approach can be quantified by microscopy (cell counting) or by measuring total fluorescence. Another approach was to assess viable cells by measuring lactate dehydrogenase (LDH) leakage, an assay that measures extracellular leakage of LDH from lysed cells. A third approach utilized by this study to assess cell viability was an MTT assay. This assay measures cell viability via enzymatic conversion of MTT. These assays can be applied to many different cell types, not specifically to cells that are associated with reproduction and development.

For a more specific evaluation, potential effects on fertility can be detected by in vitro methods measuring steroidogenesis (the production of steroid hormones such as estrogens and progestagens) in cells from adrenal gland tissue (e.g., using the H295R cell line), in male Sertoli and Leydig cells, and in female granulosa cells (Bremer et al., 2005). Wang et al. (2010), for example, complemented the cytotoxicity assay battery outlined earlier by measuring testosterone secretion in Leydig cells by radioimmunoassay (Table 1.32). The H295R steroidogenesis assay has been found a useful model for assessing the levels of steroid hormones (most often the sex steroids testosterone and 17β-estradiol but also corticosteroids) in response to chemical exposure (Hecker and Giesy, 2008; Hecker et al., 2011). The assay also has been applied for assessment of environmental samples (Gracia et al., 2008; Grund et al., 2011). Gracia et al. (2008) applied this assay with ELISA detection to evaluate

the toxicity of various effluent types (Table 1.32). The major limitation of these types of assays is their dependence on immunoassays.

Finally, Bandelj et al. (2006) used aromatase inhibition (discussed further in the following section) in rainbow trout ovaries as the endpoint for reproductive toxicity in water samples. To our knowledge, however, no in vitro bioassay exists to assess the effect of water samples specifically on reproduction.

Table 1.32. Category 1 In Vitro Cytotoxicity Assays for Reproductive Toxicity

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
FDA staining	Leydig, Sertoli, and spermatogenic cells (from Sprague−Dawley rats)	Plasma membrane integrity (fluorescent green cytoplasm) by fluorescence microscopy	None	Images taken by fluorescence microscopy (not quantified)	Wang et al., 2010
PI staining	Leydig, Sertoli, and spermatogenic cells (from Sprague−Dawley rats)	Cell viability (fluorescent red nuclei) by fluorescence microscopy	None	Images taken by fluorescence microscopy (not quantified)	Wang et al., 2010
LDH leakage	Leydig, Sertoli, and spermatogenic cells (from Sprague−Dawley rats)	LDH leakage from lysed cells by colorimetric assay (on the basis of LDH reducing NAD to NADH, which is utilized in a stoichiometric conversion of a tetrazolium dye)	None	% LDH leakage relative to control. Statistically significant or statistically insignificant	Wang et al., 2010
MTT assay	Leydig, Sertoli, and spermatogenic cells (from Sprague−Dawley rats)	Cell viability by colorimetric assay (on the basis of the cleavage of tetrazolium salt (yellow) to formazan crystals (purple) which are then solubilized)	None	Absorbance readings at 570 nm	Wang et al., 2010
Testosterone secretion in radioimmunoassay	Leydig cells (from Sprague−Dawley rats)	Testosterone secretion (concn)	None	Testosterone concn (ng/mL)	Li and Han, 2006; Wang et al., 2010
H295R steroidogenesis assay with ELISA	Human adrenocarcinoma cell line (H295R)	Steroid hormone (progesterone/pregnenolone, testosterone, 17β-estradiol) production quantified by ELISA	Several inducers (e.g., paraben, atrazine) and inhibitors (e.g., letrozole, prochloraz)	Fold induction in hormone production compared to control	Gracia et al., 2008

Note: ELISA = enzyme-linked immunosorbent assay.

1.9.1.3 Category 2 Bioassays

In addition to the steroidogenesis methods discussed in the previous section, further assays are available that have yet to be tried for water screening. Gunnarson et al. (2008) applied fluoroimmunassays to measure steroid hormone levels in mouse Leydig and granulosa cells exposed to a phthalate (Table 1.33). Furthermore, the H295R steroidogenesis assay has been applied for detection of additional steroids besides those tested with water samples (Table 1.32). Ullerås et al. (2008) used the assay with ELISA quantification to assess the effect of 30 chemicals on production of the glucocorticoid cortisol and the mineralocorticoid aldosterone, whereas Breen et al. (2011) analyzed the levels of 14 different steroids in the assay with ELISA and liquid chromatography−mass spectrometry (LC-MS) detection (Table 1.33). Again, it must be noted that, although immunoassays are commonly used in chemical assessments, these assay types are not favorable for HTS of water samples.

Development of in vitro assays to measure reproductive toxicity has been particularly challenging, given the stages at which reproduction and development are considered most susceptible to xenobiotics. Although in vitro assays have been developed to assess embryo implantation by using monolayers of the endometrium, these assays require an embryo and the monolayers are obtained as primary cells isolated from fresh tissue sections (Teklenburg and Macklon, 2009). In vitro assays that assess placental toxicity have a similar problem in that placental perfusion is required to identify the role of the placenta and of its transporters in exposing the fetus to toxic compounds (Hareng et al., 2005). Although the requirement for whole-animal testing is clearly removed, these assays are not equipped for HTS of xenobiotics that may affect reproductive toxicity.

A more elegant approach to assessing the role of xenobiotics in female reproductive toxicity may be to assess the enzymatic activity in specific cell types, for example, placental cells (JEG-3, JAR, and BeWo cells). Aromatase, for example, is a key enzyme in converting androgens to estrogens and plays an important role in maintaining the homeostatic balance between these hormone groups (Ohno et al., 2004). Despite the shortcomings of radioassays for HTS, several studies that have taken this approach to determine aromatase levels in vitro must be acknowledged (e.g., Yue and Brodie, 1997; Letcher et al., 1999). The tritium release assay (Lephart and Simpson, 1991; Drenth et al., 1998) has been used, for example, for detection of aromatase activity in JEG-3 and JAR cells exposed to a range of organochlorine chemicals (Letcher et al., 1999) (Table 1.33); however, as noted, radioassays are not suitable for HTS. Ohno et al. (2004) developed a nonradioactive method for measuring aromatase activity in a cell-based ELISA (Table 1.33). Twenty-three different compounds including flavonoids, pharmaceuticals, and pesticides were tested in the assay, and 17 of these tested positive for either inducing or inhibitory effects.

Endocrine communication is critical for successful reproduction. Assays that detect disturbance of the endocrine system are thus also highly relevant for reproductive toxicity and are further detailed in Section 1.10.

A unique approach being developed by Martin et al. (2012) is one of predictive modeling to determine the potential reproductive toxicity of chemicals. As part of the ToxCast research project, data from in vitro assay screenings were linked with the increased risk of causing adverse outcomes. A suite of HTS bioassays was used to produce a stable and robust model to predict reproductive toxicity in rats. This approach could reduce the costs of both direct and indirect testing, the time taken by authorities to reach decisions, and, importantly, the number of lab animals used. The authors of this study think that this predictive model may impact chemical testing as well as replace high-dose animal testing.

Table 1.33. Category 2 In Vitro Cytotoxicity Assays for Reproductive Toxicity

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
Steroidogenesis assay—progesterone and testosterone synthesis in fluoroimmunoassay	Mouse Leydig tumor cell line (MLTC-1) and mouse granulosa tumor cell line (KK-1)	Steroidogenesis measured as progesterone and testosterone level via fluoroimmunoassay	Mono-(2-ethylhexyl) phthalate (MEHP)	Fold induction in progesterone and testosterone levels relative to control	Gunnarsson et al., 2008
H295R steroidogenesis with ELISA for corticosteroids aldosterone and cortisol	Human adrenocarcinoma cell line (H295R)	Aldosterone and cortisol production by ELISA	Several including ketoconazole, 6-hydroxyflavone, imazalil	Aldosterone and cortisol concns relative to control	Ulleras et al., 2008
H295R steroidogenesis with ELISA and LC-MS for various steroids	Human adrenocarcinoma cell line (H295R)	Hormone (14 different steroids and cholesterol) production by LC-MS or ELISA	Metyrapone	Hormone concn	Breen et al., 2011
Aromatase activity by ELISA	Human ovarian granulosa-like tumor cell line (KGN)	Aromatase activity measured indirectly via estrone production determined by ELISA	Several flavonoids including α-naphtoflavone, pharmaceuticals including 4-hydroxy-androstenedione and pesticides including imazalil (chloramizole)	Fold increase in aromatase activity (estrone concn)	Ohno et al., 2004
Aromatase activity by tritium release assay	Human placental epithelial carcinoma cells (JEG-3,JAR)	Aromatization of 1β-^{3}H-androstenedione, which is measured indirectly via its production of $^{3}H_2O$	Several organochlorine compounds including benzo(a)pyrene and 3,3',4,4',5-pentachlorobiphenyl	Aromatase activity (pmol/h/mg of protein), fold decrease, and LOEC	Lephart and Simpson, 1991; Drenth et al., 1998; Letcher et al., 1999

Notes: ELISA = enzyme-linked immunosobent assay; LC-MS – liquid chromatography−mass spectrometry; LOEC = lowest observed effect concentration.

1.9.1.4 Conclusions

Reproductive toxicity and assessment or identification of xenobiotics that may disrupt normal reproduction are of utmost importance to ensure public health. Very few assays, however, appear to be suitable for constant HTS to assess water quality. Although a number of in vitro assays have been designed to assess the sensitivity of male reproductive cells to various chemicals (and indeed have been used to assess water samples), the cell lines utilized in these assays are primary cells. Primary cells present a significant limitation as they cannot be cultured indefinitely and therefore require constant access to animals, which, in turn, require on-site animal housing. For the purposes of this review, we were unable to find an in vitro assay, on the basis of a cell monolayer, which directly assessed reproductive toxicity. An indirect approach to assessing the integrity of female reproductive cells may be to assess enzymatic activity such as that of aromatase within the cells. The topic of reproductive toxicity therefore warrants immediate attention.

1.9.2 RAR/RXR-Mediated Developmental Toxicity

1.9.2.1 Mechanistic Background and Relevance

Retinoid signaling plays a crucial role during the early stages of development, where it is involved in cell differentiation, tissue patterning, determination of body axis formation, brain development, and limb formation (Ross et al., 2000). Excessive stimulation of this pathway can cause deformities in the form of multiple digit formation. Similarly, inhibition of retinoid signaling can cause major malformation in any of the developmental processes, from cell differentiation to limb formation (Lemaire et al., 2005). Retinoid signaling also is important for maintaining cellular homeostasis, epithelial maintenance, immune function, and reproduction (Novák et al., 2008). It is therefore of paramount importance that the distribution of retinoid ligands and of retinoid signaling is kept under tight regulation. Untimely exposure of the cell to environmental pollutants that mimic retinoid ligands or interfere with the endogenous retinoid metabolism may cause catastrophic effects during any of the processes mentioned earlier. Disruption of the retinoid signaling pathway can thus be considered a highly relevant endpoint for developmental effects and is prioritized for this review.

Retinoids are ligands that bind to a family of NRs, including the retinoic acid receptor (RAR) and the retinoid X receptor (RXR). These receptors function only as a homodimer (RXR/RXR) or as a heterodimer (RXR/RAR). Although there is some evidence that endogenous expression of retinoids occurs during early development (Wagner et al., 1992), retinoids are mainly obtained from exogenous sources (Novák et al., 2008). There are two major sources of retinoid ligands—firstly, animal sources, which are consumed as retinyl esters, and secondly, plant retinoids, which are consumed as retinoid-precursor carotenoids (Novák et al., 2008). After consumption, both forms of retinoids undergo a series of metabolic processes before being converted to retinoic acid, the most potent agonist of retinoid signaling. Retinoic acid has three isomers, all-*trans* retinoic acid (a*t*RA), 9-*cis*-retinoic acid (9-*cis*-RA), and 13-*cis*-retinoic acid, each of which can be converted to any of the other forms by isomerases or spontaneously.

The RAR can be activated by a*t*RA and 9-*cis*-RA, whereas the RXR is activated only by a*t*RA. The role of 13-*cis*-retinoic acid is not fully understood, but it is possibly a weak agonist. When retinoic acid binds to an RAR/RXR heterodimer, the heterodimer changes its conformational shape, thereby allowing it to translocate to the nucleus. Once in the nucleus, it acts as a TF by binding to the retinoic acid response element (RARE). The RXR dimerizes after ligand binding, and the homodimer translocates to the nucleus to bind to the retinoid X response element (RXRE). These response elements lie upstream of various genes involved in development and cellular homeostasis,

for example, cell differentiation, proliferation, and apoptosis, as well as in metabolism. Various environmental pollutants can act directly as ligands for the RAR or RXR, e.g., some organochlorine pesticides and tributyltin. Other xenobiotics (e.g., *p,p*'-DDE*)* perturb the RAR pathway indirectly through interference with xenobiotic metabolism and thus disturb the endogenous RA metabolism.

RXR also is a heterodimer partner to a large number of other NRs, among them the PPAR and the TR.

1.9.2.2 Category 1 Bioassays

The retinoid signaling pathway has been fully characterized and thus is utilized in in vitro assays to (1) identify both potent retinoid agonists and antagonists of the the RAR and RXR, and (2) to identify downstream genetic targets of this signaling pathway. A number of these in vitro bioassays have been adapted to detect environmental pollutants in water samples that may bind to the RAR or RXR (Table 1.34). Most in vitro bioassays used to assess RAR/RXR activity by chemicals and in water samples have employed reporter gene cells (usually involving the *lacZ* gene or *luc* gene). One of the first reporter gene assays developed to identify retinoid-signaling ligands utilized two reporter plasmids. Wagner et al. (1992) took the RARE sequence and cloned it upstream of the β-galactosidase gene and upstream of the luciferase gene. Both were then stably transfected into mammalian cells—the RARE-β-gal into F9 mouse embryonal carcinoma cells (Table 1.34) and the RARE-luc into L cells (Table 1.35). Using this reporter plasmid in vitro assay, Wagner et al. (1992) were able to show that some endogenous expression of retinoids occurred early in mammalian development. Schoff and Ankley (2002) utilized the β-galactosidase reporter gene cell line, F9S:1, to develop an in vitro assay to assess water samples collected near a pulp mill. Sample water was used as cell media for culturing the F9S:1 cells, which were subsequently evaluated for response. Although Schoff and Ankley (2002) did not detect any retinoid agonists, the presence of an antagonist was detected when cells were exposed to a potent retinoid stimulator (retinoic acid). This finding indicates that the assay may be versatile in detecting both agonists and antagonists of RARs/RXRs in water samples.

Nishikawa et al. (1999) took a different approach and established a yeast two-hybrid assay containing the *lacZ* reporter gene. Kamata et al. (2008) inserted RARγ into this yeast, optimized the assay for HTS, and used it to test 543 chemicals for RAR activity. The optimized assay also has been applied in a pilot study to assess the potential of wastewater effluents to cause RAR activity (Allinson et al., 2011). Finally, an RARα version of the yeast two-hybrid assay has seen several applications for screening of wastewater (Cao et al., 2009; Inoue et al., 2009a; Zhen et al., 2009) and of surface water (Inoue et al., 2009b, 2010). The primary advantage of using the yeast two-hybrid system rather than mammalian cells is that there will essentially be no cross-talk between the activated RAR signal and other NRs. There is, however, a strong argument for establishing these assays in a human-based, or at least mammalian-based, cell line to make the assay more relevant to human health.

Alsop et al. (2001) developed an RAR competitive binding assay, which involved the extraction of RARs and RXRs from gill and liver tissues in fish. The assay was later applied to assess the effluents from various pulp mills around Canada (Alsop et al., 2003). As this competitive binding assay applies a radiolabel, it has limited value for routine testing.

Table 1.34. Category 1 Bioassays Indicative of RAR/RXR Activation

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
RAR reporter gene assay	F9S:1 cell line with *lacZ* reporter gene	RAR activation measured via β-galactosidase activity (luminescence)	a*t*RA, 9-*cis* RA, TTNPB, retinol, retinal, retinoic acid	RLUs (luminescence divided by protein content)	Schoff and Ankley, 2002
RARα yeast two-hybrid assay	Yeast *S. cerevisiae* Y190 with *lacZ* reporter gene	RARα activation measured via β-galactosidase activity (luminescence)	a*t*RA	a*t*RA equivalents	Cao et al., 2009; Inoue et al., 2009a, 2009b; Zhen et al., 2009; Inoue et al., 2010
RARγ yeast two-hybrid assay	Yeast *S. cerevisiae* Y190 with *lacZ* reporter gene	RARγ activation measured via β-galactosidase activity (luminescence)	543 different chemicals, a*t*RA	a*t*RA equivalents	Allinson et al., 2011
RAR competitive binding assay	Receptor binding assay (ex vixo, no cell line used)	RAR binding measured via the amount of [^{3}H]-a*t*RA present	a*t*RA	% of displaced [^{3}H]a*t*RA	Alsop et al., 2003

Notes: a*t*RA = all-*trans* retinoic acid, RAR = retinoic acid receptor, RXR = retinoid X receptor, TTNPB = (synthetic retinoid) (E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthylenyl)-1-propenyl] benzoic acid.

1.9.2.3 Category 2 Bioassays

Breitman et al. (1980) showed that the HL60 cell line, derived from a patient with promyelocytic leukemia, can be induced to proliferate exponentially (500 to 160,000 times) in the presence of retinoic acid when compared to other known proliferators (Table 1.35). As external factors may induce HL60 cells to proliferate, the assay is not sufficiently specific to be used for water quality assessment. Todd et al. (1995) inserted 14 different gene constructs, including RARE, fused to the chloramphenicol acetyltransferase (CAT) gene into human HepG2 cells and applied all 14 cell lines in the CAT-Tox(L) microplate assay. This assay, however, relied on ELISA for quantification.

Reporter gene assays with easily detected markers such as β-galactosidase, luciferase, and GFP are more suitable for HTS. In addition to the Category 1 reporter gene assays discussed in the preceding section, several reporter systems are available for assessing RAR/RXR activity that have not yet been implemented for water testing. As noted in the previous section, Wagner et al. (1992) developed two reporter plasmids to assess RAR activity, one with RARE-β-gal stably transfected into F9 cells (Table 1.34) and one with RARE-luc stably transfected into L cells (Table 1.35). To our knowledge, the latter assay has not been applied for water quality assessment. A French research group has developed reporter systems for the RARα, -β, and -γ and RXR (although the latter only by transient transfection) and applied these for chemical assessments (Balaguer et al., 1999, 2001; le Maire et al., 2009) (Table 1.35). Novak et al. (2007, 2009) transfected an embryonal mouse carcinoma cell line with an ARE reporter plasmid (first established by Pachernik et al. [2005]) and tested the resulting RAR reporter gene assay with sediments and air extracts (Table 1.35). Although the air extracts did not exhibit any activity (Novak et al., 2009), the sediment extracts modulated the activity of the P15/A19 cells that were treated with 32 nM a*t*RA but exhibited no effect on the cells when exposed in the absence of a*t*RA (Novak et al., 2007). A series of PAHs, among them benzo[a]pyrene, showed the same effect if the cells were incubated with 32 nM a*t*RA but had no activating effect when dosed alone. Similar results were found in a study assessing a range of PAHs and *N*-PAHs (Benisek et al., 2008).

In addition to the yeast two-hybrid assay (Nishikawa et al., 1999) discussed under Category 1 assays, Li et al. (2008) employed a format of this assay whereby a reporter plasmid expressing RXRβ fused to a galactosidase-binding domain was inserted into yeast (Y187) (Table 1.35). Several chemicals were tested, revealing both inducers and inhibitors of RARs via RXRs.

Similar to reproductive toxicity, accurate endocrine communication is crucial for developmental processes to occur. Assays that detect disturbance of the endocrine system are therefore also highly relevant for developmental toxicity.

Table 1.35. Category 2 Bioassays Indicative of RAR/RXR Activation

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
RA-induced cell differentiation assay	HL-60 cell line (derived from patient with promyelocytic leukemia)	Cell differentiation measured colorimetrically	a*t*RA, 13-c*is*RA, retinol, retinyl acetate, retinal	% cell differentiation	Breitman et al., 1980
CAT-Tox(L) microplate reporter gene assay with ELISA	Human liver carcinoma cells (HepG2) containing 14 reporter genes, including RARE	Activation of RAR measured as concn of CAT, which is quantified by ELISA	Pentachlorophenol, a*t*RA	Fold induction compared to a*t*RA	Todd et al., 1995; Dorsey et al., 2002
RAR reporter gene assay	L cell line with *luc* reporter gene	Luciferase activity	Retinol, retinal, retinoic acid	Luciferase activity (fg of *luc*/μg of protein)	Wagner et al., 1992
RARα, -β, and -γ/RXR reporter gene assays	Cervical cancer cells (HeLa) stably transfected with RARα, -β, or -γ, and African green monkey fibroblasts (COS-7) transiently transfected with RXR	RARα, -β, -γ, or RXR activation/inhibition measured as luciferase activity (luminescence)	TTNPB and pesticides aldrin, endrin, dieldrin, chlordane, endosulfan	EC_{50} and IC_{50} (the concentrations inducing and inhibiting luciferase activity 50% compared with the activity achieved in the presence of 10 nM TTNPB (i.e., 100%)	Balaguer et al., 2001; Lemaire et al., 2005
RAR reporter gene assay	P15/A19: murine embryonal carcinoma cell line P19 stably transfected with luciferase reporter pRARE_2-TK-luc plasmid	Luciferase activity	a*t*RA, various PAHs	Luciferase activity (% of 32nM a*t*RA induction)	Novak et al., 2007, 2009
RXR yeast two-hybrid assay	*S. cerevisiae* Y187 with LacZ reporter gene	RXR activation measured via β-galactosidase activity (luminescence)	a*t*RA, 9-c*is*RA. Several chemicals including bisphenol A and DDT	EC_{20} and IC_{20}	Li et al., 2008

Notes: a*t*RA = all-*trans* retinoic acid; CAT = chloramphenicol acetyltransferase; RAR = retinoic acid receptor, RARE = retinoic acid response element; RXR = retinoid X receptor; TTNPB = (E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid.

1.9.2.4 Conclusion

The RAR/RXR signaling pathway is of high toxicological relevance because (1) it is crucial during the early stages of mammalian development as well as for maintaining cellular homeostasis, and (2) a number of environmental pollutants are available to abrogate the correct signaling. We therefore recommend the implementation of bioassays indicative of this endpoint to a test battery for water quality assessment. The F9 teratocarcinoma stem cell line used in the work by Schoff and Ankley (2002) seems very suitable because it is has endogenous RARs. The reporter gene luciferase also is very common and suitable for water quality testing. Thus far, however, this cell line has been tested only with medium made up in test-water. On the other hand, the murine cell line P15/A19 already has been successfully applied for testing of sediment extracts that contained PAHs and results were consistent between test chemicals. We recommend the evaluation of the performance of both cell lines with water extracts.

1.10 Endocrine Effects

Hormones are chemicals that carry signals from one part of the body (organism) to another. A negative feedback system controls how much hormone is released. After secretion, hormones act by binding to certain receptors. The resulting receptor−ligand complexes are then capable of activating a series of actions through cell surface or internal (cytosolic) receptors. Cytosolic receptor−ligand complexes enter the nucleus, where transcription and translation of specific gene products are induced via binding to specific DNA promoter regions (genomic pathway of hormone action [Figure 1.8]). Various xenobiotics are capable of interfering with the components of the endocrine system. Endocrine disruption can take place via agonism, where a xenobiotic mimics an endogenous hormone and via antagonism, where a xenobiotic blocks a hormone receptor (Figure 1.9).

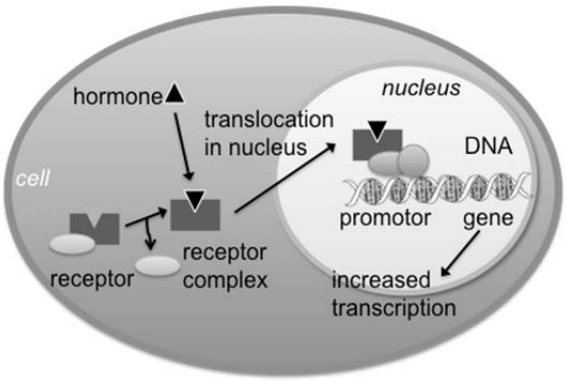

Figure 1.8. Genomic pathway of hormone action.

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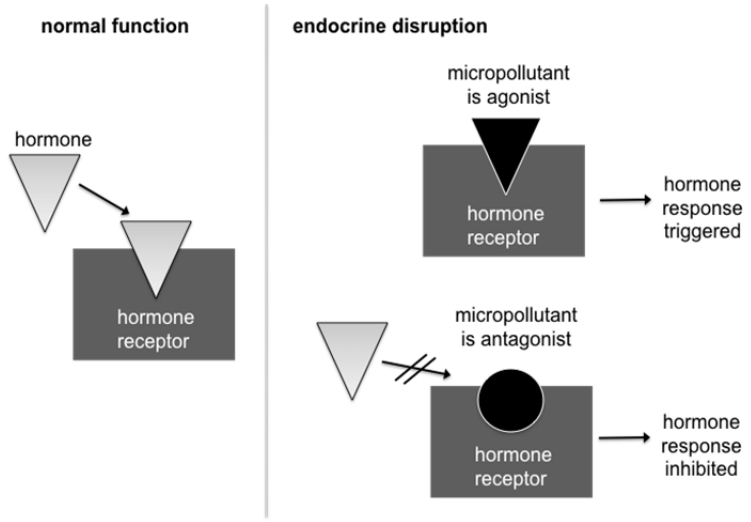

Figure 1.9. Agonistic and antagonistic effects of chemicals.
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Endocrine disruption also can occur via non-receptor-mediated pathways such as inhibition of enzymes important for hormone production (Escher and Leusch, 2012). As discussed, for example, aromatase is the enzyme responsible for coverting androgens to estrogens (Ohno et al., 2004). Aromatase can be inhibited by, for example, triorganotins and induced by, for example, atrazine (Escher and Leusch, 2012). Estrogen sulfotransferase is another example of an enzyme important for regulation of estrogen levels (Goodsell, 2006), and this enzyme can be inhibited by, for example, hydroxylated polychlorinated biphenyls (OH-PCBs) (Escher and Leusch, 2012).

In the remaining sections of this report, we review assays for detection of endocrine effects with emphasis on receptor-mediated responses, excluding competitive binding assays. Some assays relevant for endocrine disruption already have been discussed in previous sections of the report (i.e., those for PXR, CAR, and PPAR and some of the assays relevant for reproductive and developmental effects). In the following sections, we focus on hormonal activity via ERs, ARs, PRs, GRs, mineralocorticoid receptors (MRs), and TRs.

1.10.1 Estrogenicity

1.10.1.1 Background and Relevance

The potential for anthropogenic compounds to cause endocrine disruption, particularly estrogenicity, has received much attention following ongoing reports of sexual disruption and feminization in aquatic wildlife (Smith, 1981; Purdom et al., 1994; Jobling et al., 1998). Natural estrogens are sex (steroid) hormones (e.g., estrone [E1], estradiol [E2],and estriol [E3]) that act via the ER to regulate gene expression, triggering a number of cellular responses. These hormone cues are particularly important for fetal reproductive organ development, puberty and sexual maturation. During these life stages vertebrates (including humans) are particularly sensitive to endocrine disruption by synthetic estrogens (e.g., 17α-ethinylestradiol) and xenoestrogens (e.g., nonylphenol and bisphenol A), which are able to mimic natural estrogens as ligands for the ER. If such disruption takes place at the wrong time, it may have implications for fertility and reproduction, which could ultimately lead to population effects.

1.10.1.2 Category 1 Bioassays

A wide range of in vitro assays specifically targeting estrogenic effects has been developed, and many of these have been applied for water quality assessment (a selection is listed in Table 1.36 and a comprehensive review is given by F. Leusch et al. [2012]). The E-SCREEN (Soto et al., 1995) and the yeast estrogen screen (YES; Routledge and Sumpter, 1996) are commonly employed as screening tools for estrogenicity in environmental waters. The YES utilizes recombinant yeast to detect activation of the ER, whereas the E-SCREEN is based on cell proliferation in estrogen-dependent human breast cancer cells. Both assays were first applied for assessment of wastewater quality in the late 1990s (Desbrow et al., 1998; Körner et al., 1999) and have since been applied for a range of water types, including bottled mineral water (Wagner and Oehlmann, 2009, 2011). The ER- and ERα-CALUX assays (Legler et al., 1999; Sonneveld et al., 2005) are popular commercial assays for estrogenicity that have been validated for wastewater and other types of water samples (Murk et al., 2002; Van der Linden et al., 2008).

Many additional mammalian reporter gene assays are available for screening of estrogenicity in water, including the MELN assay, which was developed through stable transfection of a human breast cancer cell line (MCF7) (Balaguer et al., 1999). The MELN assay has been applied for screening of surface water and wastewaters in France in several studies (Cargouet et al., 2004; M. Muller et al., 2008; Jugan et al., 2009; Miege et al., 2009; Creusot et al., 2010; Dagnino et al., 2010) and overseas (Mahjoub et al., 2009; Leusch et al., 2010; Mahjoub et al., 2011). The MELN assay also has been optimized to include the inhibition test of MELN activation (Pillon et al., 2005). This complementary test enables differentiation between high- and low-affinity estrogens. High-affinity estrogens (e.g., free estrogens such as 17β-estradiol) bind directly to the ER, whereas low-affinity estrogens (e.g., PAHs and dioxins) activate the ER indirectly through binding to the AhR, which can then form a complex with the ER (Ohtake et al., 2003). Before one runs the inhibition test, recombinant ERα is added to compete with ER in MELN cells for binding by free estrogens, which can bind to both ERs. A reduction in ER activity in the inhibition test thus indicates the presence of high-affinity estrogens in the sample. A selection of mammalian reporter gene assays is listed in Table 1.36.

The list of assays that have been applied to detect estrogenic activity in water is long. It is not within the scope of this report to detail all available assays; however, the list includes a range of yeast-based assays such as the yeast two-hybrid assay (Nishikawa et al., 1999; Allinson et al., 2007), the recombinant yeast assay (RYA) (Garcia-Reyero et al., 2001), the Rikilt Estrogen bioAssay (REA) (Bovee et al., 2004), and the 4 h yeast assay (Riggs et al., 2003; Balsiger and Cox, 2009; Balsiger et al., 2010) (Table 1.36). Yeast assays are generally of lower sensitivity than are mammalian cell lines. In a recent effort for example, five of the most commonly used estrogen assays including the YES (plus E-SCREEN, MELN, T47D-kBluc, and ER-CALUX, Table 1.36) were subjected to an interlaboratory comparison and were tested across a range of water types (Leusch et al., 2010). The assays were found to be overall comparable with the exception of the YES assay, which was comparatively less sensitive (Leusch et al., 2010). Further, in a comparison study of three yeast assays, the REA and RYA were found to be of performance equal to that of the YES assay (Brix et al., 2010). Despite their lower sensitivity and somewhat lower relevance for human health, yeast assays do have many advantages for HTS, including low cost, simplicity, and high receptor specificity (i.e., no cross-talk between multiple endogenous NRs, as may be the case with mammalian cells) (Bovee and Pikkemaat, 2009). Depending on the water type/situation of interest, yeast-based assays may thus be favorable, although for relatively clean waters, high sensitivity is an important feature to consider when selecting an assay. Different assays have different advantages and limitations, and it is important to select an assay on the basis of predetermined selection criteria, with a full understanding of its limitations (Leusch et al., 2010).

Vitellogenin and zona radiata protein levels, which can be measured in vitro, also can be used as markers of estrogen activity (Table 1.36). Many researchers have also applied ex vivo competitive binding assays such as the ER binding assay (ERBA) using sheep uterine tissue (Tremblay et al., 2004; Leusch et al., 2005; Sarmah et al., 2006). As discussed, however, ELISAs and receptor binding assays are less practical for routine HTS. Additional assays are detailed by, e.g., GWRC (2006), Escher and Leusch (2012), and Poulsen et al. (2011).

Table 1.36. Category 1 In Vitro Bioassays for Detection of (Anti)estrogenicity in Water Samples

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
E-SCREEN and flow cytometry E-SCREEN	Human breast cancer cells (MCF7 or T47D)	Cell proliferation of estrogen-dependent breast cancer cells quantified by metabolic dye or flow cytometry	E2, 4-OP, NP, BPA, tamoxifen, benzyl-*n*-butylphthalate, 4-hydroxybiphenyl	EEQ (E2 equivalents)	Körner et al., 1999; Matsuoka et al., 2005 (T47D); Vanparys et al., 2006 (flow cytometry); Macova et al., 2011 (TEQ)
YES	Yeast (*S. cerevisiae*) stably transfected with hERα and plasmid with ERE-linked *lacZ* gene)	Activation of ER measured via induction of β-galactosidase, which metabolizes the substrate into a colored product for colorimetric measurement	E2, 4-NP, 4-OP, nonylphenol-polyethoxylate, igepal, 4-nonylphenoxy-carboxylic acid, 4-nonylphenoldiethoxylate	EEQ	Desbrow et al., 1998; Escher et al., 2008b (TEQ)
ER-CALUX	T47D stably transfected with ERE-*Luc* plasmid	Binding to ER measured as luciferase activity via luminescence	E2, 4-NP, BPA, methoxchlor, genistein, tamoxifen, chlordane, endosulfan, dieldrin, *o,p*'-DDT	E2 equivalents	Murk et al., 2002
ERα-CALUX	Human osteocarcinoma U2-OS cells stably transfected with HRE-TATA-Luc and pSG5-neo-hERα	Binding to ERα measured as luciferase activity via luminescence	EE2, NP, dibutyl-phthalate, diethylstilbestrol, *n*-butylparaben, corticosterone, genistein hexestrol, norethynodrel, 4OH-tamoxifen, *o,p*'-DDT, flutamide	E2 equivalents	Van der Linden et al., 2008; van der Burg et al., 2010b
MELN assay	MELN cells (stably transfected MCF7 cells (human breast cancer cells)	Binding to ER measured as luciferase activity via luminescence	E1, E2, E3, EE2, 4-OP, BPA, NPs, 2,4´-DDE, 4,4´-DDE, α-zearalanol, zearalenone, Δ5-androstenediol, genistein, coumestrol	E2 equivalents	Cargouet et al., 2004; Leusch et al., 2010
Inhibition test of MELN activation	MELN cells	As above but with prior addition of recombinant	E1, E2, E3, zearalenone, genistein, NPs	E2 equivalents	Pillon et al., 2005

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
		ERα for competition with binding by high-affinity estrogens			
T47D-kBluc reporter gene assay	T47D-kBluc stably transfected with a triplet ERE-promoter-luciferase reporter gene construct	Binding to ER measured as luciferase activity via luminescence	E2, EE2, 4-NP, diethylstibestrol, 5a-dihydrotestosterone, dexamethasone, genestein, tamoxifen, methoxychlor	EEQ	Wilson et al., 2004 (cell line); Leusch et al., 2010 (water application, TEQ)
Other mammalian reporter gene assays	Various including human cervical cancer cells (HeLa, HGELN, MCF7 [MVLN]), human embryo kidney cells (HEK293), monkey kidney cells (CV-1) transfected with an estrogen-responsive element linked to a luciferase (Luc) reporter gene	Binding to ER measured as luciferase activity via luminescence	Estrogens and estrogen-like chemicals	E2 or EE2 equivalents	Gutendorf and Westendorf, 2001 (MVLN, HGELN); Snyder et al., 2001 (MVLN); Gong et al., 2003 (MVLN); Pawlowski et al., 2003 (HEK293); W. Shi et al., 2009 (CV-1)
Yeast two-hybrid assay	*S. cerevisiae* (Y190) transfected with the *lacZ* gene and two plasmids, one with the ligand-binding domain for ER and one with a ligand-dependent coactivator	Binding to ER causes interaction with the coactivator and expression of *lacZ*. Galactosidase activity is measured colorimetrically	E1, E2, E3, BPA, NP, testosterone, dihydrotestosterone, β-sitosterol, stilbestrol, genistein	EEQ	Allinson et al., 2007
Other recombinant yeast assays (e.g., RYA, REA, 4 h yeast assay)	Yeast strains (BJ559, BY4741 (RYA); *S. cerevisiae* (REA), W303α [4 h yeast assay]) with plasmids for detection via, e.g., β-	Activation of ER measured via luminescence or fluorescence	E1, E2, E3, EE, BPA, NP, ethoxylated NPs, genistein	EEQ	4 h yeast assay; Balsiger et al., 2010; REA, RYA; Brix et al., 2010

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
	galactosidase (RYA, 4 h yeast assay) or GFP (REA)				
HEP-Vtg and HEP-Zrp assays	Fish primary hepatocytes from, e.g., Atlantic salmon, juvenile rainbow trout	Vtg or Zrp production quantified by ELISA as a measure of estrogenic stimulation	E1, E2, EE2, E3, mestranol, β-estradiol-17-valerate	EEQ	Tollefsen et al., 2003; Rutishauser et al., 2004

Notes: BPA = bisphenol A; DDE = dichlorodiphenyl dichloroethylene; DDT = dichlorodiphenyl trichloroethane; EEQ, estradiol equivalent; E1 = estrone; E2 = 17β-estradiol, E3 = estriol; EE2 = 17-α-ethinylestradiol; ELISA = enzyme-linked immunosorbent assay; NP = 4-nonylphenol; 4-OP = 4-*t*-octylphenol; REA = Rikilt Estrogen bioAssay; RYA = recombinant yeast assay; vtg = vitellogenin; zrp = zona radiata protein. A variety of additional Category 1 reporter gene assays are available (reviewed by GWRC, 2006, 2008; Poulsen et al., 2011; Escher and Leusch, 2012).

1.10.1.3 Category 2 Bioassays

Because of the broad variety of suitable cell-based assays that are available and validated for HTS of estrogenicity in water samples, it is considered a low priority to review further potential Category 2 bioassays for this endpoint.

The GeneBLAzer assay platform mentioned earlier (see Section 1.3.3) has both an ERα and ERβ variant (Table 1.37). This assay has been used to test approximately 3000 environmentally relevant compounds, with about 3% of these being ER agonists, whereas 5% showed ER antagonism (Huang et al., 2011b).

In the future, the Attagene multifactorial and NR reporter gene assays employed by the ToxCast program (Martin et al., 2010) may become attractive techniques for assessing multiple endpoints in the same test. The ERα component of this system proved positive for 90 of the 320 compounds tested (Martin et al., 2010). In terms of endocrine disruption, however, the AR and GR components were not responsive in the ToxCast screening and the assay does not cover the PRs and MRs. Thus, in selection of an assay battery for detection of endocrine activity, the Attagene tests may not be comprehensive.

Table 1.37. Category 2 In Vitro Bioassays for Detection of (Anti)estrogenicity in Water Samples

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
ERα and ERβ−GeneBLAzer	Human embryonic kidney cells (HEK293H)	Activated ER quantified as β-lactamase activity (*bla* reporter gene)	E2	EC_{50} or IC_{50}	Huang et al., 2011b
Attagene multifactorial reporter gene assay (ATG_ ERα_TRANS, ATG_ ERβ_TRANS	HepG2 cells transfected with 48 RTUs and 25 NRs including ERα and ERβ	PPAR binding quantified by fluorescence (via 6-carboxyfluorescein [6-FAM] labeling) and resolved from the remaining TFs by capillary electrophoresis	Various (>100) chemicals including lactofen, flusilazole, resmethrin	AC_{50} (50% of maximal response)	Romanov et al., 2008; Martin et al., 2010
ERα-EcoScreen	Chinese hamster ovary (CHO) cell lines stably transfected with a plasmid containing the cDNA of hERα	hERα activity measured via luciferase activity (luminescence)	Benzophenone and 19 hydroxylated derivatives	EEQ	Kawamura et al., 2005

Notes: EEQ = estradiol equivalent; ER = estrogen receptor.

The ERα-EcoScreen™ may be worth noting as a member of the EcoScreen assay battery, although only one study was found that applied the stable ERα-responsive cell line (Kawamura et al., 2005). The cell line responded to the majority of the 20 different compounds tested (Kawamura et al., 2005), however, and a version of the assay using transient transfection has been tested with several compound groups (Kojima et al., 2003, 2004; Takeuchi et al., 2005; Kojima et al., 2009), including 200 pesticides (Kojima et al., 2004). Again, the suitability of the EcoScreen tests as a comprehensive battery is questionable because of the limited availability of stable reporter cell lines for hormone receptors other than the ER and AR (Section 1.10.2.3).

1.10.1.4 Conclusions

A vast range of cell-based bioassays is available for assessing estrogenicity in water samples, and there is no need for adding more to the list. The E-SCREEN, ER-CALUX, and YES assays are suitable and simple assays for detection of estrogenic activity in water that complement each other well (Leusch et al., 2010). The downsides to the YES assay include the lowered relevance of using a yeast model compared to using a mammalian model for human risk assessment and the much lower sensitivity of this assay (by 2−3 orders of magnitude).

It is important that estrogen assays (and those for other NRs) can and should be run in both agonistic and antagonistic mode. Further, it is sensible to run assays for (anti)androgens alongside when screening for (anti)estrogenicity. There is considerable overlap, as estrogenic compounds are often antiandrogens and as androgen-0active compounds are often antiestrogens. It is therefore important to include androgens when screening for estrogens and vice versa.

1.10.2 Androgenicity

1.10.2.1 Background and Relevance

Androgens are natural and synthetic hormones that regulate development and maintenance of masculine characteristics via the AR. Androgens are thus of the same crucial importance as estrogens, and when the field of endocrine disruption took off for estrogenic compounds, the study of androgen-disrupting compounds quickly followed (Rogers and Denison, 2000). As noted earlier, (anti)estrogens and (anti)androgens often cross over and estrogens are often antiandrogens (Sohoni and Sumpter, 1998). It is therefore important when screening for estrogens also to look for androgens and vice versa.

1.10.2.2 Category 1 Bioassays

For many of the assays developed for estrogen-active compounds, alternative assay versions are often available to assess interference with the AR (as well as a range of other NRs including the PRs, GRs, MRs, and TRs, which are discussed in the sections below). The ability to derive an entire battery of assays using the same technique/equipment from the same laboratory/company has clear advantages.

The AR-CALUX belongs to the CALUX family of assays, which include cell lines targeting a wide range of NRs such as the ER-CALUX, which was described in Section 1.10.1.2. The AR-CALUX was first developed by Sonneveld et al. (2005) and further optimized by van der Burg et al. (2010a) using a range of test chemicals. The assay has seen many applications in water quality testing and has been tested with many different water types including drinking water, surface water, and wastewater (e.g., Van der Linden et al., 2008; NWC, 2011) (Table 1.38). The A-SCREEN, which

was developed to complement the E-SCREEN (Section 1.10.1.2), is based on human breast cancer cells (MCF7) stably transfected with human AR (Szelei et al., 1997; Soto et al., 1999) and has been applied for water monitoring (Soto et al., 2004; Havens et al., 2010) (Table 1.38).

The yeast androgen screen (YAS) assay is another popular assay that has a "partner" ER test, the YES assay. The YAS assay was developed by Sohoni and Sumpter (1998) and has been applied for testing of a range of water types, including oil field-produced water (Thomas et al., 2004), wastewater (Conroy et al., 2007), and surface water (Urbatzka et al., 2007) (Table 1.38). The yeast two-hybrid assay battery by Nishikawa et al. (1999) also comprises an assay for AR as well as for other NRs. Allinson et al. (2008) applied the AR yeast two-hybrid assay to assess androgenicity in wastewater effluents (Table 1.38), albeit with no observed response.In addition to the earlier-outlined assays, a range of AR mammalian reporter gene assays exists that have been applied for water quality screening, including the MDA-kb2 (Creusot et al., 2010), PALM (Mnif et al., 2010), AR-LUX (Blankvoort et al., 2005) and CV-1 reporter gene assays (W. Shi et al., 2009). The PALM cell line was developed along the MELN cell line (Terouanne et al., 2000) (Table 1.38). The PALM has undergone validation for chemical assessment (Freyberger et al., 2010). Further assay applications are reviewed (Poulsen et al., 2011; Escher and Leusch, 2012; Leusch et al., 2012).

Table 1.38. Category 1 Bioassays To Detect (Anti)androgenicity in Water Samples

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
AR-CALUX	Human osteosarcoma U2-OS cells stably transfected with human AR reporter gene	Binding to AR measured via luciferase activity (luminescence)	DHT, Flu, dibutylphthalate, *o,p*'-DDT, progesterone norethynodrel, prochloraz, levonorgestrel, linuron, vinclozolin	DHT-EQ for androgenicity, Flu-EQ for antiandrogenicity	Various water applications including Van der Linden et al., 2008; NWC, 2011
A-SCREEN	Human breast cancer cells (MCF7) stably transfected with AR	Cell proliferation determined by using a dye	5α-Androstan-17β-ol-3-one, R1881	Androgen equivalents	Soto et al., 2004; Havens et al., 2010
YAS	Yeast *S. cerevisiae* stably transfected with plasmid containing a human AR-responsive element and the *lacZ* gene	Activation of AR measured via induction of β-galactosidase, which metabolizes the substrate into a colored product for colorimetric measurement	DHT, Flu, bisphenol A, butyl benzyl phthalate, hydroxytamoxifen, diethylstilboestrol, DDTs, vinclozolin, 4-nonylphenol	DHT-EQ for androgenicity, Flu-EQ for antiandrogenicity	Various water applications including Thomas et al., 2004; Conroy et al., 2007; Urbatzka et al., 2007
Yeast two-hybrid assay for AR	Yeast *S. cerevisiae* transfected with a plasmid expressing human AR and galactosidase	AR activation measured via β-galactosidase	Dihydrotesosterone, ll-ketotestosterone	Androgen equivalents (no detection)	Allinson et al., 2008
PALM reporter cell line for AR	Human prostate adenocarcinoma cell line (PC-3) stably transfected with human AR, an AR-responsive element, and a luciferase reporter gene	AR activation measured via luciferase	R1881, DHT, testosterone, progesterone, estradiol, aldosterone, cortisol, dexamethasone, and many more	R1881 equivalents	Mnif et al., 2010

Notes: AR = androgen receptor; DHT = dihydroxy-testosterone; EQ = equivalents; Flu = flutamide.

