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Micropollutants, mixtures and transformation products in recycled water: how much do we really know?

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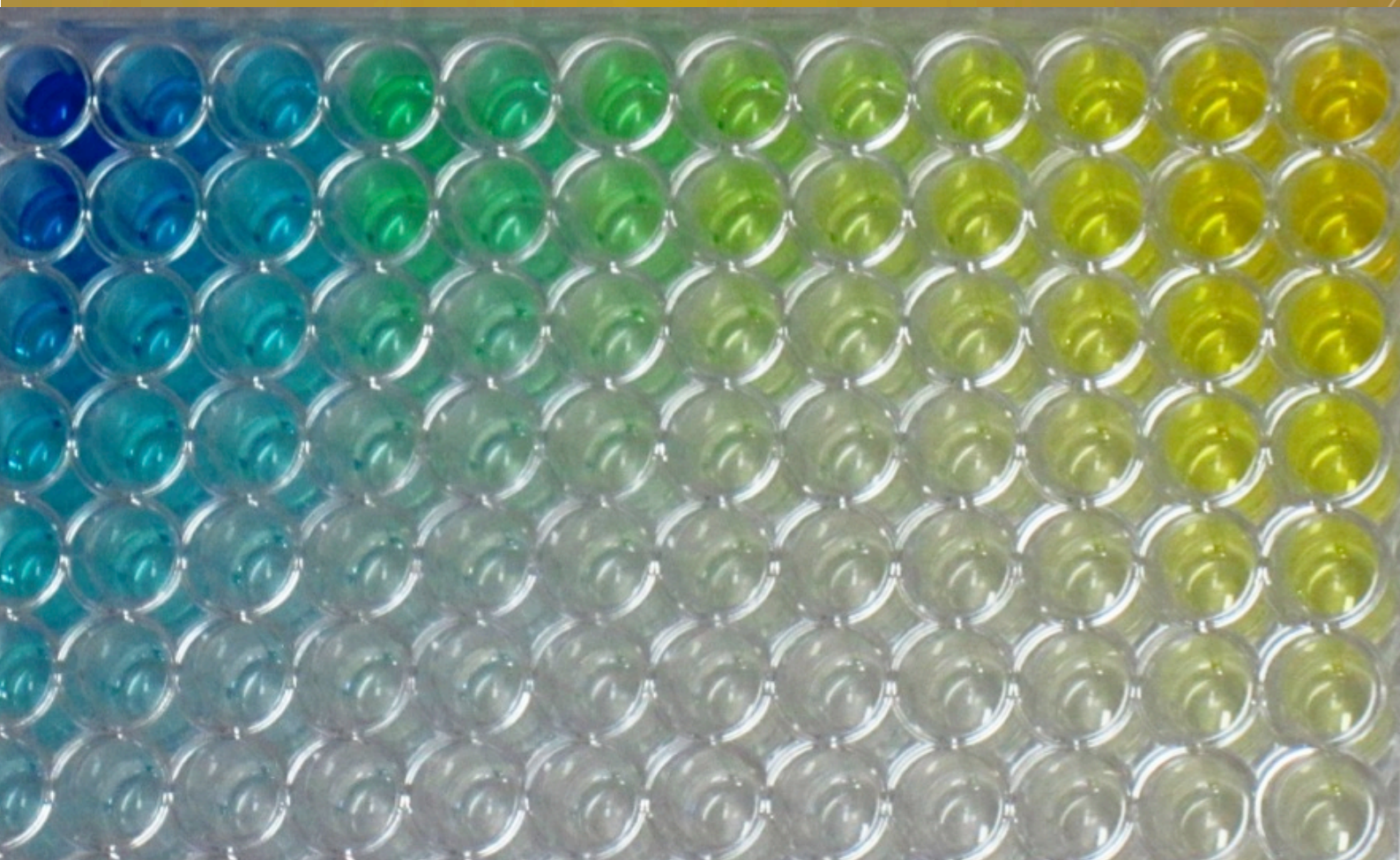

Micropollutants, mixtures and transformation products in recycled water: how much do we really know?

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The mission of the Australian Water Recycling Centre of Excellence is to enhance management and use of water recycling through industry partnerships, build capacity and capability within the recycled water industry, and promote water recycling as a socially, environmentally and economically sustainable option for future water security.

The Australian Government has provided $20 million to the Centre through its National Urban Water and Desalination Plan to support applied research and development projects which meet water recycling challenges for Australia's irrigation, urban development, food processing, heavy industry and water utility sectors. This funding has levered an additional $40 million investment from more than 80 private and public organisations, in Australia and overseas.

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CWQRC

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Executive Summary

The collaborative research project “Micropollutants, mixtures and transformation products in recycled water: how much do we really know?” was undertaken jointly by the University of Queensland and Curtin University for the Australian Water Recycling of Excellence with contributions by Water Quality Research Australia (now Water Research Australia), Watersecure, Water Corporation of Western Australia, Veolia, Department of Environment and Resource Management, Queensland Health, Melbourne Water and Seqwater.

Health and environmental risks as well as uninformed perceptions associated with micropollutants and their removal by advanced treatment processes, have, to some extent, hindered the establishment of large-scale water reuse schemes. In response, Australia has developed the single most comprehensive set of guideline values for recycled water (Australian Guidelines for Water Recycling: Managing Health and Environmental Risks (phase 2). Augmentation of Drinking Water Supplies, National Water Quality Management Strategy (NWQMS), Natural Resource Management Ministerial Council (NRMMC), Environment Protection and Heritage Council (EPHC) and National Health and Medical Research Council (NHRMC), Canberra, Australia). Uptake of the guidelines into regulations varies from State to State and Territory. The Queensland Government has adopted most of the Australian Recycled Water Guideline Values into the Public Health Regulation (Schedule 3B Standards for quality of recycled water supplied to augment a supply of drinking water, revisions in Subordinate Legislation 2008 No. 218). Western Australia has focused on 292 Recycled Water Quality Parameters.

Thousands of chemicals may be present in recycled water and it is likely that the majority of transformation products generated during water recycling processes have not been identified, let alone characterized in terms of toxicity. Although individual chemicals are typically present at very low concentrations in recycled water, they can potentially act jointly, resulting in additive or potentially even synergistic or antagonistic mixture effects. In addition, chemicals can degrade or be transformed during treatment processes. Little is known on the identity of the transformation products and their contribution to the mixture effects in water. In this project, we have performed mixture experiments with regulated chemicals and chemicals occurring in the source water intended for water recycling and have assessed the role of transformation products in the toxicity mixtures.

While a small number of individual chemicals are typically being monitored in recycled water, we do not know how many micropollutants are actually present or if the toxicological hazard can be assessed by the monitored/regulated chemicals alone. Bioanalytical tools have been used to complement water quality assessment in the past. Bioanalytical tools are cell-based *in-vitro* bioassays that can target specific mechanisms of toxicity and provide a measure of toxicity from mixtures of known and unknown chemicals, such as pesticides, industrial chemicals, pharmaceuticals and their transformation products. Bioanalytical tools can also provide measures of the cumulative effects of chemicals that exhibit the same mode of toxic action, for which the selected bioassays are indicative. In addition to this they give a measure of the cytotoxicity of all chemicals acting together in a water sample.

The following questions were addressed by this project using a combination of chemical analysis and bioanalytical tools:

1. Do mixtures matter? How do the numerous chemicals present at low concentrations (below levels where they show any individual toxicity) act together in mixtures?
2. How much of the iceberg do we see? How much do chemicals that are regulated in the Australian Guidelines for Water Recycling (AGWR) contribute to the overall toxicity of mixtures of organic micropollutants?
3. Do transformation products of micropollutants formed during relevant water recycling processes contribute to mixture toxicity?
4. Are we currently monitoring and regulating the right chemicals/chemical classes?

In recycled water, most chemicals are below the limit of detection. However, from previous work, we knew that chemicals still might be present and contribute to mixture effects. We analysed three different types of advanced water treatment trains in Water Recycling Plants in Queensland and Western Australia from the source water (wastewater treatment plant effluent) to the product water (recycled water) and also benchmarked the recycled water against other water types, including stormwater, drinking water and surface water. Advanced treatment usually consists of a combination of membrane filtration (e.g., ultrafiltration and/or reverse osmosis) and oxidation processes (e.g., advanced oxidation, UV disinfection or ozonation) to remove pathogens and chemicals—including metals, heavy metals, nutrients and organic micropollutants. Two of the advanced treatment plants investigated here, had a treatment train consisting of ultrafiltration/reverse osmosis followed by UV/H_2O_2 or UV alone, and one Water Recycling Plant applied ozonation followed by biologically activated carbon filtration.

The focus of the present study was on organic micropollutants, excluding disinfection by-products and volatile chemicals. In an initial phase almost 300 chemicals from the list of regulated chemicals in the AGWR were quantified in various water types. In a parallel project funded by the WateReuse Research Foundation, 103 *in-vitro* bioassays were applied to a selection of the samples investigated in the present project. We used these results to design a relevant bioassay battery for the present study.

In finished recycled water, no chemicals were detected but in the source water (secondary treated wastewater effluent) and in reverse osmosis reject, up to 55 chemicals listed in the AGWR were found at concentrations above analytical detection limits. We worked with these detected chemicals in designed mixture toxicity experiments. Despite the fact these chemicals were well removed during advanced water treatment, they still have the potential to be present in recycled water at low concentrations. Of the 103 bioassays applied to recycled water in our previous work, a smaller battery was recommended for assessing treatment efficacy in water recycling because these bioassays gave detectable responses and showed dynamics during treatment. These “indicator” bioassays include cytotoxicity, induction of the aryl hydrocarbon (AhR) receptor, estrogenicity, photosynthesis inhibition, genotoxicity and oxidative stress response. The associated bioassays applied were the Microtox assay for cytotoxicity, the AhR-CAFLUX assay for AhR induction, the E-CALUX for estrogenicity, the chlorophyll fluorescence assay (IPAM) for photosynthesis inhibition, the *umuC* assay for genotoxicity and the AREc32 assay for oxidative stress response. Although humans are not specifically affected by herbicides, guideline values exist for twelve herbicides in the Australian Guidelines for Water Recycling and as this study has shown they occur in source water for recycled water. Herbicides are particularly toxic to algae, and therefore algae constitute an ideal model system to quantify the effects caused by herbicides even though algal toxicity is not of direct human health relevance.

Of the evaluated bioassays, three were selected for the mixture effect studies: the cytotoxicity assay (Microtox), the photosynthesis inhibition assay (IPAM) and the oxidative stress response assay (AREc32). These three assays also represent three different modes of toxic action, namely non-specific toxicity, receptor-mediated toxicity and reactive toxicity, which is advantageous as it allows us to make some general conclusions that could be read-across to other endpoints because mixture interactions depend on the mode of action.

Do mixtures matter?

Mixture toxicity experiments were performed with (a) equipotent mixture ratio, where chemicals were mixed in ratios of their potency so each chemical should contribute equally to effect, (b) in the concentration ratios of the guideline values and (c) in the concentration ratios they occurred in water sampled at Water Recycling Plants. Sixty-six individual mixture toxicity experiments were performed and overall the mixture toxicity concept of concentration addition, which is strictly only applicable to chemicals that act according to the same mode of action proved to be a robust predictive model independent of the mode of action. This finding confirms that the bioanalytical equivalent concentration (BEQ) concept can be applied to these bioassays because one condition of the BEQ is that chemicals included must act concentration-additive in mixtures. The BEQ is the concentration of a reference

chemical that elicits the same effect as the mixture composed of unknowns. Thus it is a simple way of expressing an effect that is more intuitive than an effect concentration. It also allows us to compare measured effects with effects predicted by the analytically determined concentrations and the measured relative effect potency of the detected chemicals.

How much of the iceberg do we see?

For the "iceberg experiments" we mixed the detected chemicals and tested the designed mixtures in bioassays, then compared the results with the biological responses from wastewater treatment plant effluent, treated and recycled water. For bioassays indicative of a receptor-mediated mode of action, photosynthesis inhibition, the known chemicals could explain all biological effect in a typical water sample. This is in alignment with work in literature on estrogenicity in surface waters. In contrast, for cytotoxicity and adaptive stress responses, there remain many unknowns because the quantified chemicals could explain less than 1% of the observed biological effect. We also split the iceberg mixtures into smaller groups containing individual chemical categories. The categories were pesticides, pharmaceuticals, antibiotics, iodinated contrast media, endocrine disrupting chemicals and miscellaneous chemicals. The BEQs of the entire iceberg mixtures in the Microtox assay were dominated by an equal share of pesticides and pharmaceuticals, while the herbicides dominated, as expected, in the photosynthesis inhibition assay. The oxidative stress responses were composed of 60% contribution by pesticides, 30% by pharmaceuticals and the remaining 10% by various other groups. Good agreement between the BEQ of the iceberg and the sum of the individual group is another indication that many of these chemicals act concentration-additive in mixtures.

Do transformation products of micropollutants contribute to mixture toxicity?

The large fraction of unknown chemicals observed during the iceberg experiments not only included chemicals introduced by human activity into the wastewater stream but also those that are formed during water treatment such as biotransformation, ozonation and other oxidation processes - as this study was able to demonstrate. We performed ozonation experiments with eight micropollutants that occurred in source water (secondary treated wastewater effluent) and identified both, transformation products and mixture effects. Specific effects of the parent compound typically disappeared or were reduced with ozonation but the cytotoxicity and the oxidative stress response often remained constant despite disappearance of the parent compound, indicating that the transformation products have equal toxicity as the associated parents. For carbamazepine, diclofenac and hexazinone, the oxidative stress response increased two to four-fold, suggesting that there were reactive transformation products formed. Thus we recommend that whenever any form of oxidation process is included in a treatment train, a specific focus be set on reactive toxicity. Based on toxicity output, other oxidative treatment options (e.g., UV/H_2O_2, O_3/H_2O_2) should be investigated to mitigate/degrade the toxic transformation products formed while degrading the parent compound.

Are we currently monitoring and regulating the right chemicals/chemical classes?

In conclusion, the results obtained in this study on environmentally relevant mixtures and transformation products clearly emphasise that many unknowns remain, even if we were able to quantify for the first time which fraction of mixture effects can be explained by typically quantified and regulated chemicals. Not only does mixture toxicity matter but it is not only mixture toxicity between known micropollutants but also between parents and the transformation products. Despite amazing advances in analytical chemistry and the use of non-target analysis to detect and identify unknowns and transformation products, it will never be possible to achieve full chemical characterisation of the water. As analytical detection limits continue to be pushed lower, more and more chemicals can be detected. Detection alone is not sufficient. Relevance must be established by considering toxicity.

Thus we recommend that in the future, chemical monitoring should be complemented by a suite of indicator bioassays to account for the mixture effects of known and unknown micropollutants as well as their transformation products. We proposed a tiered approach, where in a first screening step, indicator chemicals are monitored and compared against chemical guideline values and indicator bioassays are

compared against effect-based trigger values. If either chemical guideline values or effect-based trigger values are exceeded a full monitoring would be required in a second tier.

Overall the project has widened the knowledge base and has closed important knowledge gaps particularly the relationship between analytically detectable chemicals and actually present chemicals. The results of the project will inform a firmer and weight-of-evidence based conclusion on the safety of recycled water. The proposed test battery is now ready for screening applications for the assessment of fit-for purpose recycled water as well as recycled water for indirect and direct potable reuse.

The project's results have been published in four peer-reviewed publications and have contributed to one paper. We also have reached out and participated at several workshops with our stakeholders (Veolia, Water Corporation, Seqwater etc.) and disseminated the results at international conferences. We have a better knowledge base now and can now provide tools to regulators that will allow them to better manage recycled water supplies. It is now up to the regulatory agencies in Australia and worldwide to include effect-based monitoring into a comprehensive monitoring strategy. Bioassays can be applied either occasionally to check that the chemical analysis is still targeting the toxicologically relevant chemicals or they can be applied on a regular basis for compliance monitoring and might in this respect even allow the reduction of number of chemicals in regular monitoring. Bioassays could even be used as robust tool for to benchmark against effect-based water quality trigger values.

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AO	Advanced oxidation
AOP	Advanced oxidation process
AR	Androgen receptor
ASR	Adaptive stress response
AWRP	Advanced water recycling plant
BEQ	Bioanalytical equivalent concentration
C18	Octadecyl silica
CA	Concentration addition
CAR	Constitutive androstane receptor
CT	Cytotoxicity
CYP	Cytochrome P450 mono-oxygenase
DART	Embryo toxicity test with the zebrafish *Danio rerio*
DOC	Dissolved organic carbon
DW	Drinking water
EBT	Effect-based trigger values
EC	Effect concentration
EEQ	Estradiol equivalent
Eff	Effluent
ER	Estrogen receptor
ESI	Electrospray ionisation source
DR	Glucocorticoid receptor
Frag#1-n	Daughter ions emerging from parent compound fragmentation
FTMS	Fourier-Transform Mass Spectrometer (Orbitrap)
^{1}HNMR	Proton nuclear magnetic resonance
HR	High resolution
HTS	High-throughput screening
IA	Independent action
IR	Induction ratio
ISO	International Organization for Standardization
IT	Ion trap
LC	Liquid chromatography
LR	Low resolution
MF	Microfiltration
MMC	Mitomycin
MOA	Mode of action
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry (fragmentation using a quadrupole)
MS^2	Multiple stage fragmentation (MS/MS using an ion trap)

MS^3	Multiple stage fragmentation (MS/MS/MS using an ion trap)
MTT	(3-(4,5-dimethylthiazol -2-yl)-2,5-diphenyltetrazolium bromide
m/z	Mass-to-charge ratio
NF	Nanofiltration
NIH	National Institute of Health
NRI	New Reactive SA Index
NWC	National Water Commission
NRU	Neutral red uptake
OECD	Organisation for Economic Co-operation and Development
P	Parent compound
PPAR	Peroxisome proliferator activated receptor
ppm	Parts per million
PR	Progesterone receptor
prep-LC	Preparative liquid chromatography
PXR	Pregnane-X-receptor
Q1	Quadrupole1
Q3	Quadrupole3
RAR	Retinoic acid receptor
REF	Relative enrichment factor
RFU	Relative fluorescence units
RLU	Relative light units
RO	Reverse osmosis
ROS	Reactive oxygen species
RT	Retention time
RW	River water
SPE	Solid-phase extraction
SRM	Selected reaction monitoring
SW	Stormwater
t-BuOH	Tertiary butanol
TEQ	Toxic equivalent concentration
TP	Transformation product
UV	Ultraviolet light
WRP	Water recycling plant
WWTP	Wastewater treatment plant
XM	Xenobiotic metabolism

1 Introduction

1.1 Aims of the project

Confidence in water quality is crucial for the successful implementation of water recycling schemes. While compliance with macropollutant guideline values such as nitrogen, phosphorous, and salts can easily be verified using standards and established analytical methods, the situation is less straightforward for micropollutants. Metals and inorganics can be comprehensively monitored but potentially there are thousands of organic chemicals in wastewater, a fraction of which are included in the Australian Guidelines for Water Recycling: Managing Health and Environmental Risks (Phase 2): Augmentation of Drinking Water Supplies (NRMMC & EPHC & NHMRC, 2008). These guideline values have not been adopted into regulation in all States and Territories. For example Queensland has included all suggested health-based guideline values in their Public Health Regulation (Schedule 3B Standards for quality of recycled water supplied to augment a supply of drinking water, revisions in Subordinate Legislation 2008 No. 218 (Queensland Government, 2005)). For simplicity, we refer to the chemicals that have guideline values as "regulated" chemicals, even if this not strictly the case in all States and Territories.

To date it is unclear how much of the overall burden of micropollutants is covered by these regulated chemicals. Questions remain, whether more or less chemicals need to be regulated, in order to ensure appropriate public health protection. Complementary approaches for evaluating water quality, such as using bioanalytical tools—compared to traditional analytical methods alone—ought to be considered as part of the monitoring strategy. Bioanalytical tools offer the potential for groups of chemicals acting together in mixtures to be evaluated.

Public acceptance of recycled water is hampered by a lack of evidence-based, scientifically defensible data on the effects of chemicals present in the source water and recycled water. Concerns about the risks of exposure to recycled water often include potentiation effects and synergistic interactions between chemicals. A critical knowledge gap is independent scientific evidence, which can be used to better inform decision makers. From well-designed mixture experiments we know that the likelihood of synergism decreases as the number of components increases and their concentration decreases (Funnel hypothesis, (Warne and Hawker, 1995)). However, to date, no one has been able to demonstrate this hypothesis experimentally with chemicals occurring in wastewater and recycled water and by working with real water matrices.

While a small number of individual chemicals, relative to all known, are being monitored in recycled water, we do not know how many micropollutants are actually present. Questions remain, are we monitoring the chemicals that contribute to the majority of toxicity in complex mixtures? Additionally, how much of the toxic effect in a water sample is caused by known and regulated chemicals compared to unregulated compounds (e.g. transformation products). The primary goal of this project was to evaluate whether or not currently regulated chemicals in recycled water are appropriate and determine if any identified gaps in chemical regulation could jeopardize the safe use of recycled water.

In four closely linked subprojects, this project provided evidence on the effects of mixtures of chemicals and their transformation products.

The goals were addressed in four subprojects that each addressed one key question:

1. Do mixtures matter? How do the numerous chemicals present at low concentrations (below levels where they show any individual toxicity) act together in mixtures?
2. How much of the iceberg do we see? How much do chemicals that are typically analysed in monitoring programs and are regulated as a guideline value contribute to the overall toxicity of mixtures of pollutants in recycled water (through all treatment steps)?
3. Do transformation products of micropollutants formed during relevant water recycling processes contribute to mixture toxicity?
4. Are we currently monitoring and regulating the right chemicals/chemical classes?