1.10.2.3 Category 2 Bioassays

As with assays to detect ER-active compounds, many suitable assays are already available to assess (anti)androgenicity. We therefore do not consider this endpoint of high priority in terms of implementation of Category 2 assays. We note a couple of assays in the following that may be of particular interest, mainly because these are multiendpoint assays or assays that exist in batteries of several cell lines targeting several NRs. The AR-EcoScreen™ was first developed by Satoh et al. (Satoh et al., 2004). Although this assay has not been tested with environmental samples as the DR-EcoScreen has (Section 1.4.5), it has seen several applications in assessment of chemicals (Araki et al., 2005; Kawamura et al., 2005; Satoh et al., 2005).

The GeneBLAzer β-lactamase assays also are attractive and include reporter cell lines for the AR (Wilkinson et al., 2008) as well as many more receptors and TFs reviewed throughout the report (Sections 1.3.3, 1.4, and 1.6). In a high-throughput study of 3000 environmentally relevant chemicals, roughly 2% were AR agonists and 11% were AR antagonists (Huang et al., 2011b). As noted, whereas the Attagene test appears promising for other adverse outcome pathway categories, the AR component of this assay system was not found to be responsive to any of the first 320 chemicals tested in the ToxCast program (Martin et al., 2010), which might stem from the fact that all of these chemicals are pesticides and that their androgenic effect is not established. Finally, the Rikilt yeast assay battery includes an assay for the AR that is yet to be validated for water samples (Bovee et al., 2008; Rijk et al., 2008).

Additional assays are reviewed by a GWRC report (Leusch et al., 2012).

1.10.2.4 Conclusions

Many assays are available to test for androgenicity that already have been validated for water quality screening. For the majority of these, corresponding estrogenicity assays are available. The YAS assay, for example, complements the YES assay, which has been found valuable for assessment of estrogenicity in water samples (Section 1.10.1.2) and in that sense seems a suitable Category 1 candidate for any assay battery if sensitivity is not the primary selection criterion. Mammalian alternatives, such as the PALM or the A-SCREEN, are more sensitive, although these are generally more expensive. Although commercial assays are costly, many of these are available as one assay in a suite of tests that can easily be combined in a screening battery. Such commercial assay batteries include the CALUX Category 1 assay and the Category 2 assays, the EcoScreen, and the GeneBLAzer β-lactamase assay battery.

1.10.3 Modulation of Hormonal Activity via the Progesterone Receptor

1.10.3.1 Mechanistic Background and Relevance

Progesterone is a major hormone in the progestagen (also termed progestogen) group of steroid hormones. Progesterone plays a crucial role in the development and function of the female reproductive system including the menstrual cycle, ovulation, implantation, and pregnancy (Graham and Clarke, 1997). These progestagenic functions are mediated via the ligand-dependent progesterone receptor (PR). Progestins are synthetic progesterone-like compounds (including pharmaceuticals used in female contraceptives, for example, levonorgestrel, hormone replacement, reproductive disorders, and some hormone-regulated cancers) that are able to replace progesterone as ligands for the PR, thereby causing activation or repression of gene expression (Sonneveld et al., 2011).

Production of progesterone and other progestagens can be assessed directly by measuring the hormone level in, for example, H259 cells (discussed in Section 1.9.1). In this section, we focus on in vitro assays capable of detecting the activation and/or inhibition of PR-mediated gene expression, excluding competitive binding assays.

1.10.3.2 Category 1 Bioassays

Recognition of the need to widen the array of cell-based systems for screening of endocrine activity in risk assessment has led to the development of assay batteries, often reporter cell-based, capable of targeting interference with several hormone receptors in addition to the ER and AR, including the PR. This recognition also applies to water quality assessment, and several assays have been adapted for water testing (Table 1.39).

The CALUX assay battery (discussed throughout the report; Sections 1.4.4, 1.4.5, 1.10.1−1.10.6) includes the PR-CALUX assay, an assay for measuring PR (anti)activity. The method follows the principles developed by Sonneveld et al. (2005) and was recently validated for chemical assessment against two alternative in vitro assays and one in vivo assay (Sonneveld et al., 2011). The assay has been applied to a wide range of water types, including wastewater, surface water, drinking water, and recycled water (Van der Linden et al., 2008; NWC, 2011).

The reporter cell line battery that includes the ER-responsive MELN (Balaguer et al., 1999), the AR-responsive PALM (Terouanne et al., 2000), and the HahLP for dioxins (Pillon et al., 2005) further comprises a cell line to detect PR activity. The PR-responsive cell line (HG_5LNGal4-PR) was constructed by Molina-Molina et al. (2006), on the basis of the two-step stable transfection method used by Seimandi et al. (2005), to create the PPAR reporter cell lines discussed previously (Section 1.4.4). Molina-Molina et al. (2006) applied HG_5LNGal4-PR to assess the activity of the fungicide vinclozolin and its metabolites, whereas Mnif et al. (2010) utilized the assay for evaluation of wastewater quality, albeit with no positive response observed. Another human cell-based PR reporter gene assay was able to detect PR-active compounds in Indian wastewater; however, this assay used transient transfection (Viswanath et al., 2008).

A number of yeast-based reporter gene assays have been developed for detection of PR (anti)activity in water samples (e.g., Garcia-Reyero et al., 2001; Chatterjee et al., 2008; J. Li et al., 2010). J. Li et al. (2010), for example, applied a recombinant yeast expressing a human PR response element for evaluation of wastewater treatment efficacy (the assay was first developed by Gaido et al. [1997] and also includes yeast for the ER and AR). In a later study, the wastewater samples were screened in this and three additional reporter yeasts by using S9 mix to mimic metabolism (J. Li et al., 2011). The use of S9 mix proved useful for some samples and/or reporter yeasts but not consistently for all. A second recombinant yeast assay (the RYA, Section 1.10.1) was used to evaluate the ability of wastewater components to inhibit PR binding (Garcia-Reyero et al., 2001). Further, Chatterjee et al. (2008) developed a recombinant yeast with human PR and GFP and tested this yeast with a range of chemicals as well as with industrial effluent extracts. Unfortunately, this GFP yeast was only transiently transfected. The Rikilt group assays use stable GFP yeasts for the ER (Bovee et al., 2004), AR (Bovee et al., 2008), and GR (Bovee et al., 2011); however, to our knowledge, no Rikilt assay has been developed for the PR and only the ER (REA, Section 1.10.1.2) has been validated for water samples.

Table 1.39. Category 1 In Vitro Assays To Detect Endocrine Activity via the Progesterone Receptor (PR)

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
PR-CALUX	Human osteoblastic osteosarcoma cells (U2-OS) stably transfected with a human PR-responsive element upstream of a luciferase reporter gene	(Inhibition of) binding to PR measured via luciferase activity (luminescence)	Various including progesterone, levonorgestrel, norethynodrel, org2058	org2058 equivalents	Van der Linden et al., 2008; NWC, 2011
PR reporter gene assay ($HG_5LNGal4$-PR)	Human cervical cancer cells (HeLa) stably transfected with human PR fused to a Gal4-responsive reporter gene ($HG_5LNGal4$-PR)	(Inhibition of) binding to PR measured via luciferase activity (luminescence)	Vinclozolin and metabolites, R5020 (promegestone)	RLUs, R5020 equivalents (no response with wastewater samples)	Mnif et al., 2010
PR reporter gene assay (HEK-hPR-Luc)	Human embryonic kidney cells (HEK293) transiently transfected with human PR linked to the luciferase gene (HEK-hPR-Luc)	(Inhibition of) transactivation of PR measured via luciferase activity (luminescence)	Progesterone, testosterone, estrogen, dexamethasone, *o,p*-DDT, *p,p*-DDT, *o,p*-DDE, *p,p*-DDE, nonylphemol, bisphenol A, endosulfan	Progesterone equivalents	Viswanath et al., 2008
Recombinant yeast assay−β-galactosidase	*S. cerevisiae* strain YPH500 transfected with plasmids containing human PR, a PR response element, and a gene for β-galactosidase	(Inhibition of) binding to PR measured via β-galactosidase activity (gives colored product that can be measured by absorbance)	Progesterone, RU486, estradiol, testosterone, a range of phenol compounds including bisphenol A and pentachlorophenol	Relative progesteronic activity, progesterone and RU486 equivalents for activation and inhibition, respectively	J. Li et al., 2010, 2011

Recombinant yeast assay (RYA)−β-galactosidase	Several yeast strains (BJ560, W303a, BY4741) transfected with a plasmid (pG1PRB [2μ, TRP1]) that expresses human PR (under the control of GDP1 promoter) and a gene for β-galactosidase	Inhibition of binding to PR measured via β-galactosidase activity (gives colored product that can be measured by absorbance)	Progesterone	EC_{50}	Garcia-Reyero et al., 2001
Recombinant yeast assay–GFP	*S. cerevisiae* strain YPH499 transiently transfected with plasmids with human PR, PR response element, and yeast-optimized GFP (yEGFP)	(Inhibition of) binding to PR measured via GFP (fluorescence)	Progesterone, estradiol, testosterone, dexamethasone, RU486, *o*,*p*'-DDT, *p*,*p*'-DDT, *o*,*p*′-DDE, *p*,*p*'-DDE, vinclozolin, α-endosulfan, nonylphenol	Fluorescence/transactivation relative to progesterone, EC_{50}, IC_{50}	Chatterjee et al., 2008

Notes: OD = optical density; org2058 = 16α-ethyl-21-hydroxyl-19-norpregn-4-ene-3,20-dione; RU486 = mifepristone (synthetic steroid in abortion pill and contraceptives).

1.10.3.3 Category 2 Bioassays

A range of Category 1 in vitro assays has been identified that has potential for routine water quality screening of progestagens. As many of the reviewed techniques include reporter cells for screening of the endocrine activity against a variety of NRs in addition to the PR, it is appropriate to utilize some of these prevalidated methods in the assay battery for endocrine disruption rather than attempting to implement further Category 2 assays. A few relevant Category 2 assays are, however, reviewed briefly in the following. Additional assays were reviewed by F. Leusch et al. (2012).

The aforementioned GeneBLAzer battery of β-lactamase assays includes a reporter cell line for the PR and may be an attractive candidate for implementation in water quality assessment (Wilkinson et al., 2008). In addition, two panels of luciferase reporter cell lines using human osteosarcoma cells (U2OS) were recently developed for assessment of ERα/β-, AR-, GR-, PR-, and MR-active compounds and were tested with 28 different ligand compounds (Sedlak et al., 2011). Mori et al. have developed HeLa-based cell lines expressing the PR (Mori et al., 2009) and GR (Mori et al., 2008); however, to our knowledge, these assays have not yet undergone thorough chemical validation.

The yeast two-hybrid systems by Nishikawa et al. (1999) include a yeast with a human PR, which has been used for chemical assessment, albeit thus far only for nonresponsive chemicals (Sumida et al., 2001). The ER and RAR yeast two-hybrid assays of this battery have been shown to be useful for water quality monitoring (Allinson et al., 2007, 2011). Further chemical validation is, however, recommended prior to potential implementation of the PR yeast two-hybrid assay for water testing.

1.10.3.4 Conclusions

The PR is important for comprehensive assessment of endocrine disruption through interference with NRs and should be included in any such test battery. A number of reporter assays have been developed for which ER-, AR-, and other NR-responsive techniques also are available. Many of these have been adapted to water testing, and there is no immediate need to optimize further Category 2 assays. It is therefore recommended to implement a Category 1 bioassay within the assay battery used to detect progestagenic activity.

1.10.4 Modulation of Hormonal Activity via the Glucocorticoid Receptor

1.10.4.1 Mechanistic Background and Relevance

Glucocorticoids such as cortisol (also known as hydrocortisone) are a group of steroid hormones that differ from the estrogens, androgens, and progestagens in being non-sex steroids. In contrast to the sex steroids, which are mainly expressed in the sex organs, glucocorticoids are found in all cell types (Akner et al., 1994; Bovee et al., 2011). Glucocorticoid steroids regulate an array of physiological processes crucial for, for example, the immune, cardiovascular, and nervous systems; development; metabolism; electrolyte balance; and cell proliferation and differentiation (Odermatt et al., 2006; Bovee et al., 2011). Indeed, glucocorticoid dysfunction has been associated with a range of diseases, including cardiovascular, inflammatory and immune diseases; osteoporosis; type II diabetes; and obesity (Odermatt et al., 2006). The importance of glucocorticoids for adipogenesis also is receiving increasing attention (Sargis et al., 2010). Because of the multiple functions characterizing this hormone group, glucocorticoids are produced and utilized as pharmaceuticals (e.g., anti-inflammatories and immunosuppressants) and in livestock industries also as growth promoters

(Bovee et al., 2011). The health benefits of glucocorticoid pharmaceuticals must, however, often be weighed against the many associated negative side effects (Wilkinson et al., 2008).

Despite the obvious significance and potential of glucocorticoids to be present in the environment and waterways, glucocorticoid disruption by environmental pollutants has received far less scientific interest than (anti)estrogen and (anti)androgen endocrine disrupters have. The disturbance of glucocorticoid action by environmental pollutants is reviewed by Odermatt et al. (2006). Various mechanisms are possible for chemical interference with glucocorticoidal function (Odermatt et al., 2006). In this chapter, we review assays that target disruption via the GR, excluding competitive binding assays.

1.10.4.2 Category 1 Bioassays

Although GR-active substances have received limited attention with respect to water screening, these compounds are gaining increasing attention and assay batteries for detection of endocrine disruption, including GR (ant)agonism, are also emerging in water quality assessment (Table 1.40).

Van der Linden et al. (2008) expanded the original CALUX assay battery (Murk et al., 1996; Legler et al., 1999; Sonneveld et al., 2005) to include a cell line that expresses human GR, the GR-CALUX assay. The assay was tested on a range of compounds and water sources both in its first application and subsequently, including wastewater, surface water, drinking water, and recycled water (Van der Linden et al., 2008; Schriks et al., 2010; NWC, 2011).

The research group responsible for the MELN, HahLP, and PALM reporter cell lines (Balaguer et al., 1999; Terouanne et al., 2000; Pillon et al., 2005) also developed a range of $HG_5LNGal4$ cell lines including the $HG_5LNGal4$-GR to detect GR-(in)active compounds (Seimandi et al., 2005; Molina-Molina et al., 2006). The assay was first tested with vinclozolin and its metabolites by Molina-Molina et al. (2006). Mnif et al. (2010) adapted the assay to water testing within a battery of reporter assays in a study assessing the endocrine activity of Tunisian wastewater. As with the PR- and MR-responsive cell lines (Sections 1.10.3.2 and 1.10.5.2), no GR (anti)activity was detected. The MDA-kb2 assay was developed from a breast cancer cell line (MDA-MB-453) to stably express human AR and GR (Wilson et al., 2002). The fact that this cell line expresses two NRs does make the assay less specific, and it is mainly applied to assess (anti)androgenicity, utilizing a nil GR response to verify AR as the affected receptor. Nevertheless, various compounds have been tested in the assay (Ait-Aissa et al., 2010; Y. He et al., 2011), which was recently applied for assessment of oil sand process-affected water, albeit with negative response for GR (anti)activity (Y. He et al., 2011).

Table 1.40. Category 1 In Vitro Assays to Detect Endocrine Activity via the Glucocorticoid Receptor (GR)

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
GR-CALUX	Human osteoblastic osteosarcoma cells (U2-OS) stably transfected with a human GR-responsive element upstream of a luciferase reporter gene	(Inhibition of) binding to GR measured via luciferase activity (luminescence)	Various including cortisol, cortisone, dexamethasone, polychlorinated biphenyls (PCBs)	Dexamethasone equivalents	Van der Linden et al., 2008; Schriks et al., 2010; NWC, 2011
GR reporter gene assay ($HG_5LNGal4$-GR)	Human cervical cancer cells (HeLa) stably transfected with human GR fused to a Gal4-responsive reporter gene ($HG_5LNGal4$-GR)	(Inhibition of) binding to GR measured via luciferase activity (luminescence)	Vinclozolin and metabolites, dexamethasone	RLUs, dexamethasone equivalents (no response with wastewater samples)	Mnif et al., 2010
MDA-kb2 GR/AR reporter gene assay	Human breast cancer cells (MDA-MB-453) stably expressing human GR (and AR)	(Inhibition of) binding to GR(/AR) measured via luciferase activity (luminescence)	Several including corticosterone, dexamethasone, environmental pesticides (negative), flavonoids (negative)	Fold induction relative to dexamethasone	Y. He et al., 2011

1.10.4.3 Category 2 Bioassays

Several Category 1 in vitro assays are available to screen water samples for glucocorticoid (anta)agonists. As concluded for PR-responsive assays, it is sensible to utilize one or more of these Category 1 assays, which include reporter cells for additional important NRs and do not need further validation. A few Category 2 assays are noted in the following. Additional assays were reviewed by Leusch et al. (2012).

None of the 320 chemicals tested in the Attagene multifactorial/NR reporter gene assay (Romanov et al., 2008) responded in the GR assay (Martin et al., 2010). In a recent study by the people behind the EcoScreen assays, however, 30 of 100 hydroxylated polychlorinated biphenyls (OH-PCBs) displayed antagonistic behavior toward the GR, highlighting the relevance of this NR (Takeuchi et al., 2011). The Chinese hamster (CHO) cell line used in this study was only transiently transfected with human GR, but the method has been applied for assessment of a range of chemicals (Kojima et al., 2009; Takeuchi et al., 2009, 2011). The GeneBLAzer assay battery includes a HEK293-based reporter cell line that carries human GR and has been validated for HTS with 35 pharmaceutically relevant compounds (Qureshi et al., 2003; Wilkinson et al., 2008). In a high-throughput study of 3000 environmentally relevant chemicals, roughly 0.5% were GR agonists and 7% were GR antagonists (Huang et al., 2011b). Again one can mention the recently developed two panels of human (U2OS) cell-based luciferase reporter cell lines that cover the ERα/β, AR, GR, PR, and MR and have been tested with several chemical compounds (Sedlak et al., 2011). The HeLa-based cell lines for the GR by Mori et al. still have not been validated for chemical assessment (Mori et al., 2008).

Recently, the Rikilt group added a GR-responsive assay to its battery of recombinant yeasts (Bovee et al., 2011); however, although this assay responded to as many hormone compounds as the GR-CALUX did, it was generally several orders of magnitude less sensitive than the mammalian cell-based assay (i.e., EC_{50} was in hundreds of micromolars for the Rikilt assay vs a few nanomolars for the GR-CALUX) (Bovee et al., 2011). Further, as discussed, only the Rikilt assay for detection of ER-(in)active compounds has been validated with water samples (REA assay, Section 1.10.1.2).

1.10.4.4 Conclusions

The GR has been linked to a wide spectrum of diseases and is a toxicologically very important factor for assessment of endocrine activity in water. It is thus recommended that studies targeting disturbance of the hormone system not be restricted to the sex steroids but include corticosteroid receptors such as the GR. Several reporter cell-based assay batteries are now available that incorporate all or several of these NRs and have been adapted for water testing. We recommend applying a battery that has been successfully applied with all or many of the NRs of interest. Although commercial, the GR-CALUX appears to be a suitable candidate for such an assay battery.

1.10.5 Modulation of Hormonal Activity via the Mineralocorticoid Receptor

1.10.5.1 Mechanistic Background and Relevance

Mineralocorticoids (e.g., aldosterone) together with glucocorticoids belong to the corticosteroid group of steroid hormones. Mineralocorticoids are important for regulation of electrolyte and water balance as well as of blood pressure (Bamberger et al., 1997; Gomez-Sanchez, 2011). Mineralocorticoid hormones act via the MR, which is structurally similar to the GR and therefore attracts similar ligands (Gomez-Sanchez, 2011). Although glucocorticoids have the highest affinity

for MRs and are often found in much higher concentrations than are mineralocorticoids, competition between ligands of the two corticoid hormone groups for binding to the MR is cell-specific (Gomez-Sanchez, 2011). The MR is expressed in both nonepithelial and epithelial cells of, for example, heart, vessels, and brain (Gomez-Sanchez, 2011).

Mineralocorticoids have been linked with cardiovascular diseases (Bravo, 2003; Gomez-Sanchez, 2011; Messaoudi and Jaisser, 2011). More specifically, aldosterone and chronic MR activation have been found to have a negative impact on the heart; therefore, MR inhibitors such as spironolactone and eplerenone are used to treat patients experiencing heart failure (Gomez-Sanchez, 2011; Messaoudi and Jaisser, 2011). The exact mechanisms of the MR are not fully understood, but the importance of the receptor to human health is clear. Furthermore, as both glucocorticoids and mineralocorticoids can bind to the MR, simply measuring glucocorticoid levels in samples is not sufficient to reveal through which receptor these act. In this chapter, we review assays that target (anti)mineralocorticoid (and similar ligand/inhibitor) action via the MR, excluding competitive binding assays.

1.10.5.2 Category 1 Bioassays

As the assay batteries for endocrine disruption are expanding, more batteries have become available that include MR-responsive cell lines. To our knowledge, however, only one study to date has included a cell-based assay for assessment of MR (in)activity in water samples (Table 1.41). The HG_5LNGal4-MR cell line was constructed as discussed previously (Section 1.10.4.2) (Seimandi et al., 2005; Molina-Molina et al., 2006). The cell line tested positive to antagonism by vinclozolin and its metabolites (Molina-Molina et al., 2006), but as with the PR and GR cell lines, no MR (anti)activity was detected in wastewater (Mnif et al., 2010).

Table 1.41. Category 1 In Vitro Assays to Detect Endocrine Activity via the Mineralocorticoid Receptor (MR)

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
MR reporter gene assay ($HG_5LNGal4$-MR)	Human cervical cancer cells (HeLa) stably transfected with human MR fused to a Gal4-responsive reporter gene ($HG_5LNGal4$-MR)	(Inhibition of) binding to MR measured via luciferase activity (luminescence)	Aldosterone	RLUs, aldosterone equivalents (no response with wastewater samples)	Mnif et al., 2010

1.10.5.3 Category 2 Bioassays

In addition to the HG_5LNGal4-MR assay, a number of assays are available for detection of MR-(in)active compounds that have not yet been carried out with water samples. Implementation of a cell-based assay for measuring MR-(in)activity in water samples will most likely occur as an addition to an existing bioassay battery for endocrine disruption. It is thus sensible to use one of the major assay batteries (including that comprising the HG_5LNGal4-MR cell line) discussed in earlier sections for endocrine disruption. Here one can mention the CALUX battery, which includes cell lines for a wide range of NRs (e.g., ER, AR, PR, and GR) that already have been validated for water testing (e.g., Van der Linden et al., 2008; NWC, 2011) and which may in the future offer an MR-responsive cell line. The GeneBLAzer assay battery also includes a reporter cell line for MR that has been validated for HTS with 35 pharmaceutically relevant compounds (Qureshi et al., 2003; Wilkinson et al., 2008). The MR also is included as endpoint in the two U2OS-based panels of luciferase reporter cell lines developed by Sedlak et al. (2011).

1.10.5.4 Conclusions

The MR is highly relevant in terms of human health, and its activation/inhibition has potential as an endpoint for assessing the presence of both mineralocorticoids and glucocorticoids in water samples. Of the hormone receptor-based assays discussed in previous sections, the MR is the least tested with water samples. Validation of assays that target this endpoint is thus of considerable urgency for water quality monitoring. The most common assay batteries discussed in this and previous sections include or will likely soon include a cell line for the MR and can be relatively easily implemented, once developed.

1.10.6 Modulation of Hormonal Activity of the Thyroid System

1.10.6.1 Mechanistic Background and Relevance

The thyroid hormones 3,5,3'-triiodothyronine (T_3) and thyroxine (T_4) are important for controlling growth and development and for maintaining metabolic homeostasis (DeVito et al., 1999). T_3 and T_4 act via the TR, for which several isoforms are known (Evans, 1988; Lazar, 1993). Four isoforms have been identified for mammalian TR, whereas only two isoforms are known to exist for the more studied ARs and ERs, respectively (DeVito et al., 1999; Beato and Klug, 2000). The TRs mainly act as heterodimers with RXRs (DeVito et al., 1999). As RXRs form heterodimers with one-third of the 48 receptors of the human NR superfamily (le Maire et al., 2010), TRs can be indirectly affected by other NRs competing for RXR (DeVito et al., 1999). Because of the vital functions and many facets of the thyroid hormones, this component of the endocrine system is of high relevance for water quality assessment. To date, the potential adverse effects of water-associated organic pollutants on the thyroid system have received comparatively little attention.

1.10.6.2 Category 1 Bioassays

As reviewed in previous sections of this report, screening of potential endocrine disruptive activity in waters has mainly focused on the ER and AR. Recently, an increasing number of reporter cell lines have become available for screening of (anti)thyroid activity in water (Table 1.42). The applied cell types include yeasts (J. Li et al., 2010; N. Li et al., 2010, 2011) and frog cell lines (Murata and Yamauchi, 2008; Ishihara et al., 2009) with few mammalian-based assays developed and prepared for water quality testing. Yeast two-hybrid assays have been applied to wastewater and river water in

Japan (Inoue et al., 2009b, 2011), wastewater and drinking water in China (J. Li et al., 2010; N. Li et al., 2011), and wastewater in Australia (Allinson et al., 2011).

The mammalian cell-based assays include the GH_3 assay, which utilizes a rat pituitary tumor cell line (GH_3) that is dependent on T_3 for growth (Hohenwarter et al., 1996) (Table 1.42). Cell proliferation is mediated via binding and activation of TR, and potential antagonistic effects can be determined by running the assay in the presence of T_3. Because of the similarity of this assay and the E-SCREEN (Soto et al., 1995; Körner et al., 1999), the GH_3 also is referred to as the T-Screen (Ghisari and Bonefeld-Jorgensen, 2005; Gutleb et al., 2005). The T-Screen has been applied by using metabolic activation, namely, run both in the presence and absence of S9 liver enzyme mixture (Taxvig et al., 2011). A broad range of chemicals has been tested in the T-Screen, including pesticides, parabens, phthalates, and polyhalogenated aromatic hydrocarbons (Gutleb et al., 2005; Schriks et al., 2006; Taxvig et al., 2008, 2011). Recently, the T-Screen was applied complementarily with other cell-based assays for assessing the efficacy of two WWTPs (Kusk et al., 2011). The response was reported in T_3 equivalents (T_3EQs). The T-Screen is relatively time consuming and unable to distinguish between TR-mediated cell proliferation and non-TR-mediated cell proliferation.

The TRβ-CALUX has been applied for a wide range of water samples from sewage to drinking water (NWC, 2011) (Table 1.42). Sun et al. (2009) developed a CV-1 (green monkey kidney fibroblast) based luciferase reporter gene assay (Table 1.42). In this reporter assay, cells are transfected with human TRβ1 and a galactosidase-responsive luciferase reporter plasmid, enabling luciferase activity to be measured via luminescence. After initial validation with reference compounds (bisphenol A, tetrabromo bisphenol A [TBBPA], and tetrachloro bisphenol A [TCBPA]) (Sun et al., 2009), the assay was successfully applied for testing of industrial effluents and receiving rivers in China (W. Shi et al., 2009; Shi et al., 2011). The CV-1 reporter gene technique expresses agonist and antagonist activity in T_3 and dibutyl phthalate equivalents (DBP-EQs), respectively (Shi et al., 2011). Similarly, Jugan et al. (2009) applied a luciferase reporter assay on the basis of a rat cell line with avian TRα1 (PC-DR-LUC [Jugan et al., 2007]) for testing of various water types in France (Table 1.42).

Table 1.42. Category 1 In Vitro Assays to Detect Binding to the Thyroid Receptor (TR)

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
T-Screen	Rat pituitary tumor cells (GH3)	T_3-Dependent cell proliferation	Various pesticides, parabens, phthalates	T_3-EQ	Kusk et al., 2011
TRβ-CALUX	Human osteosarcoma cells (U2OS) transfected with human TRβ and luciferase gene	TRβ-Mediated luciferase activity measured by luminescence	T_3	T_3-EQ	NWC, 2011
CV-1-based reporter assay	African green monkey kidney fibroblast cells (CV-1) transfected with human TRβ1 and luciferase	T_3-Dependent luciferase activity measured by luminescence	BPA, TBBPA, TCBPA	T_3-EQ (agonism), DBP-EQ (antagonism)	W. Shi et al., 2009; Shi et al., 2011
PC-DR-LUC reporter gene assay	Mammalian cell line expressing avian TRα1 (PC12) and luciferase gene	T_3-Dependent luciferase activity measured by luminescence	TBBPA, TCBPA, PCP, and more	T_3-EQ	Jugan et al., 2009
Yeast two-hybrid assay	Yeast cells with a hybrid reporter gene, containing the ligand-binding domain of the human TRα and a yeast GAL4 DNA-binding domain	β-Galactosidase activity	T_3, T_4, amiodarone hydrochloride (AH)	T_3-EQ (agonism), AH-EQ (antagonism)	Inoue et al., 2009b; J. Li et al., 2010; Allinson et al., 2011; Inoue et al., 2011; N. Li et al., 2011

Notes: BPA = bisphenol A; PCP = pentachlorophenol; TBBPA = tetrabromo bisphenol A; TCBPA = tetrachloro bisphenol A; T_3 = thyroid hormone 3; TRβ1= thyroid receptor β1.

1.10.6.3 Category 2 Bioassays

A number of the mammalian cell-based assays are available for evaluation of thyroid disruption but have not yet been validated for use in water quality testing. Later in the text we discuss a few of potential value for screening of water samples. Additional assays were reviewed by F. Leusch et al. (2012).

The battery of reporter cell lines (Sections 1.4.2, 1.4.5, 1.10.1, and 1.10.6) also includes an assay to assess effects on the TR. The TR-EcoScreen is a luciferase reporter gene assay developed from the TH-dependent Chinese hamster ovary cell line (CHO-K1) (ICCVAM, 2003; Kitamura et al., 2005). The cells are cloned with GFP prior to addition of luciferase substrate in order to assess cell viability (potential cytotoxicity). The assay was tested on single chemicals, including TBBPA and tetra- and dimethyl bisphenol A (TMBPA and DMBPA). To our knowledge, the TR-EcoScreen has not been applied for further testing, although a transient transfection version was tested with several PBDEs and their hydroxylated and methoxylated metabolites, of which only one compound tested positive (Kojima et al., 2009). Kitamura et al. further developed a radioactive binding assay using a rat pituitary cell line (MtT/E-2) (Kitamura et al., 2005); however, as noted throughout this report, such assays are not practical for routine screening.

Freitas et al. (2011) took the GH3 assay (T-Screen) a step further, establishing a GH3-based reporter gene assay. The new cell line, GH3.TRE-Luc, contains a TR-regulated luciferase reporter plasmid. The assay was validated by using T_3 and T_4, their metabolites Triac and Tetrac, and model compounds, including T_3 and T_4, like hydroxylated polybrominated diphenylethers (OH-PBDEs) and hydroxylated polychlorinated biphenyls (OH-PCBs), and bisphenol A. The GH3.TRE-Luc cell line demonstrated concentration-dependent sensitivity with a 20-fold increase in response induced by T_3.

The GeneBLAzer assay platform includes a TRβ variant. In a high-throughput study of 3000 environmentally relevant chemicals, roughly 0.5% were TRβ agonists and 4% were TRβ antagonists (Huang et al., 2011b).

Table 1.43. Summary of Category 2 In Vitro Bioassays to Detect Binding to the Thyroid Receptor (TR)

Assay	Cell Line	Endpoint	Reference Chemicals	Expression of Results	Method Reference(s)
TR-Ecoscreen	Chinese hamster ovary cells (CHO-K1) transfected with response elements for TRα1 and TRβ1	TR activation is measured via luciferase activity (luminescence). The construct further contains GFP for assessment of cytotoxicity	TBBPA, TMBPA, and DMBPA	IC_{50}	Kitamura et al., 2005
GH3.TRE-Luc	Rat pituitary tumor cells (GH3)	Binding of TRE induces β-galactosidase, which metabolizes substrate into colored product for measurement by luminescence	T_3, T_4, 4-OH-BDE 69, 4-OH-BDE 121, 4-OH-PCB 69, 4-OH-PCB 106, BPA, TBBPA, TCBPA	EC_{50}	Freitas et al., 2011
TRβ - GeneBLAzer	Human embryonic kidney cells (HEK293H)	Activated TR quantified as β-lactamase activity (*bla* reporter gene)	T_3	EC_{50} or IC_{50}	Huang et al., 2011b

Notes: BPA = bisphenol A; DMBPA = dimethyl bisphenol A; OH-BDE = hydroxy-brominated diphenylether; OH-PCB = hydroxyl-polychlorinated biphenyl; T_3 and T_4 = thyroid hormones 3 and 4; TBBPA = tetrabromo bisphenol A; TCBPA = tetrachloro bisphenol A; TMBPA = tetramethyl bisphenol A; TRE = TR-responsive element.

1.10.6.4 Conclusions

Because of the simplicity of establishing and running the T-Screen, this assay is an attractive candidate for application in water quality monitoring. The reporter gene assays are of higher specificity and time and cost efficiency once established. The CALUX test battery possibly has the widest range of applicability, even though there is at the moment only one study published in the grey literature on the specific TR application of the CALUX battery in water quality assessment (NWC, 2011).

A toolbox of assays may be required to properly assess thyroid activity in a sample (incorporating thyroid hormone biosynthesis and binding to thyroid hormone carrier proteins, as well as TR-mediated gene expression). This is of course true for all endocrine endpoints but specifically so for the thyroid axis because it is unclear, which of these processes is the most sensitive to environmental chemicals (Leusch et al., 2012).

Chapter 2

Understanding Bioassay Results

2.1 Introduction

Difficulties in the interpretation of results are an obstacle to the acceptance of bioanalytical tools for monitoring treatment efficacy and assessment of water quality.[2] Responses are usually reported as the percentage effect at a given sample dilution/enrichment or as an effect concentration (EC) eliciting a defined endpoint (e.g., 50% inhibition of enzyme activity or exceeding a defined effect threshold). This type of information can be very confusing for people who are not very familiar with bioassays and dose−response assessment.

Therefore, we seek to implement a uniform and simple method to interpret the toxicity measurements and to express the results of bioanalytical tools. The observed effect in most selected bioassays has been expressed as toxic equivalent concentrations (TEQs) or bioanalytical equivalent concentrations (BEQs) relative to an appropriate reference compound. The TEQ/BEQ concept lends itself in particular to receptor-mediated modes of toxic action, where there are well-defined and selective reference compounds. In contrast, for bioassays that cover nonspecific modes of action or modes of action that apply to many chemicals (e.g., ASRs), results are more typically expressed as ECs.

During this project we have ruled out any qualitative assays that do not allow dose−response assessment, as this handicap would preclude derivation of treatment efficacy (e.g., log-removal) using bioassays.

2.2 Dose−Response Assessment

A dose−response curve plots the response (e.g., death or a more subtle sublethal effect) of a population under study (e.g., rats, cells, or enzymes) against an increasing dose (or concentration) of the test chemical applied to the system, usually on a logarithmic scale (Figure 2.1).

[2]Some sections of Chapter 2 were adapted with permission from Escher and Leusch (2011). Copyright 2011, IWA Publishing.

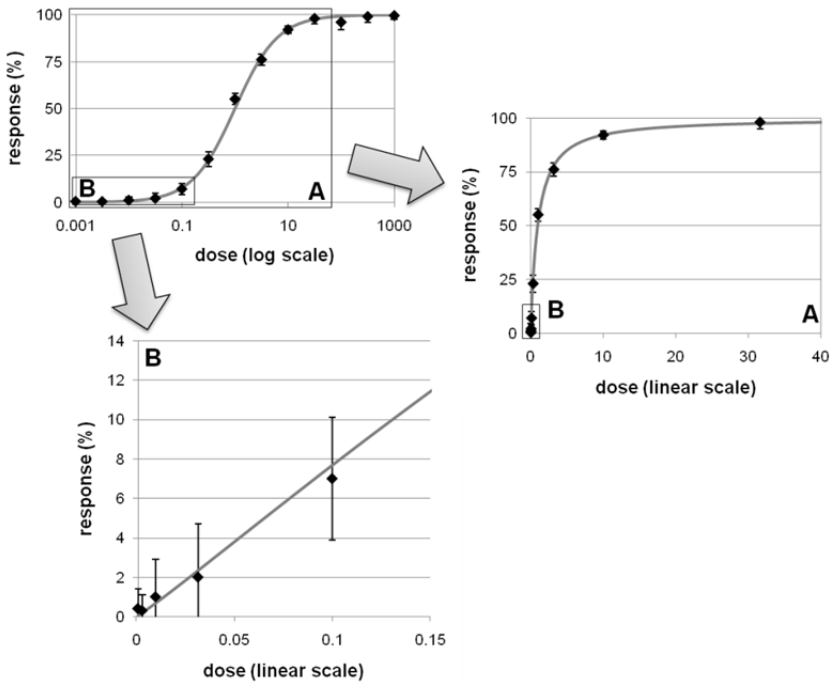

Figure 2.1. Dose–Response Curve

Notes: The top left graph depicts a typical dose−response curve with the logarithm of the dose on the x axis and the response (in %) on the y axis. To the right, the same curve is shown using a linear scale for the dose. The bottom graph illustrates the clear linear dose−response relationship that exists at the low dose range.

Source: Rep*rin*ted with permission from Escher and Leusch (2012). Copyright 2012, IWA Publishing.

The dose–response curve typically displays as a sigmoid curve. On the left, at very low dose, there is no measurable response in the test system. It is important, however, that a closer look at the lower end of the dose–response curve shows that the relationship between dose and response is in fact linear at low doses (Figure 2.1).

Figure 2.1 shows that, as the dose is slowly increased, there is initially no detectable increase in the monitored response. Eventually a detection threshold is reached, above which the response quickly increases with increasing dose in a near-linear fashion (actually log-linear, as the *x* axis is on a log-scale). The slope of the dose–response curve then plateaus to a maximum of 100%. This dose–response curve is common to all biological responses, although it can sometimes be affected when more than one type of effect co-occurs in the test system. Enzyme induction in a liver cell line will, for example, increase with growing concentration at first but decrease again at higher doses. This is a typical example of cytotoxic interference, where total enzyme activity decreases at higher doses because the toxicant is becoming cytotoxic and starts destroying the liver cells (Figure 2.2). In the same way that the sample matrix can interfere with the detectors used in standard chemical analysis and cause significant problems for highly concentrated samples, toxic interference with the biological detector can be a real concern at high chemical concentrations in bioassays. It is important to monitor the general health of the detector (whether a cell or enzyme system) concurrently with monitoring of the effect itself. Such quality assurance can be achieved by measuring cytotoxicity (e.g., cell death, growth inhibition) alongside the specific endpoint of the assay.

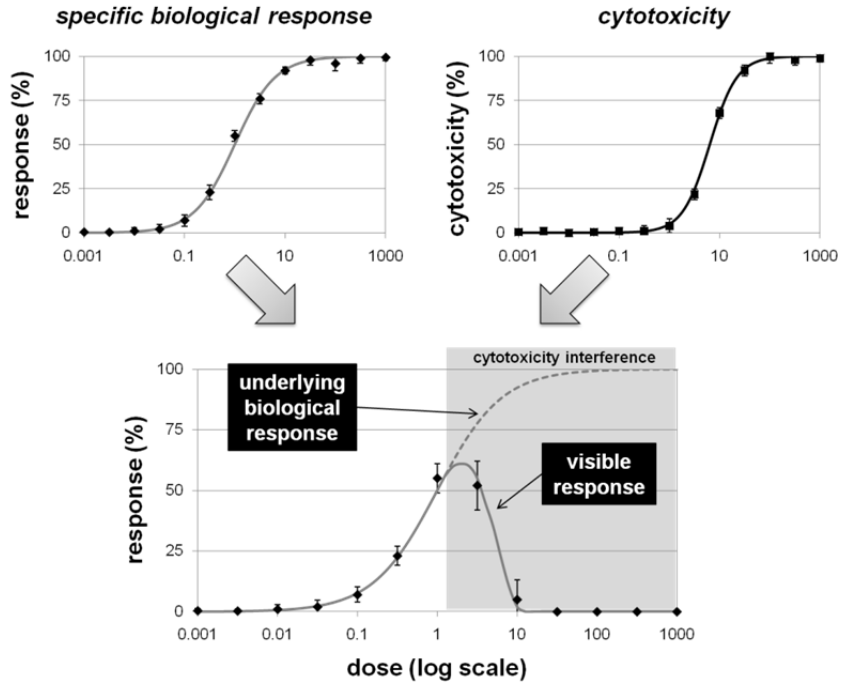

Figure 2.2. Example of a bioassay response with cytotoxicity interference.

Notes: The top graphs show the theoretical specific biological response (left) and cytotoxicity (right) as the dose is increased. The bottom graph shows how the two endpoints combine in a real bioassay, with the full line showing the response as measured in the assay, whereas the dotted line shows the dose−response for the biological effect that would be measured if there were no cytotoxic interference.

Source: Reprinted with permission from Escher and Leusch (2012). Copyright 2012, IWA Publishing.

In bioassays where a specific response is normalized to the number of cells, cytotoxicity (a decrease in cell number) will cause an increase in the response:cell ratio, thus wrongly indicating an increase in the specific response. The dose−response curve will in this case likely take an exponential shape because some of the cells found dead (decreased cell count) at the end of the exposure period will have produced a response in the earlier stages before succumbing to the cytotoxicity.

2.3 Toxicity Benchmark Values: Effect Concentrations

Several important parameters can be described by the dose−response curve. In cell-based bioassays, the exposure is usually expressed as the concentration of chemical in the medium (e.g., nanograms of test compound/liter of water) and the response is usually expressed as the EC_{50}, the concentration that causes 50% of the maximal effect.

For most assays where cytotoxicity interference is an issue (e.g., many genotoxicity assays, which eventually lead to apoptosis), linear concentration effect curves are used instead for low effect values and the effect endpoint is an induction ratio (IR) of 1.5, namely, an increase in measured activity by 50% over the control activity.

The lack of a simple method to communicate and interpret bioassay results is clearly an obstacle to the acceptance of bioanalytical tools for monitoring treatment efficacy and

assessment of water quality. Responses are usually reported as % effect at a given sample dilution/enrichment or as an EC_X eliciting a defined endpoint (e.g., 50% inhibition of enzyme activity or exceeding a defined effect threshold, such as a 50% increase in induction as compared to the baseline, IR = 1.5). This type of information can be very confusing for people who are not very familiar with bioassays and dose–response assessment, because a high number counterintuitively translates to a low toxicity. Therefore, we seek to implement a uniform and simple method to interpret the toxicity measurements and to express bioanalytical results. A more intuitive way of describing bioassay results is the bioanalytical equivalency concept.

2.3.1 Dose-Metric for Water Samples: the Relative Enrichment Factor

There are multiple options for presenting bioanalytical data and deriving ECs for water samples. In all cases, the concentrations of samples are expressed as relative enrichment factors (REFs), which is the ratio of the volume of sample to volume of bioassay:

$$\mathrm{REF} = \frac{\text{volume sample}}{\text{volume bioassay}} \tag{1}$$

The REF can be derived by the enrichment factor of the extraction process times the dilution of the extract in the bioassay. A REF of 1 is equivalent to the native sample, a REF of >1 means that the sample is enriched in the bioassays, and a REF of <1 means it is diluted in the bioassay.

The REF is expressed in units of $[L_{\text{water sample}}/L_{\text{bioassay}}]$.

$$\mathrm{REF} = \text{dilution factor}_{\text{bioassay}} \cdot \text{enrichment factor}_{\text{SPE}} \tag{2}$$

The enrichment factor of the solid-phase extraction (SPE) enrichment $\text{factor}_{\text{SPE}}$ was calculated by using Equation 3 from the volume of extracted water to the volume of resulting extract (in solvent).

$$\text{enrichment factor}_{\text{SPE}} = \frac{V_{\text{water}}}{V_{\text{extract}}} \tag{3}$$

The dilution factor of each bioassay was calculated by using Equation 4.

$$\text{dilution factor}_{\text{bioassay}} = \frac{\text{volume of extract added to bioassay}}{\text{total volume of bioassay}} \tag{4}$$

2.3.2 Selection of Concentration−Effect Model

For each bioassay, all observed responses were plotted against the sample concentration expressed in REFs. All responses were normalized to medium or solvent controls (IR), and if the maximum response in an endpoint was known, the response was converted to percentage of maximum effect (Figure 2.3).