1.2 Subprojects

1.2.1 Do mixtures matter?

The field of mixture toxicity assessment has progressed significantly over the last decade and we now have a reasonable foundation for the theoretical basis of mixture toxicity of defined mixtures with a small number of components. While lessons learned from previous theoretical and experimental work allow us to make mechanistic predictions about mixture effects in relatively simple mixtures, there has not been any experimental confirmation that these concepts hold for the far more complex mixtures such as those encountered in recycled water. In this subproject we performed a series of mixture experiments with several micropollutants to test the validity of common mixture hypotheses. One key experiment was to mix up to 50 chemicals included in the Australian Guidelines for Water Recycling and to assess their toxic effects through a series of cell-based bioassays.

Kortenkamp, Backhaus and Faust (2009) recently reviewed the state-of-the-art in mixture toxicology for the European Union and concluded that, "there is consensus in the field of mixture toxicology that the customary chemical-by-chemical approach to risk assessment might be too simplistic. It is in danger of underestimating the risk of chemicals to human health and to the environment". The issue of mixtures has not been adequately addressed in the water quality assessment field, despite its vital importance. This project helps to address this important knowledge gap.

1.2.2 How much of the iceberg do we see?

Regulated chemicals were analysed in wastewater and recycled-water samples. Mixture toxicity experiments were performed with regulated chemicals at detectable concentrations. These experiments were designed to test the contribution of regulated chemicals to the overall toxicity of recycled water and to assess how the toxicities of the mixtures change as the ratios of the component concentrations are modified.

1.2.3 Do transformation products of micropollutants formed during relevant water recycling processes contribute to mixture toxicity?

During oxidative treatment of water, transformation products and oxidation by-products are formed, only a few of which are regulated. The traditional process of identifying, isolating and quantifying hazardous transformation products and oxidation by-products is costly and time consuming. We therefore proposed to develop a novel, tiered approach, for screening organic micropollutants for their potential to form toxicologically relevant transformation products. Our method combined in parallel bioanalytical assessments with identification and quantification methods of the transformation products.

1.2.4 How much do we really know and are we currently monitoring right chemicals/chemical classes?

The question posed above, "Are we currently monitoring and regulating the right chemicals/chemical classes?" was the central focus of this subproject and this question explored the requirement to incorporate mixture effects of chemicals with the same mode of toxic action and – if deemed relevant – also include transformation products in an overall monitoring strategy. This subproject comprises a synthesis of the gained experimental experience. The question addressed which of the hundreds of micropollutants are of toxicological significance and if/how bioanalytical tools can partially replace and complement routine monitoring. The progress made in this project will ultimately lead to more cost efficient and targeted assessment tools and better assurance of water quality safety.

1.3 Application of bioanalytical tools for water quality assessment

For many decades bioanalytical tools have been applied for water quality monitoring. A comprehensive treatise of the concepts behind bioanalytical tools and a history has been recently published in a dedicated book (Escher and Leusch, 2012) and only essential information will be repeated here. More recently, we have also reviewed cell-based bioassays that have not been used previously for water quality monitoring purposes but show potential for application (Escher *et al.*, 2014b). Chapman and Leusch (2014) have recently reviewed the applications of bioanalytical tools for recycled water including detailed lists of applications.

Bioanalytical tools can be *in vitro* cell-based bioassays and low-complexity whole organism assays that describe crucial events along the adverse outcome pathway (Figure 1). An adverse outcome pathway (Ankley *et al.*, 2010) is the chain of events that lead from the uptake of a chemical to the observed toxicity in humans or ecosystems (Figure 1). The cellular response is a prerequisite for an adverse outcome to be manifested. Cellular responses are only a trigger and may not necessarily lead to adverse outcomes, however, without a cellular trigger there can be no adverse outcome (Collins *et al.*, 2008). Thus while the ultimate protection goal will be human health or ecosystem health, bioanalytical tools can be used as indicators of the hazard potential of chemicals and their mixtures. There are four major stages in the cellular toxicity pathway including metabolism, interaction with the target, defence mechanisms and ultimately cell damage.

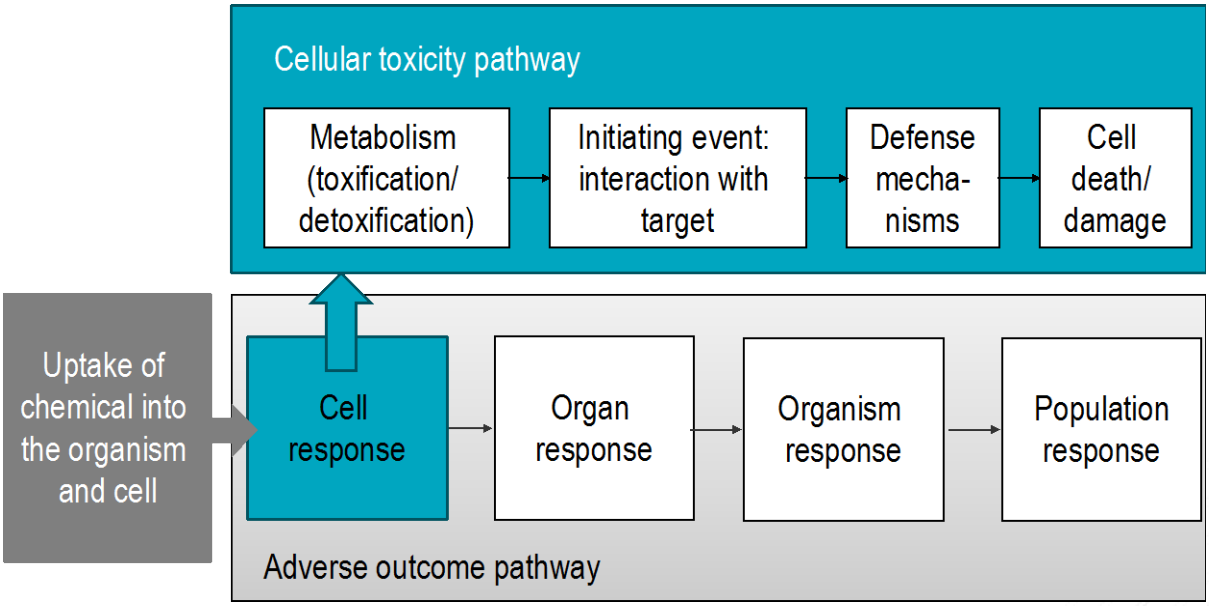


Figure 1 The concept of adverse outcome pathway and toxicity pathways provide the conceptual framework for the application of bioanalytical tools in water quality assessment. Reproduced from Escher, B. and Leusch, F. (2012). Bioanalytical tools in water quality assessment. IWA Publishing, London, UK, with permission from the copyright holders, IWA Publishing.

Molecular and cellular effects are the main targets of *in vitro* assays, which can assess metabolism, toxic mechanisms (molecular initiating event, i.e., biochemical reaction or interaction between toxicant and biomolecules) or the activation of adaptive stress response pathways (Figure 2).

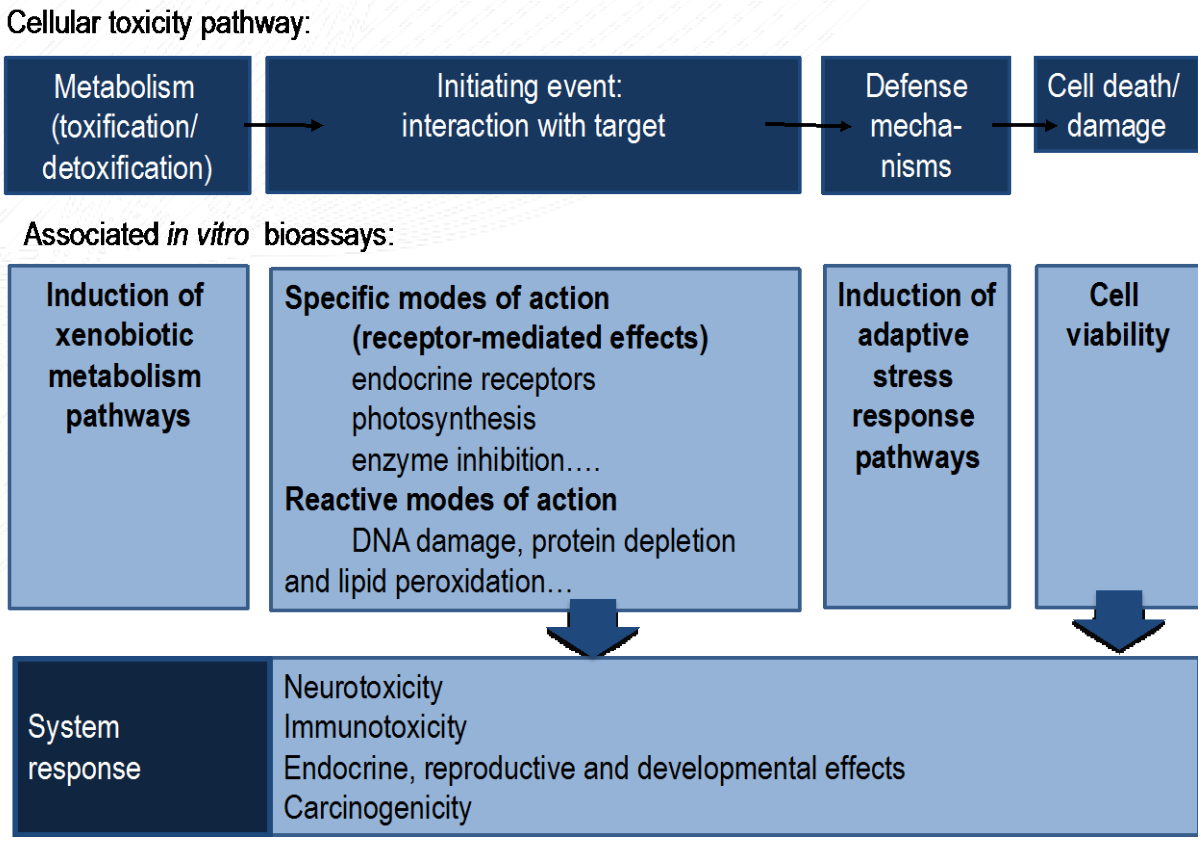


Figure 2 Classification of in-vitro bioassays according to cellular toxicity pathways. Reprinted with permission from Escher, B.I., Allinson, M., Altenburger, R., Bain, P., Balaguer, P., Busch, W., Crago, J., Humpage, A., Denslow, N.D., Dopp, E., Hilscherova, K., Kumar, A., Grimaldi, M., Jayasinghe, B.S., Jarosova, B., Jia, A., Makarov, S., Maruya, K.A., Medvedev, A., Mehinto, A.C., Mendez, J.E., Poulsen, A., Prochazka, E., Richard, J., Schifferli, A., Schlenk, D., Scholz, S., Shiraishi, F., Snyder, S., Su, G., Tang, J., Burg, B.v.d., Linden, S.v.d., Werner, I., Westerheide, S.D., Wong, C.K.C., Yang, M., Yeung, B., Zhang, X. and Leusch, F.D.L. (2014). Benchmarking organic micropollutants in wastewater, recycled water and drinking water with in vitro bioassays. Environmental Science & Technology, 48: 1940-1956. Copyright 2014 American Chemical Society.

We have identified a large number of new bioassays (Escher *et al.*, 2014b) and applied them together with well-established bioassays in a study that included a total of 100 bioassays (Escher *et al.*, 2014a). From this experience we were able to recommend a small list of indicator bioassays that lend themselves to the assessment of recycled water (Figure 3).

The nuclear xenobiotic receptors that are involved in the up-regulation of metabolism can provide early indicators of the presence of chemicals even if their activation is not necessarily linked to adverse effects (Figure 3). Activation of 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 was also highly responsive in water samples serving as source water for recycling (Escher *et al.*, 2014a).

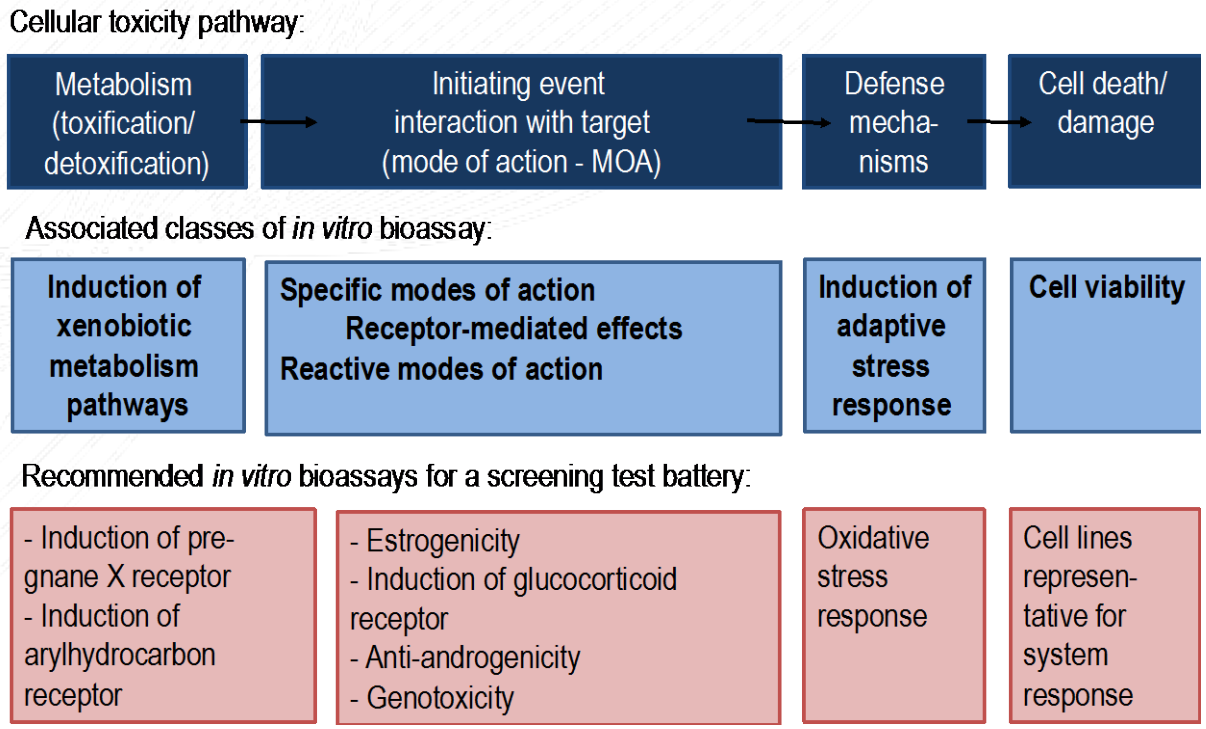


Figure 3 Recommended cell-based bioassays for monitoring of recycled water and treatment efficacy (Escher et al.*, 2014a).*

With respect to specific, receptor-mediated modes of action, the activation of the estrogen and glucocorticoid receptors were shown to be highly responsive (Escher *et al.*, 2014a). Androgenicity never tested positive, but anti-androgenicity proved to be a highly relevant endpoint (Escher *et al.*, 2014a).

For reactive toxicity, in our publication (Escher *et al.*, 2014a), we recommended 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.

The induction of defence mechanisms to compensate for damage is a logical and more sensitive alternative, which assesses the potential to do harm rather than assessing the damage. General adaptive stress response pathways, particularly the oxidative stress response pathway has proven to be a highly sensitive indicator of the presence of chemicals in water (Escher *et al.*, 2012).

2 Do mixtures matter?

2.1 Goal

Many chemicals are present in recycled water at concentrations too low to cause any observable effects. As many years of fundamental research in designed mixture toxicity experiments have shown, chemicals at concentrations that are too low to show a measurable effect on their own, may contribute to mixture effects (Silva *et al.*, 2002; Kortenkamp *et al.*, 2009). However, the transfer of basic concepts to relevant environmental mixtures, such as wastewater treatment plant effluent and recycled-water, has never been tackled. The goal of this subproject was to assess what types of mixture effects are occurring in water samples with a large number of relevant and diverse chemical micropollutant species.

The question of whether mixture effects are relevant to wastewater effluent and recycled-water samples will be tackled with three types of mixture experiments, including: (1) mixtures where all chemicals are present in proportion to their effect (equipotent mixture), (2) mixture with all chemicals at concentrations of their guideline values of the Australian Guidelines for Water Recycling (AGWR, 2008) and (3) mixtures at concentrations as they occurred in water samples from Advanced Water Recycling Plants.

2.2 Some background on mixture toxicity assessment

There is a wealth of literature available on the toxicity of mixtures that contain between two and ten components. Systematic mixture investigations with complex mixtures (i.e., more than 10 components) with individual components at very low concentrations, typical of those found in waste and recycled water, are still lacking. Unfortunately, even many of the available mixture studies provide only anecdotal evidence and lack a mechanistic understanding. For the last fifteen years, concepts from pharmacology have been adapted to toxicology. An important conceptual breakthrough is that mixture effects are now typically categorized in four classes, two of which occur more frequently and have underlying mathematical models. These are called independent action (IA) and concentration addition (CA). IA applies if chemicals have different modes of toxic action and hence have different target sites, but the chemicals do not interact in exerting their toxicity. In this case, the chemicals produce their effects absolutely independently of each other. For a multicomponent mixture with components represented by i, the biological effect of the mixture ($effect_{mixture}$) according to the IA models is

$$effect_{mixture} = 1 - \prod_{i=1}^{n} 1 - effect_i \qquad (1)$$

where $effect_i$ is the fractional biological effect of component "i" at the concentration in the mixture and Π stands for the product (multiplication).

Independent action implies that, if effects of individual components are zero, no mixture toxicity will occur. However as no observed adverse effect levels (NOAEL) in mammalian toxicology can be as high as 20% and no observed effect concentrations (NOEC) in ecotoxicology up to 40%, there is the likelihood that chemicals at the "no effect" level still act together to elicit appreciable mixture toxicity.

If chemicals act according to the same mode of toxic action, affecting the same target site and having no direct interaction with each other, they act according to the concept of dose or concentration addition. It is not the effect, which is additive, but the effective doses (mammalian toxicology) or effect concentrations (*in-vitro* toxicology and ecotoxicology). For a binary mixture, this can be rationalized as follows: If chemical A has an effective concentration causing 50% of maximum effect (EC_{50}) at 12 µg/L and chemical B at 20 µg/L, then a combination of half of the EC_{50} of A (6 µg/L) and of B (10 µg/L) will also cause 50% of the maximum effect. Any other combination of a fraction p_i (with $\Sigma\ p_i = 1$) of the EC_{50}(A) and EC_{50}(B) will also result in 50% effect. For a multicomponent mixture of "i" components the EC_{50} of the mixture $EC_{50,mixture}$ according to the CA model is:

$$EC_{50,mixture} = \frac{1}{\sum_{i=1}^{n} \frac{p_i}{EC_{50,i}}} \quad (2)$$

With these two models (IA and CA) we have the reference cases for mixture toxicity established. They hold for all effect levels, not just EC_{50} but for any EC_x with x being an effect level of choice. A large number of mixture experiments have been performed and have substantiated these two most common mixture toxicity models (Kortenkamp et al., 2009). These studies range from *in-vitro* and low complexity assays to whole animal *in-vivo* assays. Initially focused on ecotoxicological endpoints, human toxicology studies are now also more and more frequently applying these mixture concepts. All these conceptual studies confirmed that the mixture toxicity clearly exceeds the toxicity of the most potent individual chemical, be it that the CA or IA model applies.

There are exceptions to the CA and IA models and they apply if the mixture components interact. Mixture components may interact and therefore deviate from either the IA or CA models. In such cases when the resulting toxicity of the mixture is less or more than predicted, the mixture is considered to be antagonistic or synergistic, respectively. Antagonism could arise because, for example, one component activates a metabolic enzyme, which causes the other mixture component to be more rapidly detoxified. Synergism could arise because, for example, one compound facilitates the uptake of another component or suppresses detoxifying enzymes. While interactive effects are frequently observed in metal toxicology, they are rather rare for organic chemicals—especially when it comes to mixtures with increasing numbers of components and deviation from the reference models (IA and CA) become less and less frequent (Warne and Hawker, 1995).

Mixtures of compounds at concentrations well below observable effect concentrations may produce substantial mixture toxicity (Silva et al., 2002), however, there are virtually no studies in the literature addressing mixtures with more than 30 components or trying to resolve components of mixtures in environmental samples. Therefore translation of the results from designed mixture toxicity experiments to real water samples that may contain thousands of chemicals at exceedingly low concentrations remains uncertain.

2.3 Approach

The overall approach taken in this subproject is presented in Figure 4. All organic chemicals regulated by the Australian Guideline for Water Recycling (NRMMC & EPHC & NHMRC, 2008) and the Australian Drinking Water Guidelines (NHMRC, 2011) were evaluated for their suitability to serve as test chemicals in the mixture toxicity assays (Figure 4). Physicochemical properties (e.g. octanol-water partition coefficient, volatility, water solubility and, if applicable, the acidity constant(s)) of all compounds were collected and listed (see Appendix A (Tang *et al.*, 2013)). Chemicals with a tendency to escape an aqueous solution (i.e., having a high volatility combined with a low water solubility) were excluded as they pose a risk of experimental artefacts during the mixture toxicity experiments.