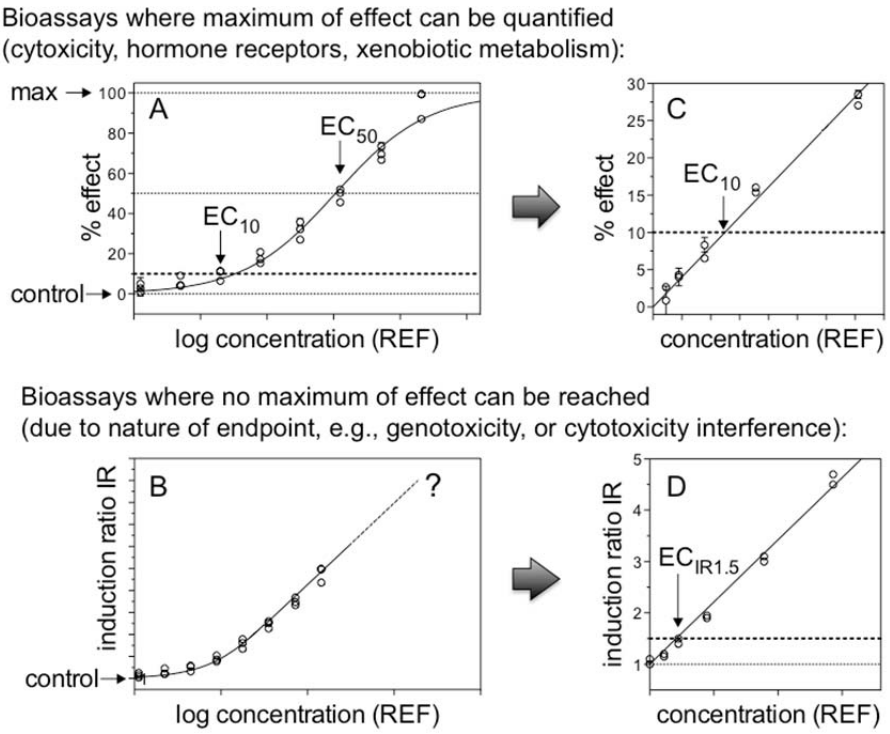

Figure 2.3. Overview of the concentration−effect models applied to derive benchmark effect concentrations (ECs) for water samples.

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A critical aspect when working with a large number of diverse biological endpoints is a consistent data evaluation process. The ideal case would be full concentration−effect curves with clearly defined maximum and minimum, which can then be converted in 0% to 100% of effect (Figure 2.3A). For all endpoints that relate to cell viability and cell population growth, the controls can be expressed as 0% effect and 100% relates to "no growth" or "all cells dead". For reporter gene assays that show the binding to a receptor, the minimum refers to the basal activity of the receptor and the maximum is defined by using an appropriate reference compound that can saturate the receptor without causing any disturbance by cytotoxicity.

If no appropriate reference compound exists, or if cytotoxicity quenches the reading of the reporter activity, then effect cannot be expressed as % effect. This problem also arises if the endpoint is inducible or, by nature, there is no clear upper limit, e.g., for DNA damage, revertants in the Ames assay, and ASR (Figure 2.3B). In these cases only the fold increase over the control, namely, the IR, can be calculated.

Any log-logistic concentration–effect curve will be linear with respect to (nonlogarithmic) concentrations at low effect level (up to 20−30% effect, as illustrated in Figure 2.3). As the water samples investigated in the present study often show very low effect levels, the linear form of the concentration–effect curves was used for derivation of the EC of the samples (Figures 2.3C and D).

2.3.3 EC_{10} (10% Effect Concentration)

EC_{10} values were reported for the cytotoxicity bioassays and for receptor-mediated effects and were obtained from a log-logistic fit of the concentration−effect curves (Figure 2.3A). The % effect was calculated with Equation 5.

$$\%\text{effect} = \frac{\text{signal}_{\text{sample}} - \text{signal}_{\text{control}}}{\text{signal}_{\text{max}} - \text{signal}_{\text{control}}} \tag{5}$$

Adjustable parameters were the slope s and the EC causing 50% reduction of maximum effect, EC_{50}.

$$\%\text{effect} = \frac{1}{1+10^{\text{slope}\cdot\left(\log EC_{50} - \log\text{concentration}\right)}} \tag{6}$$

The EC_{10}, the EC causing 10% reduction of cell viability, was derived from the EC_{50} and the slope (Equation 7).

$$\log EC_{10} = \log EC_{50} + \frac{1}{\text{slope}}\log\left(\frac{1}{9}\right) \tag{7}$$

In many cases, no full concentration−effect curves were obtained for the sample extracts. Partial concentration–effect curves can be fitted only if the slope is fixed at 1 or at the slope of the reference compound. Alternatively, because the lower portion of the log-logistic concentration effect curves is linear with respect to nonlogarithmic concentrations, the EC_{10} also can be derived from a linear concentration−effect curve up to 20% of maximum effect (Equations 8 and 9.

$$\%\text{effect} = \text{slope}\cdot\text{concentration} \tag{8}$$

$$EC_{10} = \frac{10\%}{\text{slope}} \tag{9}$$

The EC_{10} values derived with the linear method agreed well with the log-logistic derivation, and the final results of the samples were derived from the linear concentration–effect curves, although the EC_{10} values of the reference compounds were from the full log-logistic fit. Analogous to the EC_{10}, an EC_{20} also can be derived.

2.3.4 $EC_{IR1.5}$ (Effect Concentration Causing an Induction Ratio of 1.5)

The $EC_{IR1.5}$ was derived for all reporter gene assays where no maximum response could be obtained. By nature of the endpoint, the IR approach applies to genotoxicity and most ASRs such as the oxidative stress response. In addition, a few of the endpoints assessed have no reference compound, such as the FACTORIAL assay, and for those also the $EC_{IR1.5}$ was calculated.

The IR is the ratio of the measured signal (e.g., absorbance, RLU, RFU) to its control value (Equation 10). An analogous equation can be used for the number of revertants in the Ames assay, henceforth called the revertant ratio (RR).

$$IR = \frac{signal_{sample}}{signal_{control}} \tag{10}$$

Concentration–effect (IR) curves would show the typical log-logistic form, but the maximum is hard to establish because of cytotoxicity interference or because it simply does not exist (Figure 1B). Therefore, only the linear portion of the concentration−effect curves was evaluated up to an IR of 5 (Equation 11, Figure 1D).

$$IR = 1 + slope \cdot concentration \tag{11}$$

The assessment endpoint is the concentration that induces an IR of 1.5 ($EC_{IR1.5}$). The $EC_{IR1.5}$ can be derived by using the linear regression function with Equation 12 (and analogously for the RR in the Ames test with Equation 13).

$$EC_{IR1.5} = \frac{0.5}{slope} \tag{12}$$

$$EC_{RR1.5} = \frac{0.5}{slope} \tag{13}$$

The threshold of 1.5 was chosen because (1) it is chosen in several guideline documents, for example, for the *umu*C assays; (2) it is very close to the limit of detection in many cases (control plus 3 standard deviations) (Escher et al., 2012); (3) it is an interpolation; and (4) it can be applied if the maximum of the dose–response curve is not known. The disadvantage of using the IR is that, depending on the bioassay, the maximum response can be at an IR of 2 or at an IR of more than 100. If the maximum IR reaches 6, then the $EC_{IR1.5}$ is equivalent to the EC_{10}, if the maximum IR is 2, the $EC_{IR1.5}$ is equivalent to the EC_{50}, and for IRs that level off at 100 or more, the $EC_{IR1.5}$ is often close to the limit of detection.

2.3.5 $EC_{SR0.2}$ (Effect Concentration Causing a Suppression Ratio of 0.2) for All Antagonistic Effects and Chaperone Dissociation

A receptor-mediated bioassay is run in antagonistic mode if the receptors are saturated or occupied with a constant concentration of a positive control and a variable concentration of the sample—if the signal of the control is suppressed, then the sample has an antagonistic effect (Figure 2.4). The suppression ratio (SR) is defined by Equation 14. The analogous equation was used for endpoints that are based on chaperone dissociation. Again, there is the problem that often no full concentration–effect curves are obtained for antagonistic effects and that it is unclear if the bottom of the curve is at 0 or at some higher baseline. Therefore, we propose to use only the initial linear part of the concentration–effect curves and derive the $EC_{SR0.2}$ with a linear regression through the origin (0;0). The 20% effect level was chosen to derive the $EC_{SR0.2}$ by a linear regression through the origin (0;0). The 20% effect level was chosen to derive the $EC_{SR0.2}$ because the variability is typically larger in antagonistic mode than in the agonist mode and because the 10% effect level is often not above the variability of the controls, which would produce false-positive results.

$$SR = 1 - \frac{RLU}{RLU_{max}} \quad (14)$$

$$SR = slope \cdot concentration \quad (15)$$

$$EC_{SR0.2} = \frac{0.2}{slope} \quad (16)$$

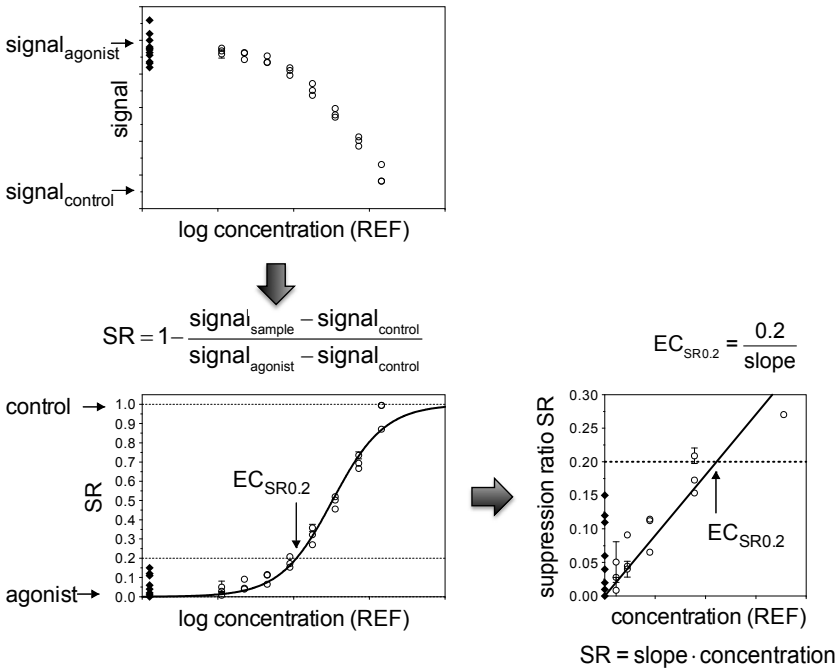

Figure 2.4. Derivation of ECSR0.2.

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2.4 Bioanalytical Equivalent Concentrations

The observed effects in bioassays can be expressed as BEQs relative to an appropriate reference compound if

- the bioassay describes a clearly defined mode of toxic action or toxicity pathway;
- there exists a reference compound of high potency that acts specifically according to the mode of action detected in the given bioassay;
- the dose–response curves of the reference compound span the entire range of possible effects; and
- no cytotoxicity or matrix/quenching effects impair the response of the sample within the range of the concentrations of reference compound used.

To derive the BEQ for those endpoints where it is applicable, the following approach was chosen: The relative effect potency (REP) of test compound i relative to the reference compound can be calculated (Villeneuve et al., 2000) in this manner:

$$REP_i = \frac{EC(\text{reference compound})}{EC(\text{compound i})} \tag{13}$$

The BEQ of a mixture of known chemicals is derived as the sum of the product of the concentration of each component i in the mixture, C_i, and its REP_i value.

$$BEQ_{chem} = \sum_{i=1}^{n} C_i \cdot REP_i \tag{14}$$

For the unresolved-mixture water samples, the EC_{50} or any other EC (for induction-based assays, we often use $EC_{IR1.5}$, which corresponds to the concentration that causes an IR of 1.5) is used to derive the BEQ_{bio}. The BEQ_{bio} is the ratio of the EC_{TRIG} (EC value at the trigger point, i.e., depending on the assay, EC_{10}, $EC_{IR1.5}$, or $EC_{SR0.2}$) of the reference compound and the EC_{TRIG} of the water sample, where the former is expressed either as a molar concentration (e.g., in micromoles per liter) or as a mass-based concentration (e.g., in nanograms per liter), whereas the latter is expressed in REFs (i.e., unitless):

$$BEQ_{bio} = \frac{EC(\text{reference compound})}{EC(\text{sample})} \tag{15}$$

The BEQ concept has some limitations for bioassays that measure effects elicited by a very large number of compounds (e.g., nonspecific or reactive effects). In that case, instead of calculating a BEQ, one can express the effect as relative toxic units (rTUs), which are calculated as 1/EC if the EC is expressed in REFs. Then a sample with an rTU of >1 is immediately toxic, whereas one with an rTU of <1 has to be concentrated before we can detect toxicity. A sample with an rTU of 10 “simply” needs to be diluted 10 times to bring it to “nontoxic”.

Chapter 3

Implementation of Novel Bioassays for Water Quality Assessment

3.1 Introduction

A thorough literature review (see Chapter 1) leads to the identification of a suite of Category 2 bioassays, i.e., those that target a relevant mode of toxic action and that have been validated with single chemicals, for example during the Tox21 program (Gibb, 2008) but have not yet been adapted to water quality assessment.

In line with the toxicity pathways chosen as structuring principles for toxicity testing, we classified the bioassays according to the main four groups of (1) induction of xenobiotic metabolism, (2) modes of action, encompassing specific, receptor-mediated, and reactive toxicity, (3) induction of ASR pathways and (4) cell viability tests that can yield some information on system responses if appropriate cell lines are chosen (Figure 3.1).

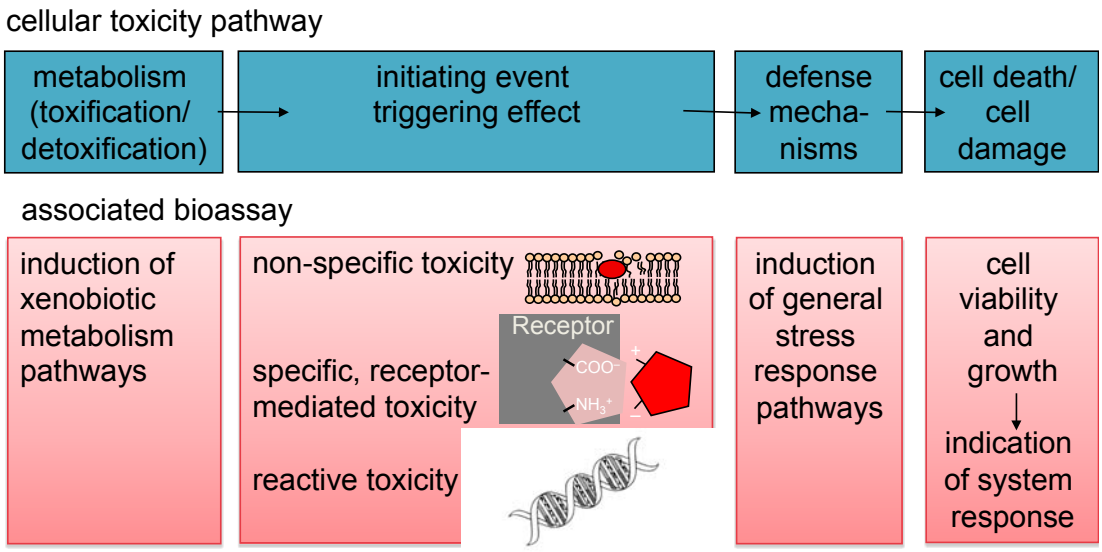

Figure 3.1. Classification of in vitro bioassays according to cellular toxicity pathways.

The project team selected three relevant bioassays for each lab from the list of Category 2 bioassays identified in the literature review in order to proceed to validation of these bioassays. The bioassays were selected according to the following criteria for their potential relevance:

- How do the selected bioassays and test endpoints relate to a human health outcome?
- Are metabolic activation/detoxification processes included in the assay, even as separate endpoint?

- Is the bioassay meaningful in the context of water reuse? Will it be able to identify mixture effects of chemicals previously identified as organic contaminants in recycled water?

This chapter summarizes the implementation and validation of the chosen Category 2 bioassays.

3.2 Choice of Category 2 Bioassays

After selection of the nine bioassays, they were validated by using reference compounds and water samples. Depending on the lab and the time when testing was performed, in some cases the samples of the interlaboratory comparison study were used for the validation. In each case one or more reference compounds were chosen to set up the test protocol. Ideally this includes one very selective potent reference compound and other reference compounds that are relevant to the recycled water matrices. Then carrier solvents were tested to identify a suitable solvent for the water extracts and the tolerable solvent concentration where there was no interference with the bioassay. In addition, if time permitted, possible matrix effects, for example, by natural organic matter, were evaluated.

Important decision criteria for including a Category 2 bioassay in a bioassay test battery apart from the relevance of the biological endpoint are as follows:

- ***Sensitivity:*** includes the size of the window between specific response and nonspecific cytotoxicity as well as detection limit for the very low concentrations of contaminants that can be expected in recycled water matrices.
- ***Reliability*****:** includes repeatability (same results day to day), reproducibility (same results in different labs), and robustness (ease of performance and low variability).

All evaluated Category 2 bioassays are listed in Table 3.1. The literature review clearly identified that bioassays that target the induction of xenobiotic metabolism are biased toward the AhR. The PPAR is activated by a number of organic micropollutants that occur in water samples and was therefore added as a new representative of xenobiotic metabolism. Although the bioassays indicative of the induction of ASR pathways appear to be promising for water quality testing, as these responses occur before damage occurs, few such bioassays have been previously used. Therefore, we propose to implement indicators for hypoxia and the oxidative stress response. For the latter, two cell lines appeared promising from the literature review and were accessible; therefore, we included the Nrf2-keap cell line and the AREc32 cell line. In addition NF-κB anti-inflammatory response was quantified by the detection of the chaperone-like inhibitory protein IκB in human lymphoblastoma cells.

A gap for assessing reactive toxicity is protein damage (in addition to DNA damage). We evaluated and adapted an assay indicative of protein damage (Tang et al., 2012), but this endpoint is not further discussed because it turned out that, despite the sensitivity of the bioassay for relevant reference chemicals, it was not compatible with water samples as natural organic matter disturbed its performance.

The vast majority of bioassays for endocrine disruption focus on the ER and the AR, despite the health relevance of other hormone receptors such as the GR (Section 3.8) and the TR (Section 3.9). Also, the RAR (Section 3.10) plays a crucial role in early stages of development and the RAR has much cross-talk with other vital functions.

Finally the literature review identified a lack of in vitro bioassays targeting more systemic responses. The cell viability of a neuroblastoma cell line was evaluated as a representative of neurotoxicity (Section 3.11).

Table 3.1. Selected Category 2 Bioassays

Chapter	Assay	Endpoint/Importance	Lab
Induction of xenobiotic metabolism pathways			
3.3	PPARγ-GeneBLAzer assay	Comprehensive endpoint (glucose, lipid, and fatty acid metabolism; obesity), no Cat 1 available	GU
Induction of ASR pathways			
3.4	Hypoxia (Switchgear)	Stress response pathway for oxygen depletion, no Cat 1 assay available	UA
3.5	Nrf2-keap cell line (luciferase assay using Nrf2-Keap1 signal pathway)	Oxidative stress, no Cat 1 assay available	UA
3.6	AREc32 cell line	Oxidative stress indicative of the keap-Nrf2-ARE (ARE pathway), no Cat 1 assay available	UQ
3.7	IκB in the Jurkat cell line	Includes endpoints for the inflammatory stress response pathway as well as for immunotoxicity	GU
Receptor-mediated effects (hormonal effects)			
3.8	GR (Switchgear)	Endocrine disruption, GR-mediated endpoint	UA
3.9	T-Screen	Endocrine disruption, effects on the thyroid system, high priority for human health	UQ
3.10	RAR	RAR-mediated developmental toxicity, very high importance for human health	UQ
Bioassays for cell viability targeting system responses			
3.11	Neuroblastoma assay	Neurotoxicity, blocking of Na^+ channels, the assay battery lacks a cell-based assay for this endpoint	GU

3.3 PPARγ-GeneBLAzer Assay

3.3.1 Introduction

The commercially available PPARγ-GeneBLAzer assay (Life Technologies, Mulgrave, Vic, Australia) was used to detect PPAR-like activity in the water samples. The assay is based on a human embryonic kidney cell line (HEK 293H cells) modified to express a fusion protein combining the ligand-binding domain of the human PPARγ fused with the DNA-binding domain of the GAL4 gene and stably transfected with a β-lactamase reporter gene downstream of a GAL4 activator sequence. When an agonist binds to the ligand-binding domain of the PPARγ-GAL4 fusion protein, the protein binds to the activator sequence and stimulates expression of β-lactamase.

3.3.2 Materials and Methods

The division arrested (DA) kit was used here (catalog no. K1419; Life Technologies). In brief, the DA cell aliquot was thawed quickly in a 37 ºC water bath, transferred to 10 mL of assay medium, and centrifuged at 200 × *g* for 5 min. The supernatant was discarded, and the cell pellet reconstituted to a cell density of 9.4×10^5 cells/mL (determined by using a Millipore Scepter Handheld Automated Cell Counter). Using a multichannel pipette, 32 µL of assay medium was added to the "cell-free control" wells, and 32 µL of the cell suspension was added to all the other wells (30,000 cells/well) of a black wall clear bottom poly-D-lysine coated 384-well plate (catalog no. 354663; BD, North Ryde, NSW, Australia). In agonist mode, 8 µL of 5× 0.5% DMSO (solvent control), 5× rosiglitazone (reference compound, final concentration range from 7 pM to 2 µM), or 5× test samples were added to their respective wells (maximum 0.1% solvent in the final well for all test samples). In antagonist mode, 10× solutions of 0.5% DMSO (solvent control), 10× GW9662 (reference compound, final concentration range from 13 pM to 3.6 µM), or 10× test samples were premixed 1:1 with 10× rosiglitazone agonist (for a final concentration in the well of 32 nM), and 8 µL of the resulting mix was added to the respective wells for solvent control, reference compound, or sample (maximum 0.1% solvent in the final well for all test samples). The plate contents were then incubated for 16 h in a humidified 37 ºC/5% CO_2 incubator. At the end of incubation, 8 µL of 6× substrate mixture (provided in the kit) was added and the plate contents were incubated for a further 2 h in the dark at room temperature. Fluorescence was then read with a plate reader (BMG Fluostar Omega; BMG Labtech, Windsor, Vic, Australia) at 460 and 530 nm after excitation at 409 nm. Background fluorescence (determined in the cell-free control wells) was subtracted from all readings, and a β-lactamase expression ratio was calculated by dividing the net fluorescence at 460 nm by net fluorescence at 530 nm. Samples were deemed positive in agonist mode when they exceeded the EC_{10} (determined from the rosiglitazone standard curve) and in antagonist mode when they exceeded the IC_{20} (determined from the GW9662 standard curve).

3.3.3 Results and Discussion

None of the samples displayed detectable agonistic (all samples < 0.25 µg/L rosiglitazone equivalents) or antagonistic (all samples < 0.55 µg/L GW9662 equivalents) activity.

Despite the failure to find any PPAR-like activity in any of the water samples tested here, other research suggests that PPAR-like activity may be detected in some water samples (Liu et al., 2005) and that the PPAR may be stimulated by compounds in drinking water (P. Shi et al., 2012). The PPAR bioassay thus appears to be a relevant assay for water quality. The PPARγ-GeneBLAzer assay tested here was sensitive, reliable, repeatable, and relatively simple and would be well suited to routine testing. This is not to say that it is the only such assay, and other assays are available that may be equally suited to the task (see the literature review in Chapter 1).

One issue is that there are three known isoforms of the PPAR: α, β (sometimes also referred to as δ), and γ. Some compounds can selectively affect one isoform but not another (F. Leusch et al., 2012). In this work, we selected the γ isoform as it appears to be affected by a wide range of environmentally relevant compounds and is expressed in a wide variety of tissues, but further work should include PPARα (which also is induced by a wide range of environmentally relevant compounds) as an additional promising endpoint.

3.4 Switchgear Assay for Hypoxia

3.4.1 Introduction

The hypoxia response pathway protects cells during oxygen depletion (hypoxia) by activating genes that trigger, for example, increased oxygen transport and glucose uptake (Simmons, 2009). The commercially available hypoxia assay kit (Switchgear Genomics, Carlsbad, CA) was used to evaluate the hypoxia response in water samples. The assay uses the HT1080 cell line with transient transfection of three reporter constructs including LDHA promoter, H1F1a promoter, and ACTB housekeeping promoter from Switchgear, in which H1F1a is a well-known hypoxia-inducible TF (Xia et al., 2009). LDHA promoter was for the first time assessed in in vitro exposure from environmental waters, and ACTB housekeeping promoter was used for cell viability monitoring.

3.4.2 Materials and Methods

In brief, transfection reagent, which contained the three plasmid constructs (LDHA, H1F1a, and ACTB), was first thawed from -20 ºC and incubated for 30 min at room temperature. The human fibrosarcoma cell line HT1080 was thawed quickly in a 37 ºC water bath from the -80 ºC freezer, and then the thawed cells were added immediately into the growth medium, which was composed of EMEM (ATCC no. 30-2003), 10% normal fetal bovine serum (FBS), 1% of GlutaMax, and 1% of PenStrep. To get 20,000 cells per well, cell density was maintained at 2.1×10^5 cells/mL. Then the transfection reagents were mixed with the cell and medium solution at a ratio of 5:95. By using a multichannel pipette, 100 µL of the transfected cell mixture was aliquoted to each well of a white 96 well tissue culture plate. In a separate clear 96 well tissue culture plate, 100 µL of cells was aliquoted to 12 wells for visual monitoring of cell viability and growth. Both plates' contents were incubated at 37 ºC in a CO_2 incubator for 12 to 16 h. After overnight culture, the medium was replaced by 90 µL of fresh charcoal-stripped FBS growth medium and 10 µL of sample, which was already diluted in 10% of stripped medium in advance. After 24 h of exposure, 10 µL of the supernatant was transferred to a secondary white 96 well tissue culture plate, and both of the plates were frozen at -80 ºC for better sensitivity. Substrate and buffer solution were then added after the plates were thawed, and luminescence was quantified as a measure of luciferase activity (LightSwitch Dual Assay System, available in the kit). Desferrioxamine (DFO) was used as the positive control. Negative control and solvent control were included for quality control.

3.4.3 Results and Discussion

None of the samples displayed detectable hypoxia activity within both the LDHA and H1F1a promoters. However, this is the first time hypoxia activity was tested in environmental water. More samples should be explored in the future to assess the feasibility of adding hypoxia into routine water quality analysis, given that this assay is relatively reliable and easy to conduct in the laboratory.

3.5 Nrf2-keap Cell Line

3.5.1 Introduction

Oxidative stress, an imbalance between production and removal of ROS, can damage biological macromolecules, including DNA, proteins, and lipids. Oxidative damage to biological macromolecules can have profound effects on cellular functions and has been implicated in cancer, inflammation, neurodegenerative diseases, cardiovascular diseases, and aging. Eukaryotic cells have evolved antioxidant defense mechanisms to neutralize ROS and maintain cellular redox homeostasis. One of the most important cellular defense mechanisms against ROS and electrophilic intermediates is mediated through the antioxidant-responsive element (ARE or electrophile-responsive element) sequence in the promoter regions of Phase II and antioxidant genes. The ARE-dependent cellular defense system is controlled by the TF Nrf2. Recent advances in the mechanistic studies of this pathway have provided the following models for Nrf2 regulation: Keap1, a key player in the activation of this pathway, has been identified as a molecular switch turning and off the Nrf2-mediated antioxidant response (Zhang, 2006). The Nrf2-Keap1-ARE activation has been used to study prevention of human disease, including cancer (Zhang, 2010), and the screening of potential carcinogens from environmental pollutants, including arsenic (Lau et al., 2013) and organic micropollutants (Escher et al., 2012).

3.5.2 Materials and Methods

In this study, the human breast cancer cell line MDA-MB-231-745, which was transfected with the ARE luciferase plasmid was donated by Prof. Donna Zhang at the Department of Pharmacy, University of Arizona. The standard growth medium was composed of MEM (no. 11095-080; Life Technologies), 10% FBS, 1% L-glutamine, 0.1% gentamycin, 6 ng of insulin/mL, 2 mM HEPES and 1.5 µg of puromycin/mL. Cells were thawed from liquid nitrogen, and passaging was carried out in 75 cm^2 flasks every 4th day. The 4th generation cells at more than 80% confluence were used in this assay. Cell density was controlled at 2×10^5 cells/mL. By using a 8-channel pipette, 100 µL of the cell solution was seeded into one white 96 well plate and one clear 96 well plate (MTS cytotoxicity test). After overnight culture in a CO_2 incubator for 16 h (5% CO_2, 90% humidity), the medium was replaced by 90 µL of fresh growth medium and 10 µL of sample, which was already diluted in 10% of growth medium in advance. All samples were tested in triplicate including the medium blank and solvent blank. *tert*-Butylhydroquinone (tBHQ) was used as the positive control, and the solvent used was methanol. After another 16 h of exposure in the CO_2 incubator, the medium in the white plates was removed and washed with phosphate-buffered saline (PBS). Twenty-five microliters of passive lysis buffer (PLB) was then added, and the plates were shaken for 15 min before luciferase analysis. A Gen5 microplate reader with a delivery pump was used for the measurement, and the luminescence was read directly by well after the luciferase buffer (pH = 7.8) was added. For the cytotoxicity test, after 16 h of exposure the medium was replaced by 100 µL of clear fresh medium (without phenol red) and 20 µL of MTS solution (No. G3580; Promega). Absorbance at 492 nm was read after 2 h of incubation.

3.5.3 Results and Discussion

All water extracts were screened in three different doses, and solvents including methanol and DMSO were also tested at different concentrations. None of the samples showed significant response (IR > 1.5) nor cytotoxicity at the concentrations tested, whereas in

several of the samples we found the response was increasing with the enrichment fold. Those samples include Australian samples Eff1 and MF and the U.S. sample GV Inf (pilot influent and secondary effluent from the GV WWTP). The results suggest that oxidative stress is still a possible preliminary screen tool for environmental pollutants.

Another interesting result was that 0.25% of DMSO (final concentration in wells) could activate the Nrf2-Keap1-ARE pathway, whereas methanol was "safe" until a 2% concentration was attained in the well. A similar solvent effect was found in the AREc32 cell line (Escher et al., 2012). Given that most laboratories prefer DMSO as the carrier because it is friendlier to cells, more attention should be paid in the future to solvent influence in Nrf2 pathway research.

3.6 AREc32 Assay Indicative of the Oxidative Stress Response

3.6.1 Introduction

The AREc32 cell line was generated by Wang et al. (2006). AREc32 cells are derived from the human breast cancer cell line MCF7, with the addition of a luciferase gene construct attached to the ARE *cis* element. The antioxidant response of the AREc32 cells can be measured by luciferase expression. On the basis of the induction of reporter genes by the reference compound tBHQ, AREc32 cells gave substantially greater levels of induction than did HepG2 cells (Wang et al., 2006). The Nrf2-ARE toxicity pathway also is relevant for skin sensitization (Natsch, 2010), and the AREc32 cell line has been used as an in vitro screen for 116 reference chemicals and potential skin sensitizers. The results from AREc32 correlated well with the in vivo local lymph node assay results from studying mice (Natsch et al., 2009).

3.6.2 Materials and Methods

A detailed account of the successful implementation and of the experimental details of this bioassay is given by Escher et al. (2012).

3.6.3 Results and Discussion

To summarize, tBHQ served as the reference compound (Figure 3.2A), and its effect showed little variability over a long experimental period. The window between specific response and nonspecific cytotoxicity was fairly wide for the reference compound, and a large number of waterborne micropollutants was tested in a subsequent project (Escher et al., 2013). Also solvents showed only moderate cytotoxicity and induction (Escher et al., 2012).

The assay was very sensitive, as shown by the example of secondary treated effluent in Figure 3.2B, where effects were already detectable at a REF of 1 (log REF = 0), which corresponds to the native sample and where more than 10-fold induction occurred before cytotoxicity quenched the induction effect.

The assay was highly reliable with a good repeatability (same results day to day) and robustness (ease of performance and low variability) and has been applied in the meanwhile to drinking water DBPs (Neale et al., 2012) and other waterborne micropollutants (Escher et al., 2013). More results are presented in Chapters 5 and 6.

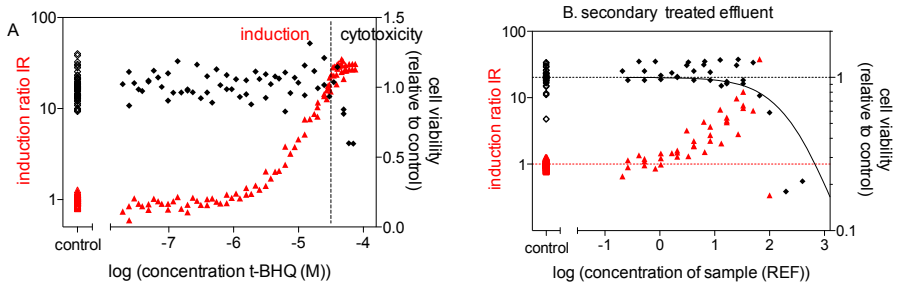

Figure 3.2. (A) Dose–response curve for the induction of the oxidative stress response by tBHQ (red diamonds) and cell viability (black empty diamonds); (B) extracted water sample.

Source: Both figures are adapted with permission from Escher et al., 2012. Copyright 2012, American Chemical Society.

3.7 NF-kappa B as a Measure of the Inflammatory Stress Response

3.7.1 Introduction

To measure the presence of compounds capable of activating the inflammatory stress response pathway NF-κB, we developed a new assay on the basis of detection of the chaperone-like inhibitory protein IκB in human lymphoblastoma cells (Jurkat E6.1 cell line). The principle of the assay is simple: under relaxed conditions, NF-κB is bound to IκB. Though bound to IκB, NF-κB is inactive. Upon inflammatory stimulation, IκB is activated by IκB kinase and degraded by ubiquitination. This process releases NF-κB, which is now free to enter the nucleus and trigger NF-κB-dependent inflammatory response genes. In the assay, the concentration of IκB is measured after exposure to the test compound. A decrease in IκB concentration indicates that the sample has initiated the inflammatory response.

3.7.2 Materials and Methods

In the assay, Jurkat E6.1 cells are resuspended in white medium (RPMI without phenol red supplemented with 5% charcoal-stripped FBS at 1×10^6 cells/mL (determined by using a Millipore Scepter Handheld Automated Cell Counter). Cells were then seeded at 200,000 cells/well by adding 200 µL of cell suspension to the 48 inner wells of a flat bottom standard 96 well plate, and the test samples were added in 50 µL of white media (maximum 0.1% final solvent concentration). The remaining wells were filled with 250 µL of PBS to act as a humidity barrier, and the plate contents were incubated for 24 h in a humidified 37 ºC/5% CO_2 incubator. A geometric dilution series of phorbol-12-myristate-13-acetate (PMA) was used as a reference compound, with final concentrations in the well ranging from 0.2 nM to 0.2 µM. After incubation, the content of each well was gently mixed and 200 µL was transferred to a v-bottom 96 well plate. The plate was centrifuged at 300 × *g* for 5 min, and 150 µL of the supernatant was discarded (paying particular attention not to disturb the cell pellet). The pellet was rinsed with 100 µL of warm sterile PBS, and the plate was centrifuged again at 300 × *g* for 5 min. After centrifugation, 100 µL of the supernatant was discarded (again paying particular attention not to disrupt the cell pellet). The IκB concentration in the cell pellet was then determined by using a commercially available ELISA kit (IκBα Total InstantOne ELISA, catalog no. 85-86061; eBioscience), with minor modifications. In brief, cells were lysed with 1.5× lysis mix added in the v-bottom 96 well plate directly and mixed

by aspirating/dispensing with a multichannel pipette and then placed on an orbital shaker at 300 rpm for 10 min at room temperature. Then 50 µL of cell lysate was transferred into the InstantOne assay plate (provided with the kit) followed by 50 µL of IκB antibody cocktail (provided with the kit). Negative and positive IκB controls, provided with the kit, were also tested with every ELISA run. The plate was covered with an adhesive seal, and its contents were incubated for 1 h at room temperature on a microplate shaker at 300 rpm. The wells were washed with 200 µL of wash buffer (provided with the kit), all liquid was removed by inverting on a paper towel, and 100 µL of detection reagent (provided with the kit) was added to each well. The plate contents were incubated for 10 min at room temperature on a microplate shaker at 300 rpm, and the reaction was stopped by adding 100 µL of stop solution. The absorbance of each well was then measured with a plate reader (BMG Fluostar Omega; BMG Labtech, Mornington, Vic, Australia) at 450 nm. Samples were deemed as positive when they exceeded the IC_{20} (determined from the PMA standard curve).

3.7.3 Results and Discussion

Low activity was detectable in several of the samples (for more details, see Chapters 5 and 6), and although it could be quantified in only four of the samples (because it was too low in the remaining samples for a reliable estimate), there was a clear trend of lower activity with increased treatment (in other words, microfiltration (MF) > reverse osmosis (RO) > advanced oxidation (AO) = ultrapure water laboratory blank). Four samples induced quantifiable activity in the assay: Eff1 (Australian treated secondary effluent) with 3 µg of PMA equivalents/L, sample DW (Australian drinking water) with 2.8 µg of PMA equivalents/L, sample SW (Australian stormwater) with 3.3 µg of PMA equivalents/L, and sample GV Inf (U.S. treated wastewater effluent) with 4.2 µg of PMA equivalents/L. All other samples had activity of <2.5 µg of PMA equivalents/L.

This assay measures an inflammatory cellular stress response, which could be induced by a variety of chemicals. This is a very novel assay, and it is not known what compounds are likely to induce this inflammation pathway. As is the case with other ASR pathways, it is likely to be induced by a wide range of compounds and is more useful as a rough measure of water quality and as a comparative tool rather than as an absolute response. In other words, it is useful here to show that advanced water treatment effectively removes the biological activity to levels comparable with ultrapure laboratory-grade water. On the basis of the results obtained here, this assay certainly seems relevant to water quality assessment, although it should be noted that it is relatively difficult to perform and can be relatively expensive (mostly because of the cost of the ELISA kit).

Additional experiments may further fine-tune the assay, specifically to address two issues: (1) sensitivity and (2) duration of exposure. In relation to sensitivity, as previously indicated there was a clear trend in activity measured in the samples, with treated secondary effluent > MF > RO > AO = ultrapure water. To avoid solvent toxicity, we dosed a maximum of 0.1% final solvent in the assay. This means a 1000× dilution in the assay, which combined with the 1000- to 2000× SPE enrichment factor resulted in in a REF of only 1−2. This REF could be improved (to allow reliable quantification in the cleaner samples) to 10−20 by either adding more solvent (if the assay allows it without adverse effect) or by concentrating the sample further (i.e., reconstituting the final aliquot in 100 µL instead of 1 mL, thus achieving an additional 10× from the SPE). Given that most of the assays require only 1−2 µL of each sample, this arrangement would still allow for several assays on the same sample (although some assays such as the Microtox or Ames do require significantly more volume). The issue of duration of exposure should also be investigated. For example, the IκB degradation is

expected to occur relatively quickly, and shorter exposure times may be possible (resulting in quicker turnaround). Furthermore, one of the genes induced by the NF-κB response is IκB itself. This means that the assay may be more sensitive with shorter exposure times. We tested the response at 1 h and 24 h and found that there was no significant effect on IκB after 1 h, whereas there was a clear concentration−effect curve after 24 h. It may be worth testing for an intermediate time (e.g., 6−12 h).

3.8 Glucocorticoid Receptor

3.8.1 Introduction

Glucocorticoids are a group of steroid hormones that regulate an array of physiological processes crucial for development, metabolism, electrolyte balance, cell proliferation, and differentiation (Odermatt et al., 2006; Bovee et al., 2011). Glucocorticoid dysfunction has been associated with a range of diseases including cardiovascular, inflammatory and immune diseases, osteoporosis, Type II diabetes, and obesity (Odermatt et al., 2006). The importance of glucocorticoids for adipogenesis also is receiving increasing interest (Sargis et al., 2010). Recent studies have demonstrated the potential ecotoxicological effects of glucocorticoid compounds on fish, including inhibited locomotion and aggressive behavior of rainbow trout. Natural and synthetic glucocorticoids have been widely applied as therapeutic pharmaceuticals as well as veterinary medicines, which are often used to restore muscle strength or as growth promoters. Despite the obvious significance and potential for environmental occurrence, glucocorticoid disruption has received far less interest than have estrogens and androgens.

In this phase of the study, a commercially available GR assay kit (Switchgear Genomics) was used to evaluate the GR activity in water samples. The GR-Switchgear assay integrates the signal from four validated pathway-specific reporter vectors by using the RenSP reporter gene. This is important, and unique to this assay, because there are numerous endogenous promoters for the gene and because no single promoter can respond to all potential agonists and antagonists. Multiple validated housekeeping reporters, using the CLuc reporter gene, also are applied to monitor cell "health" during the assay, also unique to this particular assay. The assay is a transient transfection assay, which means that plasmids containing the reporter genes are freshly transfected each time the assay is performed.

3.8.2 Materials and Methods

A human fibrosarcoma cell line (HT1080) was maintained in standard growth medium composed of 500 mL of minimal essential medium (MEM), 5 mL of GlutaMax, 50 mL of FBS (heat inactivated) and 5 mL of Pen/Strep. Cells were thawed from liquid nitrogen, and passaging was carried out in 75 cm^2 flasks every 2 to 3 days. The 2nd generation cells were used in this assay when they reached greater than 80% confluence. Cell density was controlled at 1×10^5 cells/mL in stripped growth medium (charcoal-stripped FBS without antibiotics). The transfection reagent containing the four GR plasmid constructs and housekeeping constructs was thawed, mixed, and incubated for 30 min at room temperature. The transfection mix was then added to cell medium and thoroughly mixed, and 100 µL of the cell mixture was then added to each well of a 96 well white tissue culture plate. In a separate 96 well clear tissue culture plate, an aliquot of 100 µL was also added in 12 wells for monitoring of cell viability and growth. Both plates' contents were incubated at 37 °C in a CO_2 incubator for 12 to 16 h (90% humidity). After overnight culture, the medium was replaced by 90 µL of fresh stripped FBS growth medium and 10 µL of water sample extract

diluted in 10% of stripped medium in advance. After 18 to 24 h of exposure, 10 µL of the cell supernatant was transferred to a secondary white 96 well tissue culture plate and both plates were frozen at -80 ºC. Substrate and buffer solutions were added after the plates' contents were thawed. Luminescence was measured to determine for the luciferase reporter gene activity (LightSwitch Dual Assay System). Dexamethasone (DEX) was used as the positive control, and negative control and solvent control were always included for quality control.

3.8.3 Results and Discussion

The GR-Switchgear assay results were highly reliable with good repeatability across the sampling events. The reference compound DEX exhibited an EC_{20} of 0.65 nM in the GR-Switchgear assay, which is similar to another commercially available GR kit also evaluated (GR-GeneBLAzer) (Figure 3.3).

Of the samples evaluated, four U.S. samples and three Australian samples were found to exhibit significant activity when the GR-Switchgear assay was used. Those U.S. samples include GV Inf, Chlor, Ozone, and RR Eff (see Chapter 5 for sample description), whereas the Australian samples include Eff1, MF, and Eff2 (see Chapter 6 for sample description). These results indicate that GR activity can be significant in wastewater effluents, even after powerful oxidative treatment such as ozonation. Additional results for GR activity are provided in Chapters 5 and 7 of this report.

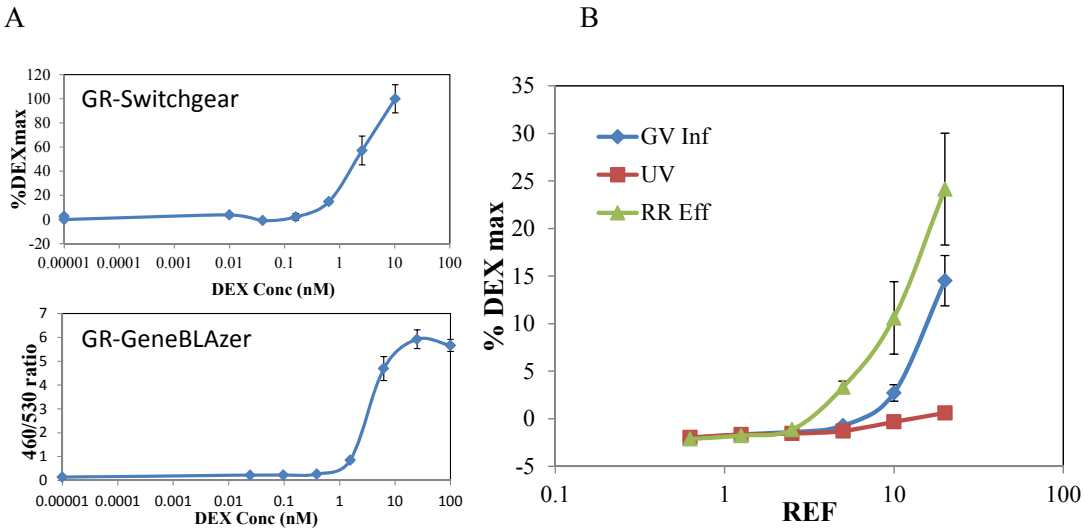

Figure 3.3. (A) GR dose–response of reference compounds DEX; (B) GR-Switchgear dose–response of three detected U.S. samples.

Notes: GV Inf = Green Valley WWTP influent; UV = Green Valley UV treated; RR Eff = Roger Road WWTP effluent.