As a next step, up to 300 chemicals were analysed in representative water samples (see Chapter 3) and the detected chemicals were selected for the mixture toxicity studies. In addition, we profiled the activity of chemicals that were shown to act according to a relevant mode of action and some of these were then included in the mixture toxicity studies even if they were not found in wastewater treatment plant (WWTP) effluent sample (Figure 4).

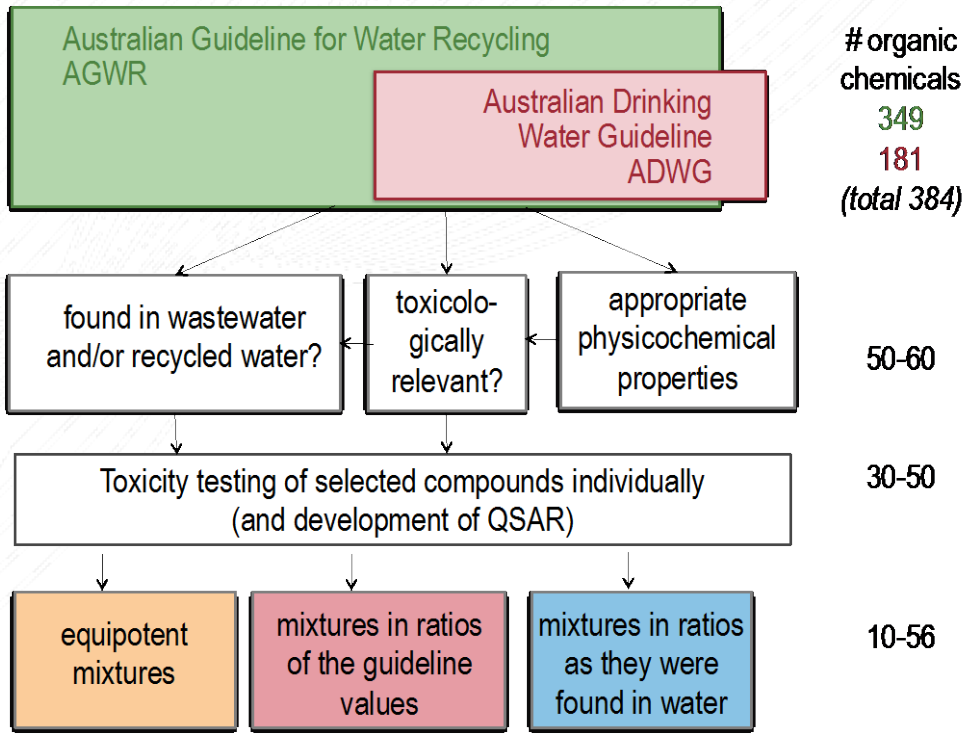


Figure 4 Selection process of chemicals investigated in this study and type of mixture experiments.

Three types of mixture experiments were performed on chemicals that were found in recycled water and its source water (Figure 4):

1. The selected chemicals were mixed at **equipotent concentration ratios** (i.e. each present at the same ratio of the EC_{50} values of the individual chemicals) to determine
    a. if the observed toxicity can be explained with mixture toxicity concepts;
    b. if despite various different modes of toxic action, the mixture acts concentration-additive.
2. The selected chemicals were mixed at the **concentrations that correspond to the guideline values** and full concentration-effect curves were measured to assess
    a. the absolute effect measured at the sum of guideline values;
    b. if despite various different modes of toxic action, the mixture acts concentration-additive.
3. The selected chemicals were mixed at **concentration ratios as they occur in environmental samples** and a full concentration response curve will be measured to
    a. identify the chemicals dominating the mixture toxicity at these very low concentrations;
    b. identify those chemicals that do not contribute to the mixture toxicity at all under these conditions;
    c. verify the validity of mixture toxicity concepts at very low concentrations below observable effect levels of the individual chemicals and outside the classical and less environmentally relevant equipotent mixture ratios.

The overall approach taken was to compare the experimental EC_{50} value for this mixture with predicted EC_{50} values using the CA and IA models to evaluate consistency with or deviation from theoretical expectations. A measure of the deviation between the observed and predicted mixture effect is the index on prediction quality IPQ (Altenburger *et al.*, 1996). The IPQ is zero if there is perfect agreement

and is positive if the prediction for CA has a higher EC than the experiment and negative if the prediction for CA has a lower EC than the experiment (Escher *et al.*, 2013).

If $EC_{CA} > EC_{IR1.5,experimental}$ then

$$IPQ = \frac{EC_{CA}}{EC_{experimental}} - 1 \quad (3)$$

If $EC_{CA} < EC_{IR1.5,experimental}$ then

$$IPQ = 1 - \frac{EC_{experimental}}{EC_{CA}} \quad (4)$$

The IPQs are only reported in the Appendices, in the main report, the comparison between experimental and model is only presented graphically.

Bioassays for the mixture experiments were a subset of the recommended indicator bioassays (Figure 3). Of those three assays were selected that had the most pronounced response in the investigated samples. These bioassays were also used for the iceberg experiments in Chapter 3 (for a detailed discussion on the choice of the bioassays see Chapter 3).

The Microtox assay on bioluminescence inhibition served as representative bioassay for cytotoxicity. Unfortunately it was not possible to use a eukaryotic cell for this purpose due to the relatively low sensitivity. Microtox is an assay that is widely used in water quality assessment and there exist many mixture toxicity studies that have applied this assay due to its simplicity (Backhaus and Grimme, 1999; Backhaus *et al.*, 2000). There are already several QSARs available for this assay (Cronin and Schultz, 1997; Vighi *et al.*, 2009; Aruoja *et al.*, 2011). The 30 min bioluminescence inhibition assay is reported to detect nonspecific effects (baseline toxicity) and specific effects on energy production (as bioluminescence is ATP dependent) but due to the short-term nature of the assay we can safely assume that the nonspecific aspects dominate the overall response, which was also confirmed by the QSAR analysis described in Appendix A.

As example of adaptive stress response, we have selected the response to oxidative stress. Cellular stress response pathways play a key role in maintaining cell homeostasis and/or for repairing damage by transcriptional activation of cytoprotective genes (Simmons *et al.*, 2009). Stress response pathways are only induced by chemicals or other stressors, therefore they are referred to as adaptive. Activation and detection of an adaptive stress response pathway is much more sensitive than cytotoxicity and thus provide early warning signals of exposure to chemicals (Escher and Leusch, 2012). The NF-E2-related factor 2 (Nrf2) regulates the cellular defence mechanism against oxidative stress through activation of detoxification and antioxidant genes (Nguyen *et al.*, 2009; Giudice *et al.*, 2010; 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 reactive oxygen species and environmental carcinogens. 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 MCF-7, 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. There have been no studies undertaken assessing mixture effects with this endpoint.

The only bioassays indicative of specific modes of action showing a measureable response on samples throughout the treatment train were the photosynthesis inhibition assays—ones that mainly target herbicides.

2.4 Experimental Methods

A detailed account of all experimental methods is given in Appendices A to C.

2.5 Results and Discussion

2.5.1 Nonspecific toxicity/ cytotoxicity

Twenty-five chemicals from the AWGR were profiled with the Microtox assay (Appendix A). The experimental EC_{50} values were mostly within the applicability domain of the baseline-toxicity QSAR (Quantitative Structure Activity Relationship) derived for this endpoint. Therefore the effects of the additional mixture components were predicted by the QSAR and all mixture toxicity models were parameterised with the QSAR data.

Thirteen equipotent mixtures of 10 to 56 chemical (in methanol) were prepared and the experimental EC_{50} values were compared to the predictions for IA and CA. The mixtures were composed of pharmaceuticals only, of pesticides only and of mixtures of the two groups. All chemicals were found in some samples that we had investigated (see Chapter 3). In all cases the experimental EC_{50} of the equipotent mixtures were lower by a factor of 2 to 6 (i.e., higher cytotoxicity) than the CA and IA predictions (Figure 5). This systematic deviation is most likely due to the uncertainty of the QSAR prediction of EC_{50} values of single compounds. In particular, the less hydrophobic compounds had a tendency to be more toxic than the QSAR predicted, which would translate into lower experimental mixture EC_{50} values, compared to predicted values (Figure 2 in Appendix A).

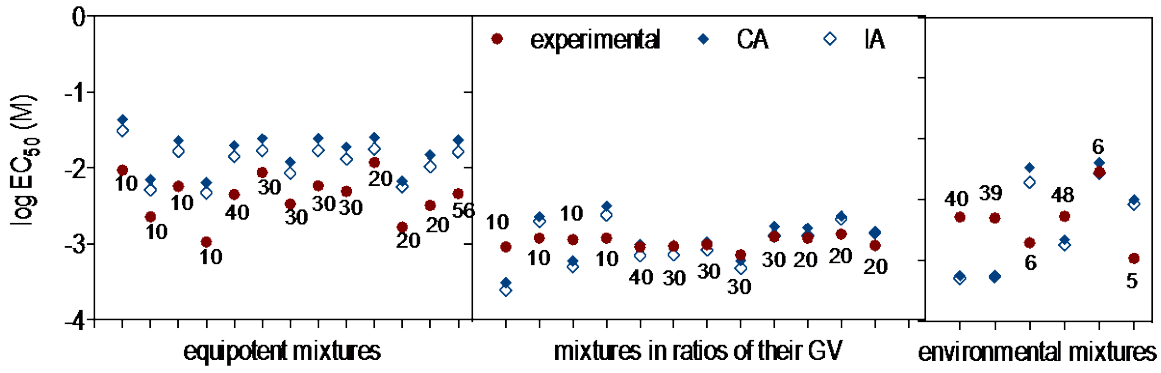


Figure 5 Comparison between experimental and predicted mixture EC_{50} for the cytotoxicity measured with the Microtox assay. The numbers refer to the number of components in a given mixture. GV = guideline value, CA = concentration addition, IA = independent action.

Twelve different mixtures were composed of between 10 and 40 components in concentration ratios of the guideline values of the AGWR. The predicted EC_{50} for the guideline value (GV) mixtures were very close to the experimental EC_{50} (Figure 5). The same chemicals as for the equipotent mixtures were applied but the concentration ratios were quite different from the ones used for the equipotent mixtures. For example, atenolol made up more than 80% of a 10-component equipotent mixture, while it was only 10% of the total concentration in a GV mixture. In this particular GV mixture, carbamazepine made up 45% of the molar fraction, while in the corresponding equipotent mixture it contributed only 0.4 %. The detailed composition of all mixtures and their effects are given in Appendix A. The contribution of each component to the mixture effect in the 40-component AGWR mixture is shown in Figure 6. Figure 6 demonstrates that nonylphenol and galaxolide dominate the mixture effect while compounds like caffeine and 5-methyl-1H-benzotriazole have a negligible contribution. This contribution is a combination of GV and potency: the dominant chemical in the mixture, galaxolide, had a high potency and a high GV of 1800 µg/L, while the second ranked nonylphenol has a high potency and a low GV of 0.5 µg/L. At the bottom end is 5-methyl-1H-benzotriazole with a very low GV of 0.007 µg/L and a very low potency.

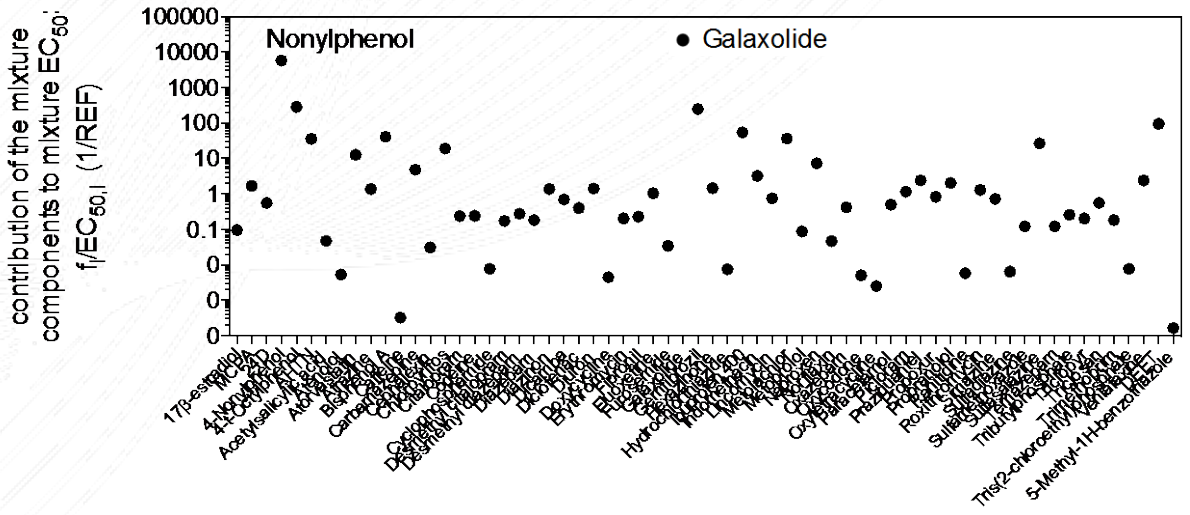


Figure 6 Contribution of individual mixture components to the predicted mixture EC_{50}

Finally, 5 to 48 chemicals were mixed in concentration ratios as they were detected in various samples including the advanced water recycling plant (AWRP) samples discussed in Chapter 3, plus a surface water sample. In four of these samples the deviation between experimental and modelled EC_{50} values was much larger than in any other experiment but in those mixtures a small number of chemicals were included, so an error in the EC_{50} of a single component would skew the result of the prediction and also there were individual components that dominated the mixture effects, for example galaxolide was present in the 40- 39- and 48-component mixture in concentrations of more than 1 µg/L and dominated the mixture effects in all cases. A detailed analysis similar to Figure 6 is given in the Appendix A.

In all 66 mixture experiments, the prediction for IA would result in similar or only slightly lower effects than the prediction for CA (Figure 5). Comparing prediction and experimental data, we could not make a conclusion as to which mixture model was more appropriate. From a theoretical point of view, we can favour CA over IA because the same mode of toxic action applies. As all individual chemicals tested were within the prediction range of the QSAR one can conclude that they act together in these assays with a similar mode of action and therefore CA applies.

2.5.2 Reactive mode of action: oxidative stress response

The oxidative stress response is a very different case from the cytotoxicity assay discussed above. Firstly, we used a reporter gene assay that only gives a response if the oxidative stress response is induced. Thus the only reference model for mixtures is the model of concentration addition, while IA is not applicable. Prior to this there have been no studies on mixture effects on the oxidative stress response and in general on transcription factor-based assays.

Initially we screened 15 pharmaceuticals and 20 pesticides for their ability to induce oxidative stress. These 35 chemicals were primarily from the AGWR list and chosen according to literature on their ability to induce oxidative stress (Martin *et al.*, 2010). We selected chemicals at both ends of the potency spectrum using a multifactorial assay based on HepG2 cells. Ten out of 15 pharmaceuticals were active and had an $EC_{IR1.5}$ between 5 µM and 3.7 mM (Figure 7). Seven out of 20 pesticides were active. The pesticides were generally more potent than the pharmaceuticals with $EC_{IR1.5}$ between 8 and 100 µM (Figure 7). The details are given in Table 1 in the Appendix B. Compounds inactive up to 5 mM included ranitidine, gemfibrozil, ibuprofen, norflaxin, salicylic acid and warfarin. Compounds inactive up to 100 µM included 2-methyl-4-chlorophenoxyacetic acid (MCPA), adicarb, dicamba, hexazinone, methomyl, dimethoate, pirimiphos-methyl, dichlorprop, fluometuron, fenitrothion, dieldrin, ethion and piperonyl butoxide.

There was a generally good consistency between the AREc32 reporter gene assay used in the present study and the literature with the exception of a few pesticides that were active in HepG2 and inactive in AREc32. AREc32 lacks metabolic activity while the liver cell line HepG2 is more metabolically active which can explain the difference.

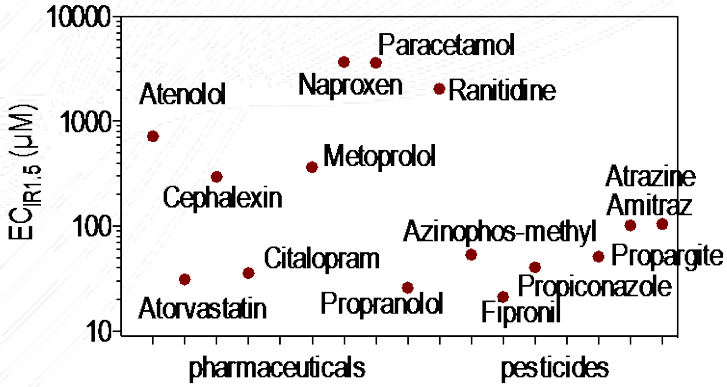


Figure 7. EC$_{IR1.5}$ of all single compounds tested in AREc32. Detailed results are given in Appendix B.

There was a very good consistency between the experimental EC$_{IR1.5}$ and the prediction for CA for the equipotent mixtures containing five to 15 chemicals at equipotent concentration ratios (Figure 8). This is the first time that CA was shown to be applicable for the endpoint of oxidative stress response.

In contrast, the agreement between the experimental EC$_{IR1.5}$ and the prediction for CA was less satisfactory for the mixtures in the ratios of their guideline values, in particular for the 20-component mixtures where chemicals that induced the oxidative stress response and others that did not were combined (Figure 8). We hypothesised that this was due to mixture components such as amitraz and atrazine that alone did not have any activity in AREc32 or activity was masked by cytotoxicity but they still had a low intrinsic potency. Amitraz and atrazine, showed an upward trend in their concentration-IR curves but did not exceed the threshold of IR 1.5 thus were assigned as inactive and were not included in the mixture effect calculations, where in reality they might have contributed with to the overall mixture effect. A more comprehensive discussion of the mixture studies with AREc32 is given in Appendix B.

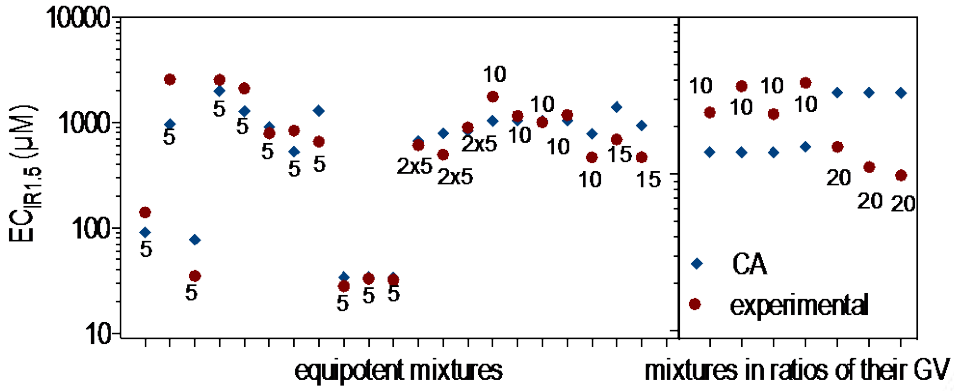


Figure 8 Comparison between experimental and predicted mixture EC$_{50}$ for oxidative stress response determined with the AREc32 assay. The numbers refer to the number of components in a given mixture.

2.5.3 Specific (receptor-mediated) mode of action: inhibition of photosynthesis

There is a wealth of experimental evidence available on the concentration additivity of PSII herbicides (Faust *et al.*, 2001b; Junghans *et al.*, 2003; Junghans *et al.*, 2006). We confirmed that CA also accurately predicted the experimental mixture effect of the 12 photosystem II inhibitors included in the AGWR (NRMMC & EPHC & NHMRC, 2008) (Figure 9, the blue diamond for the EC_{50} for CA is hidden behind the red circle of the experimental EC_{50}).

In environmental mixtures, there are not only herbicides but also many chemicals that act according to other modes of action in algae. Therefore we also explored how herbicides and non-herbicides act together in mixtures. A more detailed description is given in Appendix C, which includes details relevant for answering the question of whether mixtures matter. It is possible to perform two-stage prediction where all chemicals that act according to the same mode of action are grouped together and modelled with CA and then all groups are modelled as individual component of an IA mixture.

We first separated the analytically detected chemicals (from Chapter 3) into two groups: herbicides that inhibit photosynthesis by binding to the photosystem II (PSII) and all other chemicals (termed "non-herbicides" hereafter), then the CA prediction model was applied to the experimental values of the detected PSII herbicides and a CA prediction based on effect concentrations estimated with a Quantitative Structure-Activity Relationship (QSAR) model was used for non-herbicides assuming that all non-herbicides act as baseline toxicants. Then we used an IA model to predict the combined effect of PSII herbicides and non-herbicides to evaluate whether the two-stage prediction model could explain toxicity of the real environmental mixtures. As is described in more detail in Appendix C, this model adequately predicted the mixture effects but it was actually not necessary to invoke the two-stage model because the contribution of the IA effect by the baseline toxicants was negligible.

Therefore only the CA prediction of the herbicides is presented in Figure 9 and compared with the experimental mixture effect. There was excellent agreement for the mixtures with 40, 39 and 48 components, while the CA predictions for the mixtures with 5 and 6 component over– and underpredicted the experimental mixture EC by a factor of 3.3 and 3.5, respectively. This discrepancy is due to experimental variability, as only two and one herbicide(s), respectively, were present in those mixtures with a small number of components.