3.9 T-Screen for Endocrine Disruption of the Thyroid Receptor

3.9.1 Introduction

The thyroid hormones (THs) 3,5,3'-triiodothyronine (T_3) and thyroxine (T_4) are important for controlling growth and development and for maintaining metabolic homeostasis (DeVito et al., 1999). Several classes of environmental contaminants or their metabolites can alter TH homeostasis through interference with the TH signal transduction pathway and associated cellular functions (Gutleb et al., 2005). These compounds or metabolites are known to interact at the TH gland, TH metabolism, or TH receptor or with TH transport proteins. For most chemicals and mixtures, the potential effects on TH functions are still unclear, and there are no standard methods to analyze whether a chemical or an environmental sample has TH disrupting potential. Gutleb et al. (2005) developed an in vitro bioassay on the basis of TH-dependent cell proliferation by using a rat pituitary tumor cell line GH3 to study the interference of xenobiotics with T_3–receptor interaction at the cellular level.

3.9.2 Materials and Methods

In this assay, GH3 cells were obtained from American Type Culture Collection (ATCC) and were maintained in Dulbecco's MEM (DMEM) with sodium pyruvate, L-glutamine, and high glucose (Gibco no. 11995065) and were supplemented with 10% (v/v) fetal calf serum. Passaging was carried out in 75 cm^2 flasks every 4th day, and 48 h before seeding of the cells into 96 well microplates for the dosing experiment, the culture medium was changed to serum-free PCM medium. PCM medium is made from DMEM:F12 (Gibco no. 1041025) supplemented with 10 µg of bovine insulin/mL, 10 µM ethanolamine, 10 ng of sodium selenite/mL, 10 µg of human apotransferrin/mL, and 500 µg of bovine serum albumin/mL. Cells were released by using a cellscraper and were aspirated several times with a pipette as they detach easily and are sensitive to trypsine. We tested different cell densities (2500, 5000, and 10,000 cells/well per 100 µL of medium) for the optimum seeding procedure as the cells

do not attach well onto the 96 well microplates. We then added 800 µg of fetuin/mL to the PCM medium to promote attachment, spreading, and growth of cells after consultation with Dr. Merijn Schriks (Schriks et al., 2006). All samples were tested in triplicate, including a medium blank and solvent blank. The exposure period was 96 h, and the cell proliferation was measured with the resazurine assay. The dye resazurine is nonradioactive, nontoxic, and water soluble and is commonly used for cell proliferation determination. Enzymes in the mitochondria of GH3 cells reduce oxidized blue resazurine to the highly fluorescent pink complex resorufin, and the fluorescence is a measure for the number of viable cells present. Eight microliters of a 400 µM resazurine solution prepared in PBS was added to each well after the 96 h of exposure. The plate contents were then incubated in the dark for 4 h, and the fluorescence was measured with an excitation wavelength of 530 nm and an emission wavelength of 590 nm with a Fluostar Omega plate reader.

3.9.3 Results and Discussion

The reference compound T_3 had an EC_{50} of $7.40 \pm 1.82 \times 10^{-10}$ M, which is similar to the reported values in the literature (e.g., 1.90×10^{-10} M in Gutleb et al. [2005] and 2.10×10^{-10} M in Schriks et al. [2006]). However, the environmental samples showed variable results during the four replicate experiments. Induction of T-Screen activities was found in Eff1 and MF samples in one set of experiments; however, the effects could not be reproduced during the other exposure experiments. This might occur because PCM medium uses only bovine serum albumin and fetuin as protein sources and because the cells did not attach well during the 96 h exposure. We also tried different types of 96 well microplates to enhance the attachment of GH3 cells in PCM medium but did not find any improvement. We noticed that one major drawback of this assay was the long incubation period of environmental extract in a small volume of PCM medium, and cell proliferation was perhaps not as sensitive as found in other receptor-mediated bioassays. In the last few years a number of luciferase reporter gene assays specifically targeting the TH receptors were developed for water quality assessment (Freitas et al., 2011; Kusk et al., 2011; Chinathamby et al., 2013) (Huang et al., 2011a; Shi et al., 2011; Sun et al., 2012). These assays required only a small volume of environmental extract, and the response could be detected after 24 h of exposure. These luciferase reporter gene assays may be more appropriate for environmental samples for future testing.

3.10 P15/A19 Assay for Induction of the Retinoic Acid Receptor (RAR)

This study was performed in collaboration with Klara Hilscherova of RECETOX, Brno, who also provided the cell line.

3.10.1 Introduction

Novak et al. (2007) transfected an embryonal mouse carcinoma cell line with a RARE reporter plasmid (first established by Pachernik et al. [2005]) and tested the resulting RAR reporter gene assay with sediments and air extracts. Although the air extracts did not exhibit any activity (Novak et al., 2009), the sediment extracts modulated the activity of the P15/A19 cells that were treated with 32 nM a*t*RA but exhibited no effect on the cells when exposed in the absence of a*t*RA (Novak et al., 2007). A series of PAHs, among them benzo[a]pyrene, showed the same effect if the cells were incubated with 32 nM a*t*RA but had no activating

effect when dosed alone. Similar results were found in a study assessing a range of PAHs and N-PAHs (Benisek et al., 2008).

3.10.2 Materials and Methods

Cells were grown in Dulbecco's MEM with sodium pyruvate, and L-glutamine, high glucose, 10% FBS, 1% penicillin−streptomycin, and 1.6% nonessential amino acid (NEAA) were obtained from Gibco, Mulgrave, Australia. Cells were grown in T75 flasks in 11 mL of DMEM and were incubated at 37 ºC and 5% CO_2 and passaged every 2−3 days when cells were 70% confluent.

For an exposure experiment, the cell concentration was adjusted to 100,000 cells/mL and 100 µL was transferred to each well of a white polystyrene tissue culture treated 96 well microplate (Corning). The plate contents were then incubated for 24 h at 37 ºC and 5% CO_2 and were dosed with the appropriate amount of chemical or extract. Each plate should include one serial dilution of a*t*RA ($3.23E^{-11}$ M to $3.85E^{-18}$ M) or 9-*cis* RA ($2.43E^{-07}$ M to $2.89E^{-14}$ M) as positive control and one row of medium only. The plates were then covered with PCR-SP plate sealer from Axygen, and their contents were incubated for 24 h before cytotoxicity or induction was assessed. A typical experiment consisted of two steps, each of which was performed in duplicate. First, a range finder with a serial (twofold) dilution series was performed, where induction of the RAR pathway and cytotoxicity were evaluated. Interference by cytotoxicity suppresses the induction signal, and those concentrations cannot be used for the induction data evaluation. Second, concentrations/dilutions of the water sample were chosen that were not cytotoxic and a linear dose−response curve was measured for induction only. Often the window between induction and cytotoxicity was small and no maximum induction could be reached; therefore, concentrations should be chosen in a way that the maximum IR is 5.

As a control, the cell viability was assessed with the MTS assay. MTS (tetrazolium) is bioreduced by cells into an aqueous, soluble formazan product by dehydrogenase enzymes found in metabolically active cells (Mosmann, 1983). When cells die, they rapidly lose the ability to reduce these products because of mitochondrial dysfunction. The absorbance of the formazan product at 490 nm can be measured directly from 96 well assay plates without additional processing, and the amount is directly proportional to the number of living cells in culture. After 24 h of incubation, the medium in each plate was replaced by 120 µL of MTS (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay [Promega] with MTS and phenazine methosulfate as the electron coupling reagent) in Hyclone DMEM without phenol red (Thermo Scientific) and absorbance at 492 nm was read after 2 h of incubation.

As no log-logistic concentration effect curves could be obtained with reference compounds, it was not possible to calculate the samples' BEQ.

3.10.3 Results and Discussion

We started with the establishment of concentration−effect curves for positive and negative controls. a*t*RA and 9-*cis*-RA served as reference compounds for the derivation of the toxic equivalencies. Other known inducers of the RAR signaling pathway were also tested. All of these reference compounds showed good and reproducible concentration−effect curves, and 9-*cis*-RA was chosen as the reference chemical for this bioassay.

Also, the solvents methanol and DMSO were tested to evaluate the appropriate carrier for sample extracts and to ensure that the solvents had no effect on the cell viability and induction of the RAR. It was observed that none of the solvents were inducers of the RAR, exhibiting an average IR value of about 1.0. The EC_{10} for cytotoxicity was about 3.2% for methanol and 1.2% for DMSO. Both are extrapolations and subject to high uncertainty, but we can clearly see that, above 1% of solvents, the cell viability started to be impaired. Thus, among the two tested solvents, methanol proved to be an appropriate choice of carrier solvent for the chemical/water samples, without interfering with the induction of the samples at levels lower than 0.1%.

To assess the effect of environmental chemicals, we merged the list of chemicals regulated in the Australian Drinking Water Guidelines (ADWG) (NHMRC and NRMMC, 2011) and the Standards for Quality of Recycled Water Supplied to Augment a Supply of Drinking Water (NWQMS, 2008) with the list of active inducers of the RAR reported by Martin et al. (2010) by using the "FACTORIAL" assay developed by Attagene, Inc. that can simultaneously screen 25 NRs or 48 TF response elements in HepG2 human liver carcinoma cell lines. Those included lindane, propioconazole, fipronil, and prometryn. Further experiments were done with benzo[a]pyrene (BaP). Experiments with these compounds were done alone and in the presence of a constant concentration of the potent inducer 9-*cis*-RA.

Piperonyl butoxide had an IR of 1.7 in Martin et al. (2010) but did not exceed the threshold of effect (IR = 1.5) in our assay. There was a slight increase of IR, but it was masked by cytotoxicity setting in at 10 µM. The same observation was made for propionconazole (maximum IR of 5.1 in Martin et al. [2010]). Dicamba was not very active (maximum IR of 1.6 in Martin et al. [2010]), which was consistent with our observation of an IR of 2 before cytotoxicity started to take over. Lindane showed a maximum IR of 4.2 in our experiments, which was only slightly lower than the IR of 5.9 in Martin et al. (2010). Fipronil and prometryn both did not cross the threshold value of IR at 1.5. A slight tendency to increase in IR was masked by cytotoxicity setting in. In contrast, the maximum IRs were 2.9 and 2.4, respectively, in Martin et al. (2010).

Benzo[a]pyrene (BaP) was shown in previous work to have no effect on its own but increased the effect of a*t*RA (Benisek et al., 2008) (Figure 3.4). We observed a weak effect of BaP alone with an $EC_{IR1.5}$ of 12 ± 6 µM (CV 56%, 3.1 ± 1.7 mg/L). This concentration exceeds the solubility limit of BaP of 2 µg/L, but the medium proteins were sorbing a large fraction and thus stabilising BaP in solution. There was a clear enhancing effect of BaP in the presence of 0.76 nM 9-*cis*-RA (Figure 3.4), thus replicating the findings of Benisek et al. (2008).

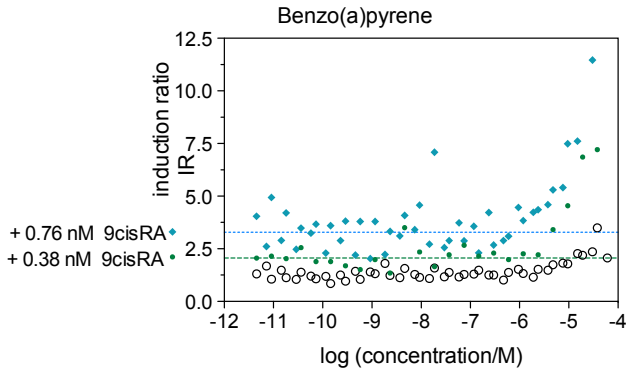

Figure 3.4. Effect of benzo[a]pyrene alone (black circles) and in the presence of a constant background concentration of 0.38 and 0.76 nM 9-cis-RA in the P15/A19 assay.

None of the water samples tested showed any response in the P15/A19 assay as is detailed in Chapter 6. However, if a constant concentration of the reference compound 9-*cis*-RA was added to the bioassay, an increasing effect with increasing concentration of the water sample could be observed.

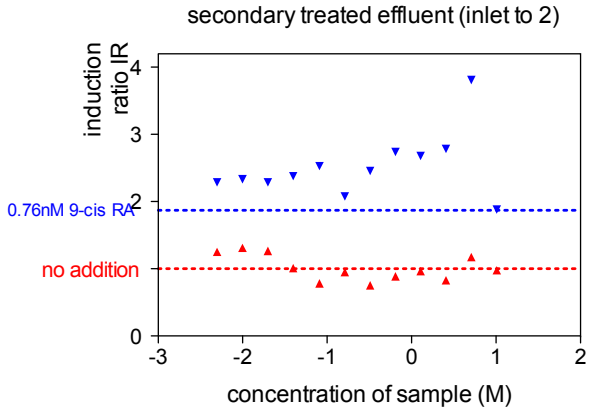

Figure 3.5. Effect of a water sample (secondary treated effluent) in presence (blue) and absence (red) of 0.76 nM 9-cis-RA in the P15/A19 assay.

The effects are subtle and visible only in more polluted samples (e.g., wastewater); therefore, we do not recommend this assay for application in water quality assessment.

3.11 Human Neuroblastoma Cells (SK-N-SH Cells)

3.11.1 Introduction

Initially, the aim was to develop a cell-based bioassay to detect interference with sodium channels as a measure of neurotoxicity. A similar bioassay has been developed previously to detect sodium channel blockage caused by exposure to cyanobacterial toxins such as saxitoxin in mouse (Neuro-2a) cells and human (SK-N-SH) neuroblastoma cells (Manger et al., 2003). The assay relies on the neurotoxicity of veratridine, a natural compound that forces and keeps sodium channels open, eventually causing cell death from ion imbalance. In the assay, neuroblastoma cells are coincubated with the sample and a high concentration of veratridine. Exposure to veratridine should cause cell death, but if the sample contains sodium channel blockers (such as saxitoxin), the cells are "rescued" from veratridine-induced cell death. We had hoped to use this mechanism to detect chemical sodium channel blockers (such as lidocaine or carbamazepine). Although there was some initial concern about differences in the binding site of the blocker, with natural toxins binding on the extracellular side and most pharmaceuticals on the intracellular side, it seemed that both veratridine and anesthetic drugs affected the same subunit of the sodium channel (Wang and Wang, 2003) and that it would thus be possible to block the effect of veratridine with chemical sodium channel blockers. Unfortunately, that was not the case, and we were unable to rescue veratridine- or brevetoxin (another natural toxin that opens sodium channels)-induced cell toxicity in human neuroblastoma cells (SK-N-SH) with any of the compounds we tested even at concentrations as high as their limit of solubility (millimolar range): the pharmaceuticals lidocaine and carbamazepine and the pesticide pyrethrum.

As an alternate measure of neurotoxicity, we defaulted back on measuring acute cytotoxicity on neuronal cells as a measure of "neurotoxicity", as has been done previously (Ba et al., 2003; Lee et al., 2005). The idea is that measuring cytotoxicity with neuronal cells may provide a measure of acute toxicity specific to neuronal cells and hence a (coarse) measure of neurotoxicity.

3.11.2 Materials and Methods

In the assay, human neuroblastoma cells (SK-N-SH) were resuspended in white media (DMEM/F12 without phenol red supplemented with 5% FBS, 1× nonessential amino acids and 2 mM Glutamax (Life Technologies) at 1×10^5 cells/mL (determined by using a Millipore Scepter Handheld Automated Cell Counter). By use of a multichannel pipette, 200 µL of cell suspension was added to every well (20,000 cells/well) of a standard flat bottom 96 well plate, and the plate contents were incubated for 24 h in a humidified 37 ºC/5% CO_2 incubator. The media were then removed by aspiration and were replaced with 200 µL of fresh white media containing the test sample (maximum solvent concentration of 0.5%), and the plate contents were incubated 21 h in a humidified 37 ºC/5% CO_2 incubator. The media were then aspirated, the wells rinsed with 150 µL of PBS, the PBS was aspirated, and 150 µL of neutral red media (50 µg of neutral red solution/mL, prepared fresh) was added. The plate contents were then incubated a further 3 h in a humidified 37 ºC/5% CO_2 incubator. At the end of the incubation, the media was aspirated, the wells rinsed with 150 µL of PBS, the PBS was aspirated, and 150 µL of neutral red desorbing fixative (1% acetic acid, 50% ethanol, prepared in ultrapure water) was added. The plate was placed on an orbital shaker at 600 rpm for 10 min, and the absorbance was read at 540 nm in a plate reader (BMG Fluostar Omega; BMG Labtech, Mornington, Vic, Australia). DMSO was used as a reference compound, with

an IC_{10} and IC_{50} of approximately 50 and 500 mM, respectively. Samples were deemed as "neurotoxic" when cytotoxicity exceeded IC_{10} (determined from the DMSO standard curve).

3.11.3 Results and Discussion

None of the water samples (see interlaboratory studies in Chapters 5 and 6) displayed detectable "neurotoxic" activity, with all samples causing less than 10% cell death.

A recent study has shown that, whereas acute toxicity using cell lines was able to detect a range of hepato- and neurotoxicants, cells sourced from different tissues did not appear to show tissue-specific toxicity observed with whole animals (Hu et al., 2013). This finding would suggest that a cytotoxicity assay with a neuronal cell line is not a specific measure of "neurotoxicity" but simply a measure of basal cytotoxicity in a different cell line. This arrangement means that the assay used here is of limited use, when other, simpler cytotoxicity assays are available. There is, however, still a need for a cell-based assay specific for neurotoxicity. In this project, we were unable to apply a well-validated neurotoxicity assay for cyanobacterial toxins, possibly because of a difference in sites of action of the cyanobacterial toxins and of chemical sodium channel blockers. It may be possible to find a chemical alternative to veratridine that competes with the same site as the chemical sodium blockers (such as carbamazepine and lidocaine), which would then allow an adaptation of this assay relevant for environmental chemicals (instead of cyanobacterial toxins) that act as sodium channel blockers.

Chapter 4

Sample Preparation for Bioanalytical Assessment

4.1 Introduction

Natural water samples contain not only the trace organic pollutants that are our target of interest but also salts, metals, and natural organic matter. Prior to testing, water samples (especially less polluted samples such as surface water, recycled water, and drinking water) must be enriched to allow quantification of a measurable response, which can then be back calculated to effects or toxic equivalents of the original water sample.

Enrichment can be done by liquid-liquid extraction or SPE (Escher and Leusch, 2012). As a positive side effect of extraction, matrix components such as salts and metals and most, but not all, of the natural organic matter are removed. This residual natural material can disturb the functioning of cell-based bioassays. Cell-free bioassays are not suitable for applications in water quality monitoring because they have no means to differentiate specific effects from nonspecific effects, namely, the specific inhibition of an enzyme from its nonspecific denaturation by the water matrix or other trace organic compounds (Neale and Escher, 2013). In cell-based bioassays, in contrast, the cell viability may serve as control and only concentrations of a water extract that are not cytotoxic will be used for evaluation of the specific effect, such as the induction of a certain toxicity pathway.

Bioanalytical tools can detect all biologically active compounds in a water sample but only if those compounds are successfully extracted from the water phase and recovered during sample concentration. Previous studies have looked at the extraction efficiency of different methods but are usually focused on specific classes of compounds (such as pharmaceuticals [Escher et al., 2005]) or a particular bioassay endpoint (such as estrogenic activity [Leusch et al., 2006]).

For this component of the project, we looked at the recovery efficiency of various SPE material and liquid-liquid extraction for a wide range of micropollutants. This understanding is critical to our appreciation of bioanalytical results.

The project was carried out in two stages: Stage 1 was designed to compare the recovery of different SPE and liquid-liquid extraction techniques to allow selection of an optimal method, and Stage 2 was designed to test the influence of a natural matrix (in this case river water) on the extraction efficiency of the selected method.

4.2 Materials and Methods

The water samples were spiked with a wide variety of pesticides, pharmaceuticals, hormones, and industrial compounds to determine the recovery efficiency of the method for compounds with a wide range of physicochemical properties (Figure 4.1).

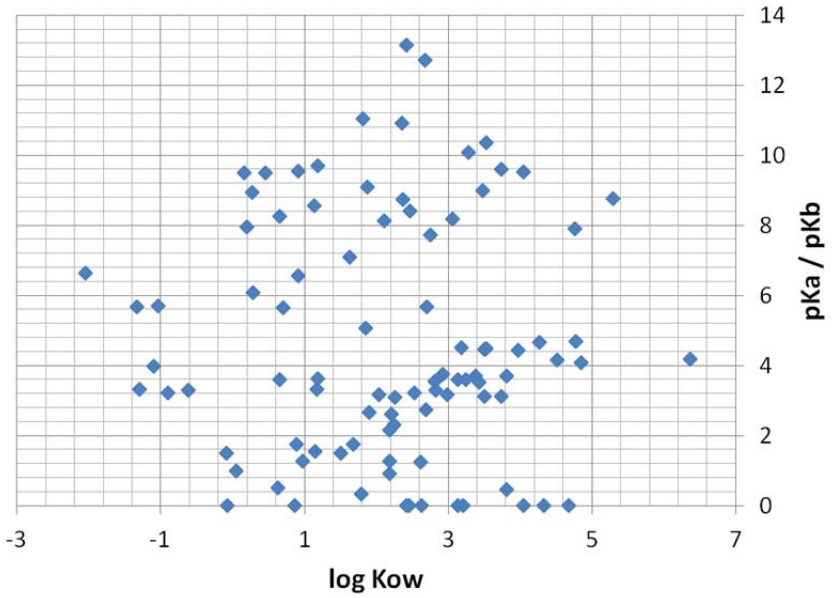

Figure 4.1. Acid dissociation constant (pKa) vsersus octanol−water partition coefficient (K_{ow}) for the pesticides spiked in this study, showing the wide range of physicochemical properties of the spiked compounds.

4.2.1 Stage 1—Comparison of Different Material and Extraction Methods with Pure Water

In the first stage, ultrapure laboratory water was spiked with 179 pesticides at 1 µg/L and 84 pharmaceuticals and herbicides at 20 ng/L. The pH of the spiked water was adjusted to pH 2 or pH 7, and the samples were extracted in duplicates using eight different extraction methods: six SPE (Table 4.1) and two liquid-liquid extraction methods.

Table 4.1. Solid-Phase Extraction (SPE) Cartridges Used in This Study

Cartridge	Size (Sorbent/ Cartridge)	Distributor	Catalog No.
Oasis HLB	200 mg/6 mL	Waters Corp., Rydalmere, NSW, Australia	WAT106202
Supelco SupelSelect HLB	200 mg/6 mL	Sigma-Aldrich, Castle Hill, NSW, Australia	54183-U
Varian Bond Elut PPL	500 mg/6 mL	Agilent Technologies, Mulgrave, Vic, Australia	12255001
Strata X	500 mg/6 mL	Phenomenex, Lane Cove West, NSW, Australia	8B-S100-HCH
Supelco Supelclean coconut charcoal	2 g/6 mL	Sigma-Aldrich, Castle Hill, NSW, Australia	57144-U
Varian Bond Elut Carbon	500 mg/6 mL	Agilent Technologies, Mulgrave, Vic, Australia	12252201

For the SPE, the SPE cartridges were preconditioned by passing 2 × 5 mL of acetone:hexane 50:50, 2 × 5 mL of methanol, and 2× 5 mL of ultrapure water by gravity. One liter of the spiked water was then passed by vacuum (up to 2.6 kPa) through the 6 mL cartridges (Table 1). After passage of the full 1 L, the cartridges were air dried on the manifold for a minimum of 30 min until visibly dry and were stored at 4 ºC until ready for the next step. The cartridges were eluted with 2 × 5 mL methanol and 2 × 5 mL acetone:hexane 50:50, allowing the solvent to pass through the sorbent bed by gravity first, and were finished by applying a vacuum to pull all the solvent off the cartridge. The 20 mL eluate was pooled and evaporated to dryness at 40 ºC under a gentle nitrogen stream, reconstituted in 2.5 mL of methanol, and split into three aliquots for the different analysis methods: 1 mL for LC-MS/MS analysis, 1 mL (solvent exchanged into dichloromethane) for gas chromatography (GC)-MS/MS analysis, and 0.5 mL for archiving.

For the liquid-liquid extraction, 500 mL of the spiked water was mixed with 200 mL of either ethyl acetate (EthA) or methyl *tert*-butyl ether (MTBE) on a shaker for 30 min. The solvent was recovered by using a separatory funnel, and the operation was repeated twice more with 50 mL of solvent. The pooled 300 mL solvent was evaporated to dryness in a rotary evaporator, reconstituted in 2.5 mL of methanol, and split into three aliquots for the different analysis methods: 1 mL for LC-MS/MS analysis, 1 mL (solvent exchanged into dichloromethane) for GC-MS analysis, and 0.5 mL for archiving.

4.2.2 Stage 2—Performance of the Selected Method with Spiked Drinking and River Water

After selection of a combination of Waters Oasis HLB and Supelco Supelclean coconut charcoal SPE methods on the basis of the results of Stage 1 and those from a previous project (NWC, 2011), the performance of this method was tested in more-relevant environmental matrices such as drinking and river water.

Metropolitan tap water and river water samples were collected, the river water was filtered (Millipore AP20), and half the samples were spiked with 12 endocrine disrupting compounds (hormones and industrial xenoestrogens) at 50 ng/L, 215 pesticides at 0.8 µg/L, and 88

pharmaceuticals and herbicides at 30 ng/L. The pH of the river water samples was adjusted to pH 2 or pH 7 (only pH 7 for the drinking water), and the samples were extracted in duplicates by using the following SPE method.

The SPE cartridges were preconditioned separately by passing 2× 5 mL of acetone:hexane 50:50, 2 × 5 mL of methanol, and 2 × 5 mL of ultrapure water by gravity. The cartridges were then stacked, with a Waters Oasis HLB cartridge on top of a Supelco Supelclean coconut charcoal cartridge. One liter of the spiked and unspiked water samples was passed by vacuum (up to 2.6 kPa) through two cartridges in series. After passage of the full 1 L, the cartridges were separated and air dried on the manifold for a minimum of 30 min until visibly dry and were stored at 4 ºC until ready for the next step. The cartridges were eluted with 2 × 5 mL methanol and 2× 5 mL acetone:hexane 50:50, allowing the solvent to pass through the sorbent bed by gravity first, and were finished by applying a vacuum to pull all the solvent off the cartridge. The two 20 mL eluates were pooled and evaporated to dryness at 40 ºC under gentle nitrogen stream, reconstituted in 3 mL of methanol, and split into four aliquots for the different analysis methods: 1 mL for LC-MS/MS analysis, 0.5 mL (solvent exchanged into dichloromethane) for pesticide GC-MS analysis, 0.5 mL (solvent exchanged into dichloromethane) for endocrine disrupting compound GC-MS analysis, and 1 mL for archiving.

4.2.3 Chemical Analysis

All samples from stages 1 and 2 were analyzed by using standard methods at Queensland Health Forensic and Scientific Services (QHFSS) laboratory.

Pesticides were analyzed by gas chromatograph equipped with quadrupole mass spectrometer (GC-MS) for multiscreening of organochlorine, organophosphorus, synthetic pyrethroid pesticides, and some herbicides by using a standard protocol (QHFSS Document No. 16315: Organochlorine, Organophosphorus and Synthetic Pyrethroid Pesticides, Urea and Triazine Herbicides and PCBs in Water). Surrogates used included 2-nitro-*m*-xylene, decachloroPCB, triphenylphosphate, pyrene-D10, and dibromobiphenyl.

Pharmaceuticals and herbicides were analyzed by LC-MS/MS by using a standard protocol (QHFSS Document No. 27701: PPCP in Water, Preparation and Analysis by SPE and LCMSMS).

Endocrine disrupting compounds (spiked only in Stage 2) were derivatized with *N,N*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) and were analyzed by GC-MS by using a standard protocol (QHFSS Document No. 25391: Determination of Endocrine Disrupting Compounds in Effluent, River, and Recycled Water).

Total (and dissolved) organic carbon was measured by using a Shimadzu TOC-V CSH total organic carbon analyzer at the Smart Water Research Centre.

4.3 Results and Discussion

4.3.1 Stage 1—Comparison of Different Materials and Extraction Methods with Pure Water

The results show that most compounds are well recovered by most of the SPE materials selected for comparison and confirm the wide retention spectrum of HLB sorbent (Figure 4.2). The Supelco Supelclean coconut charcoal cartridge retained the fewest compounds and had the lowest

median recovery but had been previously shown to be relatively effective at capturing amines such as NDMA (NWC, 2011). For this reason, we decided to combine an Oasis HLB cartridge with the Supelco coconut charcoal cartridge in Stage 2.

Lowering the pH to 2 resulted in a minor improvement in both median extraction recovery and the number of compounds recovered (Figure 4.2).

Both liquid-liquid extraction (LLE) techniques yielded an average extraction recovery for the compounds selected in this study similar to that of the SPE methods (Figure 4.2); however, the recovery efficiency was significantly more variable between different compounds (as indicated by the larger standard error with the LLE samples, Figure 4.2, left) and twice as many compounds were not recovered with LLE as with SPE methods (Figure 4.2, right). The LLE methods also used significantly more solvent than did the SPE methods (600 mL/L vs 20 mL/L) and left an insoluble residue after evaporation. LLE also can create emulsions at the interface between the solvent and the water, which can make extraction of some water samples difficult (Wells, 2002).

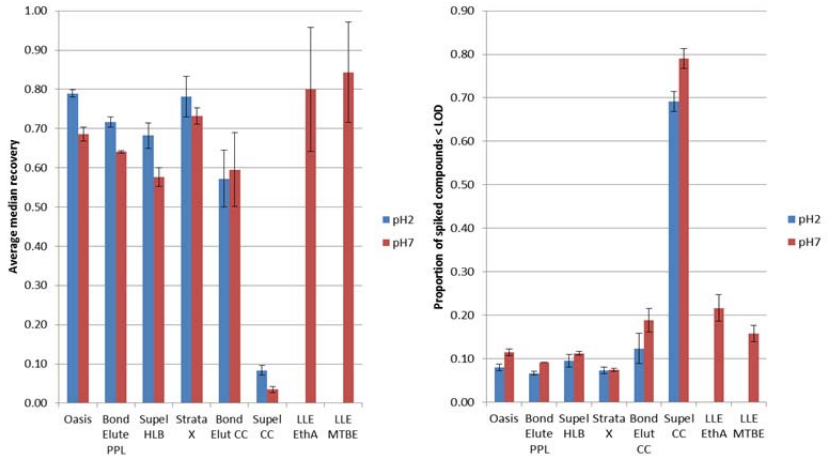

Figure 4.2. Average median recovery (left) and proportion of spiked compounds below the limit of detection (right) ± standard error for the different solid-phase media and liquid extraction methods at pH 2 (blue) and pH 7 (red).

4.3.2 Stage 2—Performance of the Selected Method with Spiked Drinking and River Water

A few pesticides and pharmaceuticals were detected at low nanograms-per-liter concentrations in the river water sample (data not shown), and the spike recovery is therefore calculated as (spiked–unspiked)/spiked concentration.

The recovery of the combined Oasis HLB/Supelco CC method was very good, with an average recovery of 89 to 93% in both river and drinking water (Figure 4.3). The more complex river water sample did not affect the recovery efficiency, suggesting that the extraction method is sufficiently robust to deal with a moderate level of organic matter (Table 4.2).

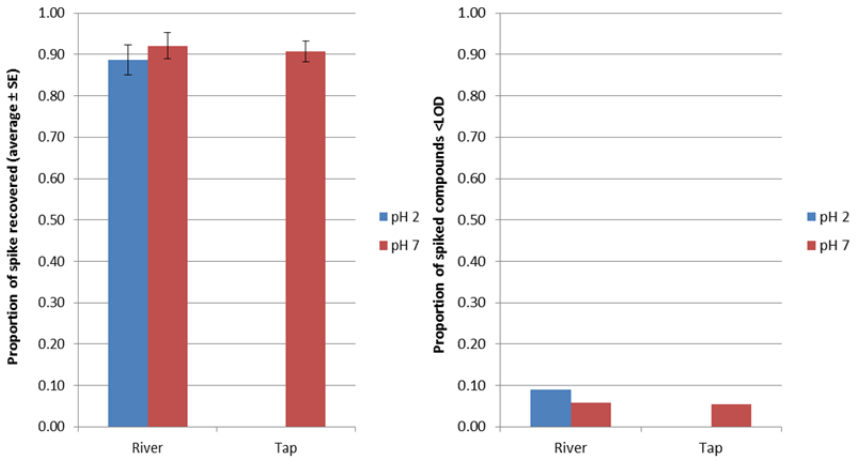

Figure 4.3. Average recovery ± standard error (left) and proportion of spiked compounds below the limit of detection (right) for compounds spiked in river and tap water at pH 2 (blue) and pH 7 (red) using a tandem Oasis HLB/Supelco coconut charcoal solid-phase extraction cartridge.

Table 4.2. Total Organic Carbon in the Water Samples Used in This Study

Sample Type	TOC Concn
Ultrapure laboratory water	0.15 mg/L
Tap water	2.05 mg/L
River water (filtered)	8.31 mg/L

Note: TOC = total organic carbon.

4.4 Conclusions

Using a combination of Oasis HLB and Supelco coconut charcoal cartridges in series results in good recoveries of a wide spectrum of micropollutants even in environmental water samples. This extraction technique provides a sound method for extraction and concentration of water samples for bioassay analysis.

Chapter 5

Stage 1 Interlaboratory Study: 10 U.S. Water Samples, 3 Laboratories, 39 Bioassays

5.1 Introduction

The core of the validation plan was an interlaboratory comparison study. This interlaboratory comparison study did not focus on a strict interlaboratory comparison of one assay with a shared standard operating procedure (SOP) but rather aimed at a comparison of the performance of similar bioassays targeting the same endpoint/toxicity pathway and/or mode of toxic action.

The interlaboratory comparison proceeded in two stages: in Stage 1, only the core laboratories were involved and a larger number of water reuse matrices and more quality assurance/quality control controls (e.g., mixtures of reference chemicals, mixtures of regulated chemicals, etc.) were used. Overall 20 samples were included in Stage 1: 10 samples collected in Australia and 10 samples collected in the United States. In Stage 2, the 10 Australian samples were sent out to additional 17 laboratories worldwide and these laboratories ran between 1 and 10 bioassays on each of the samples. In this report the results of the application of the bioassays in the three core labs of the samples collected in the United States are discussed in Chapter 5, whereas the results of the three core labs on the Australian samples were integrated in the larger interlaboratory comparison and Chapter 6 discusses the results of the interlaboratory comparison of the 10 Australian samples involving a total of 20 laboratories that performed 137 individual experiments in a total of 100 distinctly different bioassays.

As membrane processes are relatively well characterized with respect to removal of micropollutants and also are included in the samples described in Chapter 6, the focus of the samples taken in the United States was on the one hand on a classical water reclamation facility (WRF) that has been in operation for more than 50 years and on the other hand on a novel innovative AO processes (AOPs) for water recycling. The first site site was the Roger Road WRF, which was established in 1951. It has a capacity to treat 41 MGD and serves Tucson, AZ. The Roger Road WRF is comprised of headworks for primary treatment, clarifiers, biotowers and secondary clarifiers. After chlorination the water is used for irrigation of golf courses or recharged into the Sweetwater Recharge Facilities that are comprised of several recharge basins and the Sweetwater Wetlands. Samples were taken from the Roger Road WWTP and from two monitoring wells of the Sweetwater Recharge Facilities. The second investigated site was a pilot AOP plant, where secondary effluent from Green Valley WWTP was treated with various AOPs.

5.2 Participating Laboratories and Choice of the Bioassays

The three core laboratories participated in this study, covering five large categories of modes of action and conducting 39 different bioassays. The detailed information is provided in Table 5.1.

Table 5.1. Bioassays by the Categories Assigned in Figure 3.1

Lab	Xenobiotic Metabolism	Specific Modes of Action	Reactive Modes of Action	Induction of ASR Pathways	General Cytotoxicity and Models for System
GU	PPARγ GeneBLAzer, Anti-PPARγ GeneBLAzer	ER-CALUX, anti-ER_CALUX, AR-CALUX, anti-AR-CALUX, PR-CALUX, anti-PR-CALUX, GR-CALUX, anti-GR-CALUX, GR-GeneBLAzer, TR-CALUX		Jurkat E6-I	Caco-2 NRU, SK-N-SH, THP1 cytokines
UA		YES, YAS, GR-Switchgear	Ames TA98 -S9, Ames TA98 +S9, Ames TAmix -S9, Ames TAmix +S9	Hypoxia-Switchgear, nrf2-MDA-MB	Nrf2 cell viability, GR-Switchgear CLUC cell viability, Hypoxia Switchgear ACTB cell viability
UQ	AhR-CAFLUX	Algal photosynthesis inhibition, E-SCREEN, T-Screen	*umu*C -S9, *umu*C +S9, Ames TA100 -S9	AREc32	Microtox, AREc32 cell viability, Algal growth inhibition

Notes: GU = Smart Water Research Centre, Griffith University; UA = University of Arizona; UQ = Entox, The University of Queensland.

5.3 Water Samples

5.3.1 Sample Collection

Ten grab samples were collected during February and April 2012 from various sites in Pima County, AZ. Green Valley WWTP secondary effluent (GV Inf) serves as the inlet to the AOP pilot plant (Wedeco). Two samples were taken from this WWTP, including GV Inf and Chlor (WWTP secondary effluent after chlorine), and four samples were taken from the pilot plant after UV, ozone, ozone/UV, and UV/H_2O_2 treatment (Table 5.2).

The final effluent from Roger Road Wastewater Reclamation Facility (sample RR Eff) is from Tucson, AZ. TW1 and TW2 were collected from two Tucson water monitor wells after infiltration of tertiary municipal wastewater at the Sweetwater Recharge Facility, which receives the effluent of the Roger Road WWTP (Table 5.2). The field blank was ultrapure water (MilliQ water), which was held in the same storage condition, and the same SPE process was used as for the samples.

Table 5.2. Sample Name, Collecting Site, and Date for U.S. Samples

Name	Description	Location	Sampling Date	Vol Sampled
FB	Field blank	Green Valley pilot plant	2012/2/22	4 L
GV Inf	Pilot influent (secondary effluent from GV WWTP)	Green Valley WWTP	2012/2/22	4 L
UV	Pilot flow rate 2000 L/h, UV 500 mJ/cm^2	Green Valley pilot plant	2012/2/22	4 L
UV/H_2O_2	Pilot flow rate 2000 L/h, UV 500 mJ/cm^2, 10 mg of H_2O_2/L	Green Valley pilot plant	2012/2/22	4 L
O_3	3 mg of O_3/L	Green Valley pilot plant	2012/2/22	4 L
O_3/H_2O_2	O_3/UV (3.0 mg of O_3/L followed by 500 mJ of UV/cm^2)	Green Valley pilot plant	2012/2/22	4 L
Chlor	Chlorinated (10 mg of Cl_2/L, 2 h contact time)	Green Valley WWTP	2012/2/22	4 L
RR Eff	Roger Road WWTP secondary effluent	Roger Road WWTP	2012/3/22	4 L
TW1	Tucson Water Monitoring Well no. 1 WR-068B	Sweetwater Recharge Facility	2012/4/16	4 L
TW2	Tucson Water Monitoring Well no. 2 WR-092B	Sweetwater Recharge Facility	2012/4/16	4 L

Figure 5.1. Sampling sites (from Google Maps).

5.3.2 Sample Extraction

The SPE was performed according to Macova et al. (2011) with the sorbent material validated by NWC (2011). Samples containing chlorine were quenched after collection with sodium thiosulfate (50 mg/L), and 1 g of sodium azide/L was added, and then the samples were stored at 4 ºC. All samples went through a glass fiber filter (GF/A; Whatman) before extraction. One-liter batches of samples were passed through two 6 mL tandem solid-phase cartridges in series, first an Oasis® HLB (500 mg, catalog no. 186000115; Waters) and then a Supelclean coconut charcoal cartridge (2 g, catalog no. 57144-U; Sigma-Aldrich). Both types of cartridges were individually preconditioned prior to extraction with 10 mL of 1:1 acetone:hexane mixture, followed by 10 mL of methanol and 10 mL of MilliQ water. After SPE, 2 × 5 mL of ultrapure water was used to rinse the cartridges, and a vacuum was applied for 2 h to dry the sorbent bed. All cartridges were then stored at -20 °C until elution. For elution, 10 mL of methanol and 10 mL of acetone:hexane (1:1) were used, and then the eluate of two cartridges was combined and evaporated under purified nitrogen gas. The resulting mixture was solvent exchanged to methanol and brought to a final volume of 1 mL. The extracts were split and then sent to GU and UQ by overnight shipping.

5.3.3 Data Evaluation

The experimental data were evaluated as described in Chapter 2.

In this study, $EC_{IR1.5}$ evaluation was used in the mutagenicity assay, genotoxicity assay, and oxidative stress assay where either EC_{10}/EC_{20} was difficult to obtain or the dose−response curve was unclear.

For the Ames II mutagenicity test, we replaced the control value ($signal_{control}$) with a baseline value for calculation, on the basis of the definition of mutagenicity itself (revertant numbers falling below twofold induction of the baseline were generally not considered positive). In order to maintain data consistency, we chose $EC_{IR1.5}$ as the result output. Baseline values were

calculated as the sum of the mean number of positive wells in negative control and the standard deviation of positive wells in negative control.

5.4 Results and Discussion

5.4.1 EC Values of Reference Compound

Typical known bioactive compounds, which showed response in the literature evaluated, were chosen as the positive controls as well as the reference compound for each assay. For the Caco-2 and SK-N-SH cytotoxicity test, because absolute positive control was not confirmed, we used the solvent itself (DMSO or methanol) as the reference compound. For the specific assay cell viability test (Nrf2-MTS, SG Cluc, SG ACTB), the same reference compounds as the assay were used. In the Ames II mutagenicity test, given the potential binomial distribution character of this assay (Heringa et al., 2011), the U.S. lab reported only the $EC_{IR1.5}$ as the final evaluation without EQ transformation. But for TA100 and *umu*C conducted in UQ, the BEQ calculation remains because the entire dose curve was obtained for the reference compounds.

Different assays were conducted for the same endpoints in the three labs, which makes drawing parallel comparisons between the lab results more difficult, but the EC values of the reference compounds allow BEQ values to be calculated from each assay. From the results, we see that the sensitivity of the human cell-line receptor assay is much higher than that of the yeast-based assay (ER vs YES, AR vs YAS). For the same endpoint with different cell lines, the sensitivity was also different but on a more similar level (E-SCREEN vs ER-CALUX, GR-CALUX vs GR-Switchgear vs GR-LifeTech). The EC values of the reference compounds in most assays were nanomolar levels (or even lower), which allowed the toxicity test of real water samples where many compounds occur in the nanograms-per-liter range.

Table 5.3. EC Value of Reference Compound in Different Bioassays for U.S. Samples

Bioassay	Endpoint	Reference Compounds	EC Value of Ref.	Result Expression
Bioluminescence inhibition assay	Bacterial cytotoxicity	Phenol	EC_{10} = 10.1 µM	Baseline-TEQ
Caco-2 NRU	Human cytotoxicity	MeOH	EC_{10} = 0.38 M	Baseline-TEQ
SK-N-SH	Neurotoxicity	DMSO	EC_{20} = 0.28 M	Baseline-TEQ
THP1-cp	Immunotoxicity	DEX	EC_{20} = 4.98 nM	DEX-TEQ
I-PAM	Phytotoxicity	Diuron	EC_{50} = 6.0 nM	DEQ
Nrf2-MTS	Human cytotoxicity	tBHQ	EC_{10} = 57.1 µM	tBHQ-TEQ
SG Cluc	Human cytotoxicity	DEX	EC_{10} = 0.15 nM	DEX-TEQ
SG ACTB	Human cytotoxicity	Desferrioxamine (DFO)	EC_{10} = 1.5 µM	DFO-TEQ
*umu*C	Genotoxicity	w/o S9: 4-NQO	$EC_{IR1.5}$ = 63.5 nM	TEQ
		w/ S9: 2-AA	$EC_{IR1.5}$ = 0.29 µM	
TA98	Ames II mutagenicity test	w/o S9: 4-NQO		
		w/ S9: 2-AA		
TAmix		w/o S9: 4-NQO		
		w/ S9: 2-AA		
TA100		w/o S9: Nitrofurantoin (NF)	$EC_{IR1.5}$ = 80 µM	NF-TEQ
E-SCREEN	Estrogenic effects	17β-Estradiol (E2)	EC_{20} = 0.118 pM	EEQ
ER-CALUX		17β-Estradiol (E2)	EC_{20} = 0.226 pM	EEQ
ER-CALUX-Anta		Tamoxifen (TMF)	EC_{20} = 2 nM	TMF-EQ
YES		17α-Ethynylestradiol (EE2)	EC_{20} = 455.6 pM	EE2-EQ

Bioassay	Endpoint	Reference Compounds	EC Value of Ref.	Result Expression
AR-CALUX	Androgenic effects	Dihydrotestosterone (DHT)	EC_{20} = 2 pM	DHT-EQ
YAS		Testosterone (TTR)	EC_{20} = 3.2 nM	TTR-EQ
AR-CALUX-Anta		Flutamide (FLU)	EC_{20} = 0.9 µM	FLU-EQ
GR-CALUX	Glucocorticoid effects	DEX	EC_{20} = 1.0 nM	DEX-EQ
GR-Life Tech			EC_{20} = 0.8 nM	
GR-Switchgear			EC_{20} = 0.65 nM	
GR-CALUX-Anta		Mifepristone (MIF)	EC_{20} = 3.03 nM	MIF-EQ
GR-Life Tech-Anta		Mifepristone (MIF)	EC_{20} = 0.09 nM	
PR-CALUX	Progesteronic effects	Levonorgestrel	EC_{20} = 0.75 nM	LEV-EQ
PR-CALUX-Anta		Mifepristone (MIF)	EC_{20} = 8.5 pM	MIF-EQ
T-Screen	Thyroid effects	Triiodothyronine (T3)	EC_{20} = 3.0 nM	T3-EQ
TRβ-CALUX			EC_{20} = 0.85 pM	
AhR-CAFLUX	Binding to Ah receptor	2,3,7,8-TCDD	EC_{20} = 0.65 pM	TCDD-EQ
PPAR	Glucose, lipid, and fatty acid metabolism	Rosiglitazone	EC_{20} = 0.83 nM	ROS-EQ
PPAR-Anta		GW9662	EC_{20} = 6 nM	GW-EQ
Hypoxia	Stress response pathway for Oxygen depletion	Desferrioxamine (DFO)	EC_{20} (H1F1a) = 15 µM	DFQ-EQ
Jurkat cell line	Immunotoxicity	PMA	EC_{20} = 5 nM	PMA-EQ
MDA-MB cell line assay	Oxidative stress indicative of the keap-Nrf2-ARE pathway	tBHQ	$EC_{IR1.5}$ = 44 µM	tBHQ-EQ
AREc32 cell line assay			$EC_{IR1.5}$ = 2.3 µM	

Note: EEQ = estradiol equivalent.

5.4.2 Bioassay Activity of U.S. Samples

5.4.2.1 Activity Summary

The detailed EC and EQ results for all U.S. samples are listed in Table 5.4. The bioassay response was determined not only by the assay's intrinsic sensitivity but also by the sample enrichment factor (REF). If there was no significant response found under the applied REF in this research, the EC values were expressed as greater than the maximum REF used in this project, whereas "ND" implied "not detected under current maximum REF".

Table 5.4 Summary of All EC and EQ Data of the U.S. Samples

Bioassay	Effect Concentration	Value for:									
		FB	**GV Inf**	**UV**	**UV/H_2O_2**	**O_3**	**O_3/H_2O_2**	**Chlor**	**RR Eff**	**TW1**	**TW2**
Bioluminescence	EC_{10}	23.2	7.1	24.7	47.1	4.7	12.0	9.7	4.1	9.6	12.4
	BEQ (µM)	0.70	3.53	0.99	0.96	4.57	1.29	1.93	3.96	1.34	0.96
NRU	EC_{10}	>10	>10	>10	>10	>10	>10	>10	>10	>10	7.3
	BEQ (M)	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.05
I-PAM	EC_{50}	>333	210	110	105	260	240	>333	68	>333	>333
	BEQ (nM)	ND	0.029	0.055	0.057	0.023	0.025	ND	0.088	ND	ND
SK-N-SH	EC_{20}	0.9	>1	>1	>1	>1	>1	>1	1.0	1.0	>1
	BEQ (M)	0.32	ND	ND	ND	ND	ND	ND	0.32	0.28	ND
THP1-cp	EC_{20}	>1	0.8	>1	>1	1.0	>1	>1	>1	>1	>1
	BEQ (nM)	ND	6.7	ND	ND	5.5	ND	ND	ND	ND	ND
Nrf2-MTS	EC_{10}	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
	BEQ (µM)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SG Cluc	EC_{10}	>10	12.6	15.6	>10	>10	>10	9.4	7.8	>10	>10
	BEQ (nM)	ND	0.012	0.009	ND	ND	ND	0.016	0.017	ND	ND
SG ACTB	EC_{10}	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
	BEQ (µM)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
*umu*C w/o S9	$EC_{IR1.5}$	>139	105	73	60	90.5	60	130	50	105	110
	BEQ (nM)	ND	0.605	0.87	1.058	0.702	1.058	0.488	1.27	0.605	0.577
*umu*C w/ S9	$EC_{IR1.5}$	>139	34	23	10.5	27	23	44	110	110	>139

Bioassay	Effect Concentration	Value for:									
		FB	**GV Inf**	**UV**	**UV/H_2O_2**	**O_3**	**O_3/H_2O_2**	**Chlor**	**RR Eff**	**TW1**	**TW2**
	BEQ (µM)	ND	0.009	0.013	0.028	0.011	0.013	0.007	0.003	0.003	ND
Ames II TA98 w/o S9	$EC_{IR1.5}$	>100	>100	>100	>100	>100	>100	>100	30.5	>100	>100
Ames II TA98 w/ S9	$EC_{IR1.5}$	>100	>100	72	>100	>100	>100	>100	3.5	>100	>100
Ames II TA100 w/o S9	$EC_{IR1.5}$	>20	0.57	0.85	0.45	0.35	0.35	7.2	2.7	>20	4.3
	BEQ (µM)	ND	140.3	94.1	177.8	228.6	222.2	11.1	29.6	ND	18.6
Ames II TAmix w/o S9	$EC_{IR1.5}$	>100	83	35	69	59	48	69	8.5	>100	>100
Ames II TAmix w/ S9	$EC_{IR1.5}$	>100	66	36	>100	75	55	48	29.5	>100	84
E-SCREEN	EC_{20}	>27	0.35	4	2.4	0.50	4.7	1.05	0.85	5.6	>15
	BEQ (pM)	<DL	0.525	0.011	0.019	0.269	0.094	0.105	0.233	0.023	<DL
ER-CALUX	EC_{20}	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	BEQ (pM)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
YES	EC_{20}	>200	>200	>200	>200	>200	>200	>200	60.1	>200	>200
	BEQ (pM)	ND	ND	ND	ND	ND	ND	ND	7.576	ND	ND

Bioassay	Effect Concentration	Value for:									
		FB	**GV Inf**	**UV**	**UV/H_2O_2**	**O_3**	**O_3/H_2O_2**	**Chlor**	**RR Eff**	**TW1**	**TW2**
Anti-ER-CALUX	EC_{20}	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	EQ (nM)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AR-CALUX	EC_{20}	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	BEQ (pM)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Anti-AR-CALUX	EC_{20}	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	BEQ (µM)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
YAS	EC_{20}	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200
	BEQ (nM)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
GR-CALUX	EC_{20}	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	BEQ (nM)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Anti-GR-CALUX	EC_{20}	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	BEQ (nM)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
GR-Life Tech	EC_{20}	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	BEQ (nM)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Anti-GR-Life Tech	EC_{20}	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	BEQ (nM)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
GR-Switchgear	EC_{20}	>10	11.2	>20	>10	16	>10	10.2	16	>10	>10
	BEQ (nM)	ND	0.058	ND	ND	0.041	ND	0.064	0.041	ND	ND
PR-CALUX	EC_{20}	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	BEQ (nM)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Bioassay	Effect Concentration	Value for:									
Anti-PR-CALUX	EC_{20}	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	BEQ (pM)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
TRβ-CALUX	EC_{20}	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	BEQ (pM)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AhR-CAFLUX	EC_{20}	>100	32	10.1	33	34	51	16	6.1	>100	>100
	BEQ (pM)	ND	0.020	0.064	0.020	0.019	0.013	0.041	0.107	ND	ND
PPAR	EC_{20}	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	BEQ (nM)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Anti-PPAR	EC_{20}	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
	BEQ (nM)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Hypoxia	EC_{20}	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
	BEQ (μM)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Jurkat	EC_{20}	>1	1	>1	>1	>1	>1	>1	>1	>1	>1
	BEQ (nM)	ND	5.0	ND	ND	ND	ND	ND	ND	ND	ND
Nrf2-MDA-MB	$EC_{IR1.5}$	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
	BEQ (μM)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AREc32	$EC_{IR1.5}$	>67	22	>67	37	60	67	30	12	>160	>160
	BEQ (μM)	ND	0.105	ND	0.062	0.038	0.034	0.077	0.192	ND	ND

Table 5.4 provides a list of the bioassays applied. Initial screening of two WWTP effluents (GV Inf and RR Enf) shows that, compared with field blanks (experiment control), several of the assay provided positive responses. Because 1/EC values were used here, the closer to the middle of the cycle, or the shorter the distance to the field blank, the weaker the response (Figure 5.2). Given that two WWTPs were municipal WWTPs that received predominantly domestic water, the results provides evidence of limited bioactivity within the assays evaluated. It should be noted that, on the basis of the limited enrichment fold for some assays (maximum REF = 1), more studies should be conducted using higher enrichment factors.

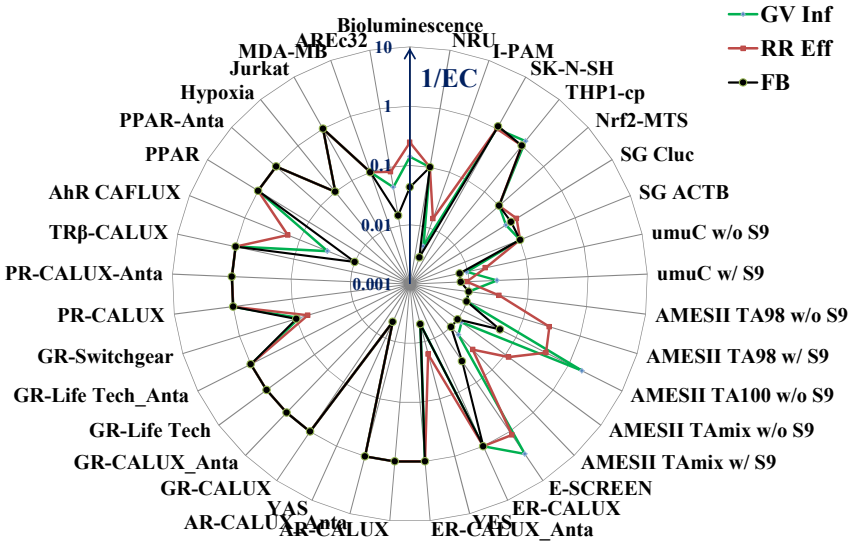

Figure 5.2. Comparison of all EC values between two WWTP effluents with field blank.

Notes: For those ND samples in Table 5.4, use the maximum REF as replacement. The 1/EC is plotted to have a large signal for higher toxicity.

Given the attenuation effects of the treatment process employed, the bioluminescence inhibition test, umuC (w/S9), Ames II (TA100 w/o S9), E-SCREEN, and AhR all appeared to have positive detections within the enrichment factors utilized (Figure 5.5).

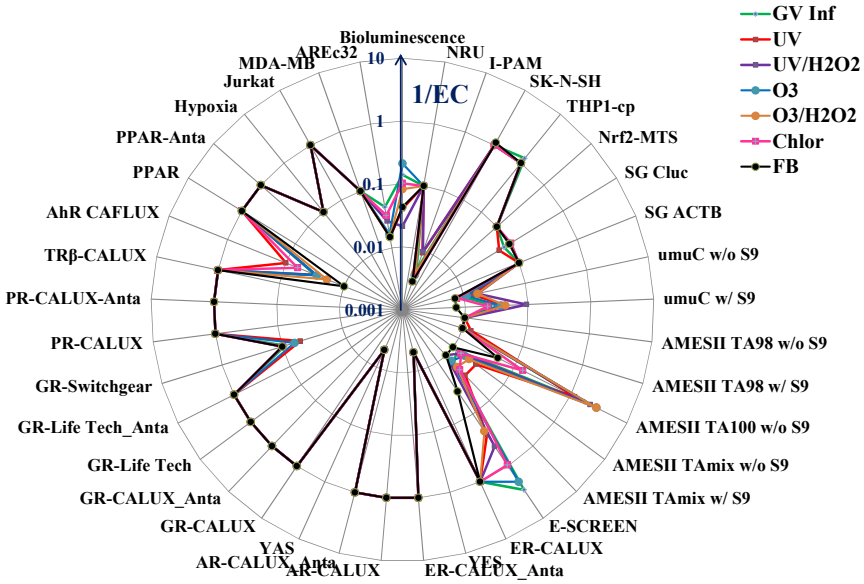

Figure 5.3. Comparison of all EC values between Green Valley pilot plant and field blank.

Note: For those ND samples in Table 5.4, use the maximum REF as replacement.

Although groundwater infiltration appeared to be effective for the removal of AREc32, AhR, Ames II, and E-SCREEN indicated bioactivity (Figure 5.4).

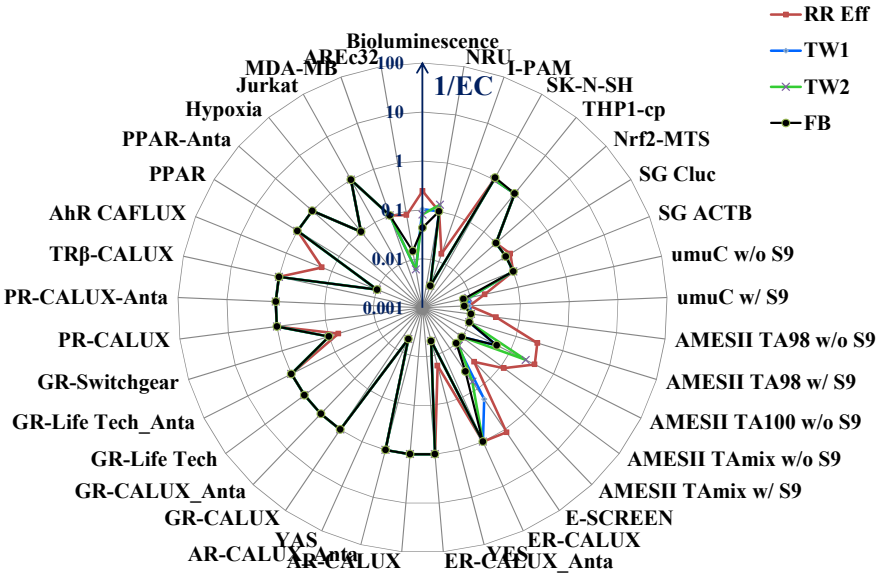

Figure 5.4. Comparison of all EC values between Roger Road effluent and the receiving infiltration waters and the field blank.

Note: For those ND samples in Table 5.4, use the maximum REF as replacement**.**

5.4.3 Cytotoxicity

Cytotoxicity is the most common and direct measurement of cell viability. It also is used to assist the induction assay for the judgment of positive/negative response. In this research, if the cytotoxicity (cell viability inhibition) was larger than 50%, then the induction data were not used for that specific dose or for higher doses. Thus, in those induction assay-related cytotoxicity tests including Nrf2-MTS, SG-Cluc, and SG-ACTB, we did not always achieve the entire dose curve because of cytotoxicity at higher doses. For two specific cytotoxicity tests including bioluminescence inhibition and I-PAM phytotoxicity, we were able to achieve the entire dose–response. It is interesting that UV and UV-based treatment (UV/H_2O_2) increased the phytotoxicity (lower EC_{50} values) while decreasing bacterial cytotoxicity (bioluminescence inhibition). However, ozone, ozone/H_2O_2, and chlorination treatment did not significantly impact cytotoxicity.

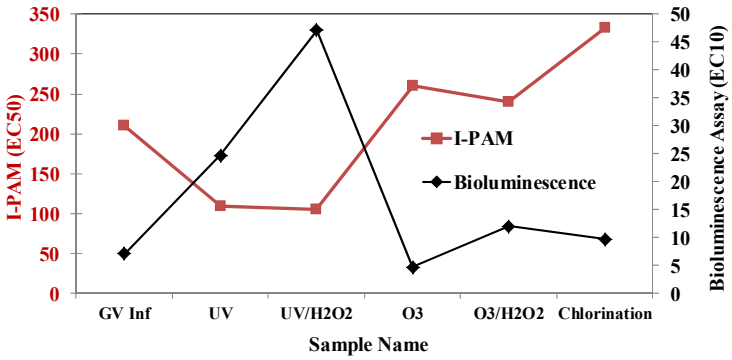

Figure 5.5. Comparison of all EC values of Green Valley samples in bioluminescence inhibition and I-PAM phytotoxicity test.

5.4.4 Induction of Xenobiotic Metabolism

In the xenobiotic metabolism study, two bioassays were carried out: AhR-CAFLUX and PPAR. The AhR assay showed a positive response in samples, whereas the PPAR assay had no response in any of the samples tested. One possible reason was the relatively low concentration factors (REF) of the samples in the PPAR assay as compared to those in the AhR-CAFLUX assay. The highest dose (sample REF) in AhR-CAFLUX was about 100, whereas in PPAR it was conducted below 1. Another reason was the different detection limits, with an EC_{20} for AhR-CAFLUX being approximately 1000× lower than for the PPAR assay.

Xenobiotic metabolism was active in seven out of nine samples, and the EC_{20} values ranged from 6.1 to 51 REF. Tucson Water samples (TW1 and TW2) did not yield a positive response, which indicated that the infiltration process could reduce the xenobiotic metabolism activity from the original wastewater source.

5.4.5 Mutagenicity and Genotoxicity

Mutagenicity and genotoxicity exhibited significant responses in U.S. samples. Both assays rely upon *S. typhimurium* bacterial strains; however, *umu*C is related to DNA repair (Oda et al., 1985) and AMES to mutation revertance (Ames et al., 1975). Three different bacterial

strains were tested for Ames II; however, for TA98 (frameshift mutation) no positive results were found in most of the U.S. samples except RR Enf (Table 5.4). Mutagenic responses were found in both TA100 and TAmix strains, which targeted different locations of base-pair substitution. The $EC_{IR1.5}$ of TA100 was much lower than for TAmix (w/o S9) and showed that TA100 had higher sensitivity than TAmix did in detecting mutagenicity for the U.S. samples. This finding possibly was attributable to the diluted strain density of TAmix (a six-strain mixture from TA7001 to TA7006).

The similar EQ values achieved among the different Green Valley samples suggest that AOPs using ozone and UV were ineffective in reducing mutagenicity and genotoxicity using both *umu*C and Ames II tests. However, chlorination appeared to significantly attenuate the effects (Figure 5.6).

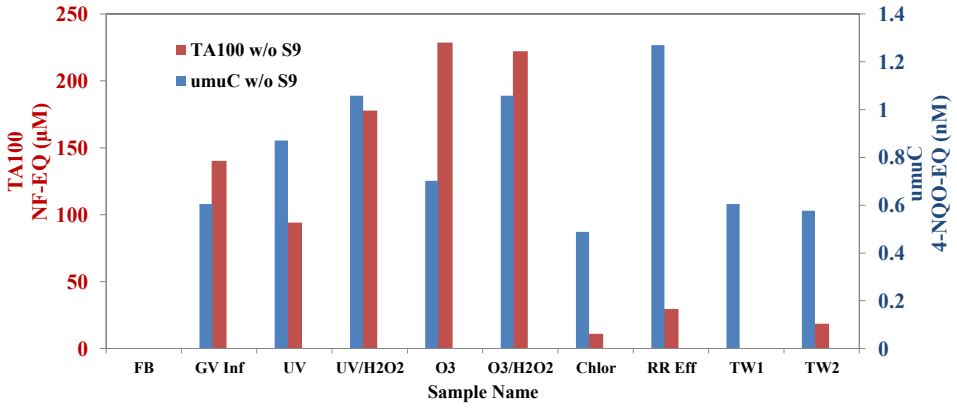

Figure 5.6. EQ values of all U.S. samples in umuC and Ames II-TA100 (w/o S9) tests.

The rat liver enzyme S9 also showed differences between the Ames II and *umu*C tests. The lower ECIR1.5 values obtained with the presence of S9 in the *umu*C test compared with values found in the test without S9 indicated that more compounds showed genotoxicity after metabolic activation. However, no significant difference was observed with the Ames II TAmix test with and without S9.

In the absence of S9, a potential correlation existed between the *umu*C and Ames II tests in all of the U.S. samples (Figure 5.7). The result suggests that, if one of these two assays was conducted, it had a high potential that the other one might also show a positive result. However, the linear relationship between results with and without S9 under TAmix strains and the poor relationship between results with and without S9 under the *umu*C test suggested that *umu*C with S9 and Ames II without S9 might have more relevance for water quality monitoring.

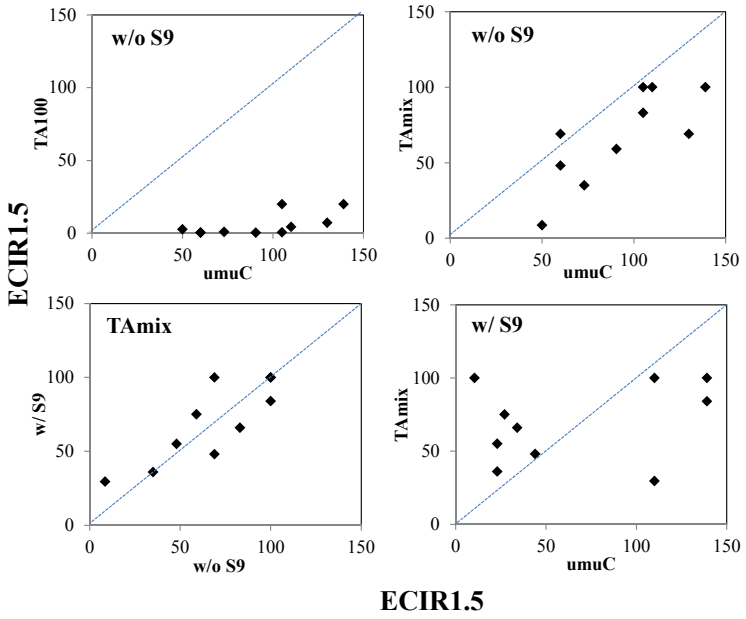

Figure 5.7. Relationship between mutagenicity and genotoxicity.

5.4.6 Endocrine Disruption: Estrogen Activity

The various assays have unique advantages and limitations due to the estrogenicity detection mechanisms. The YES assay utilizes recombinant yeast to detect activation of the human ER, whereas the E-SCREEN is based on cell proliferation in estrogen-dependent human breast cancer cells. The ER-CALUX (Legler et al., 1999; Sonneveld et al., 2005) measures regulation of the human ER through luciferase activity via luminescence (Murk et al., 2002; Van der Linden et al., 2008). In this study, the E-SCREEN was the only bioassay that showed positive responses among the four estrogenic assays evaluated. One reason may be the detection limit for the E-SCREEN being lower than those of other bioassays in the same category. The EC_{20} value for the E-SCREEN was as low as 0.118 pM E2 equivalent, whereas the EC_{20} value of YES was 455.6 pM EE2 equivalent (~4000-fold that of E-SCREEN).

Another possible reason was the relatively low concentration factors (REFs) of the samples used for the ER-CALUX compared with those for the E-SCREEN, although their detection limits were similar. The highest dose (sample REF) in the E-SCREEN was about 27, whereas in the ER-CALUX it was conducted below 1.

In all bioassays for estrogenicity, the field blank was under the detection limit. The bioassay screening also demonstrated that UV-based treatment (UV and UV/H_2O_2) could reduce ~98% of estrogenicity from the pilot influent (GV Inf), whereas O_3 and O_3/H_2O_2 could reduce 20~50% of estrogenic activity. The results suggest that UV and UV/H_2O_2 treatment was more efficient in estrogenic compound elimination than was ozone; however, actual doses and operating conditions would need to be carefully evaluated to draw meaningful conclusion.

5.4.7 Endocrine Disruption: Glucocorticoid Activity

Glucocorticoid activity was detected in one of the three GR cell line assays. Given their similar sensitivity for the reference compound DEX, a possible reason for the difference in

detection might have been the relatively lower sample enrichment factor (REF) in the other two assays.

Four of the 10 U.S. samples showed glucocorticoid activity including GV Inf, Ozone, Chlor, and RR Eff, with the EC_{20} ranging from 10 to 16 and the DEX-EQ value ranging from 0.041 to 0.064 nM. UV and/or UV-based treatment including hydrogen peroxide (H_2O_2) appeared to be the most efficacious treatment for GR activity attenuation.

Glucocorticoid activity has been detected in the United States over the past year with different effluents from Tucson at several sampling events (beyond the scope of this project), and similar results were consistently obtained. Given the biological importance of these compounds and their potential risk to the ecological system and to human health, more investigation of the causal compounds of observed GR activity was conducted (Chapter 7).

5.4.8 Adaptive Stress Response

AREc32 and Nrf2-MDA-MB cell lines were used for bioassays representing the ASR. The assay using AREc32 showed a positive response in samples, whereas the Nrf2-MDA-MB cell had no response in any of the samples tested. One possible reason was the relatively low detection limits in the Nrf2 assay, with the EC_{20} for AREc32 10 times lower than for the Nrf2-MDA-MB assay.

Adaptive stress was active in six out of nine samples. Like the xenobiotic metabolism, Tucson Water samples (TW1 and TW2) did not provide any significant response, which again showed that the infiltration process could reduce this effect from its source water (RR Eff).

5.5 Conclusion

With a burgeoning population and diminishing availability of freshwater resources, the United States continues to expand the use of alternative water resources for drinking. Potable water reuse is growing, both from indirect potable reuse and from direct potable reuse. In many cases, potable water reuse relies on natural and/or engineering barriers for water purification. AOPs have been proven to effectively remove organic contaminants from water and generally rely on UV with hydrogen peroxide or ozone with or without hydrogen peroxide. Although knowledge of practical application of AOP technology is common, additional information is needed regarding possible side effects from transformation products because AOPs generally do not remove contaminants but rather transform them into other substances. Here we select nine representative U.S. water samples and one blank. These samples were extracted with an optimized extraction method (see Chapter 4), and then the resulting extracts were shared among the three core laboratories for toxicity testing. This study provides further evidence as to which bioassays are most robust and sensitive and which may be ready for deployment in water monitoring. For comparison, another WWTP effluent and two drinking water samples as well as a blank (MilliQ water) were also assessed

A bioassay battery consisting of 39 endpoints was built to screen different qualities of water. The results showed that one single bioassay cannot represent all potential bioactivity but that there must be always a combination of bioassays representing different steps in the cellular toxicity pathway. Genotoxicity and mutagenicity deserve additional attention given their human health relevance. The *umu*C with S9 and Ames II without S9 are reliable assays for these endpoints. For specific mode of action (i.e., endocrine disruption related), estrogen

activity and glucocorticoid activity showed positive results in some waters. For xenobiotic metabolism induction, the AhR seems to have the highest induction, whereas for adaptive stress the AREc32 pathway provided the largest number of positive results. In the end, the cytotoxicity test is always important and should be considered with all endpoints.

Water treatment can be efficient to eliminate part of the activity, although this is process and operation related and bioassay dependent. Chlorination appears to be the most efficient treatment for mutagenicity and genotoxicity, whereas ozone and UV did not significantly attenuate the activity. Conversely, UV- and/or UV-AOP-based treatment appear to be most efficacious for attenuation of estrogenicity and glucocorticoid activity. Groundwater infiltration can significantly decrease xenobiotic metabolism and the adaptive stress mode of activity.

Chapter 6

Stage 2 Interlaboratory Study: 10 Australian Water Samples, 20 Laboratories, 103 Bioassays

6.1 Introduction

Although bioanalytical assessment has become very popular in the last years (Escher and Leusch, 2012), many studies rely on a small set of bioassays and evidently each study uses different types of water samples, sample preparation methods, bioassays and protocols, and data evaluation methods.[3] It is thus difficult to compare different techniques just from a literature analysis, as different samples and sample preparations were used. Here we selected 10 representative water samples (nine water samples and one blank), extracted them with a common and optimized extraction method (see Chapter 5), and sent them around the world to 17 laboratories. Together with the three core labs, the samples were tested in 20 laboratories. Most of the participating laboratories did not receive funding. They voluntarily offered the bioassays they already had set up in-house. It was not the goal of this study to directly compare bioassay protocols and performances of bioassays but to get a good overview on which biological endpoints were sensitive to water samples and which should be further investigated and prioritized in monitoring programs.

Standardizing the operating procedure itself was not the goal of this study, as it is generally impossible because of differences in available consumables and equipment in different countries and because the focus was not on identical assays but on overlapping endpoints. Our previous experience with interlaboratory comparison has demonstrated that development of universal SOPs may not be practical but that on the other hand it is critically important to ensure consistency of the bioassay data analysis (Leusch et al., 2010), and this approach will be implemented in this project. This statement implies that all labs derived full dose−response curves, using reference compounds. One goal was to report the results in terms of TEQs and BEQs, but this was not possible for all endpoints.

For each mode of toxic action/step in the toxicity pathway identified as relevant in the literature review, one or more bioassays were evaluated (endpoint comparison). Several assays were run in two or more laboratories (interlaboratory comparison), and most labs performed the tests twice on separate occasions (intralaboratory reproducibility). The study shed further light on which bioassays were most robust and sensitive amongst the responsive

[3] This study was partially funded through WRRF-10-07. Additional financial contributions were made by the California Water Resources Control Board (Agreement No. 10-096-250) and the European Union, project Demeau, grant agreement no. 308339, as well as by additional in-kind contributions of the participating laboratories listed in Table 6.1. This chapter provides a summary of the complete study (Escher et al., 2014), including contributions not funded by WRRF-10-07.

biological endpoints. The outcome of this exercise includes recommendations on a robust screening test battery using indicator bioassays.

The results were used to benchmark water quality and to compare the treatment efficiency of different recycling processes. For this reason, the samples assessed came from two treatment trains. The first treatment train consisted of MF, RO, and polishing with H_2O_2/UV, and the second treatment train used ozonation followed by biologically activated carbon filtration. For comparison, also river water, stormwater, and drinking water as well a blank (MilliQ water) were assessed.

6.2 Participating Laboratories and Choice of the Bioassays

A total of 20 laboratories participated in this study: their names, addresses, and names of responsible scientists, as well as a code for each lab used throughout the report, are given in Table 6.1. The bioassays were selected to cover many crucial steps in the toxicity pathways: xenobiotic metabolism, specific and reactive modes of action, induction of ASR pathways, and bioassays for cytotoxicity, some of which are models for system responses (Figure 3.1). Crucial pathways and modes of action were typically covered by several bioassays as listed in Table 6.2**.**

Table 6.1. Participating Laboratories and Codes for the Laboratories Used Throughout This Report

Lab Code	Laboratory	Participants
ATG	Attagene, P.O. Box 12054, Research Triangle Park, NC 27709	Alex Medvedev, Sergei Makarov
AWQC	Australian Water Quality Centre, 250 Victoria Square, Adelaide SA 5001, Australia	Andrew Humpage
BDS	BioDetection Systems, Science Park 406, 1098 XH, Amsterdam, The Netherlands	Sander van der Linden, Bart van der Burg
CAPIM	CAPIM: Centre for Aquatic Pollution Identification and Management, DPI Queenscliff, 2A Bellarine Hwy, Queenscliff, Vic 3225 Australia	Mayumi Allinson, Fujio Shiraishi
CSIRO	CSIRO Land and Water, Private Bag No. 2, Glen Osmond, SA 5064, Australia	Anu Kumar, Peter Bain
GU	Smart Water Research Centre, Griffith University, Edmund Rice Dr, Griffith University Gold Coast Campus Southport QLD, 4222 Australia	Frederic Leusch, Erik Prochazka
HK	Department of Biology, Croucher Institute for Environmental Sciences, Level 10, Science Tower, Ho Sin Hang Campus, Hong Kong Baptist University, 224 Waterloo Rd. Kowloon Tong, Kowloon, Hong Kong	Chris K. C. Wong, Boniie Yeung
IRCM	Cancer Research Institute Montpellier, CRLC Val, d'Aurelle, Parc Euromédecine, 34298 Montpellier, Cedex 5, France	Patrick Balaguer, Marina Grimaldi
IWW	IWW Muehlheim, IWW Water Centre, Department of Toxicology, Moritzstrasse 26, 45476 Mülheim/Ruhr, Germany	Elke Dopp, Jessica Richard

Lab Code	Laboratory	Participants
NJU	The Lab of Ecotoxicology and Environmental Health, School of the Environment, Nanjing University (Xianlin Campus), 163 Xianlin Avenue, Nanjing, Jiangsu, 210046 China	Xiaowei Zhang
RCEES	State Key Lab. of Environmental Aquatic Chemistry, Research Center for Eco-Environmental Sciences, Chinese, Academy of Sciences, P. O. Box 2871, Beijing, 100085 China	Min Yang
RECETOX	Research Center for Toxic Compounds in the Environment (RECETOX), Masaryk University, Kamenice 753/5, 62500, Brno, Czech Republic	Klara Hilscherova, Barbora Jarosova
SCCWRP	Southern California Coastal Water Research Project (SCCWRP), 3535 Harbor Blvd., Suite 110, Costa Mesa, CA 92626-1437	Keith Maruya, Alvina Mehinto
SWISS	Center for Applied Ecotoxicology (oekotoxzentrum), Eawag-EPFL, Überlandstr. 133, 8600 Dübendorf, Switzerland	Inge Werner
UA	University of Arizona, 1133 E. James E. Rogers Way, Harshberger 108, Tucson, AZ 85721-0011	Shane Snyder, Ai Jia
UCR	Aquatic Ecotoxicology, Department of Environmental Sciences, University of California−Riverside, Riverside, CA 92521	Daniel Schlenk, Jordan Crago
UFL	University of Florida, Department of Physiological Sciences, PO Box 100144 1600 SW Archer Rd, Gainesville, FL 32610-0144,	Nancy D. Denslow, Sumith Jayasinghe Balanapanage
UFZ	Helmholtz Center for Environmental Research – UFZ, Permoserstr. 15, 04318 Leipzig, Germany	Rolf Altenburger, Stefan Scholz, Wibke Busch
UQ	The University of Queensland, National Research Center for Environmental Toxicology (Entox), 39 Kessels Rd, Brisbane 4108, Australia	Beate Escher, Janet Tang
USF	Department of Cell Biology, Microbiology and Molecular Biology, University of South Florida, 4202 E Fowler Ave, BSF218, Tampa, FL 33620	Sandy Westerheide, Jamie Mendez

Table 6.2. Bioassays Tested in the Different Laboratories, Categorized According to the Categories Assigned in Figure 3.1

Lab Code	Xenobiotic Metabolism	Specific Modes of Action	Reactive Modes of Action	Induction of Adaptive Stress Response Pathways	General Cytotoxicity and Models for System Response
ATG	PXR-cisFACTORIAL, PXR-transFACTORIAL, CAR-transFACTORIAL, PPARα-transFACTORIAL, PPARγ-transFACTORIAL, AhR-transFACTORIAL	ERE-cisFACTORIAL, ERα-transFACTORIAL, AR-transFACTORIAL, GR-transFACTORIAL, THRα1-transFACTORIAL, RORβ-transFACTORIAL		HSE-cisFACTORIAL, HIF-1a-cisFACTORIAL, NF-κB-cisFACTORIAL, NRF2/ARE-cisFACTORIAL, p53-cisFACTORIAL	
AWQC			Micronucleus assay		
BDS	CALUX-PPARα, CALUX-PPARγ	ER-CALUX, AR-CALUX, PR-CALUX, GR-CALUX, TR-CALUX		NF-κB-CALUX, nrf2-CALUX, p53-CALUX, p53-CALUX+S9	
CAPIM	CAR-yeast, AhR-yeast	hER-yeast, medER-yeast, hRAR-yeast			
CSIRO	CALUX-PPARγ	ER-CALUX, anti-ER-CALUX, YES, YAS, PR-CALUX, GR-CALUX	RTG2 oxidative stress		RTG2 MTT

Lab Code	Xenobiotic Metabolism	Specific Modes of Action	Reactive Modes of Action	Induction of Adaptive Stress Response Pathways	General Cytotoxicity and Models for System Response
GU	PPARγ GeneBLAzer	ER-CALUX, anti-ER-CALUX, AR-CALUX, anti-AR-CALUX, PR-CALUX, anti-PR-CALUX, GR-CALUX, GR-GeneBLAzer, anti-GR-GeneBLAzer, TR-CALUX		Jurkat E6-1 IκB	Caco-2 NRU, SK-N-SH, THP1, CPA
HK	MCF7-PPAR, MCF7-DRE	MCF7-ERE, MCF7-ARE, MCF7-RARE			
IRCM	HG5LN PXR, HELN-PPARγ	HELN-Erα, HELN-ERβ, HELN-AR, HELN-TR			
IWW		ER-CALUX	Ames TA98 and 100, +/-S9	p53-CALUX	
NJU		Steroidogenesis, induction of progesterone, induction of 17α OH-progesterone			
RCEES			*umu*C NM5004, *umu*C TA1535/pSK1002		*P. phosphoreum* T3
RECETOX	AhR-CAFLUX, H4IIEluc	hERα-HeLa-9903, MDA-kb2, anti-MDA-kb2, GR-MDA-kb2 (AR suppressed with Flutamide), P15/H19	SOS chromotest		
SCCWRP		ER-GeneBLAzer, AR-GeneBLAzer, GR-GeneBLAzer			

Lab Code	Xenobiotic Metabolism	Specific Modes of Action	Reactive Modes of Action	Induction of Adaptive Stress Response Pathways	General Cytotoxicity and Models for System Response
SWISS		Algal photosynthesis inhibition, YES			Microtox, algal growth inhibition
UA		YES, YAS, GR-Switchgear	Ames TA98 -S9, Ames TA98 +S9, Ames TAmix -S9, Ames TAmix +S9	Hypoxia-Switchgear, nrf2-keap	
UCR		ER-GeneBLAzer, AR-GeneBLAzer, GR-GeneBLAzer			
UFL		ER-GeneBLAzer, AR-GeneBLAzer, GR-GeneBLAzer		p53-GeneBLAzer	
UFZ	DART cyp1a induction	DART cyp19a1b (aromatase)		hspb11 induction in DART after 120 h	DART 48 h lethality, DART 120 h sublethal
UQ	AhR-CAFLUX	Algal photosynthesis inhibition, AChE inhibition, E-SCREEN, T-Screen	*umu*C -S9, *umu*C +S9, Ames TA100 -S9, protein damage *E. coli* $GSH^{+/-}$	NF-κB-GeneBLAzer, AREc32	Microtox, AREc32 cell viability, algal growth inhibition
USF		ER-GeneBLAzer, AR-GeneBLAzer, GR-GeneBLAzer			

6.3 Water Samples

6.3.1 Selection of Water Samples

The selected water samples were a subset from 50 samples characterized previously with a smaller set of bioanalytical tools (Macova et al., 2011). The subset was chosen to represent two types of WRPs and reference samples from other water sources for benchmarking purposes.

Sample Eff1 is a secondary treated effluent that serves as inlet to a WRP that is based on membrane technology (see Figure 6.1). Three samples were taken in the plant, after MF, RO, and AO using H_2O_2/UV (Escher et al., 2011; Macova et al., 2011). In the second WRP investigated, secondary treated sewage effluent (Eff2) was ozonated followed by biologically activated carbon filtration (O_3/BAC) (Reungoat et al., 2010, 2011, 2012b).

For comparison and benchmarking, the following additional samples were evaluated: river water (RW) and drinking water (DW) samples are the inlet and outlet of a drinking water treatment plant (Macova et al., 2011; Neale et al., 2012). Sample SW was taken from a stormwater collection site in the northern suburb of Brisbane, Fitzgibbon, after a rain event on January 25, 2012. The sampling site is situated in a stormwater drain that receives runoff from a surrounding 290 ha residential catchment. The lab blank was ultrapure water (MilliQ water, abbreviated in the following as H_2O) run through the same SPE process as the samples.

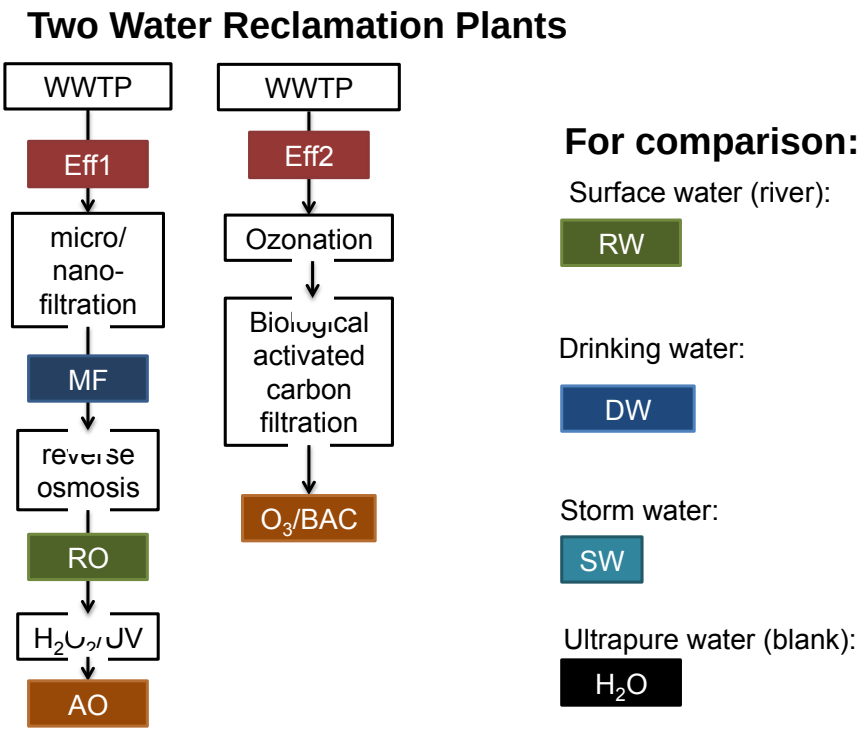

Figure 6.1. Sample codes from two water reclamation plants using different treatment technologies.

Note: For comparison we also assessed samples from a river, drinking water, stormwater, and a blank (ultrapure water).

6.3.2 Sample Collection

Ten grab water samples were collected in December 2011 and January 2012 from various sites in Australia. Twenty-eight liters each was collected for Eff1, MF, Eff2, and SW; for all remaining samples 14 L were collected.

6.3.3 Solid-Phase Extraction

The SPE was performed according to Macova et al. (2011) with the sorbent material validated in a report for the Australian National Water Commission (NWC [2011] and Chapter 5). All samples were acidified to pH 3. Samples containing chlorine were quenched with sodium thiosulfate (1 g/L) and were filtered with a glass fiber filter (GF/A; Whatman) before extraction. Twenty-eight 1 L batches of Eff1, MF, Eff2, and SW and 28 0.5 L batches of RO, AO, O_3/BAC, RW, DW, and Blank were extracted by each passing through two 6 mL solid-phase cartridges in series, first an Oasis® HLB (500 mg, catalog no. 186000115; Waters) and then a Supelclean coconut charcoal cartridge (2 g, catalog no. 57144-U; Sigma-Aldrich). Both types of cartridge were individually preconditioned prior to extraction with 10 mL of 1:1 acetone:hexane mixture, followed by 10 mL of methanol and 10 mL of 5 mM HCl in MilliQ water. All cartridges were sealed individually and were kept at -20 °C until elution. Before elution the cartridges were defrosted and dried completely under vacuum; then they were eluted with 10 mL of methanol and 10 mL of acetone:hexane. The eluate of 8/4 pairs of cartridges per sample was combined and evaporated under purified nitrogen gas before being solvent exchanged to methanol at a final volume of 1 mL. These extracts were aliquoted and tested in four labs (ATG, GU, UA, and UQ). The extracts were dried as described later to send to ATG and were sent as methanolic extracts to UA. After the initial positive results, the remaining 20/10 pairs of cartridges, which had been stored for 5 months, were eluted,. Their extracts were combined, and the extracts were aliquoted for the 16 remaining laboratories and were evaporated under purified nitrogen gas before being solvent exchanged to DMSO at a final volume of 2 µL for shipping. The 2 µL samples in Agilent high-recovery HPLC vials (catalog no. 5183-2030) were flushed with purified argon gas. The samples were shipped at room temperature by express mail to all laboratories, where they were reconstituted upon arrival (after 1 day [Australia] to 3−5 days [overseas]) with appropriate solvent and stored at -20 °C until bioanalysis.

6.3.4 Bioassays

The experimental methods of all bioassays applied (Table 6.2) are referenced or described in detail by Escher et al. (2014).

6.3.5 Data Evaluation

The experimental data were evaluated as described in Chapter 2. The EC values are presented in scatter plots that allow us to discuss and compare the different assays as well as different treatment trains and samples investigated. The ECs are plotted from high to low ECs (Figure 6.2), so that samples/bioassays with high effect are plotted on the top and low effect on the bottom. The lines in Figure 6.2 indicate what the EC values mean.

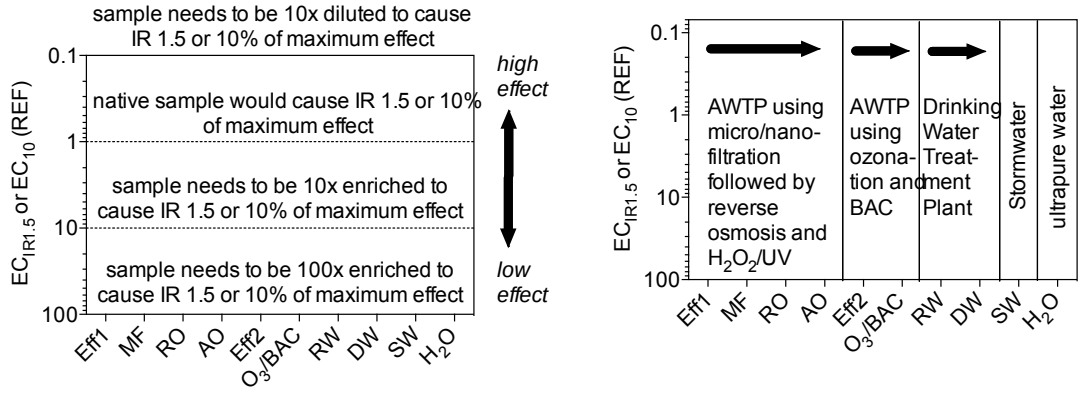

Figure 6.2. Explanation of the data presentation.