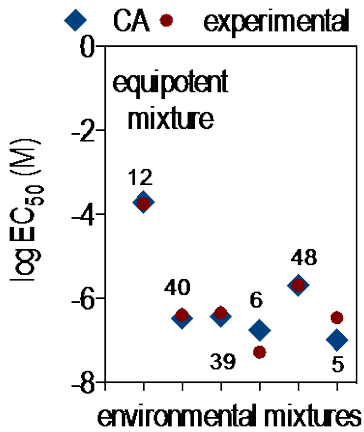


Figure 9 Comparison between experimental and predicted mixture EC_{50} for the algae test on inhibition of photosynthesis in designed iceberg mixtures that reflect concentration ratios observed in the water samples tested. The numbers refer to the number of components in a given mixture.

Thus the overall the specific toxicity of herbicides dominated the mixture effects in the realistic mixtures evaluated here. More details are given in Appendix C (Tang and Escher, 2014).

This conclusion cannot be generalised as it clearly depends on the composition of each sample. Nevertheless we can make some general conclusions. For receptor-mediated modes of action, the

toxic ratio, i.e., the ratio of how much more toxic a chemical is in comparison to its baseline toxicity, often was above 1000. For the pesticides tested, the toxic ratio ranged from 1250 to 11500. For a baseline toxicant to have a measurable impact in a mixture, it would need to be present at 1000 to 10000 times higher of a concentration, compared to the herbicide. The likelihood is small that this case occurs and it is not expected that non-herbicides would significantly influence the mixture toxicity.

The same is likely to be the case for endocrine-disrupting compounds, which have very high TR-values and especially for endpoints that are selective for endocrine effects such as reporter gene assays.

2.6 Conclusions

Our extensive mixture toxicity results with chemicals that are occurring in WWTP effluents, and potentially recycled water, have supported previous suggestions that the mixture toxicity concept of concentration addition is a suitable concept to describe and predict the effects of organic micropollutants in environmentally relevant mixtures (Kortenkamp *et al.*, 2009). Unlike previous studies, which used mainly reference chemicals and mixture ratios guided by theoretical considerations (Kortenkamp *et al.*, 2009), the present study focused on environmental relevance. The chemicals included in this study were chosen because they were detected in WWTP effluents (Tang *et al.*, 2013). Furthermore, the concentration ratios evaluated in the mixtures were not only equipotent but they were also mixed in the ratios as they occur in the environment. This part is taken up in Chapter 3, where these mixtures were used to interpret the results of the iceberg mixtures.

The mixture toxicity studies on cytotoxicity using the Microtox assay also provided practically relevant insights into mixture effects of a large number of chemicals. The Microtox assay, presumably due to its short exposure time of 30 min, is rather indiscriminate for MOAs. The majority of the tested chemicals could be classified as baseline toxicants independently of their MOA in the Microtox assay apart from antibiotics, which have high toxic ratios (Tang *et al.*, 2013). In all 31 tested mixtures, CA was an adequate prediction model for toxicity. The Microtox assay thus provides as a good sum parameter of the underlying concentration-additive baseline toxicity of all chemicals in a mixture.

Our previous work on the application of cell-based bioassays has shown that adaptive stress responses –in particular the oxidative stress response–are suitable indicators for micropollutant occurrence in water as well as hazard potential. To the best of our knowledge, there were no studies that dealt with the mixture effects on oxidative stress response. By again applying equipotent, and environmentally relevant concentration ratios, we were able to demonstrate that organic micropollutants that induce an oxidative stress response, act as concentration-additive. Chemicals that did not cause oxidative stress, did not influence the effects, apart from a few exceptions that were rationalised in the Appendix B. This observation was very interesting from a scientific perspective despite the magnitude of effect was rather minor, but for environmental assessment the CA concepts proved to be sufficiently robust to describe and predict mixture effects.

A large number of designed mixture toxicity studies on endocrine disruption have confirmed that CA is applicable for chemicals that act according to specific modes of action, and that are receptor mediated (Kortenkamp, 2007). This conclusion holds also for photosynthesis inhibition by herbicides that act via the quinone-binding site on photosystem II by blocking the electron transport chain (Faust *et al.*, 2001a; Junghans *et al.*, 2003). The present study confirmed the concentration additive effects of photosynthesis inhibitors in equipotent mixtures and mixtures as they occur in WTTP effluent. In addition, we were able to show that chemicals that are not photosynthesis inhibitors, but act according to diverse MOAs, are not substantially contributing to the mixture toxicity in algae under environmentally realistic conditions.

In summary, in 66 mixture experiments of variable design in three different bioassays representing three types of MOAs, CA was a robust mixture toxicity concept that can be applied to micropollutants occurring in WWTP effluents. We cannot extrapolate to other mixtures and other bioassays but the

agreement justifies the use of CA as a modelling tool. The applicability of CA is highly relevant for the iceberg mixtures discussed in Chapter 3. In addition, these findings imply that the BEQ concept is not limited to receptor-mediated MOAs but can be extended to a wider range of effects including adaptive stress responses and cytotoxicity.

3 How much of the iceberg do we see?

3.1 Goal

The goal of this subproject was to understand which fraction-of-effect observed in cell-based bioassays can be explained by known chemicals. The answer to this question will be important when deciding whether bioassays are required for water quality monitoring or if the regulated and regularly monitored chemicals are sufficient to assure water quality.

3.2 Approach

Representative recycled water samples were analysed for almost 300 chemicals with a focus on chemicals regulated in the Australian Guidelines for Water Recycling (NRMMC & EPHC & NHMRC, 2008). Authentic water samples were collected from an AWRP and based on the analytes detected, experimental mixture samples were formulated using authentic standards at the same concentrations as those detected in the water samples ("tip-of-the-iceberg" mixtures)—in an attempt to recreate the authentic mixture of known compounds. Mixture effects were experimentally determined by serially diluting reformulated samples to derive a full concentration-response curve (Figure 10). The mixture effect concentrations (EC) obtained with our designed mixtures were then compared to the toxicity of the respective authentic water sample, in order to assess how much of the overall toxicity can be explained by the typically analysed and quantified chemicals (Figure 10).

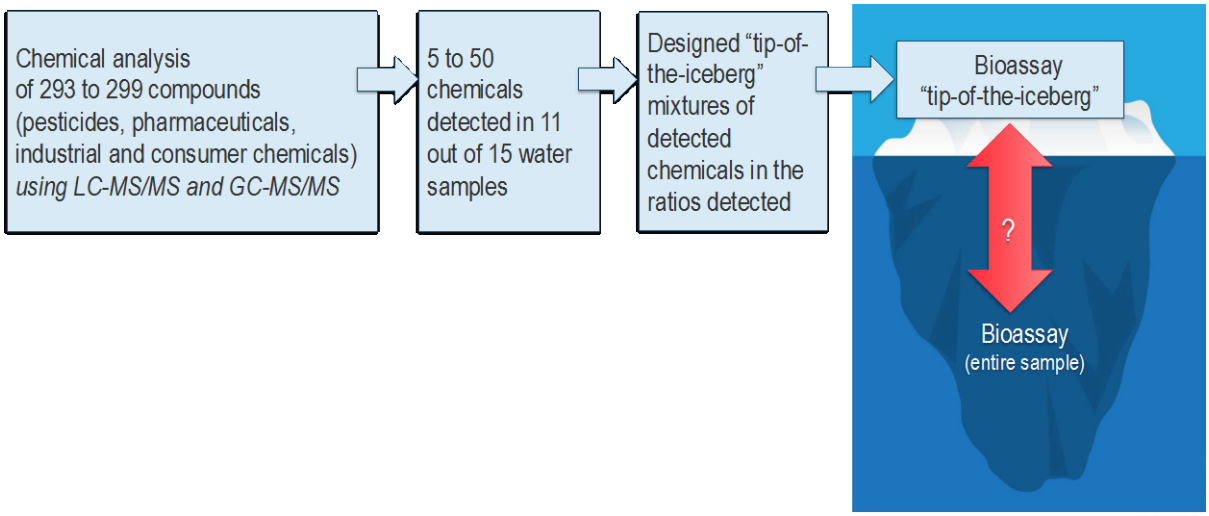


Figure 10. Outline of "iceberg" experiments. 299 chemicals were analysed in AWRP1 (Appendix D) and 293 chemicals were analysed in samples from AWRP2 and AWRP3 (Appendix A). The detected chemicals were mixed in ratios of the detected concentrations and assessed together with the entire water samples using the cell-based bioassays.

3.3 Water Samples

Three Advanced Water Recycling Plants (AWRP) were investigated in this study (Figure 11). Not only recycled water but also samples from the source water and samples taken throughout the treatment train were included because the concentrations of micropollutants in recycled water were typically below the limit of detection. Using source water, we know that the chemicals investigated are of environmental relevance and the concentrations are within detection limits of the analytical methods applied in this study.

AWRP1 has a treatment process with ultrafiltration (UF1), reverse osmosis (RO1), and UV disinfection (AO1). Additional samples included: a sample from the WWTP influent (raw), a sample from the mixing tank (MT) and a reverse osmosis reject (ROC1) sample (Tang *et al.*, 2014). AWRP2 starts with secondary treated sewage effluent Eff2 (Escher *et al.*, 2011). Three samples were taken from this plant: one after ultrafiltration (UF2), one after reverse osmosis (RO2) and one after advanced oxidation (AO2). From the third AWRP, a secondary treated sewage effluent (Eff3) sample and a sample following ozonation/biologically activated carbon filtration (O_3/BAC) were collected (Reungoat *et al.*, 2010; Reungoat *et al.*, 2011; Reungoat *et al.*, 2012) (Figure 11).

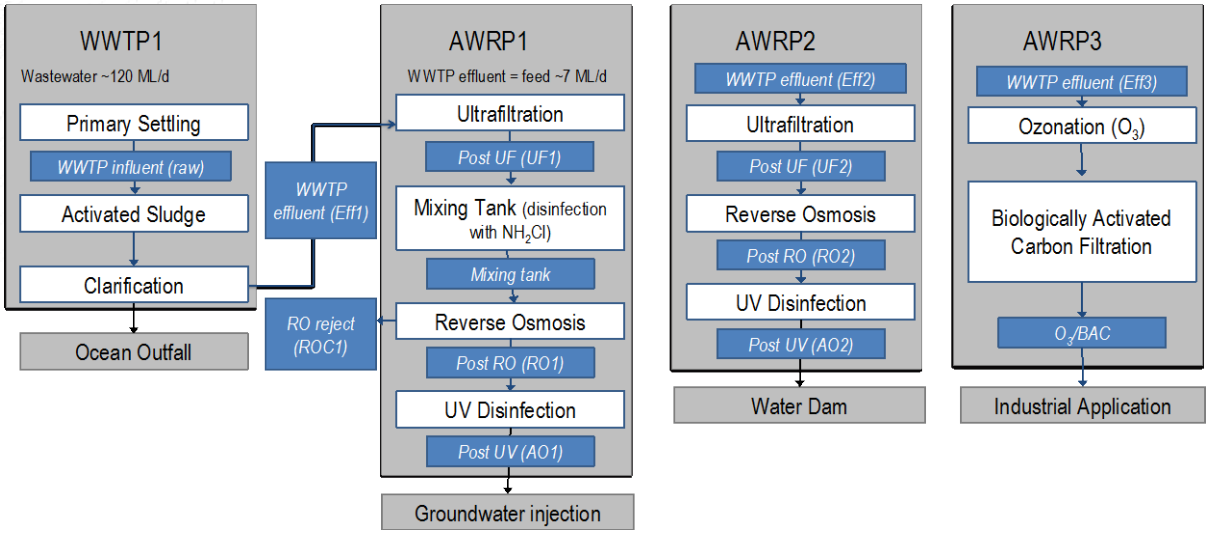


Figure 11 Three Advanced Water Recycling Plants investigated. In the blue boxes the sample codes are defined.