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An EC of REF 1 means that the native sample would cause the benchmark effect (10% of maximum effect or IR = 1.5), a lower EC means that the sample would need to be diluted to cause this effect, and a higher REF_{EC} means that the sample needs to be enriched to show the effect. On the right of Figure 6.2, the two advanced water treatment trains are depicted each from left to right, and for comparison river water, drinking water, stormwater, and a blank are included.

6.4 Results and Discussion

6.4.1 Technical Matters

6.4.1.1 Two Batches of Sample Extracts Showed Consistent Results

For practical reasons, whereas all water samples were taken together and enriched on SPE cartridges together, the cartridges were eluted in two batches. The first batch was used in four labs (the core labs UQ, UA, and GU as well as ATG for the initial screening to define the target endpoints). Only after the appropriateness of the 10 samples was assessed in this initial stage by comparison with historic data was the second larger batch extracted, aliquoted, and sent out to the remaining 16 laboratories. To ensure that the storage of cartridges had not changed the samples, the Microtox assay was performed with both batches, which led to good agreement, as evidenced in Figure 6.3 and by a paired *t* test that concluded that the pairing was effective with a *P* of 0.0001 and $r = 0.9351$ and a log-log linear regression with an r^2 of 0.9162.

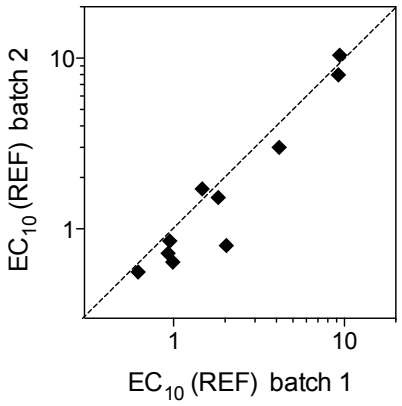

Figure 6.3. Comparison of the EC10 values between Batch 1 and Batch 2 extracts in the Microtox assay.

6.4.1.2 How Robust Were Bioassays Performed in Different Labs?

A number of bioassays were performed in multiple laboratories. In the following chapters only mean results per bioassay are reported and a detailed account of the results obtained by multiple laboratories is given by Escher et al. (2014).

6.4.1.3 Blanks and Dynamic Range of Response

The first two questions that we have to answer to judge the suitability of bioassays for water quality assessment are (1) is there a response in a polluted sample? and (2) is the effect low or nonexistent in a control sample? Even small impurities leaching out of the material or present in the solvent would likely contribute to the nonspecific effect of the blank. Here we applied two different SPE sorbent materials (HLB and coconut charcoal), which were eluted separately and required twice the amount of solvent.

Figure 6.4 gives an overview of all results of two samples in all 103 different bioassays tested. The WWTP effluent Eff2 served as a moderately "polluted" sample, and ultrapure water extracted with SPE served as the negative control. Sixty-five bioassays showed a response in at least one of the tested samples, and in Eff2 the number of positive results (IR > 1.5 or >10% effect) was 53. In contrast, the ultrapure water showed an effect in only five bioassays.

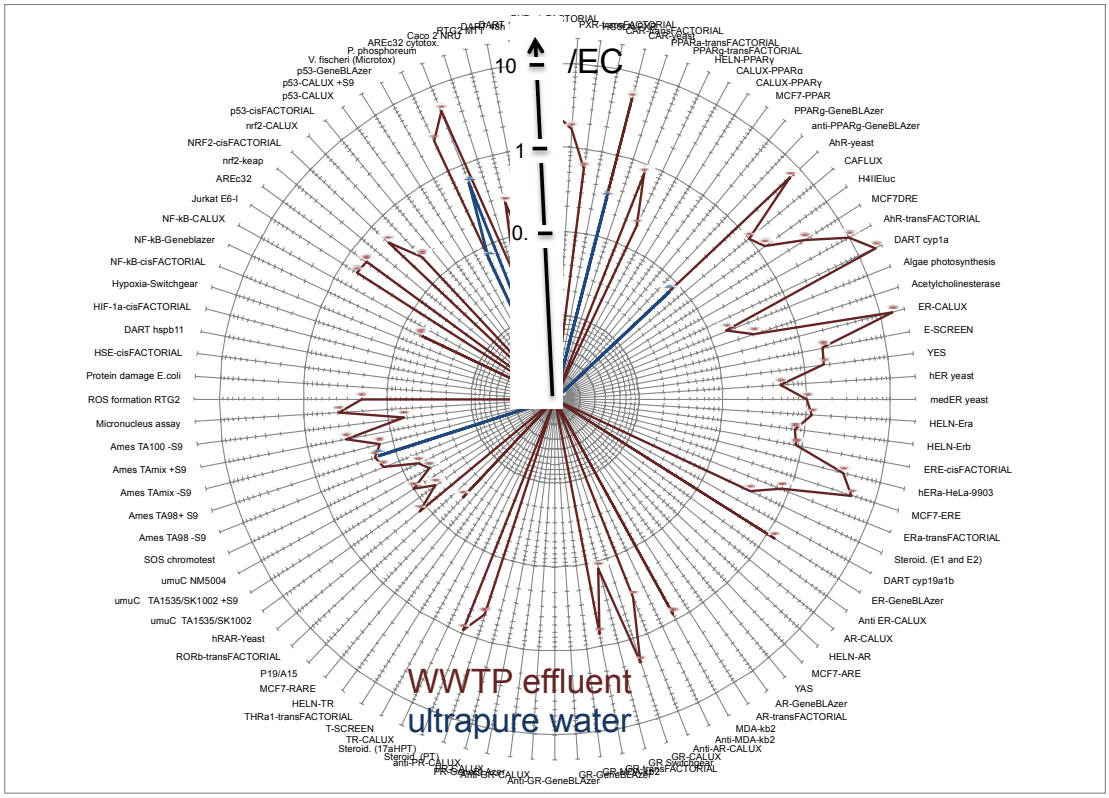

Figure 6.4. Comparison between all EC values (plotted as 1/EC; thus, a high value means high effect) for the blank (blue) and Eff2 (red).

The bioassays with positive results in the ultrapure water were the two bioluminescence inhibition assays with marine luminescent bacteria. They were fast screening tests (15 to 30 min of incubation), and they responded rapidly and very sensitively, but their effects were of low human health relevance. They were very nonspecific, as anything can impair the energy production and thus decrease the bioluminescence. The magnitude of effect is somewhat higher than what we have previously observed when only one type of solid-phase material was applied. Here we applied both HLB and coconut charcoal and eluted them separately, which required twice the solvent used, and any small impurity leaching out of the material or in the solvent contributed to the nonspecific effect of the blank. Nevertheless, the dynamic range for the Microtox assay was still a factor of 100 between the Eff2 and the blank, and the sensitivity was amongst the highest for all endpoints (indicated by one of the highest 1/EC values), allowing the use of this endpoint for monitoring purposes.

Furthermore, the yeast-based assays AhR-yeast and hRAR-yeast showed a response in the blanks but only at much higher REF than the samples. An additional positive blank value was observed in one of the various Ames assays and was most likely attributable to measurement uncertainty, as this value was derived from only one data point.

6.4.2 Screening of 25 Nuclear Receptors and 48 Transcription Factors with 1 Multiplexed Assay

Although a large number of Category 1 bioassays have been applied for water quality assessment and were tested here for the first time on one common set of water samples, we

identified some biological endpoints as relevant during the literature review, but the associated bioassays have not yet been applied to water samples. Therefore, before final selection of the test battery, we screened a large number of NRs and TFs to test if they were induced by water samples. The novel biosensor system "FACTORIAL" developed by Attagene, Inc. can simultaneously screen 25 NR or 48 TF response elements in HepG2 human liver carcinoma cell lines (Martin et al., 2010). The limitation of single-reporter gene assays, where only one NR or one TF can be tested, has been overcome by the construction of uniform RTUs, which are a common plasmid with individual TF-inducible promoters fused to a reporter sequence that varies only slightly between the different RTUs. The different reporters can be separated and quantified by capillary electrophoresis (Romanov et al., 2008). The same technology was also adopted for NRs, but only those that are expressed endogenously in the HepG2 cell line (25 NRs) were implemented in this assay (Martin et al., 2010).

The FACTORIAL bioassays were applied here for the first time to screen water samples. The raw water samples did not show any effects (data not shown); the following responses relate to water samples after enrichment by SPE to a REF of 4. As no reference compounds were measured and as the maximum response was not known, only IRs could be calculated from the raw response data.

The highest induction was seen for the PXR both in the NR and TF assays and in all samples but the blank (H_2O) (Escher et al., 2014). As expected, ERα was activated but not the estrogen-related receptors ERRα and ERRγ in the NR assay, and the estrogen response element (ERE) was activated in the TF assay (Escher et al., 2014).

In the NR assay, the PPARγ was active but with a lower IR about or below 2 in the samples Eff1, Eff2, and MF. The GR responded weakly in the NR assay (IR of 1.4 at a REF of 4) but showed no response in the TF assay (Escher et al., 2014).

The highest induction in the TF assay was observed for the AhR element, which did not come as a surprise, as a large number of chemicals activate this xenobiotic metabolism pathway (Escher et al., 2014). The next highest activity was caused by the response element associated with the PXR, and this finding was consistent with the high activity in the NR assay (Escher et al., 2014). Third in activity was the ARE, which was activated through the Keap-Nrf2 pathway (Escher et al., 2014).

In response to these results, additional endpoints related to the positive response in this screening were included in the test battery.

6.4.3 Overview of Results

The summary of all 103 EC values in each of the 10 samples was presented by Escher et al. (2013a), and the results of two samples, Eff2 and the blank H_2O, are shown in Figure 6.4. Evidently, quantitative comparison is difficult because ECs were expressed as EC_{10} or $EC_{IR1.5}$. These two values are directly comparable only if the maximum IR is about 6 (see Chapter 2).

Here follows a summary of the responsive and nonresponsive endpoints in relation to the associated step in the toxicity pathway. Responsiveness is determined on one hand by the presence of triggering organic micropollutants in the water extracts: in the absence of chemicals that trigger a certain toxicity pathway, even the most sensitive bioassay will not

respond to a given water sample. On the other hand, the responsiveness is directly related to the sensitivity of a given bioassay. Absolute sensitivity can be assessed only by comparing the ECs and limits of detection of reference chemicals, but the results obtained in the present study can give some indication of the suitability of bioassays for monitoring purposes.

6.4.4 Induction of Xenobiotic Metabolism Pathways

Induction of metabolic pathways is not per se an indicator of toxicity, but it gives an indication of the presence of chemicals. Metabolism can detoxify chemicals, but some chemicals are actually activated by metabolism. In a recent review, Omiecinski et al. (2011) stressed the relevance and the toxicological implications of a number of xenobiotic metabolism pathways and associated NRs, including the PXR, CAR, PPAR, and AhR (Table 6.3).

Table 6.3. Bioassays Indicative of the Induction of the Xenobiotic Metabolism Pathways

NR	Function	Inducing Chemicals	No.	No. +	No. -	Positive Bioassays	Negative Bioassays
PXR	Induction of various Phase I enzymes	Steroids	3	3	0	PXR-cisFACTORIAL, PXR-transFACTORIAL, HG5LN PXR	-
AhR	Induction of CYP (CYP1A1)	PAHs, PCDDs, coplanar PCBs	6 (1)	6	0	AhR-yeast, AhR-CAFLUX, H4IIEluc, MCF7-DRE (transient), AhR-transFACTORIAL, DART cyp1a induction	-
CAR	Induction of various Phase I and II enzymes	Indirectly activated by phenobarbital, various pharmaceuticals	2 (1)	1	1	CAR-yeast	CAR-transFACTORIAL
PPAR	Glucose, lipid, and fatty acid metabolism	Phthalates, fibrate pharmaceuticals	7 (1)	2	5	PPARγ-transFACTORIAL, HELN-PPARγ	PPARα-transFACTORIAL, CALUX-PPARα, CALUX-PPARα, PPARγ GeneBLAzer, MCF7-PPAR (transient)

Notes: No. of bioassays (replicates). no. +: number of positive bioassays, i.e., those that exceed the effect threshold of IR = 1.5 or 10% of maximum effect in one or more measured dilutions. no. -: number of negative bioassays, i.e., those that remain below the effect threshold of IR = 1.5 or 10% of maximum effect even in the highest REF. CYP = cytochrome P450 mono-oxygenase enzymes, PAH = polycyclic aromatic hydrocarbon, PCB = polychlorinated biphenyl, PCDD = polychlorinated dibenzodioxins.

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Three and six bioassays were evaluated for the PXR and AhR, respectively, and all showed positive responses in more polluted samples and were negative in recycled water and the blank (Table 6.3, Figure 6.5). For PXR the FACTORIAL assays were most responsive with an $EC_{IR1.5}$ below 1; namely, even the native sample would show an effect. The HG5LN-hPXR cell line was constructed from cervical cancer (HeLa) cells in a two-step stable transfection with the intermediate cell line HG5LN (Seimandi et al., 2005) and expresses an hPXR ligand-binding domain fused to the GAL4 DNA-binding domain (Lemaire et al., 2006). The HG5LN-hPXR initiates a luciferase response when a PXR ligand binds to the hPXR ligand-binding domain, which is followed by the GAL4 DNA-binding domain binding to GAL4RE5, which, in turn, initiates the expression of luciferase. This reporter gene assay has been applied widely in water quality monitoring, including for testing of wastewater, surface water, and reclaimed water (Mahjoub et al., 2009; Creusot et al., 2010; Kinani et al., 2010; Mnif et al., 2010, 2011). It was responsive to the same samples as the FACTORIAL endpoints, and sensitivity was smaller but proportional. Samples did not need to be enriched beyond 10 times to see a response.

The most sensitive endpoint related to the induction of the AhR was the induction of CYP1A transcription in the zebrafish embryo measured by RT-PCR. Only the four samples Eff1, MF, Eff2, and SW were tested, and the $EC_{IR1.5}$ ranged from 0.06 to 0.16 REF; thus, the effect was obvious already in diluted samples. Although this assay is highly sensitive, it is based on an in vivo test with the zebrafish embryo (which is considered an in vitro assay in some regulations) and cannot be used for routine monitoring. All five other bioassays showed consistent effect patterns for the different samples, with the AhR-transFACTORIAL being most responsive in most samples followed by MCF7-DRE, AhR-CAFLUX (chemically activated fluorescence expression [Nagy et al., 2002]), and H4IIEluc (Murk et al., 1996), the three of which behave very similarly. The AhR-yeast (Miller, 1999; Kamata et al., 2009) had the lowest sensitivity, but the results observed were consistent with previous monitoring work by Allinson et al. (2010) in wastewater.

In contrast, there was no response detected in the CAR-transFACTORIAL assay up to a REF of 4, and the CAR-yeast showed induction only after more than 10-fold enrichment. CAR plays a role in both Phase I and II metabolism and plays a protective role against toxicity induced by bile acids as well as regulation of physiological functions. The target chemicals are less clearly defined, and whereas a few pesticides, for example, methoxychlor, carbaryl propazine, and 6-deisopropylatrazine, induced the CAR in the CAR-transFACTORIAL assay, their effects were only marginal.

For the PPAR, only two out of seven bioassays gave signals in the four most polluted samples. Only the PPARγ was active in the PPARγ-transFACTORIAL and HELN-PPARγ (Seimandi et al., 2005). The PPAR is less important for xenobiotic metabolism and is rather involved in the regulation of glucose and lipid metabolism (Scarsi et al., 2007). In a high-throughput study of 3000 environmentally relevant chemicals, roughly 1% were PPARγ agonists and 8% were PPARγ antagonists (Huang et al., 2011b). In the present study no PPAR antagonism was detected, but the REF did not exceed 2. Also, organotins (le Maire et al., 2009) and polyhalogenated bisphenol A (Riu et al., 2011) were found to induce the PPAR. The activity of the PPARγ being greater than that of the PPARα is consistent with 146 out of 309 ToxCast Phase I chemicals being active in PPARγ-transFACTORIAL and fewer in the other isoforms (Martin et al., 2010).

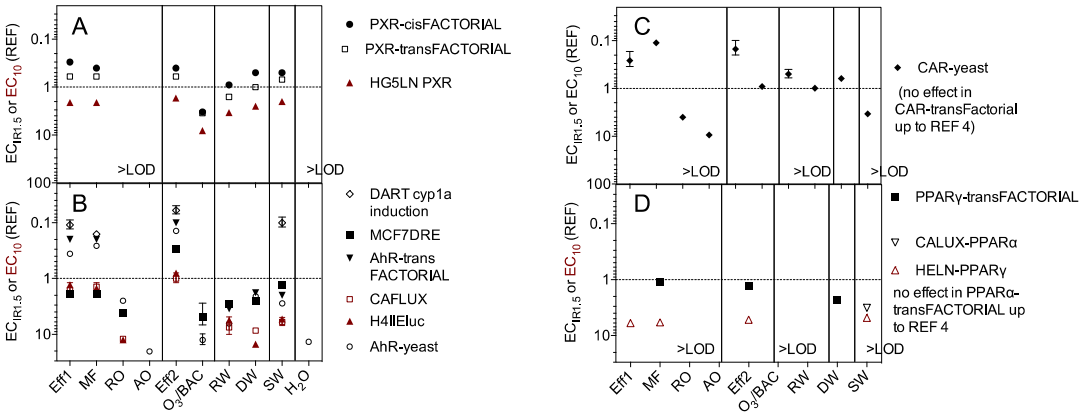

Figure 6.5. Results from bioassays indicative of the induction of the xenobiotic metabolism pathways.

Notes: The red symbols are EC10 values; the black symbols are ECIR1.5 values.

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In summary, the induction of xenobiotic metabolism is a highly relevant and responsive parameter in water quality assessment with highest responsiveness from the AhR, followed by the PXR and PPAR and low relevance to the CAR, which also is shown by a direct comparison on those endpoints in one common bioassay, the FACTORIAL assay (Figure 6.6).

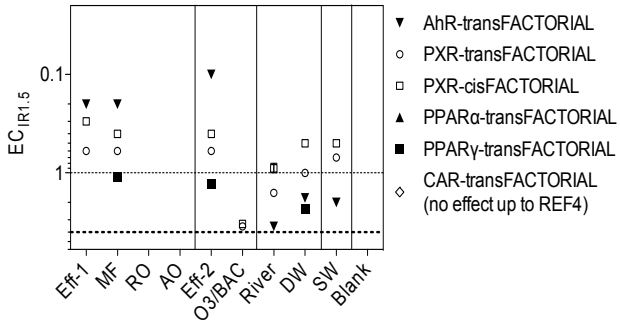

Figure 6.6. Direct comparison of all NRs involved in xenobiotic metabolism that were tested in the FACTORIAL assay.

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6.4.5 Specific Modes of Toxic Action

Most specific modes of action involve binding to receptors or inhibition of enzymes. Direct enzyme inhibition assays were popular in the past for water quality testing, but more-recent work in our group on the influence of dissolved organic matter (DOM) on the AChE assay has demonstrated that DOM nonspecifically impacts the assay at relatively low concentrations (Neale and Escher, 2013), which means that the concentrations of water samples cannot be increased in most cases above a REF of 2. Thus, only the two wastewater samples gave a valid response in this assay, and this assay (and possibly other cell-free

enzymatic assays) is not suitable to investigate recycled water samples, despite the high relevance of this biological endpoint that applies for many insecticides.

Another important group of pesticides are herbicides that inhibit photosynthesis. Although photosynthesis is not an endpoint relevant to human health, herbicides are still regulated in recycled and drinking water. The most sensitive assays to detect herbicides use algae, and the inhibition of the photosystem by triazines and phenylurea herbicides can be highly specifically measured by pulse-amplitude modulated fluorometry (Muller et al., 2008). This test also can be applied on a microtiter plate, and both labs that applied this assay used the same protocol (Escher et al., 2008a) and obtained very consistent results with EC_{10} values for wastewater Eff1 and MF in the range of 2−3 REFs and Eff2 being about 2 to 3 times lower in photosynthesis inhibition. SW was positive at one lab only at a REF of 6.5, and all other samples were below the limit of detection (LOD >20 REF).

Table 6.4. Bioassays Indicative of Specific Modes of Action (Excluding Endocrine Effects)

Enzyme or Receptor	Function	Inhibiting Chemicals	No. of Bioassays (Replicates)	No. +	No. -	Positive Bioassay	Negative Bioassay
AChE	Chemical transduction of nerve signal	Insecticides	1	0	1	-	AChE enzyme inhibition
Photosystem II	Herbicides	Herbicides	1 (1)	1	0	IPAM	-

Notes: No. +: number of positive bioassays, i.e.,those that exceed the effect threshold of IR = 1.5 or 10% of maximum effect in one or more measured dilutions; no. -: number of negative bioassays, i.e., those that remain below the effect threshold of IR = 1.5 or 10% of maximum effect even in the highest REF.

The most relevant receptor-mediated effects are related to endocrine disruption. Estrogenic effects are by far the most prominent and environmentally relevant effects, and the large amount of work on this endpoint is reflected in the fact that 14 different bioassays indicative of estrogenic effects were evaluated here (Table 6.5). All but the two yeast-based assays were active in four or five samples. The absolute sensitivity was highest for the ER-CALUX and MCF7-ERE, but the relative sensitivity was similar for all bioassays (Figure 6.7). A comparison of the associated estradiol equivalent (EEQ) concentrations for those bioassays, where dose−response curves for reference compounds are available, is given further later.

In all bioassays for estrogenicity the blanks and the sample AO did not induce any in vitro effects, and only one bioassay yielded very minimal effects at a high enrichment factor in RO and drinking water (DW) (Figure 6.7). The results are consistent with a previous more thorough review and comprehensive interlaboratory comparison study of five different bioassays for estrogenicity (GWRC, 2008), where the effects in the ER-CALUX, the YES, the E-SCREEN, and the T47KBluc (not assessed here) were highly correlated. Consistent with our findings, the results of GWRC (2008) showed that the yeast-based assays were as reliable as other assays but they had higher detection limits and therefore were not suitable for highly treated water but that the overall response expressed as EEQ concentration was very similar. An advantage of the yeast assays is that they are highly selective because there is no cross-talk that complicates mammalian cell assays. The steroidogenesis assays showed a

clear induction of estrone and estradiol, which can be connected to a decreased estradiol metabolism. In this assay the formation of steroid hormones is assessed, which is an aspect different from receptor binding to the ER, but it will also modulate endocrine effects. A similar effect was observed when oil sand product water was assessed with the steroidogenesis assay. The raw water increased the estradiol levels, and the effect disappeared after ozonation of this water (He et al., 2010) as the effect of sample Eff2 disappeared when it was ozonated.

The anti-ER test, which quantifies how the sample influences the effect of a constant concentration of estradiol, typically spiked at concentrations that would elicit 50 to 80% of maximum effect. If the effect of the constant concentration of estradiol was suppressed and if the sample was not cytotoxic, the sample can be considered to act antiestrogenically. No antiestrogenic activity could be detected in any of the samples (Table 6.5).

Table 6.5. Bioassays Indicative of the Endocrine Disruption

NR	No. of Bioassays (Replicates)	No. Positive	No. Negative	Positive Bioassay(s)	Negative Bioassay(s)
ER	14 (8)	14	0	ER-CALUX, E-SCREEN, YES, HELN-ERα, HELN-ERβ, ERE-cisFACTORIAL, hERα-HeLa-9903, MCF7-ERE, ERα-transFACTORIAL, steroidogenesis, DART cyp19a1b (aromatase), ER-GeneBLAzer, hER yeast, medER yeast	
ER	1	0	1	-	Anti-ER-CALUX
AR	7 (6)	1 (but coexpression with GR)	6	MDA-kb2	AR-CALUX, HELN-AR, MCF7-ARE (transient), yeast androgen screen (YAS), AR-GeneBLAzer, AR-transFACTORIAL
AR	2	2	0	Anti-AR-CALUX, anti-MDA-kb2	
PR	4 (5)	2	2	Steroidogenesis and induction of progesterone and of 17α OH-progesterone	PR-CALUX, PR-GeneBLAzer
GR	5 (6)	5	0	GR-CALUX, GR-Switchgear, GR-transFACTORIALGR-MDA-kb2 (AR suppressed), GR-GeneBLAzer	-
TR	3 (1)	0	3	-	TR-CALUX, T-Screen, THRα1-transFACTORIAL
RAR/RXR	4	0	4	-	MCF7-RARE, P15/H19, RORβ-transFACTORIAL, hRAR-yeast assay

Notes: No. + = number of positive bioassays, i.e., those that exceed the effect threshold of IR = 1.5 or 10% of maximum effect in one or more measured dilutions. no. - = number of negative bioassays, i.e., those that remain below the effect threshold of IR = 1.5 or 10% of maximum effect even in the highest REF.

The AR showed reciprocal features. Of seven bioassays (AR-CALUX, HELN-AR, MCF7-ARE [transient], yeast androgen screen [YAS], AR-GeneBLAzer, AR-transFACTORIAL, and MDA-kb2) only one (MDA-kb2) gave positive results, but in MDA-kb2 the ER was coexpressed with the GR and therefore the activity could be caused by something besides androgenic effects. However, both of the bioassays for antiandrogenicity (anti-AR) were positive but only at very high concentrations.

The two receptor-binding assays for the PR, PR-CALUX and PR-GeneBLAzer, were negative, whereas the bioassay for steroidogenesis showed that progesterone and 17α hydroxyprogesterone were enhanced in human H295R cells. The steroidogenesis pathway is, strictly speaking, an assay indicative of xenobiotic metabolism and quantifies formed hormones and intermediates, but as it affects the hormone system function, we classify it with receptor-mediated hormonal effects. None of the steroids quantified in H295R cells was induced except for E1 and E2 (discussed previously) and progesterone and 17α hydroxyprogesterone. These increased progesterone levels are most likely due to an inhibitory effect on CYP21A. The effect pattern of the Eff1 samples was very similar to what has been observed when dosing with bisphenol A (Zhang et al., 2011).

ER, AR, and PR are important for the development and functioning of the reproductive system and are mainly expressed in the sex organs. The GR is more abundant and found in all cell types. It has been linked to a wide spectrum of diseases, including cardiovascular disease, inflammatory and immune disease, diabetes, and obesity. Therefore, it has high potential relevance (see literature review, Chapter 1). Accordingly, five bioassays indicative of activation of the GR were included in this study. All of them were active in two or more samples. The GR-CALUX was the most sensitive assay, followed by the GR-transFACTORIAL. These were roughly by a factor of 10 more sensitive than the GR-Switchgear and GR-MDA-kb2 assay. The GR-GeneBLAzer was positive, but the potency did not correlate well with the other assays, and there was more variability in the results from different labs, indicating that optimization of the protocol might possibly improve the performance of this assay, which was applied for the first time in the labs.

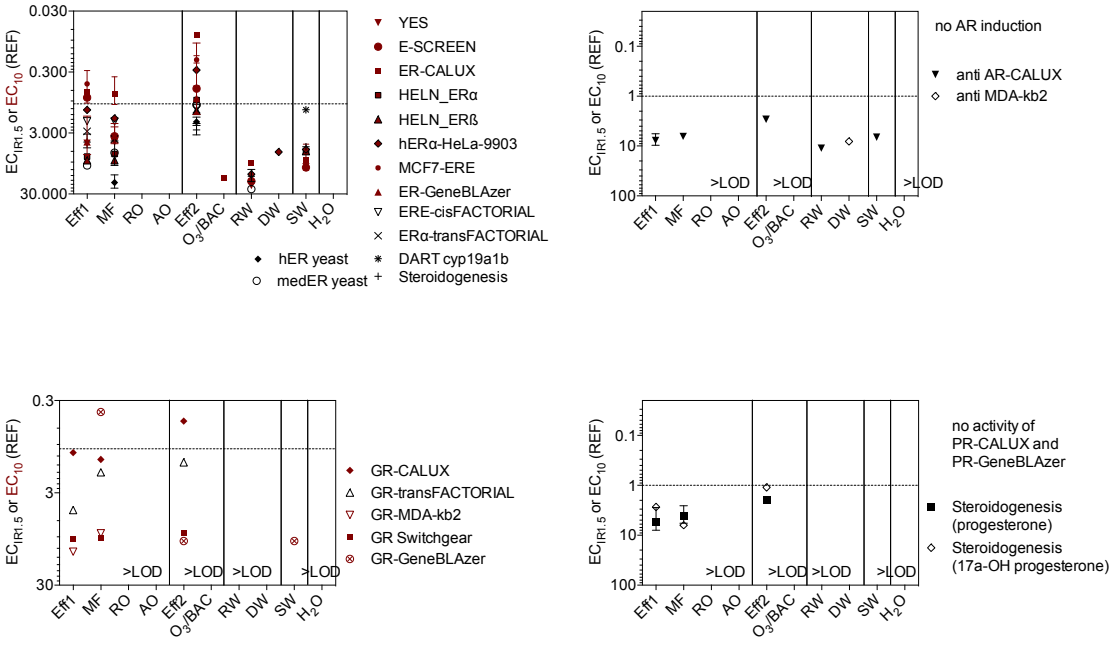

Figure 6.7. Results from bioassays indicative of the endocrine disruption.

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None of the assays indicative of modulation of the thyroid hormone system showed any response with any of the water samples, even at high REF. The T-Screen is a cell proliferation assay where the cells proliferate only in the presence of thyroid hormones (Gutleb et al., 2005). This assay has been mainly applied for chemicals, and only few showed activity. Accordingly, it was not surprising that no effects were detected in the water samples. Many of the individual chemicals need metabolic activation; thus, a combination with a system for metabolic activation might be beneficial (Taxvig et al., 2011). Also a novel reporter gene assay based on the GH3 cell lines used for the T-Screen was recently developed (Freitas et al., 2011), but because of delays in the preparation of the Material Transfer Agreement, it could not be incorporated in the present study. Inoue et al. (2009b) had observed some activity but less than 10% of maximum effect at a REF of 100 with a two-hybrid yeast assay in surface waters. They observed significant effects (>10%) in WWTP influent, which disappeared in the effluent (Inoue et al., 2011). In a different yeast-based assay, N. Li et al. (2011) did not observe any TR agonistic effect in water samples but were able to trace anti-TR activity to phthalates (N. Li et al., 2010).

The retinoic acid signaling pathway is crucial for reproduction and development as well as for cell homeostasis and immune function (Novák et al., 2008). Two receptors are key to this pathway, the RAR and the RXR, which is a heterodimer partner not only for the RAR but also for other NRs, including the PPAR and TR. We tested four bioassays that are connected to the retinoic acid signaling pathway, but none of them showed a response for the water samples. Only the two-hybrid assay where RARγ is inserted into yeast with *lacZ* as reporter gene showed activity in wastewater effluents. The FACTORIAL assay used ROR-trans as NR, and although about a third of the ToxCast I chemicals were active in this endpoint, no effect could be observed in the water samples. The P15/H19 cell line was developed by transfecting an embryonal mouse carcinoma cell line with a plasmid carrying the retinoic acid response element (Novak et al., 2007). This cell had not been tested with water samples prior to this study, but sediment samples showed activity in the presence of the reference compound a*t*RA, when they enhanced the signal of the reference compound. The water samples showed the same effect: they were not active on their own but enhanced the effect of 9-*cis*-RA (for more details, see Section 3.10).

6.4.6 Reactive Toxicity

The focus of reactive toxicity testing was laid on genotoxicity and mutagenicity (Table 6.6). Only one test, the MN assay, detected DNA damage directly; the Ames test relies on back mutations and the *umu*C assay on detection of DNA repair. The MN assay could not be evaluated with either the IR or the % maximum EC effect model. Therefore, the EC_{LOD} was interpolated from a linear regression of concentration versus the percentage of micronuclei formed and the LOD was defined as a 2.7% increase in micronuclei. Three samples were active in the MN assay: Eff2, RW, and DW (Figure 6.8). This is a different profile from that of the receptor-mediated modes of action, and the activity in the DW sample presumably stems from the DBPs formed during chlorination. Tests for genotoxicity can be run in the presence and absence of a rat liver metabolic enzyme mix to differentiate between chemicals that must be metabolically activated to become genotoxic and those that are detoxified by metabolism. The SOS chromotest is based on induction of SOS repair in *E. coli* (Quillardet et al., 1982) and on the *umu*C assays that use *S. typhimurium* (Oda et al., 1985). Both are reporter gene assays, whereas the Ames test uses histidine-deficient *S. typhimurium* that grows only if a mutation occurs. There are different strains of *umu*C and various Ames strains, so the impressive number of eight genotoxicity assays in Table 6.6 boils down to only four different assay types. All *umu*C assays had very similar sensitivity and were active only at REFs of about 20. There was no large difference between the test with and without S9 (Figure 6.8). The SOS chromotest gave responses very similar to those of *umu*C. The Ames assay was generally more sensitive, but there was a large variability between the different Ames strains applied (Figure 6.8). No clear picture emerged if +S9 or –S9 was

more active. One problem with the Ames assay was that samples like RO, AO, SW, and the blank, which were not positive in all other genotoxicity assays, showed some but inconsistent activity in the Ames assay. The dynamic range of these genotoxicity assays was relatively small, and effects were observed only at relatively high enrichments (REFs = 2−20).

Table 6.6. Bioassays Indicative of the Reactive Modes of Action

Reactive Modes of Action	No. of Bioassays (Replicates)	No. +	No. -	Positive Bioassay	Negative Bioassay
Genotoxicity	11 (4)	8	0	*umu*C +/-S9, SOS chromotest, Ames +/-S9, MN assay	
Oxidative stress	1	1	0	Oxidative stress in RTG2 cells	-
Protein damage	1	0	1	-	Protein damage *E. coli* $GSH^{+/-}$

Notes: No. + = number of positive bioassays, i.e., those that exceed the effect threshold of IR = 1.5 or 10% of maximum effect in one or more measured dilutions; no. - = number of negative bioassays, i.e., those that remain below the effect threshold of IR = 1.5 or 10% of maximum effect even in the highest REF.

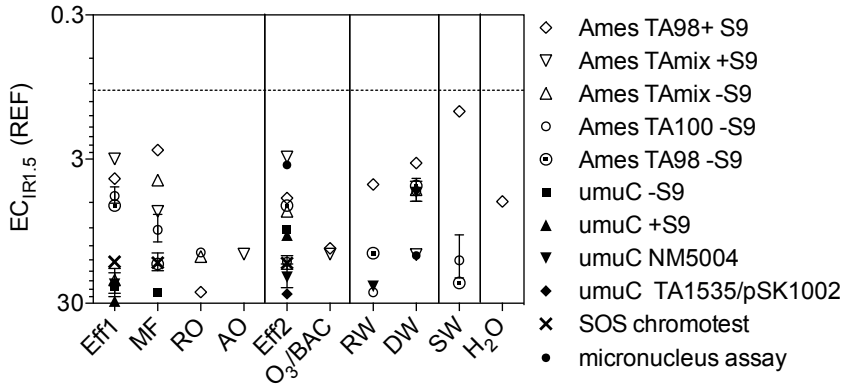

Figure 6.8. Results from bioassays indicative of the reactive modes of action.

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The *E. coli* assays for DNA damage turned out not to be suitable for samples with high organic matter content; therefore, no effects could be registered, although from qualitative observation, there was a difference in growth inhibition between the GSH^- and the GSH^+ strains. Only one assay attempted successfully to quantify ROS, but the oxidative stress response pathway that is discussed in Section 3.6 appears to be an indicator of oxidative stress that is experimentally more easily accessible.

6.4.7 Induction of Adaptive Stress Response Pathways

Both the HSR and the hypoxia induction were negative in all assays tested (Table 6.7). No bioassay for EpR stress could be identified. Response to inflammation was tested in the human T-lymphoblast cell line Jurkat E6-1 by quantifying IκB, which is a chaperone for NF-κB, with an ELISA. Five samples tested positive in this assay (Figure 6.9). In contrast, the NF-κB-CALUX, NF-κB-

GeneBLAzer, and NF-κB-cisFACTORIAL did not respond to any of the samples. These later assays are fairly new and have not yet been applied for water quality assessment. More work will be required to validate these assays and to improve their detection limits.

Three out of four bioassays indicative of the oxidative stress response were active in six to eight samples, which is an indication of the importance of this stress response pathway. The AREc32 cell line, NRF2/ARE-cisFACTORIAL, and the nrf2-CALUX all showed high sensitivity, with the effluent samples needing not much enrichment to show an effect, and a wide dynamic range, which makes them ideal water quality indicators.

P53 plays an important role as a tumor suppression factor, but most evaluated assays did not show an effect (p53-cisFACTORIAL, p53-CALUX +S9, and p53-GeneBLAzer). Only the p53-CALUX without metabolic activation showed induction, albeit at a REF of >10 and with unusual fingerprints. For example, the effect increased from Eff1 over MF to RO and from Eff2 to O_3/BAC.

Table 6.7. Bioassays Indicative of Adaptive Stress Response Pathways

Adaptive Stress Response Pathway	Inducing Chemicals	No. of Bioassays (Replicates)	No. +	No. -	Positive Bioassays	Negative Bioassays
HSR	Oxygen depletion	2	0	2	-	HSE-cisFACTORIAL, hspb11 induction in DART after 120 h
Hypoxia	Tunicamycin, thapsigargin, caplain, brefeldin A	2	0	2	-	HIF-1a-cisFACTORIAL, Hypoxia-Switchgear
EpR stress	High salt, glycol	0	0	0	-	-
Inflammation	Metals, PCBs, smoke, particles	4	1	3	Jurkat E6-1	NF-κB-CALUX, NF-κB-GeneBLAzer, NF-κB-cisFACTORIAL
Oxidative stress	Chemicals that produce ROS	4	3	1	AREc32, NRF2/ARE-cisFACTORIAL, nrf2-CALUX	nrf2-keap
DNA damage	Electrophilic chemicals, UV radiation	4 (1)	1	3	p53-CALUX -S9	p53-cisFACTORIAL, p53-CALUX +S9, p53-GeneBLAzer

Notes: No. + = number of positive bioassays, i.e., those that exceed the effect threshold of IR = 1.5 or 10% of maximum effect in one or more measured dilutions; no. - = number of negative bioassays, i.e., those that remain below the effect threshold of IR = 1.5 or 10% of maximum effect even in the highest REF.

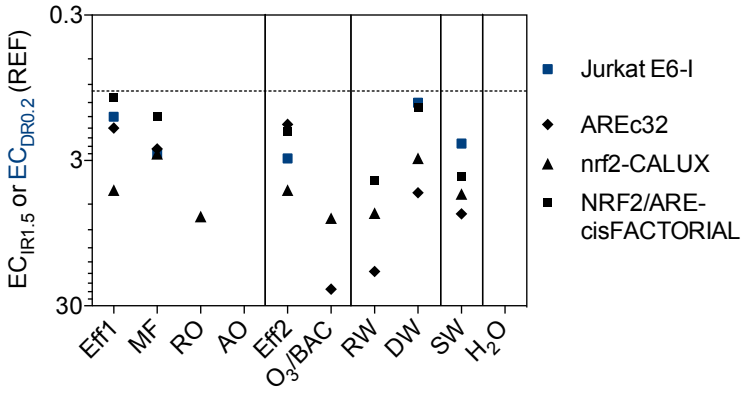

Figure 6.9. Results from bioassays indicative of adaptive stress response pathways.

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6.4.8 General Cytotoxicity and Models for System Response

The overarching effect overlying each of the cellular toxicity pathways is cytotoxicity (Figure 3.1). The bacterial screening assays were used as a quick initial screening tool only as they are rapid but have no human health relevance. Despite them being very short-term tests (15 min of incubation for the Microtox assay), they respond very sensitively, often at concentrations where the sample does not even have to be enriched.

Cytotoxicity normally manifests at higher concentrations than does induction of response pathways, so cytotoxicity is usually measured as the control on all induction assays. We did not do full dose−response curves for cytotoxicity in all mammalian reporter gene assays apart from AREc32, the cytotoxicity of which was similar to the targeted cytotoxicity assays in a human colon cancer cell line, Caco-2 NRU. The fish cell line (RTG2 MTT) was of relatively low sensitivity. Acute toxicity in the zebrafish embryo (DART 48 h lethality) was observed in two only samples, Eff2 and SW, but at low enrichment factors (REF = 5−6).

Cytotoxicity assays also can give some information about system toxicity if appropriate cell lines are used. Here we considered the sublethal endpoint in the zebrafish embryo toxicity test after 120 h of incubation as a endpoint for reproductive effects. This effect was clearly more responsive than the 48 h acute lethality endpoint in the zebrafish embryo. The SK-N-SH neuroblastoma cell line (Ba et al., 2003) is sensitive to chemicals that block the sodium channels, and this assay has been so far mainly used to evaluate paralytic shellfish poisons caused by neurotoxic freshwater cyanobacteria (Humpage et al., 2007). This endpoint was not active with the water samples of the present study, but the highest REF applied was only 2.

Expression of various cytokines in the human acute monocytic leukemia cell line THP-1 gives an indication of potential immunotoxicity (NWC, 2011). Here we measured interleukin 1β (IL-1β). We applied the assay in the antagonistic mode, but there was no detectable effect up to a REF of 2.

Table 6.8. Cytotoxicity Bioassays as Indicators of System Responses

Cytotoxicity Indicative of System	Function	Inducing Chemicals	No. of Bioassays (replicates)	No. +	No. -	Positive Bioassays	Negative Bioassays
Bacterial screening assay		All	2 (1)	2	0	Microtox, *P. phosphoreum* T3	-
Algal cells		All (+herbicides)	1 (1)	1	0	Algal growth inhibition	-
Mammalian cells		All	4	4	0	AREc32 cell viability, Caco-2 NRU, RTG2 MTT, DART 48 h lethality	-
	Develop-ment	All	1	1	0	DART 120 h sublethal	-
	Neuro-toxicity	Neurotoxicants	1	0	1	-	SK-N-SH
	Immuno-toxicity	Immuno-suppressive chemicals	1	0	1	-	THP1 cytokines

Notes: No. + = number of positive bioassays, i.e., those that exceed the effect threshold of IR = 1.5 or 10% of maximum effect in one or more measured dilutions. No. - = number of negative bioassays, i.e., those that remain below the effect threshold of IR = 1.5 or 10% of maximum effect even in the highest REF.

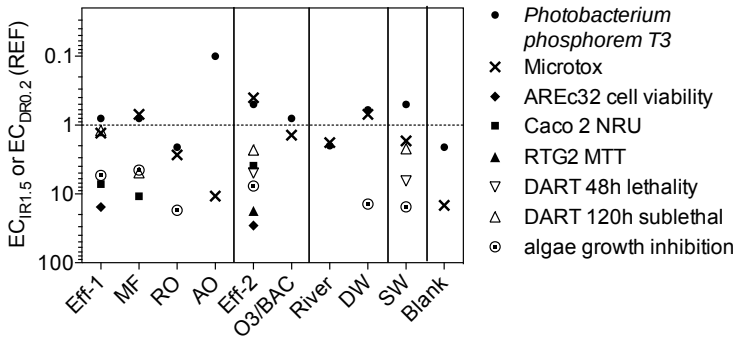

Figure 6.10. Results from cytotoxicity bioassays as indicators of system responses.

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6.4.9 Benchmarking Treatment Efficacy

In this section we discuss the bioassays in the light of their suitability to serve as process monitoring tools. One cannot say a priori that a bioassay is "good" or "sensitive" if it still measures an effect in treated waters. Effects can disappear if all chemicals that are responsive in this endpoint are well removed in the particular treatment process. The dynamic range between the effect of the product water and that of the blank is decisive for the suitability of an assay for assessing treatment efficacy

(provided that reproducibility, repeatability, and sensitivity already have been established with reference chemicals).

6.4.9.1 Advanced Water Treatment Plant Using Reverse Osmosis

The first advanced water treatment plant investigated uses microfiltration followed by RO and finished with AO. The micropollutant flow in this plant has been characterized in much detail in previous work by both chemical and bioanalytical tools (Escher et al., 2011; Macova et al., 2011). In the present study, we selected only four sampling points before and after critical treatment steps: the inflow (WWTP effluent, Eff1), after MF, after RO, and after AO combining hydrogen peroxide and UV irradiation (AO).

Effects were detected in Eff1 in 53 out of 100 bioassays (Figure 6.11). Treatment greatly reduced the effect burden caused by micropollutants. After MF, 52 bioassays tested positive (not exactly the same ones), but RO decreased the number of positives sharply to 11. After AO, only three bioassays tested positive and these also tested positive in the ultrapure water blank.

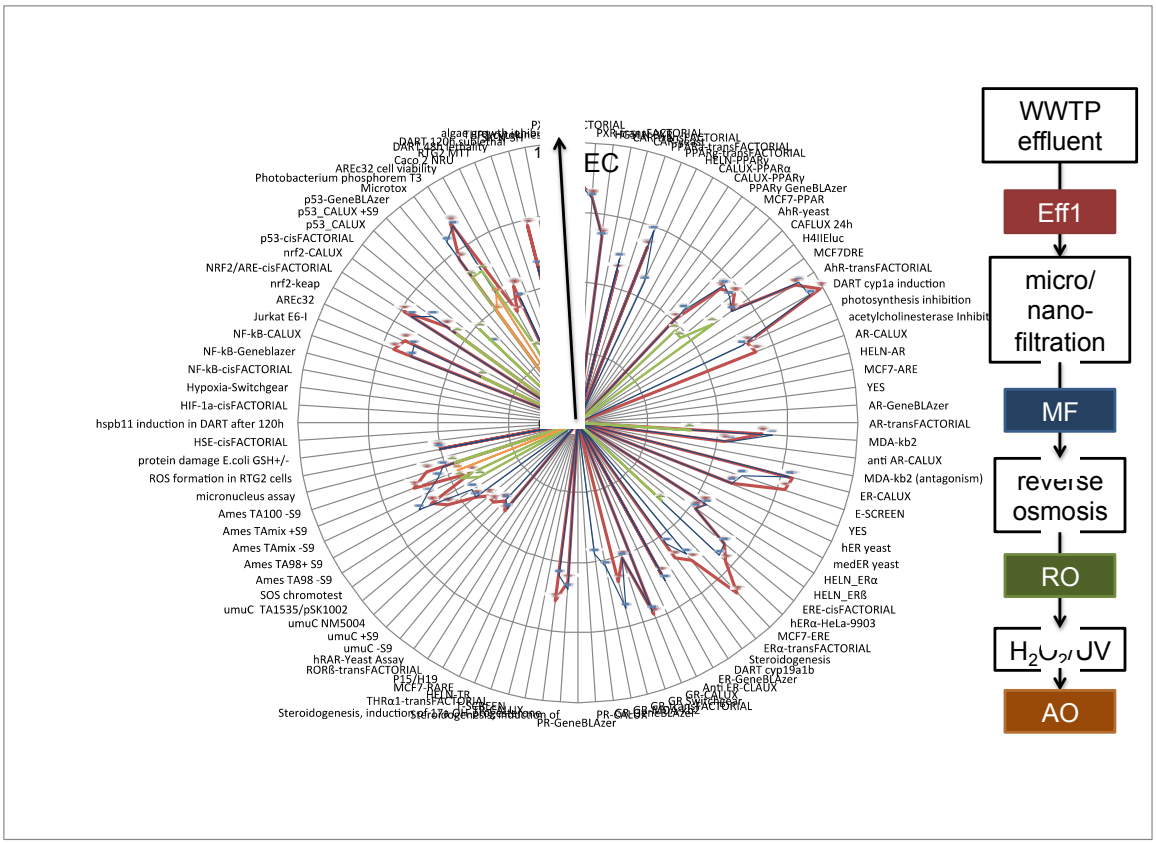

Figure 6.11. Bioanalytical fingerprint of the AWTP process.

Notes: The red diamonds represent the Eff1 sample, blue squares are MF, green triangles RO, and yellow circles AO.

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In 12 of 51 bioassays, the effect after MF increased by more than 20%, namely, more than the variability of the assay response. In 20 bioassays the effect remained constant (±20%), and in 15 bioassays, the effect decreased substantially already in the MF step. The fivefold increase in effect in the Ames TA_{mix}–S9 is presumably an artifact of the large variability of the results of this endpoint.

To avoid biofouling, the MF membranes are chloraminated, which causes the formation of DBPs that can cause effects in some of the bioassays for reactive modes of action (Neale et al., 2012).

The effects were greatly reduced after RO (Figure 6.11). In 34 out of 51 bioassays, the effect disappeared below detection limit, and in an additional seven bioassays, more than 20% of the effective micropollutant burden was removed. There was no preferred type or group of effects removed. The bioassays with high variability, for example, the Ames assay, seem not to be suitable for a reliable assessment. The bioassays that showed reduction of effect but were sensitive enough to still show something in RO, are best suited as indicator bioassays. They included three nonspecific cytotoxicity assays, *P. phosphoreum* T3, Microtox, and algal growth inhibition. Among xenobiotic metabolism indicators, the AhR-CAFLUX, H4IIEluc, and MCF7-DRE also fell into this category. Among specific receptor-mediated modes of action, the MDA-kb2 and hERα-HeLa-9903 were able to show the dynamics of treatment. In the group of ASRs, the AREc32 and nrf2-CALUX showed a distinct reduction pattern but were still above the LOD in RO water and thus were suitable as a sensitive screening tool for process control.

6.4.9.2 Water Reclamation Plant Using Ozonation and Biologically Activated Carbon Filtration

The second investigated water treatment plant produces recycled water from secondary treated wastewater plant effluent using ozonation and activated carbon filtration (van Leeuwen et al., 2003). The plant has a capacity of 10 ML day^{-1} and provides water to industry for nonpotable uses. Although the plant provides water for nonpotable applications, it has been designed to meet drinking water standards. The treatment process incorporates biological denitrification, preozonation, coagulation/flocculation/dissolved air flotation−sand filtration (DAFF), ozonation, biological activated carbon treatment, and ozone disinfection. The removal efficacy of micropollutants has been analyzed in detail in a series of studies that combined chemical analysis with bioanalytical tools (Macova et al., 2010; Reungoat et al., 2010, 2011, 2012a, 2012b). In the present study only two samples were incorporated: the secondary treated effluent (Eff2) and the water produced after ozonation and biological activated carbon treatment (O_3/BAC).

As is shown in Figure 6.12, 60 bioassays gave results above detection limit in the Eff2, similar to what was found with Eff1. The treatment reduced the number of responses by 47 to 13, and the effects in those 13 positive bioassays were also greatly reduced (Figure 6.13). As was the case for the other recycling plant, those bioassays that still showed an effect in the treated water are suitable as indicator bioassays to benchmark treatment efficacy.

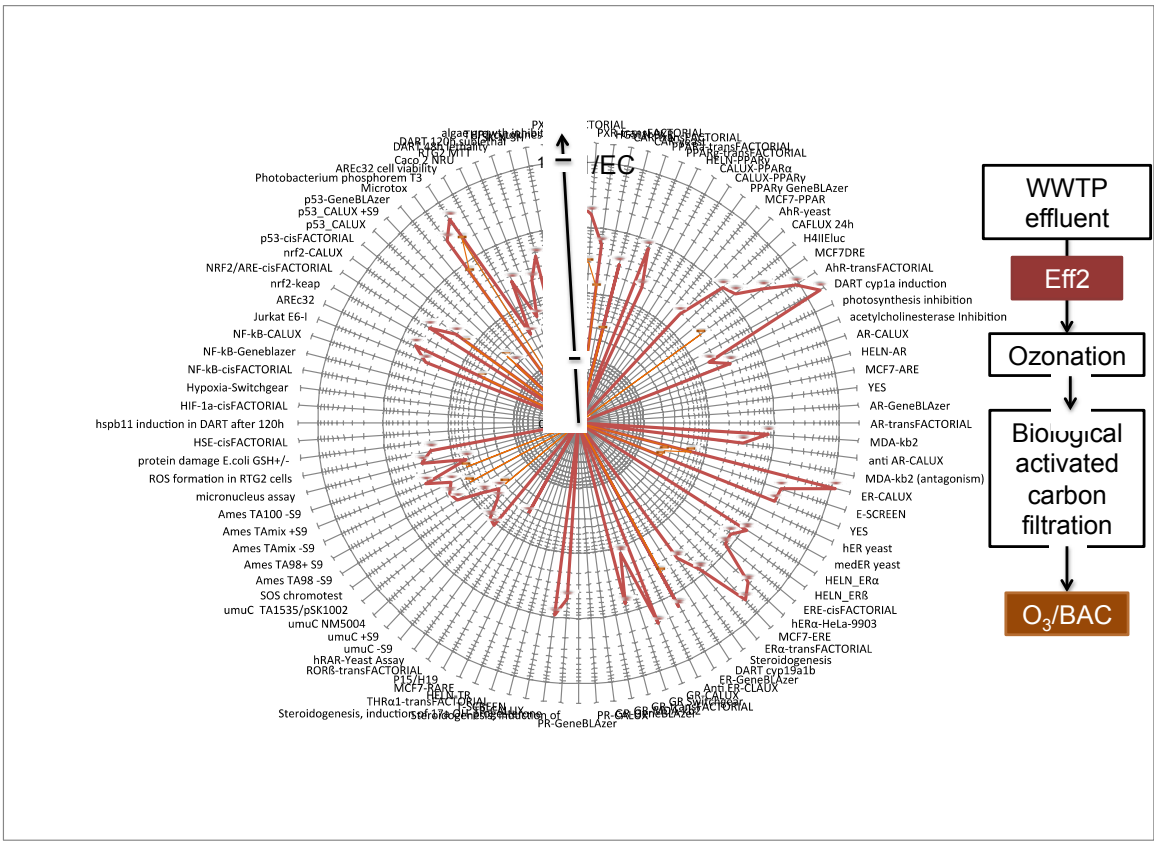

Figure 6.12. Bioanalytical fingerprint of the water treated with ozonation and biological activated carbon.

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Figure 6.13. % treatment efficacy in the 11 bioassays that did not fall below LOD after treatment.

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6.4.9.3 Drinking Water Treatment Plant

For comparison, we also assessed treatment in a drinking water treatment plant. This plant had been evaluated previously (Macova et al., 2011; Neale et al., 2012). The feed water is drawn from a river, and the levels of micropollutants (Tang et al., submitted for publication) and effects (Figure 6.14) were low. In the RW and DW samples (meaning river water and drinking water), 27 and 24, respectively, out of 103 bioassays were positive, but only 17 bioassays were identical to those positive in the advanced water treatment plants. For the remaining bioassays, remaining different biological endpoints were triggered.

The effects in the E-SCREEN, AhR-CAFLUX, and MCF7-DRE remained the same or were reduced. This observation is consistent with literature findings that chlorination degraded or did not change existing micropollutants but did not produce specifically acting compounds with enhanced specific toxicity. However, drinking water treatment with chlorination and chloramination increased the nonspecific and reactive toxicity (Figure 6.14), presumably because of the formation of DBPs, which is consistent with previous findings and chemical analysis of formed DBPs (Neale et al., 2012).

Of the bioassays that increased in the toxicity, there was just one for a specific mode of action, the hERα-HeLa-9903, but the majority targeted, as expected, xenobiotic metabolism, reactive modes of action, and ASRs. The cytotoxicity in the assays with *P. phosphoreum* T3, Microtox, and AREc32 cell viability increased by a factor of 2.1 to 4.4. The increase was most pronounced in the reactive modes of action (Ames TA98 + and -S9, Ames TA100 -S9, *umu*C NM5004, and MN assay). The effect was small, but there was a detectable increase by up to a factor of 2 for the bioassays indicative of xenobiotic metabolism, with a preference for the PXR (HG5LN PXR, PXR-transFACTORIAL, PXR-cisFACTORIAL, and AhR-transFACTORIAL). The response in all three bioassays for the oxidative stress response (nrf2-CALUX, NRF2/ARE-cisFACTORIAL, and AREc32) increased by a factor of 2.4 to 4.2.

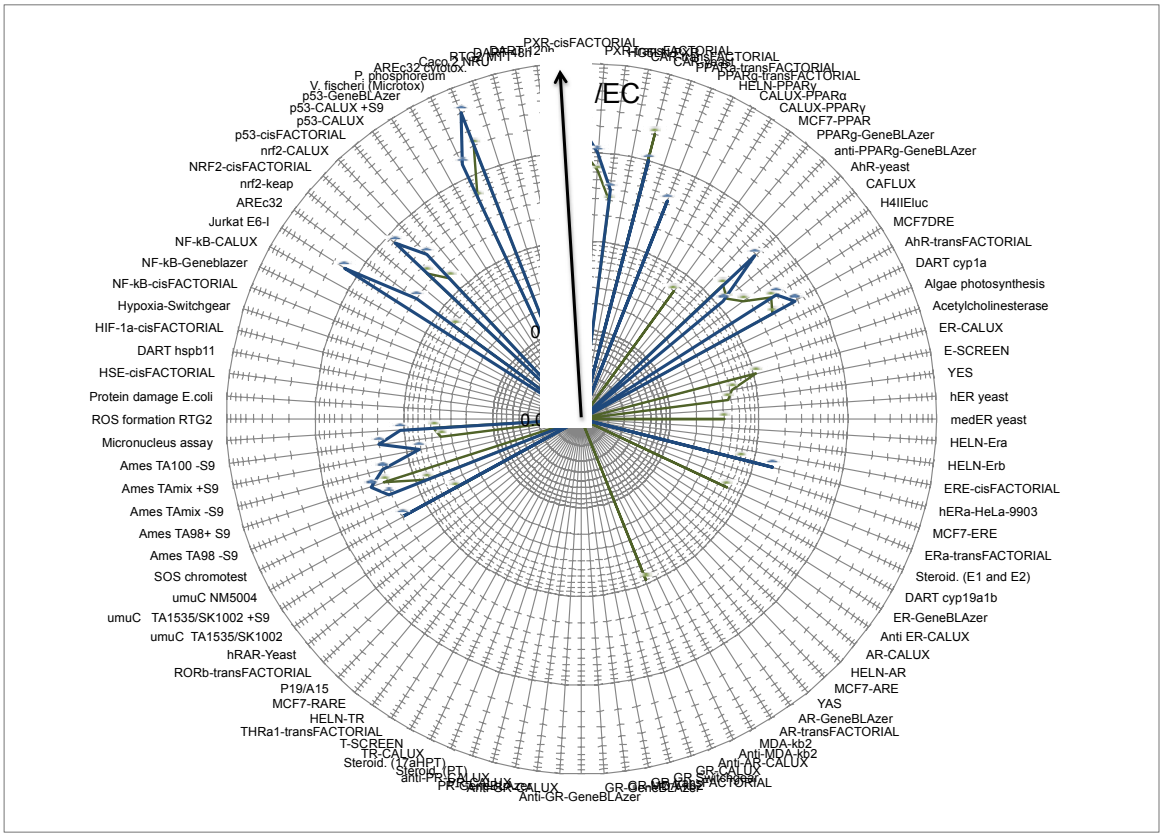

Figure 6.14. Bioanalytical fingerprint of the drinking water treatment (green RW; blue DW).

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6.4.10 Benchmarking Water Quality

BEQs are best suited for benchmarking water quality across different bioassays. Differences in the sensitivity of the various bioassays applied should be normalized by using the same reference chemical, provided that the relative potencies of all chemicals contributing to mixture effects do not vary too much between different in vitro assays.

To calculate BEQs, one needs a reference compound for which a full concentration−effect curve is available. Because this condition was not fulfilled for all endpoints of this study, we applied the BEQ concept to illustrate the concept for a few bioassays where we had experimental data for reference chemicals available (Table 6.9). These include the CAFLUX assay, algal photosynthesis inhibition, and the ER-CALUX, AR-CALUX, PR-CALUX and GR-CALUX, and E-SCREEN.

Table 6.9. Reference Chemicals and Their Effect Concentrations

Mode of Action	Reference Chemical	EC_{10} (pM)	BEQ
AhR-CAFLUX	2,3,7,8-Tetrachlorodibenzodioxin	1.7	TCDD-EQ
Algal photosynthesis inhibition	Diuron	3.8	DEQ (µg/L)
AR-CALUX	Dihydroxytestosterone	95.4	DHT-EQ (ng/L)
PR-CALUX	Progesterone/Org 2058	62.5/6.6	Org2058-EQ (ng/L)
GR-CALUX	DEX	364	DEX-EQ (ng/L)
ER-CALUX	17β-Estradiol	3.4	EEQ (ng/L)
E-SCREEN	17β-Estradiol	1.5	EEQ (ng/L)
ER-GeneBLAzer	17β-Estradiol	15.1	EEQ (ng/L)

The BEQs calculated for all samples with the EC_{10} values of the reference compounds are listed in Table 6.10. BEQs are good for communication of results compared to their effect-based trigger BEQs (EBT-BEQs) for drinking and recycled water, which is further discussed in Chapter 8. All drinking and recycled water samples were "compliant", whereas wastewater treatment effluent would be "noncompliant," indicating that the trigger values are appropriate to this real-world scenario (see more details in Chapter 9).

Table 6.10. BEQ Values of the Water Samples

Bioassays	Bio-analytical equivalent	Value for:										
		Eff1	MF	RO	AO	Eff2	O_3/BAC	RW	DW	SW	Blanks	LOD (BEQ)
Algal photosynthesis inhibition	DEQ (µg/L)	0.41	0.35	<0.03	<0.03	0.14	<0.03	<0.03	<0.03	0.14	<0.03	0.03
ER-CALUX	EEQ (ng/L)	1.14	1.10	<0.01	<0.01	13.32	<0.01	<0.01	<0.01	<0.01	<0.01	0.01
E-SCREEN	EEQ (ng/L)	0.53	0.12	<0.01	<0.01	0.75	0.01	0.02	0.01	0.04	<0.01	0.01
AR-CALUX	DHT-EQ (ng/L)	<0.48	<0.48	<0.48	<0.48	<0.48	<0.48	<0.48	<0.48	<0.48	<0.48	0.48
PR-CALUX	Org2058-EQ(ng/L)	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	0.08
GR-CALUX	DEX-EQ (ng/L)	15.51	17.3	<9.2	<9.2	32.09	<9.2	<9.2	<9.2	<9.2	<9.2	2.28

6.5 Conclusion

This is the first time that such a large set of bioassays was applied for water quality assessment. Characteristic fingerprints were obtained for the nine water samples (plus blank). These bioanalytical fingerprints allowed for a clear differentiation of apparent water quality (e.g., from treated wastewater to reclaimed water, as well as drinking and river water) and provided information about the modes of action of the micropollutants present in complex water samples. This is an excellent database suitable for future use in benchmarking water quality, for example, to interpret results from a smaller number of bioassays suitable for routine monitoring.

Several bioassays can be used for monitoring the efficacy of micropollutant removal by wastewater treatment and advanced water treatment. For this purpose, a small number of indicator bioassays would be sufficient to characterize the performance of unit processes that compose a treatment train in wastewater, water reclamation, and/or drinking water treatment facilities.

Chapter 7

Chromatographic Fractionation for Identification of Bioactive Compounds on the Example of the Glucocorticoid Receptor

7.1 Introduction

Because GR activity was ubiquitously detected in several extracts of water samples, an analytical study was undertaken to investigate potential causes for observed toxicity. High-pressure LC-MS analysis was coupled with fraction collecting for effect-directed structure identification for the water samples collected in the United States (Chapter 5).

7.2 Materials and Methods

In order to provide greater extract volume for instrument analysis and to increase assay sensitivity, an HPLC fractionation method with large injection volume and a large-diameter chromatography column was developed. This method utilized a ZORBAX Eclipse XDB-C18 (9.4 × 250 mm, 5 μm) column with mobile-phase composition of acetonitrile (A) and ultrapure water (B). The flow rate was 3 mL/min, and the column temperature was 30 ^{o}C. The HPLC gradient was as follows: 0−2 min at 5% A, from 2−30 min A linearly increased to 95%, from 30.1−36 min 100% A, and from 36−41 min at 5% A for equilibration. The injection volume was 1.5 mL. Fractions were collected every 2 min until 36 min, resulting in 18 separate fractions.

LC−quadruple time-of-flight (QTOF) was used to screen for all possible GR agonists in each water sample extract that demonstrated GR activity along with the corresponding fractions. The separation was accomplished by using an Agilent 1290 UHPLC system combined with an Agilent 6540 QTOF accurate MS system. A ZORBAX Eclipse Plus C_{18} (2.1 × 150 mm, 1.8 μm) column was used, and the QTOF system was operated in scan mode by using both ESI positive and ESI negative ionization modes. The mobile-phase A was methanol, and mobile-phase B was ultrapure water with 0.1% formic acid. A flow rate of 0.3 mL/min was used with a column temperature of 35 ^{o}C. The gradient profile was 0−0.5 min at 5% A; 0.5−15 min linearly increasing from 5 to 100% A; 15−18 min 100% A; and from 18−20 min at 5% A for equilibration. The injection volume was always 10 μL.

As opposed to LC-QTOF scans for unknown GR agonists, LC-MS/MS was used for the identification and quantitation of target (known) GR compounds. Separation and detection were achieved by using an Agilent 1290 UHPLC system combined with an Agilent 6490 triple quadruple MS system (LC-MS/MS). The MS source was polarity switching ESI, and the instrument was operated in multiple reaction monitoring (MRM) mode. The separation column was a ZORBAX Eclipse Plus C18 (2.1 × 50 mm, 1.8 μm). The mobile-phase profile was methanol (A) and ultrapure water with 0.1% of acetic acid (B). The flow rate was maintained at 0.3 mL/min with a column temperature of 35^{o}C. The elution gradient was as follows: 0 to 1 min, 10% A linearly increasing to 25%; 1 to 7 min, 25% A linearly increasing

to 95%; 7 to 9 min, 95% A; and 9.1 to 11.5 min, 10% A for equilibration. The injection volume was 10 µL.

7.3 Results and Discussion

7.3.1 Fractionation

Four common glucocorticoid compounds were used as positive spike controls, namely, DEX, cortisol (COR), cortisone (CORT), and prednisolone (PRED). These were used for fractionation method validation relying on HPLC with UV detection (Figure 7.1). The results showed that consistent separation was obtained with the fractionation method.

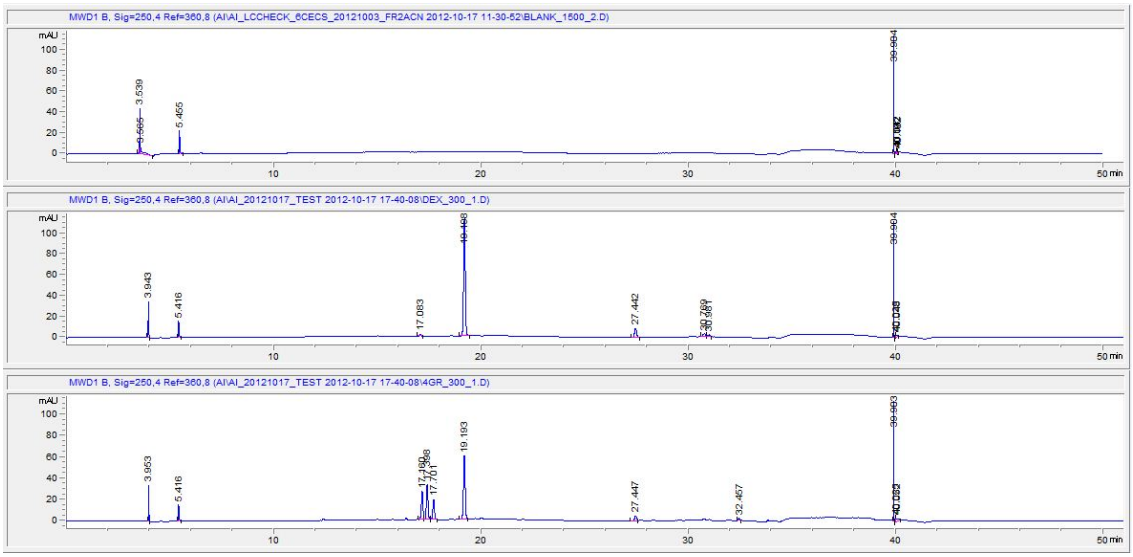


Figure 7.1. Fractionation method validation using HPLC-DAD.

Notes: Top: MeOH blank; Middle: DEX (RT = 19.2 min); Bottom: 4 GR mixture standards (RT from 17.1 to 19.2 min).

Several iterations of fractionation and subsequent bioassay evaluation using the GR-Switchgear bioassay were conducted over 1 year. Results were consistent, showing that fractionation resulted in GR activities with four- to fivefold decrease in receptor activity as compared with that in unfractionated samples. For instance, the GV Inf sample from our intial sampling event had an IR of 23, whereas the IR of the first fraction dropped to 6.8 (Figure 7.2).

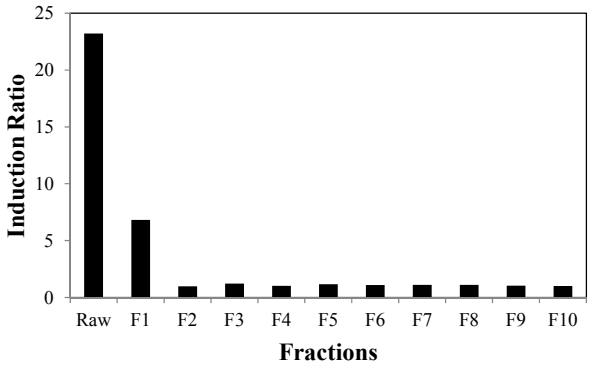


Figure 7.2. The GR-Switchgear activity of Sample GV Inf fractions compared with the unfractionated samples using unimproved fractionation method.

Because of the apparent lack of retention, we modified the fractionation method by changing the HPLC column, increasing the injection volume, and increasing enrichment of the fractions from 10-fold to 25-fold. The GR-Switchgear assay of those fractions showed that three fractions (F1, F9, and F17) displayed significantly higher GR activity than did other fractions. The middle fraction F9 was the fraction where cortisol, cortisone, and prednisolone were eluted on the basis of our HPLC-UV study (Figure 7.3). This finding indicates that structurally similar compounds are concentrated in this fraction. Conversely, F1 and F17 indicate that some highly polar and relatively nonpolar compounds, respectively, also contribute to the glucocorticoid activity.

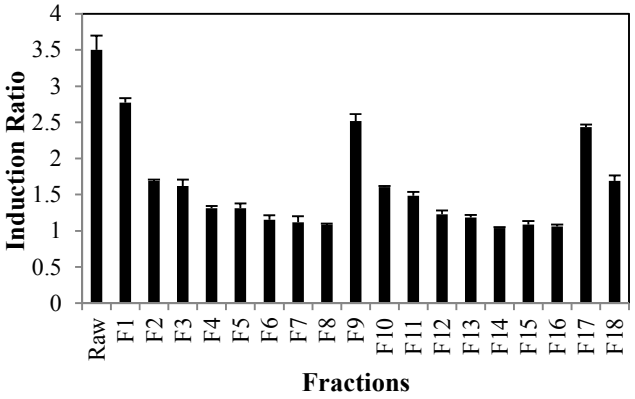


Figure 7.3. The GR-Switchgear activity of Sample GV Inf fractions compared with the unfractionated samples using improved fractionation method.

7.3.2 Instrument Analysis

Samples as well as their fractions were all injected into the LC-QTOF in both positive and negative product ion scan mode. Figure 7.4 shows a typical chromatogram comparison between the different samples.

Figure 7.4. Baseline peak chromatography (BPC) of different U.S. samples under HPLC-QTOF positive scan mode.

The results indicate that there was no apparent chromatogram change between those samples with and without GR activity. Under further molecular feature extraction, however, we were able to extract all of the product ions from these samples. Three injections for each sample were made in order to ensure that accurate mass could be obtained reproducibly and that ions were further screened by principal component analysis (Figure 7.5). The heat map shows mass profile differences between different samples.

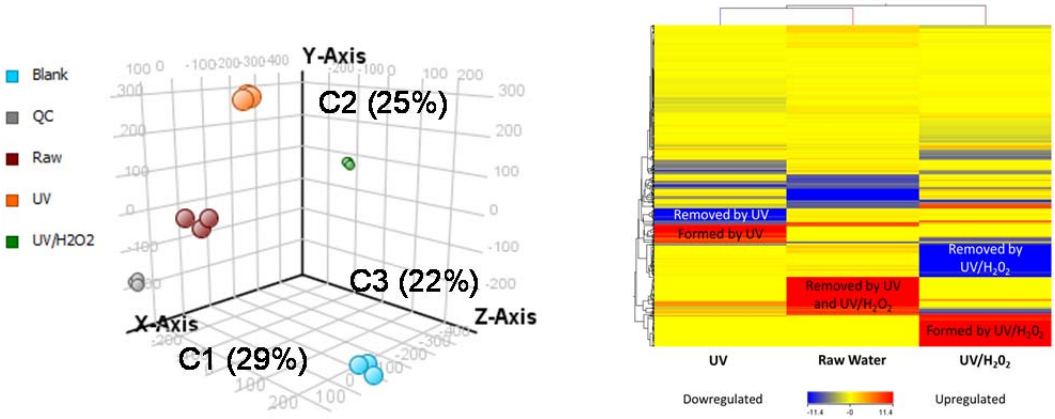
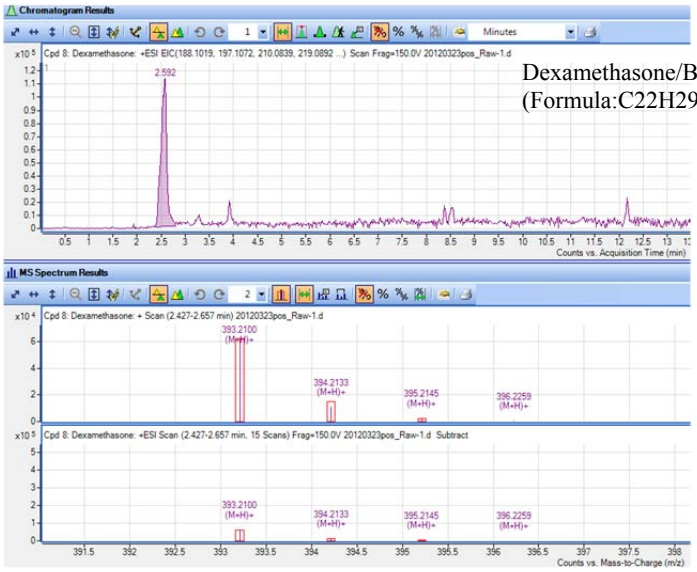


Figure 7.5. Principal component analysis screen (left) and heat map for different sample profiles (right).

Using the QTOF, we were able to decrease the target compound list from more than 1000 to 170, which marks a significant difference between the GV Inf sample and the UV-treated sample. Because of the limited source of compounds represented by the LC-MS database, we were unfortunately able to identify only a few as known potential glucocorticoid chemicals. DEX/betamethasone was one of the compounds identified (Figure 7.6).

As the QTOF data indicated that GR agonists such as DEX and betamethasone were indeed present within the extracts showing GR activity and absent in those samples not exhibiting GR activity, 28 commercially available standards for known GR agonists were procured for LC-MS/MS analysis (Table 7.1).



Dexamethasone/Betamethasone
(Formula:C22H29FO5)

Figure 7.6. Possible glucocorticoid compounds in GV Inf sample.

Table 7.1. Glucocorticoid Compounds Quantified by LC-QQQ

Formula	Mass (g/mol)	Compound
C21H28O5	360.193674	Aldosterone
C23H32O4	372.2300595	Deoxycorticosterone acetate
C23H31FO6	422.2104669	Fludrocortisone acetate
C21H28O5	360.193674	Cortisone
C22H30O5	374.2093241	6-α-Methylprednisolone
C22H29FO5	392.1999022	DEX
C21H27FO6	394.1791668	Triamcinolone
C21H30O5	362.2093241	Hydrocortisone
C21H26O5	358.1780239	Prednisone
C21H28O5	360.193674	Prednisolone
C22H29FO5	392.1999022	Betamethasone
C21H30O4	346.2144095	Corticosterone
C24H32O4S	416.2021302	Spironolectone (aldactone)
C28H37ClO7	520.2227813	Beclomethasone dipropionate
C25H34O6	430.2355388	Budesonide
C25H31NO6	441.2151377	Deflazacort
C24H31FO6	434.2104669	Flunisolide
C28H35FO7	502.2366817	Amcinonide
C26H32F2O7	494.2116098	Fluocinonide
C25H31F3O5S	500.1844294	Fluticasone propionate
C27H30Cl2O6	520.1419441	Mometasone furoate
C22H29FO4	376.2049876	Fluorometholone
C22H29ClO5	408.1703518	Beclomethasone
C22H28F2O5	410.1904804	Flumethasone
C24H31FO6	434.2104669	Triamcinolone acetonide
C24H30F2O6	452.2010451	Fluocinolone acetonide
C25H32ClFO5	466.19223	Clobetasol propionate
C26H32ClFO5	478.19223	Clobetasone butyrate
Surrogate compounds		
Hydrocortisone-9,11,12,12-d4		
DEX-d4		
Prednisone-d4		

Under primary detection, prednisone was found in two samples with GR activity at 20 and 59 ng/L. Triamcinolone and hydrocortisone were each detected in one sample showing GR activity, with concentrations of 24 and 7 ng/L, respectively. These concentrations are similar to those reported by Schriks et al. (2010), who concluded that these three compounds were responsible for most of the GR activity in European wastewater samples.

7.4 Conclusion

Prednisone, triamcinolone, and hydrocortisone are GR agonists that were detected in water samples with positive GR bioassay results. Thus far, all other GR agonists were not detectable; however, with additional instrumental optimization, far lower detection limits should be possible. Future research will focus on additional fractionation and comparisons for known GR agonists to complete mass balance.

Chapter 8

Interpretation Guidelines: Toward the Development of Effect-Based Trigger Values

8.1 Introduction

Although ECs and BEQs are well suited to benchmark water quality of waters from different sources and to assess the treatment efficacy of wastewater treatment and advanced water treatment, it remains uncertain what the observed in vitro effects actually mean in terms of environmental and health risk. Chemical guideline values, namely, acceptable concentrations, are defined for many types of waters and for many individual chemicals. Bioassays respond to mixtures of chemicals with a common mode of toxic action; therefore, a one-to-one translation from chemical guideline values to bioassay-based guideline values for all compounds would not be possible. Instead, we sought to make initial steps toward the establishment of bioassay-based trigger values by using the approaches outlined later. Exceeding these trigger values would indicate that a more detailed analysis, including chemical analysis or more-definitive toxicity assessment, is warranted.

In this report, we present some information on how traditionally health-based chemical guideline values are derived, making particular reference to the Australian Guidelines for Water Recycling: Augmentation of Drinking Water Supplies (AGWR) (NRMMC & EPHC & NHMRC, 2008) and the ADWG (NHMRC and NRMMC, 2011). After this literature review we discuss three approaches on how to derive bioassay-based trigger values and illustrate the concept with experimental data from this project. We conclude with a recommendation on a way forward in this area.

Bioassay-based trigger values will not and should not replace guideline values. They merely serve as a first-tier screening tool. Exceeding these trigger values will indicate that a more detailed analysis, including chemical analysis or more-definitive toxicity assessment, is warranted.

8.2 Chemical-Based Guideline Values for Individual Chemicals

Health-based water quality guideline values (GVs) are defined as concentrations of chemicals in drinking water that do not pose an appreciable risk over a person's lifetime of exposure. GVs are based on acceptable (or allowable) daily intake (ADI) or the reference dose (RfD). The RfD for threshold (i.e., non-cancer) endpoints is extrapolated from "No Observed Adverse Effect Levels" (NOAELs) or benchmark doses (BMDs) derived from a set of animal studies divided by an uncertainty factor. The uncertainty factors can usually range from 10 to 10,000 and account for extrapolation from animal studies to humans, from individual people to a whole population, for differences in exposure duration, as well as quality and comprehensiveness of the database and for all other uncertainties related to extrapolation from a model system to a human population (Figure 8.1).

Existing GVs for individual chemicals are defined, for example, in the AGWR (NRMMC & EPHC & NHMRC, 2008) and in the ADWG (NHMRC and NRMMC, 2011).

Schriks et al. (2010) recently proposed a pragmatic approach to derive provisional drinking water GVs for unregulated chemicals once they have been detected in drinking water: in the absence of statutory GVs, available ADI or RfD values are prioritized, and if those are not available, TDIs are calculated from available literature. This is similar to the scheme applied in the AGWR (NRMMC & EPHC & NHMRC, 2008) and matches the approach proposed by Snyder et al. (2008) to derive drinking water equivalent levels for pharmaceuticals by using the minimum therapeutic dose.

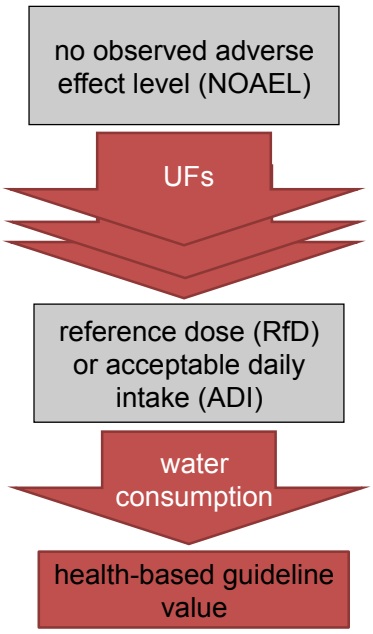


Figure 8.1. Derivation of health-based water quality GVs.

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8.3 Derivation of Health-Based Trigger Value for Bioassays

Brand et al. (2013) proposed very recently an approach using the example of the CALUX bioassays on how to derive effect-based trigger (EBT) values from the ADI of the reference compound, which also are often the point of departure for the derivation of chemical-based GVs (e.g., in the AGWR). This approach is based on the idea of Punt et al. (2013) that compounds act similarly (thus fulfilling the prerequisite of the TEQ concept of concentration-additive mixture effect) but have different toxicokinetics and therefore must be corrected for the toxicokinetic differences.

The toxicokinetic differences between in vivo and in vivo are considered by accounting for oral bioavailability and binding to plasma proteins (Figure 8.2). Probably the most important toxicokinetic modifier, metabolism, is not included in the model at this stage.

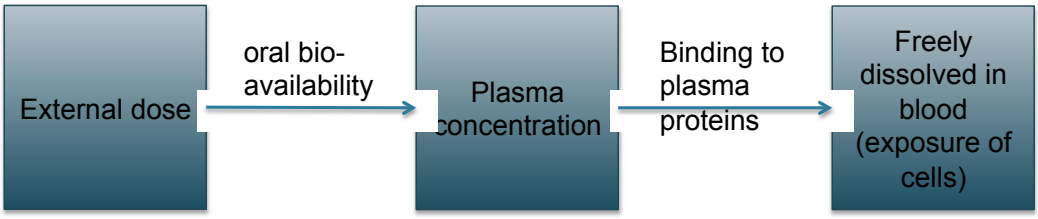

Figure 8.2. In vivo to in vitro extrapolation.

The approach proposed by Brand et al. (2013) was illustrated on the example of hormonal effects that can be quantified by various CALUX assays. The point of departure is the ADI of a potent reference compound. The toxicokinetic factors of oral bioavailability and fraction unbound to plasma proteins of the reference compound are used to estimate the acceptable target concentrations of the reference compound. Those are then translated to acceptable target concentrations of a compound other than the reference compound in the associated CALUX assay by the relative effect potency in vitro $REP_{in\ vitro}$, and then the conversion is back calculated for the other compound to its external equivalent concentration. Then the water consumption and an allocation factor are used just like in the ADWG to derive the trigger value of the "other" compound, which can then be directly assessed with the CALUX (Figure 8.3). The thus-derived EBT-BEQs are listed in Table 8.1.

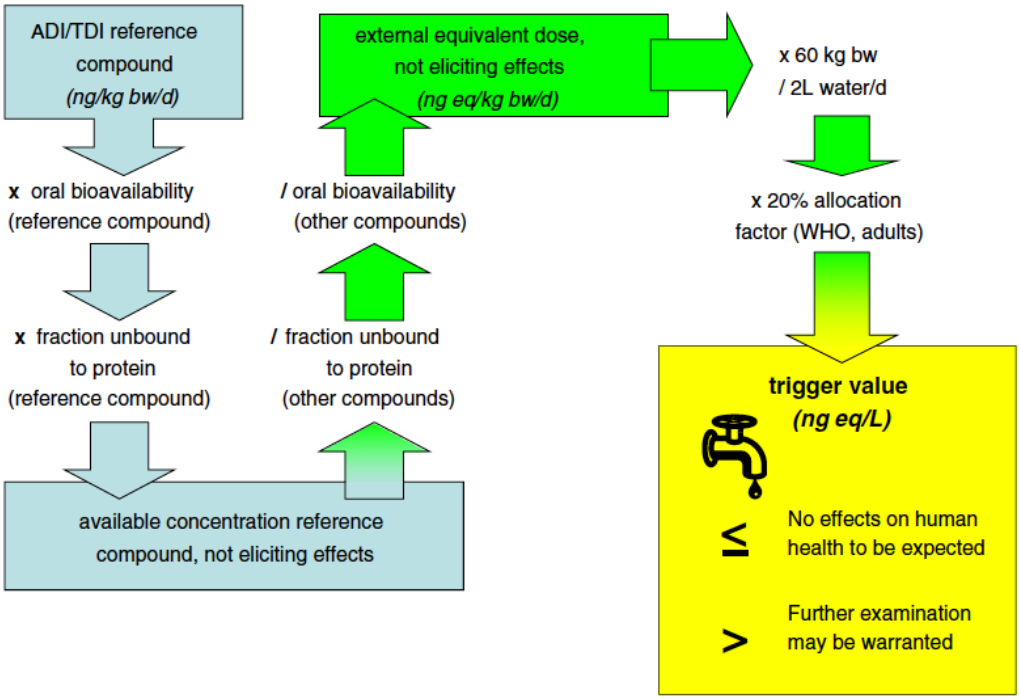

Figure 8.3. Derivation of effect-based triggers from ADI.

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Table 8.1. EBT-BEQ Directly Derived from ADI

Bioassay	Reference Compound	EBT-BEQ	Numerical Value	Unit
ER-CALUX	17β-Estradiol	EBT-EEQ	3.8	ng/L
AR-CALUX	Dihydroxytestosterone	DHT-EQ	11	ng/L
PR-CALUX	Org2058	Org2058-EQ	333	ng/L
GR-CALUX	DEX	DEX-EQ	21	ng/L

Source: Brand et al., 2013.

8.4 Effect-Based Trigger Values

To derive bioassay-based trigger values, it may not be necessary to do a full derivation from first principles as is done for the derivation of GVs for individual chemicals and outlined in Section 8.3 but rather to adopt whatever chemical GVs already exist and cross over from these existing values to bioassay-based trigger values as proposed in Figure 8.4.

There is no single EBT value, but an individual EBT value must be defined for each bioassay. Depending on the biological endpoint, we have to differentiate between two approaches to define EBT values.

- *For receptor-mediated effects*, where a reference chemical can be defined with clear maximum and minimum potency, the BEQs can be used to define EBT-BEQs.

- *For nonspecific toxicity and ASRs*, and in all cases where all or a large fraction of the present chemicals contribute to the effect, the BEQ concept is not applicable. In this case, mixture toxicity concepts can be applied to derive ECs of the sample in units of relative enrichment factors that equate to the triggers, i.e., effect-based trigger ECs (EBT-ECs). Per definition the EBT-EC must be derived for mixtures and must include consideration of the chemicals' interaction in mixtures (concentration additive or via independent action).

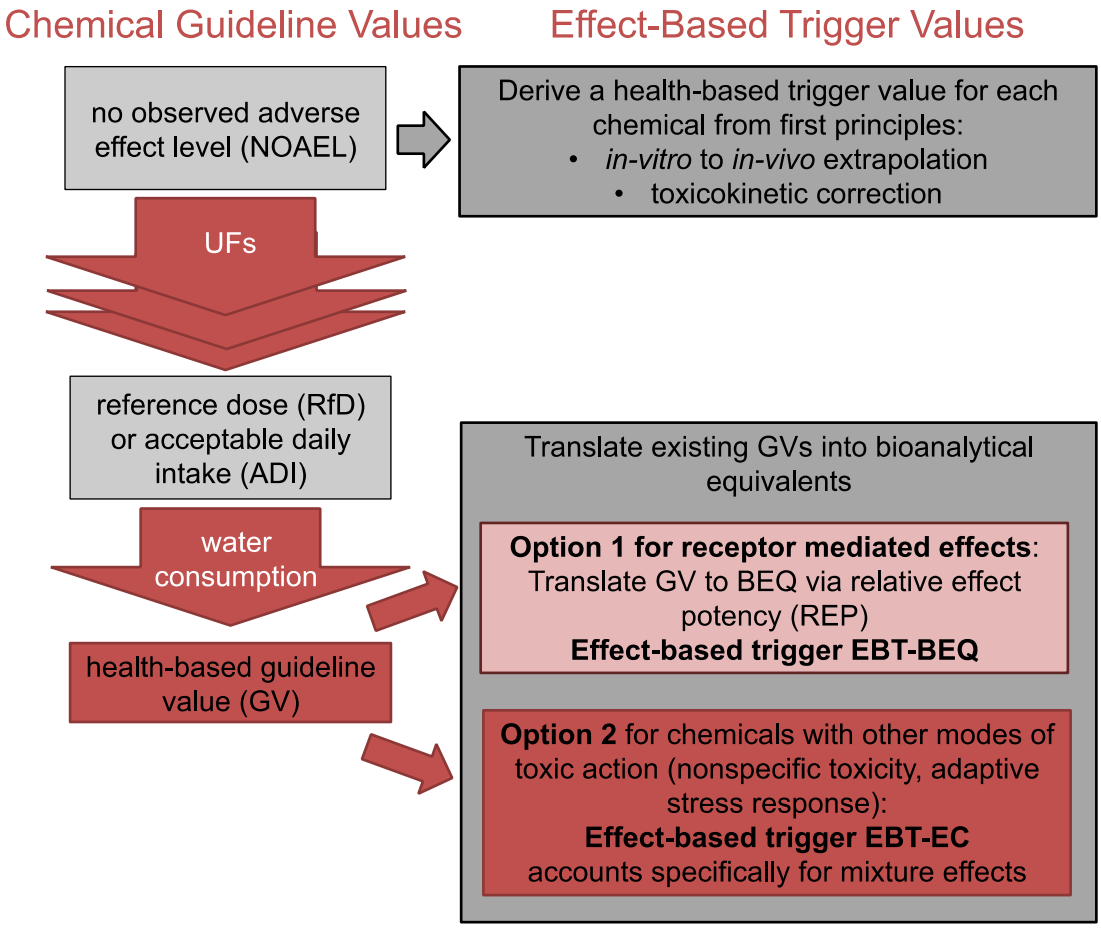

Figure 8.4. Options to derive effect-based trigger values for bioassays from GVs.

8.5 Effect-Based Trigger Bioanalytical Equivalent Concentration (EBT-BEQ)

EBT-BEQs are most suitable for bioassays for receptor-mediated effects such as estrogenicity or activation of the AhR, where there are well-defined and potent reference chemicals. Bioassays respond to a mixture of chemicals with a common mode of toxic action, in reporter gene assays equivalent to the target mode of action.

In principle, chemicals that act according to the same mode of action elicit a concentration-additive mixture effect. It does not really matter if this effect is caused by compound A or compound B, as both affect the same mode of action. Thus, a GV for a single compound should also be protective for the mixture, as long as the GV has been derived from toxicity data on the basis of the mode of action monitored in the in vitro assay. For example, a guideline for 17β-estradiol on the basis of its carcinogenicity will not be useful when comparing it to an EEQ value measured from an estrogenicity bioassay, but a guideline for 17β-estradiol on the basis of an estrogenicity assay will be applicable.

If the $REP_{in\ vitro}$ values are known, the different GV_i values can all be converted to a common BEQ—in the example of estrogenic chemicals, the EEQ. For chemicals other than the

reference chemical, the GVs need only to be multiplied by the relative effect potency $REP_{in\ vitro}$ in the in vitro assay.

$$BEQ_i = REP_{in\text{-}vitro} \cdot GV_i \tag{17}$$

Although chemicals are present in mixtures, the mixture effects are not relevant because each GV should be protective for a mixture of compounds with the same mode of action, provided its derivation was based on the mode of action that the bioassay measures. However, as discussed earlier, this is not always the case; otherwise, all BEQ_i values would be the same. In reality as is demonstrated in the examples later, the BEQ_i values are distributed and the EBT-BEQ can be chosen from the distribution of $logBEQ_i$ values.

The EBT-BEQs can then be derived from a chosen percentile of the distribution of $logBEQ_i$ values. A precautionary approach would use the 5th percentile, assuming that the lower BEQi values are likelier to be related to the target mode of action. Alternatively, the 50th percentile represents the median, the likeliest value of the distribution.

8.5.1 Example 1: EBT-EEQ for E-SCREEN Assay

This approach is illustrated on the example of estrogenic compounds in the E-SCREEN in Table 8.2. There are GVs for 10 estrogenic chemicals in the AGWR (none of them in the ADWG) and the corresponding BEQ_i values are EEQs.

In theory, the EEQ_i should be very similar for different compounds *i* if their GV_i values are based on the same mode of action. This is not the case for all chemicals (Table 8.2). This clearly highlights that the GVs for those compounds are not always based on the target mode of action of the bioassay (i.e., estrogenicity).

Table 8.2. Derivation of EEQ_i from GV_i for the E-SCREEN Assay for Estrogenicity

Compound	$REP_{in\ vitro}$	GV_i (ng/L) in AGWR[d]	Basis for the GV	Correspond-ing EEQ_i in the E-SCREEN (ng/L) (Equation 17)
17β-Estradiol (βE2)	1	175	NOAEL for changes in hormone-dependent parameters in postmenopausal women	175
17α-Estradiol (αE2)	0.008[a]	175	On the basis of βE2 assessment and assuming equipotency	1.40
Estrone (E1)	0.012[b]	30	On the basis of LDTD	0.36
Estriol (E3)	0.071[b]	50	On the basis of LDTD	3.55
Ethinylestradiol (EE2)	1.25[b]	1.5	On the basis of LDTD	1.88
Mestranol (Mes)	0.013[c]	2.5	On the basis of LDTD	0.033
4-Nonylphenol (4NP)	0.000078[b]	500,000	NOAEL for reproductive effects in rats consistent with estrogenic disruption	39
4-*t*-Octylphenol (4tOP)	0.000065[b]	50,000	NOAEL for effects on the kidney in rats. The NOAEL for reproductive effects was ~10× higher.	3.25
Bisphenol A (BPA)	0.00003[b]	200,000	NOAEL for effects on the liver in rats (NOAEL for reproductive effects >10×)	6.0
di-*n*-Butylphthalate (Di-nBP)	0.00000034[b]	35,000	NOAEL for reproductive effects in rats	0.012

Notes: [a]Data collected from Fang et al. (2000).

[b]Data collected from Leusch et al. (2009).

[c]Data collected from Körner et al. (2001).

[d]GVs from the AGWR from (NRMMC & EPHC & NHMRC, 2008). LDTD = lowest daily therapeutic dose.

To determine in a more systematic way which values can be used, one can plot the ratio of GV (i.e., GV for 17β-estradiol divided by GV of the other compound) to the $REP_{in\ vitro}$ values (Figure 8.5). From Figure 8.5, it is clear that the GV_{E2}/GV ratios for the weak xenoestrogen di-*n*-butylphthalate and for the estrogen mestranol are about 5 orders of magnitude higher than the $REP_{in\ vitro}$ and that, for all other xenoestrogens, the difference is at least 10 to 100. This finding may indicate that the GV, though derived from toxicological data on reproductive effects, may not be via estrogenic activity and thus that an EEQ trigger on the basis of these compounds may not be suitable for the purpose of deriving an EBT for estrogenicity. Therefore, we have removed the strong outliers di-*n*-butylphthalate and mestranol from the data set.

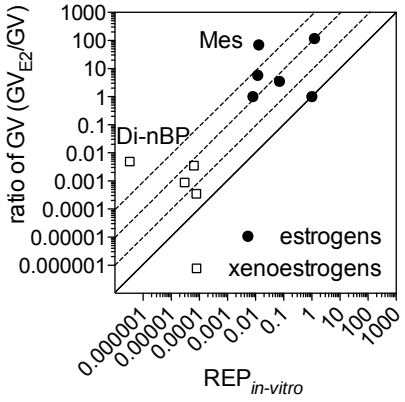

Figure 8.5. Comparison of REPin vitro with the ratios of the GVs (i.e., GV for 17β-estradiol divided by GV of the other compound).

Because we typically do not know the full chemical composition in a water sample (of estrogens in the example), we cannot say a priori which of the EEQs is representative for the water sample. The most sensitive EEQ_i (for estrone) is 3 orders of magnitude lower than the highest EEQ_i, which also happens to be the value for the reference compound estradiol. Therefore, we propose to calculate the EBT-EEQ from the distribution of EEQ_i values given in Table 8.2. Such values are typically log-normally distributed, not normally distributed. The distribution of the logEEQ_i is shown in Figure 8.6 for the E-SCREEN. As a precautionary approach, one could use the 5th percentile of the distribution as the EBT value. The 5th percentile of this distribution is thus our proposed EBT-EEQ for the E-SCREEN at 0.17 of EEQ/L.

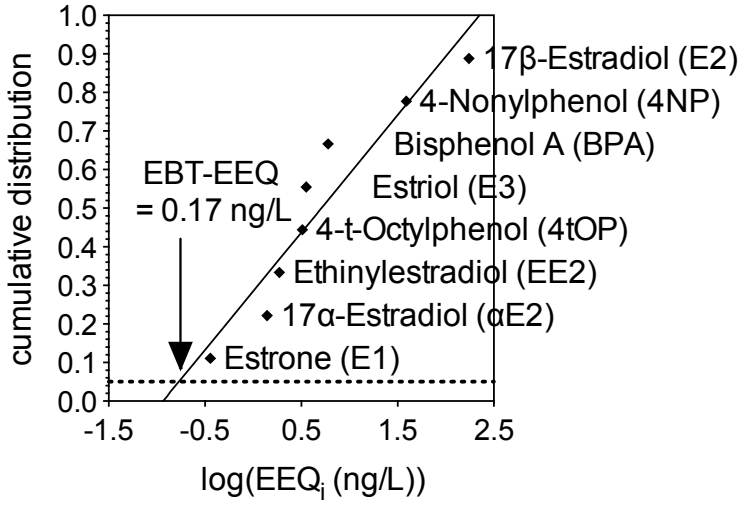

Figure 8.6. Cumulative frequency distribution of EEQi and estimated EBT-EEQ (5th percentile).

8.5.2 Example 2: EBT-DEQ for Inhibition of Photosynthesis

For herbicides the situation is different from that of estrogens because the human health-based GV is clearly not derived from the target mode of action, given that the latter is inhibition of photosynthesis. The combined algal assay gives a targeted measure of the inhibition of photosynthesis by herbicides, and although nonherbicides also are active in this assay, the potency is so much lower that nonherbicides can be neglected (Tang and Escher, 2013). Therefore, we can directly convert the GV in the two guidelines (ADWG and AGWR) with the experimental REP (Tang and Escher, 2013) into EBT-DEQ (Table 8.3). The distribution of the $\log DEQ_i$ and the thereby-derived 5th percentile, namely, the EBT-DEQ, is shown in Figure 8.7. The EBT-DEQ was estimated from the 5th percentile of the distribution to be 0.4 μg of DEQ/L for drinking water (ADWG) and 0.6 μg of DEQ/L for recycled water (AGWR).

Table 8.3. GVs in the ADWG and the AGWR and Their Translation into DEQ_i

Herbicide	GV_i (µg/L) ADWG	GV_i (µg/L) AGWR	REP[a]	DEQ_i (µg/L) ADWG	DEQ_i (µg/L) AGWR
Atrazine	20	40	0.12	2.4	4.8
Bromacil	400	300	0.50	200	150
Diuron	20	30	1.00	20	30
Fluometuron	70	50	0.02	1.2	0.8
Hexazinone	400	300	0.26	102	77
Metribuzin	70	50	0.34	23	17
Prometryn	n.d.	105	0.36	n.d.	38
Propanil	700	500	0.08	54	39
Propazine	50	50	0.05	2.3	2.3
Simazine	20	20	0.04	0.7	0.7
Terbuthylazine	10	n.d.	0.32	3.23	n.d.
Terbutryn	400	300	0.51	206	154

Note: n.d.= not determined

Source: Data from Tang and Escher (2013).

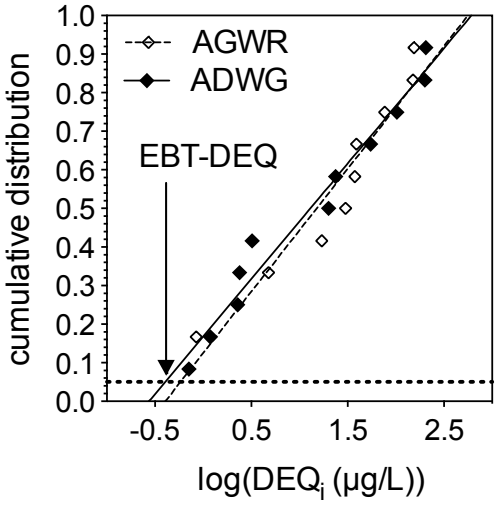

Figure 8.7. Cumulative frequency distribution of DEQi and the thereby estimated EBT-DEQ.

8.5.3 Comparison of the Proposed EBT-BEQ with Results Obtained in This Study

8.5.3.1 Estrogenic Chemicals

As the ADWG contains no GV for estrogenic chemicals, we cannot illustrate the approach for drinking water. Therefore, only the EBT-EEQs for recycled water are discussed later, but the experimental data for drinking water also are shown for comparison.

The EEQs were measured experimentally with the E-SCREEN assay in three recycled water samples (Table 8.4). All recycled water samples were below the EBT-EEQ, which was derived from the distribution of individual EEQ_i values.

Some other water types also are tabulated for comparison. They are as important as the recycled water for validating the approach, as indeed water of lesser quality should not be able to meet the derived EBT-BEQs. In other words, secondary treated effluent should not be "compliant" with the EBT-EEQ for recycled water. This stipulation is important because, if the EBT-EEQ were defined so high that it would be permissible to drink wastewater, its acceptance and utility would be highly questionable. The fact that these samples' readings were higher than the EBT-EEQ suggests that the derived EBT-EEQ is appropriate and relevant.

Table 8.4. Application of the Derived EBT-EEQ for the E-SCREEN Assay to Recycled Water Samples in Australia

Sample	Experimental EEQ (ng/L)	"Compliant" with an EBT-BEQ of 0.17 ng/L?
Secondary treated effluent 1*	0.53	x
After membrane filtration*	0.12	✓
After reverse osmosis	**<0.01**	✓
After advanced oxidation	**<0.01**	✓
Secondary treated effluent 2*	0.75	x
After ozonation and biologically activated carbon filtration	**0.01**	✓
Drinking water plant inlet (river)*	0.02	✓
Drinking water plant outlet*	0.01	✓

Notes: Effluent and drinking water are shown for comparison. ✓ = compliant, x = noncompliant. *, not recycled water, shown only for comparison. The green shaded areas are the samples where compliance testing would be appropriate.

8.5.3.2 Herbicides

The DEQs were measured experimentally with the combined algal assay in three recycled water samples and one drinking water sample (Table 8.5). All recycled and drinking water samples were compliant with EBT-DEQ. Additional validation samples (e.g., secondary treated effluent and the sample after microfiltration) were not compliant, supporting the derivation of the EBT-DEQ as outlined earlier in Section 8.5.3.1.

Table 8.5. Application of the EBT-DEQ for the Photosynthesis Inhibition Endpoint of the Combined Algal Assay to Recycled Water and Drinking Water Samples in Australia

Sample	Experimental DEQ for 2 h inhibition of photosynthesis (µg/L)	EBT-DEQ (µg/L) ADWG (0.4 µg/L)	EBT-DEQ (µg/L) AGWR (0.6 µg/L)
Secondary treated effluent 1*	1.71	x	x
After membrane filtration*	2.39	x	x
After reverse osmosis	<0.01	✓	✓
After advanced oxidation	<0.01	✓	✓
Secondary treated effluent 2*	3.67	x	x
After ozonation and biologically activated carbon filtration	<0.01	✓	✓
Drinking water plant inlet (river)*	<0.01	✓	✓
Drinking water plant outlet*	<0.01	✓	✓

Notes: Effluent and drinking water are shown for comparison. ✓ = compliant, x = noncompliant, * = not recycled water or drinking water, shown only for comparison. The green shaded areas are the samples where compliance testing would be appropriate.

8.6 Effect-Based Trigger Effect Concentration (EBT-EC)

The derivation of EBT-ECs takes an entirely different approach from that of the EBT-BEQs in that it is not concerned with different sensitivities and effects on the organism level but simply treats bioassays as a measure to detect complex mixtures of chemicals. This approach lends itself more to nonspecific modes of toxic action and more-generic response such as the activation of ASR pathways, where many different chemicals are activators and where there is no clearly most potent reference compound. When the EC_{50} values of regulated chemicals in the bioluminescence inhibition test with *V. fischeri* (Microtox) (Tang et al., submitted for publication) are plotted against the GV (Figure 8.8), then it becomes evident that there is no relationship whatsoever between the EC_{50} and the GV, as the endpoint of the biotest is not related to the point of departure for the derivation of the health-based GV.

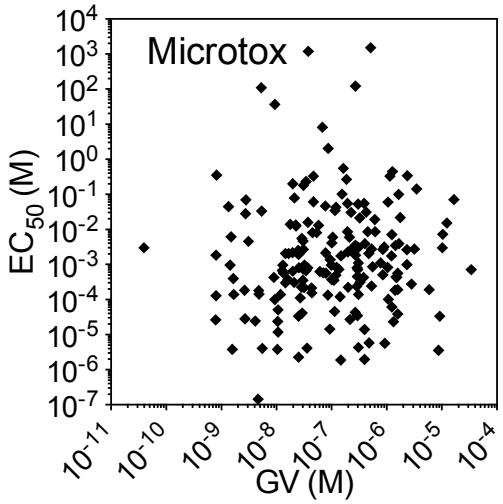

Figure 8.8. Comparison of EC50 values and GV for recycled water in Australia
Source: NRMMC & EPHC & NHMRC, 2008

Thus, all existing GVs of a given regulatory framework have to be used to derive the EBT-EC for a given biological endpoint and a large number of chemicals need to be fingerprinted and assessed in the target bioassay and tested for mixture effects before mixture-based trigger values can be proposed. In the following we present the principle of derivation of EBT-ECs and apply them to two bioassays, the nonspecific bioluminescence inhibition assay with *V. fischeri* (Microtox) and the AREc32 assay for the induction of the oxidative stress response.

8.6.1 Mixture Toxicity Concepts

For bioassays that detect nonspecific effects, reactive effects, and ASRs, it is typical that only a very small fraction of effects can be explained by known and identified chemicals (Reungoat et al., 2012b; Tang et al., submitted for publication). Thus, there is a need to account for mixture effects in the derivation of EBT values. Also, for these types of responses, there is no clear-cut reference chemical available, as often many different chemicals induce the effect.

Therefore, we propose an alternative approach that targets the development of EBT-EC from mixture studies with regulated chemicals. The approach is outlined in Figure 8.9.

As a first step, as many chemicals with GVs as possible are tested in the given bioassay system. In some cases, data gaps can be closed with the quantitative structure–activity relationship (QSAR); in other cases, enough representative chemicals must be fingerprinted and the EC extrapolated to a larger number of chemicals.

As the next step, mixture experiments should be conducted to assess if the tested chemicals indeed act as a concentration additive in mixtures of various composition. Once that is established, the effect-based trigger ECs EBT-EC_x can be calculated from existing water quality GVs for water contaminants with Equation 18.

$$EBT - EC_x = \frac{EC_{x,mixture}}{EF}\left(\frac{1}{n}\sum_{i=1}^{n} guideline\ value_i\right)^{-1} \quad (18)$$

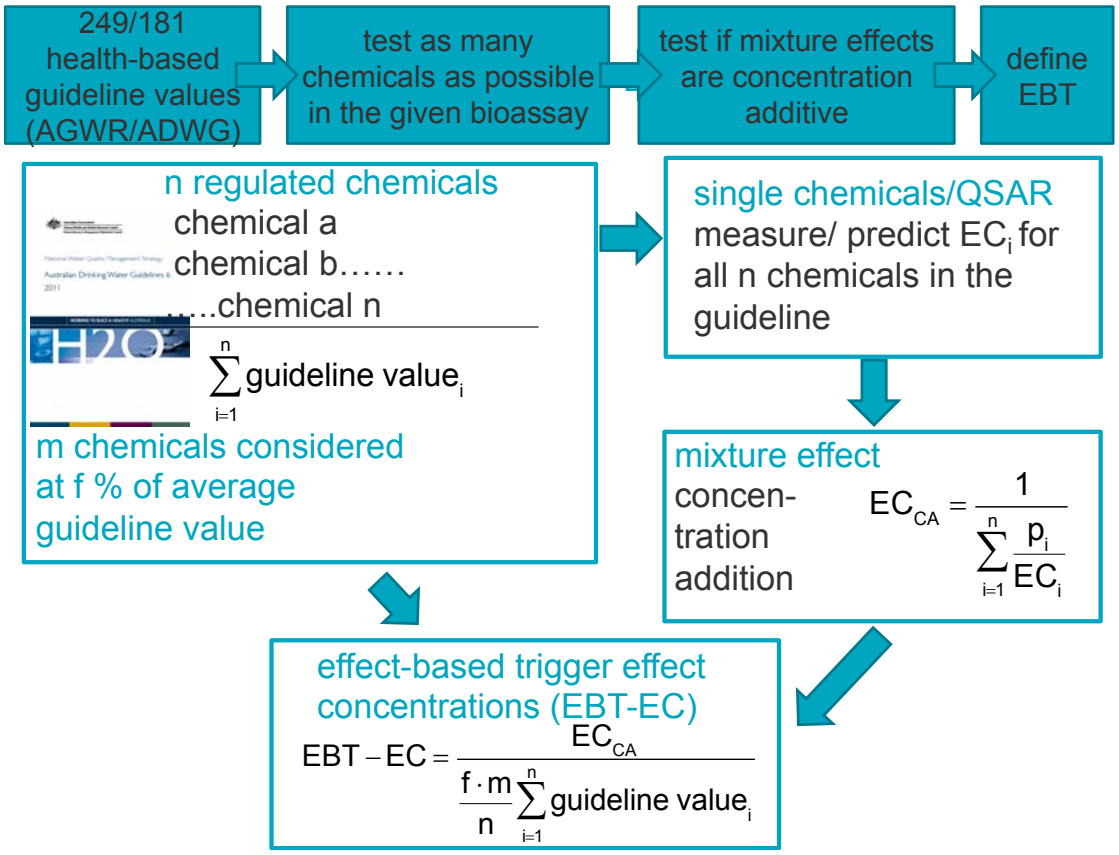

Figure 8.9. Derivation of EBT-EC values.

Source: Reprinted with permission from Tang et al., 2013. Copyright 2013, Elsevier.

The sum of all concentrations for the *n* chemicals in a guideline, and $EC_{x,mixture}$ refers to either the predicted mixture EC_x of the *n*-component mixture predicted with the combined QSAR and CA model (e.g., Microtox) or the experimental mixture values (e.g., AREc32). The EC_x values can either be EC_{50} values of a log-logistic concentration–effect curve (Microtox and algae) or $EC_{IR1.5}$ for the IR of a linear concentration–effect curve (AREc32). Because each guideline contains a different number of chemicals and because a guideline containing a

larger number of chemicals will yield a higher effect level, an extrapolation factor (EF) is needed to account for the number of chemicals in the mixture model (m) and the acceptable fraction of individual chemicals related to the guideline concentration (f). The EF equates to the product of f times m and is a science policy decision, which should be defined on the basis of a management decision for regulatory acceptance and also should account for model uncertainties; e.g., an EF of 50 corresponds to m = 1000 chemicals, which would be included at f = 0.05 (i.e., it could mean 5% of their guideline concentrations, or it could equally apply to m = 500 and f = 0.10 or any combination of m and f that corresponds to an EF of 50).

8.6.2 Application

We have evaluated the concept on the example of two bioassays, the bioluminescence inhibition test with *V. fischeri* (Tang et al., submitted for publication) and the oxidative stress response (Escher et al., 2013), and have applied it to water samples collected for this project (Chapters 6 and 7).

8.6.2.1 Nonspecific Cytotoxicity on the Example of the Bioluminescence Inhibition Test with V. fischeri *(Microtox)*

A detailed account of this example is given by Tang et al. (2013). A brief summary follows here.

A QSAR for baseline toxicity was set up by using a set of six established baseline toxicants. The experimental EC_{50} of 19 pharmaceuticals and 27 pesticides agreed with a QSAR parameterized independently for baseline toxicants; only 4 out of 60 chemicals were outliers (Figure 8.9). Therefore, in the derivation of the EBT-EC_{50}, the predicted EC_{50} of all 249/181 regulated chemicals could be calculated.

Up to 56 regulated chemicals were mixed in three types of mixtures: (1) equipotent concentration ratios, (2) ratios of the GVs, and (3) ratios of concentrations detected in the water sample. In all experiments, the mixture toxicity concept of concentration addition adequately described the experimental data (Figure 8.9). We also tested if the number or type of chemicals had any effect on the mixture toxicity and if there was no irregularity. Overall, the sum of the concentration of all 181 organic chemicals regulated in the ADWG (0.179 mM) would elicit approximately 11% of effect (inhibition of bioluminescence). The predicted mixture $EC_{50,CA}$ of all 181 ADWG chemicals that were mixed in ratio of their GV was 147 µM. With an f = 0.05 and m = 1000 (i.e., 1000 chemicals present at 5% of their GV) the EBT-EC_{50} comes to 3 (in units of REF) for drinking water and 2.8 for recycled water. This meant that, if a sample that was three times enriched has 50% or less effect, it was compliant.

All recycled water and drinking water samples were compliant with the proposed EBT-EC_{50} (Table 8.6). As discussed earlier, comparison with the other types of water sample is important for validating the approach. Secondary treated effluent would not be compliant (Table 8.6). This is important because if the EBT-EC_{50} were defined as so high that it would be permissible to drink wastewater, its acceptance and utility would be highly questionable.

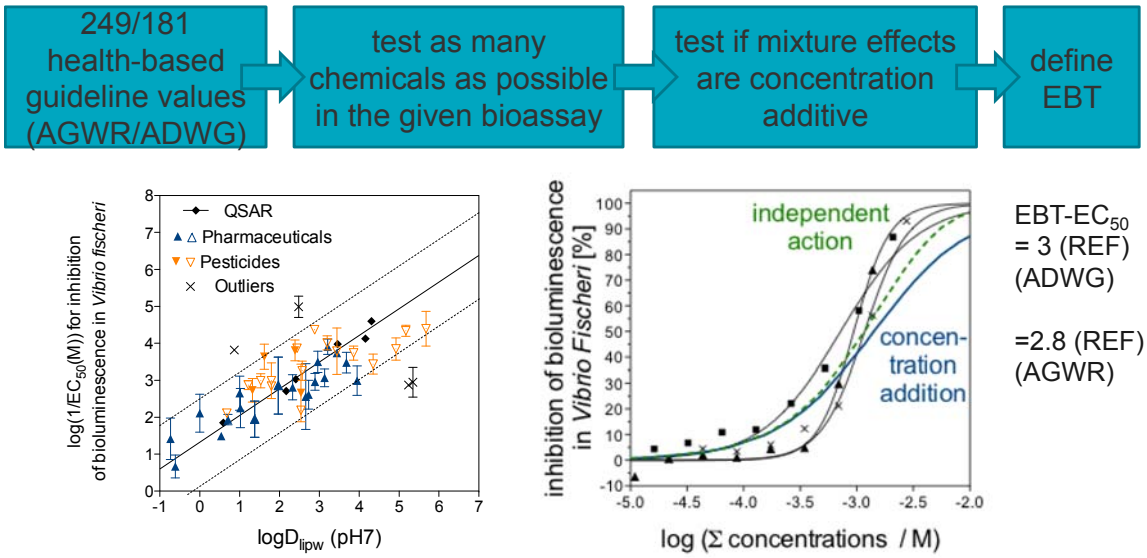

Figure 8.10. Derivation of an EBT-EC50 value for the bioluminescence inhibition test with V. fischeri.

Source: Adapted with permission from Tang et al., 2013. Copyright 2013, Elsevier**.**

Table 8.6. Compliance Testing for All Recycled Water and Drinking Water Samples in the Microtox Assay

Sample	Experimental EC_{50} (REF)	EBT-EC_{50} =3 (ADWG)	EBT-EC_{50} = 2.8 (AGWR)
Secondary treated effluent 1*	4.2	✓	✓
After membrane filtration*	6.1	✓	✓
After reverse osmosis	6.1	✓	✓
After advanced oxidation	50.6	✓	✓
Secondary treated effluent 2*	3.0	x	x
After ozonation and biologically activated carbon filtration	10.3	✓	✓
Drinking water plant inlet (river)*	12.9	✓	✓
Drinking water plant outlet*	3.4	✓	✓

Notes: ✓= compliant, x = noncompliant, * = not recycled water or drinking water, shown only for comparison. The gray shaded areas are the samples where compliance testing would be appropriate.

8.6.2.2 Activation of the Oxidative Stress Response on the Example of the AREc32 Bioassay

A detailed account of this example is given by Escher et al. (2013). A brief summary follows here.

In the AREc32 bioassay for oxidative stress response, we used a subset of pharmaceuticals and pesticides from the AGWR. Ten out of 15 pharmaceuticals and 5 out of 20 pesticides

were identified as inducers of Nrf2 in AREc32. All active inducers acted in accordance with concentration addition (CA in Figure 8.11, right panel) in equipotent mixtures and in guideline mixtures. However, mixtures of inducers and noninducing chemicals sometimes gave higher effects than expected from CA modeled for the active compounds only. This is presumably because of a contribution by the noninducers that had not reached the threshold of effect before cytotoxicity took effect but could contribute to the mixture effects in concentrations below their cytotoxicity.

After CA was confirmed for inducers of the oxidative stress response, the mixture effect was just upscaled from the mixture effect of the active ingredients (empirically tested in various combinations of up to 56 components) and an EBT-$EC_{IR1.5}$ was proposed for this endpoint (Figure 8.11). All recycled water samples were compliant with the proposed EBT-$EC_{IR1.5}$ (Table 8.7). There was a problem with the drinking water samples as DBPs formed were highly active in the AREc32 and therefore the finished drinking water would be noncompliant. The EBT is derived for micropollutants, and we know that no new micropollutants are formed during drinking water treatment, only DBPs. Therefore, we suggest using the DWTP inlet for compliance checking of micropollutants.

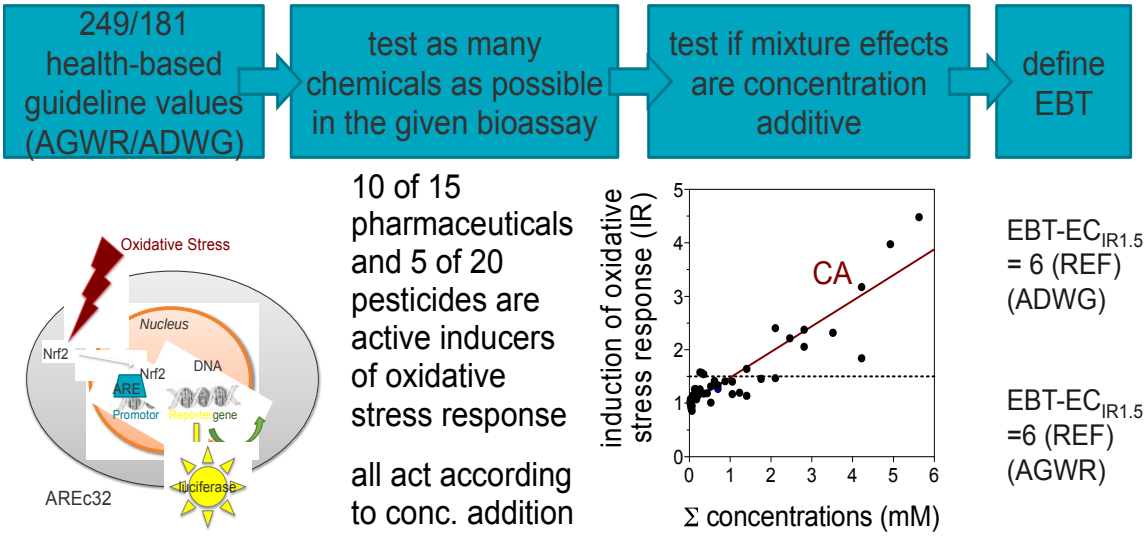

Figure 8.11. Derivation of an EBT-$EC_{IR1.5}$ value for the AREc32 bioassay for induction of oxidative stress.

Source: Adapted with permission from Escher et al., 2013b. Copyright 2013, American Chemical Society.

Table 8.7. Compliance Testing for All Recycled Water and Drinking Water Samples in the AREc32 Bioassay for Induction of Oxidative Stress

Sample	Experimental $EC_{IR1.5}$ (REF)	EBT-EC_{50} =6 (ADWG)	EBT-EC_{50} = 6 (AGWR)
Secondary treated effluent 1*	1.8	x	x
After membrane filtration*	2.5	✓	✓
After reverse osmosis	30.8	✓	✓
After advanced oxidation	>80	✓	✓
Secondary treated effluent 2*	1.7	x	x
After ozonation and biologically activated carbon filtration	23.1	✓	✓
Drinking water plant inlet (river)*	17.4	✓	✓
Drinking water plant outlet*	5.0	x	x

Notes: ✓ = compliant, x = noncompliant, * = not recycled water or drinking water, shown only for comparison. The gray shaded areas are the samples where compliance testing would be appropriate.

8.7 Conclusion

The proposed concepts to derive EBT values are simple and can be calculated with available data (or data easily generated by in vitro experiments). The preliminary EBT values produced here appear relevant and achievable (on the basis of the data produced in this project) with current treatment technologies, and all final recycled water samples were below the derived trigger values (and would thus have been deemed compliant).

The adoption of EBT values will be a first step toward the use of bioanalytical data in a preventive scheme. It must be stressed, though, that responses triggered by bioanalytical tools should be considered first-tier screening level alerts and not hard standards. Bioassays should be chosen that are indicators of initial triggers in toxicity pathways. These initial triggers are conditional but not sufficient indicators that an adverse health outcome will occur, so it is possible that false-positive responses will occur. False positives are acceptable in a precautionary framework, where it is important to avoid false negatives. Bioassays can be used to define the probability of the presence of chemicals that may be cause for concern and thus should be further investigated and evaluated.

Chapter 9

Conclusions

9.1 What Have We Learned?

In the literature review, we collated information on adverse effects of organic pollutants on human health, focusing on toxicity pathways, which are the molecular events from uptake of a chemical to its ultimate effect. Only toxicity pathways that are linked to relevant adverse outcomes for human health from exposure to potential toxicants in recycled water were considered.

The literature review has revealed a number of additional biological endpoints for inclusion into effect-based water quality assessment. A single bioassay will be unlikely of being capable on its own to assess water quality comprehensively, but a small number of relevant biological endpoints that are sensitive to water samples can be used as indicators.

From the literature review we selected nine new bioassays for implementation, some of which are variations of previously used bioassays and some of which were used for the first time on water samples. Not all of the additional endpoints were responsive with water samples, but two endpoints that have been rarely evaluated in the past were identified as high priority. These are the activation of the PXR that regulates the production of metabolic endpoints and the oxidative stress response via the keap-nrf2-ARE pathway.

One of the major difficulties encountered was a common way of evaluating bioassay data to allow a direct comparison of results. Whereas the many different ways of treating data could be somewhat streamlined, the problem of having a minimum of two different types of data evaluation remains.

1. For cytotoxicity and modes of action that involve binding to receptors, the effect range from 0 to 100% and toxicity can be translated to BEQs that relate the effect of a complex environmental sample of unknown composition to the effect of a known reference chemical. This method can be applied both for agonistic and antagonistic modes of receptor-mediated modes of action.

2. For reactive modes of action and ASR, an upper limit of effect often cannot be defined and therefore it is difficult to apply the BEQ concept, although at low effect levels it is possible to derive BEQs (Escher et al., 2013). For these bioassays, we can use the $EC_{IR1.5}$ and we can, in principle, also derive a BEQ value from $EC_{IR1.5}$ values.

Extensive quality control is imperative for application of cell-based bioassays. Quality control includes a full dose−response curve of a reference compound on each plate but also a sufficient number of control measurements (medium or solvent controls). The controls serve also to quantify the limit of detection of each bioassay.

Despite limitations, this study has clearly demonstrated that bioassays are a valid tool for water quality assessment that complements but does not replace chemical analysis.

9.2 Setting Up a Test Battery of Screening Assays

A battery of bioassays should always include relevant examples of the different categories of endpoints related to different steps in the cellular toxicity pathway. It can never comprehensively cover all health-relevant endpoints, but one should select "indicator bioassays" that are responsive and health-relevant and show a wide dynamic range of responses.

For induction of axenobiotic metabolism, the activation of the AhR, the most applied endpoint so far in water quality assessment, serves well. Our study also has confirmed that this endpoint is a good indicator. In addition the PXR shows high responsiveness and should be further explored for the development of more-routine bioassays.

The PPAR has been linked to environmental chemicals, but the few bioassays tested here were less responsive to the water samples, most likely because of the lighter chemical burden rather than because of low sensitivity of the bioassays per se. More work would be required to develop a routine bioassay for the PPAR. Finally, the CAR appears to be of lowest responsiveness. It was tested in only two bioassays in our two case studies, but if we directly compare the results of the four NRs relevant for xenobiotic metabolism, it becomes clear that the CAR is of lowest relevance for water samples.

There are only a few assays for specific modes of toxic action that are not receptor-mediated. Assays with "naked" enzymes have a limited applicability because of interference with coextracted natural organic matter (Neale and Escher, 2013) and therefore were omitted from the analysis. Specific receptor-mediated modes of action include estrogenic, androgenic, and progestagenic effects. They are the endpoints that have received the most attention so far in water quality assessment. The results of the present study align with previous work in that estrogenic and antiandrogenic activities are most pronounced. An endpoint that has not yet received much attention in water quality assessment so far is the glucocorticoid pathway, but recent work on environmental chemicals (Odermatt et al., 2006) and on the GR-CALUX applied to various water samples (Van der Linden et al., 2008) foreshadows our findings that this endpoint could be detected in secondary treated effluent with all five bioassays used in the present study. Thus, in future work, if resources are limited, priority for endocrine disruption assays should be given in the following order: estrogenicity, glucocorticoid activity, and progestagenic/androgenic effects. It is vital to test not only agonistic effects but also antagonistic or suppressive effects.

Genotoxicity is the most relevant class of reactive modes of action. Established bioassays such as *umu*C or the SOS chromotest serve the purpose, although they are based on bacteria and bacterial DNA repair systems. Bioassays derived from mammalian cells would be preferable, but the applicability of the MN assay is limited for these types of water samples because of the lower sensitivity and also because of the fairly complicated test setup. A high-throughput mammalian cell-based bioassay for genotoxicity would be desirable.

Of the ASR pathways, the oxidative stress response appears to be a highly sensitive but still selective indicator. This finding is consistent with previous chemical testing in Nrf2/ARE-cisFACTORIAL, where almost 50% of the ToxCast chemicals were active (Martin et al., 2010). Surprisingly, no effects were observed with any of the p53 bioassays despite some environmentally relevant chemicals inducing this stress response pathway.

The bioassays for cytotoxicity could be divided into three categories. The bacterial cytotoxicity assays (Microtox and *P. phosphoreum*) are very fast and sensitive screening assays but have low health relevance. In contrast cytotoxicity assays with mammalian cells are very insensitive, which limits their applicability. Specific cell lines can be used as indicators of system response. However, the cell lines selected in the present study for this purpose did not meet expectations. Clearly, further work has to be invested in the selection of appropriate test systems and protocols for cell-based bioassays for system responses as they are much less developed than nonspecific cytotoxicity assays and bioassays targeting cellular toxicity pathways.

Apart from the choice of the endpoint, we recommend that in the future more attention should be paid to the basal activities of the cell lines in use. As metabolism is the most crucial modifier of toxicity, detoxifying many chemicals but activating others, the metabolic capacity of the cell line in use needs to be taken into account when choosing or designing a bioassay. Many available cell lines have low metabolic activity, and for those it is advisable to run each experiment in addition in the presence of an exogenous metabolic mix, for example, liver S9, which is commercially available.

9.3 Toward the Development of Effect-Based Trigger Values

In Chapter 8 we took first steps toward EBT values. Clearly, for receptor-mediated effects, there is enough supporting evidence available to propose EBT-BEQs. In Table 9.1 the EBT-BEQ derived in Chapter 8 are compared with the water samples collected in Australia (Chapter 7). On the basis of these data, all potable recycled water and drinking water from this study would be compliant with the EBT-BEQ (highlighted in green in Table 9.1). In contrast, the BEQ levels of the WWTP effluents would not be compliant with a wide range of endpoints (highlighted in red in Table 9.1), which is good, as this confirms the selectivity of the proposed EBT-BEQ.

Table 9.1. BEQ Values of the Water Samples Collected in Australia (Chapter 7) Compared to Effect-Based Trigger BEQs (EBT-BEQs) (Chapter 8)

Bioassay	EBT-BEQ	BEQ	Values for:						
			Eff1	AO	Eff2	O_3/BAC	RW	DW	Blank
Algal photosynthesis inhibition	0.4[a]	DEQ (µg/L)	0.41	<0.03	0.14	<0.03	<0.03	<0.03	<0.03
ER-CALUX	3.8[b]	EEQ (ng/L)	1.14	<0.01	13.32	<0.01	<0.01	<0.01	<0.01
E-SCREEN	0.17[a]	EEQ (ng/L)	0.53	<0.01	0.75	0.01	0.02	0.01	<0.01
AR-CALUX	11[b]	DHT-EQ (ng/L)	<0.48	<0.48	<0.48	<0.48	<0.48	<0.48	<0.48
PR-CALUX	333[b]	Org2058-EQ (ng/L)	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08
GR-CALUX	21[b]	DEX-EQ (ng/L)	15.51	<9.2	32.09	<9.2	<9.2	<9.2	<9.2

Notes:

[a]EBT-BEQ derived in Chapter 8. Compliant samples are shaded in green; only the ones that are true recycled water or drinking water are marked. The noncompliant samples are shaded in red; these are actually only wastewater samples to which the EBT-BEQs do not apply.

[b]EBT-BEQ derived from ADI for single compound (Brand et al., 2013).

In addition, for two endpoints where there were no appropriate reference compounds available, we defined trigger ECs, EBT-EC. These included the Microtox assay (Chapter 8 and Tang et al.,2013) and the AREc32 for oxidative stress response (Chapter 8 and Escher et al., 2013b). For these endpoints all recycled and drinking water samples would be below the trigger value.

We can provide only the scientific underpinning for the derivation of EBT values. Ultimately, it is the responsibility of regulators to define these triggers. However, the concepts and databases developed in this study will provide an important input in discussions with regulators, although the path to acceptance is most likely still very long.

9.4 The Future of Bioanalytical Tools for Water Quality Assessment

This project has demonstrated the capabilities and limitations of bioanalytical tools for water quality assessment. Especially when it comes to highly treated water such as recycled water, it is important that the chosen bioassays are highly sensitive and that they target groups of modes of toxic action and toxicity pathways that are triggered by chemicals that are expected to occur in the respective water samples.

An ideal test battery should contain highly sensitive bioassays that cover all different aspects of cellular toxicity pathways (Figure 9.1). To represent the induction of xenobiotic metabolism pathways, we recommend the AhR and PXR. There is a multitude of bioassays available for AhR activation, and all evaluated ones appear to be suitable. However, only a few bioassays exist for the PXR, so future efforts should be directed to developing robust bioassays for the PXR. For specific modes of action, the receptor-mediated hormonal effects related to the estrogenic, glucocorticoid, and antiandrogenic pathways appear to be most relevant as most responsive in water samples. The oxidative stress response clearly sticks out as a highly responsive defense mechanism. Cell viability assays should be further developed to favor in the future those that are representative for system responses.

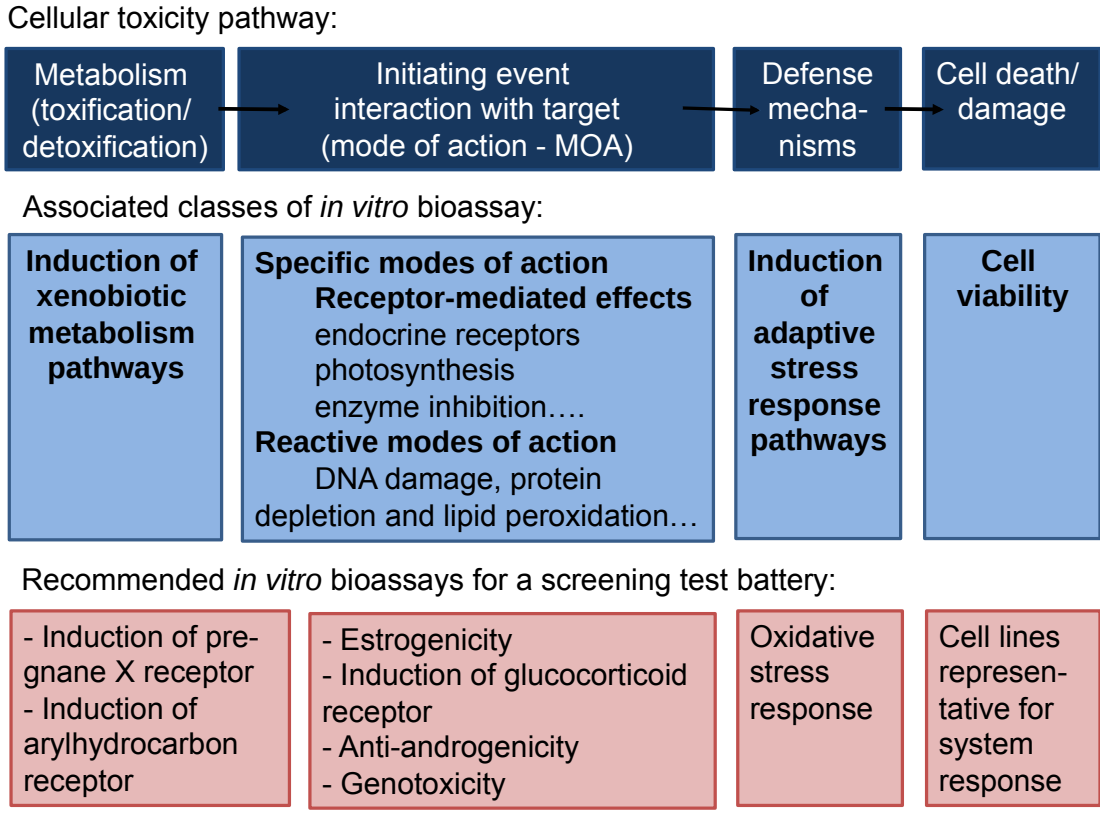

Figure 9.1. Setup of a screening test battery for recycled/reclaimed water.

This project has demonstrated the potential of cell-based bioassays for water quality monitoring, but at the moment not all test systems have reached maturity yet. Clear guidelines for the performance of assays, data evaluation, and interpretation are needed for regulatory application of these tools. In particular a more coherent data evaluation will facilitate comparability between different bioassays and different endpoints. The work presented in this report shows the way in all three main areas but evidently could not usher every step in the process to a final solution.

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Practical Solutions for Water Scarcity

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