The detailed results for AWRP 2 and 3 are described in Appendices A, B and C, and for AWRP1 in Appendix D. The selected target analytes differed between the studies. For logistic reasons, we began our work by evaluating AWRP2 and AWRP3 and based on those results, we designed the mixture experiments reported in Chapter 2. AWRP1 was comprehensively sampled at a later stage in the project so that lessons learned from the first experience could be incorporated and the set of target analytes was extended. Below we present an overview of key results, with comprehensive data reported in the Appendices.

3.4 Chemical Analysis

As each subproject encompassed a different list of target analytes for chemical analysis a direct comparison between the numbers of detected chemicals in different AWRP is not possible but a comparison can be made of the numbers of detected chemicals within a treatment train. Figure 12 indicates that secondary treatment and ultrafiltration did not reduce the number of detected analytes. Reverse osmosis and ozonation had a dramatic effect both in terms of reducing the overall number of analytes detected as well as decreasing the concentrations of detectable compounds. Final recycled water had less than compounds above the limit of detection.

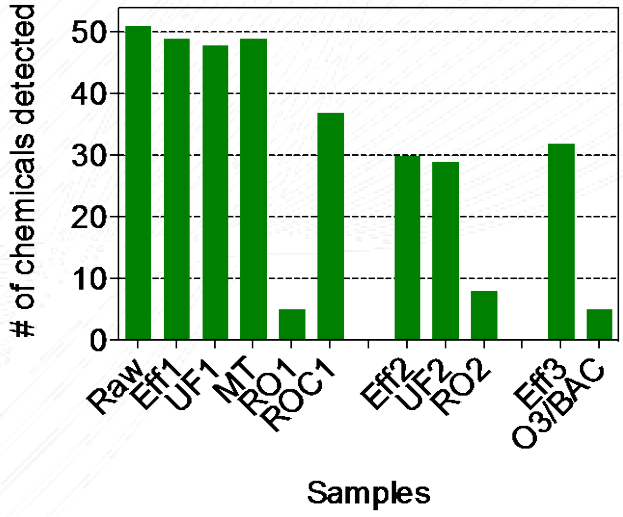


Figure 12 Number of chemicals that were detected in the water samples.

A detailed account of the chemical analysis in all AWRP is given in the appendices and we report in the following only the subset of 65 chemicals that overlapped in all studies. The black lines indicate the GV. The upper panel highlights all source water (Figure 13A). A few chemicals would have exceeded the GV prior to treatment, such as diatrizoic acid, tolytriazole and the pesticide MCPA (Figure 13A). Most chemicals were already below the GV in the source water, treated WWTP effluent. The disinfected recycled water samples (AO1 and AO2) did not have any chemicals above the detection limit and the few detected chemicals after reverse osmosis (RO1 and RO2) and after ozonation (O_3/BAC) were all well below the GV (Figure 13B).

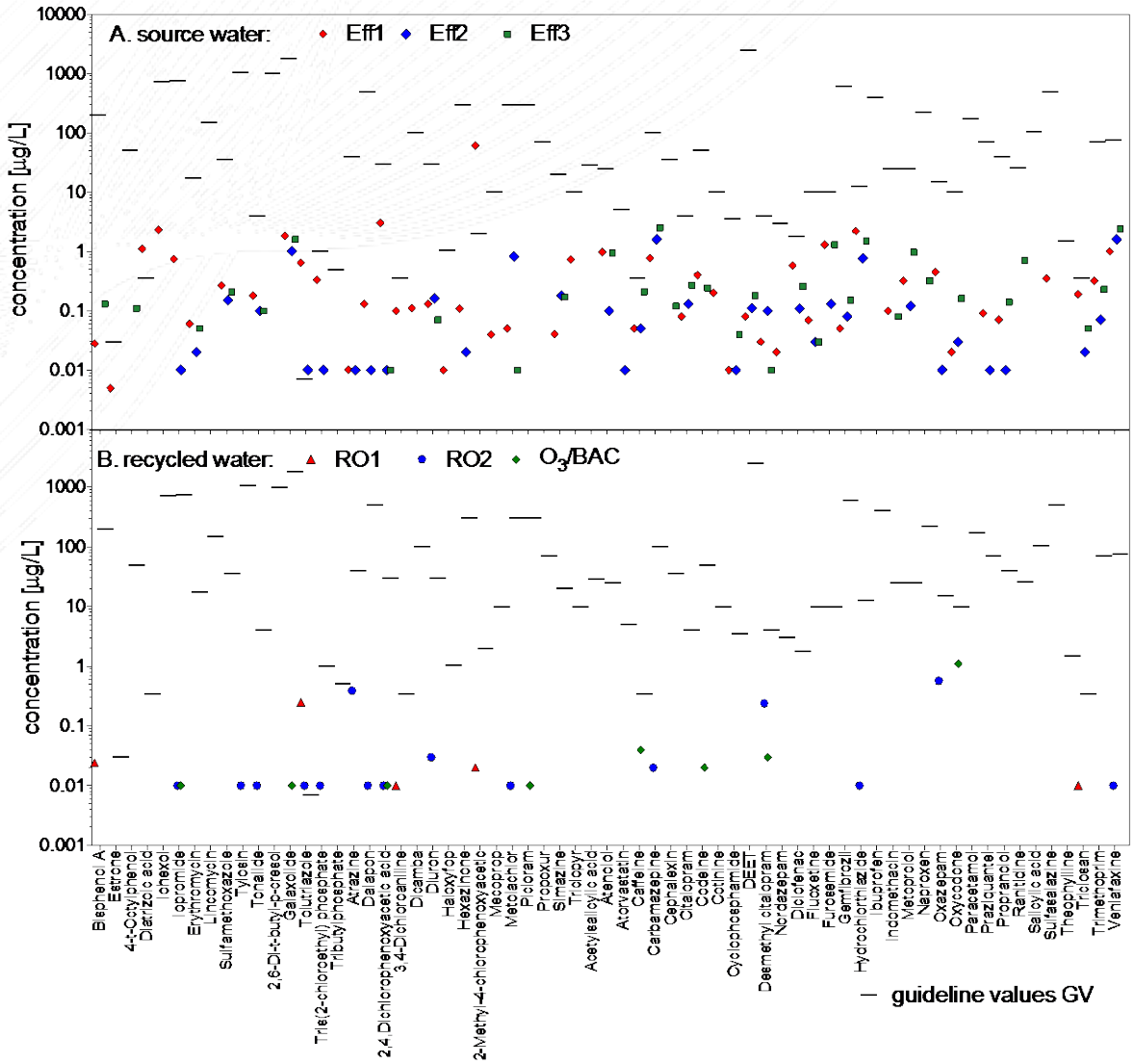


Figure 13 Summary of 65 chemicals included in the AGWR and in both experimental series. For full lists of chemicals, detected concentrations and limits of detection refer to (Tang et al., 2013; Tang et al., 2014) in Appendices A to D. The red symbols refer to samples taken in AWRP1, blue to AWRP2 and green to AWRP3.

3.5 Bioassays

Overall 103 bioassays were applied in AWRP2 and AWRP3 (Escher *et al.*, 2014a). Table 1 reports selected results of bioassays that were recommended by Escher *et al.* (2014) as suitable indicator bioassays and which included: one assay for cytotoxicity (*V. fischeri* (Microtox)), one assay for metabolism (AhR-CAFLUX), two assays for receptor-mediated specific effects (estrogenicity: ER-CALUX; Algae photo-synthesis inhibition with *Pseudokirchneriella subcapitata)*, and two assays for reactive toxicity/adaptive stress response (umu C for genotoxicity and AREc32 for oxidative stress response). In AWRP1 only these six bioassays were performed.

Apart from the Microtox assay all recycled water sample (AO1, AO2) resulted in effect concentrations below the limit of detection. Recycled water following ozonation showed small effects in ER-CALUX and AREc32 bioassays (Table 1 and Figure 14).

Table 1. Effect concentrations for all samples in the six bioassays. EC = effect concentration, REF = relative enrichment factor. The sample code is given in Figure 11.

		Cytotoxicity	*Metabolism*	*Specific toxicity*		*Reactive toxicity*	
	Bioassay	*V. fischeri* (Microtox)	AhR-CAFLUX	ER-CALUX	Algae photo-synthesis	umuC	AREc32
	Sample	EC_{10} (REF)	EC_{10} (REF)	EC_{10} (REF)	EC_{10} (REF)	$EC_{IR1.5}$ (REF)	$EC_{IR1.5}$ (REF)
AWRP 1	Raw	0.3	>30	>20	3.5	17	4.7
	Eff1	0.8	>30	>30	7.8	>30	8.4
	UF1	1.6	>30	>30	10.0	>30	19.5
	MT	1.4	>30	>30	14.8	>30	29.9
	RO1	20.2	>30	>30	>30	>30	>30
	AO1	11.1	>30	>22	>30	>30	>30
	ROC1	0.2	>30	>30	2.28	16	2.2
AWRP 2	Eff2	1.3	1.6	0.6	2.2	22.6	1.8
	UF2	0.7	1.4	0.7	2.6	25.3	2.5
	RO2	2.7	12.1	>25	>20	>30	>30
	AO2	10.7	>30	>25	>20	>30	>30
AWRP3	Eff3	0.4	1	0.1	6.3	17.5	1.7
	O3/BAC	1.4	>30	16.6	>20	>30	23.1
	Blank	>30	>30	>25	>20	>30	>30

The EC values in Figure 14 are plotted from high to low numbers, i.e., on an inverse scale, so that the most "toxic" samples are higher up on the y-axis and low effect samples are lower on the y-axis. Increasing treatment gradually reduced the biological effects. For clarity the data points below the limit of detection were omitted in Figure 14 but the highest tested concentrations without an effect are listed in Table 1.

The data collated in Figure 14 were used to select the bioassays for the iceberg experiments. Another selection criterion was to choose one bioassay each of cytotoxicity, specific receptor-mediated toxicity and reactive toxicity. Microtox was the most sensitive assay with a dynamic range over two orders of magnitude and was chosen as the first bioassay for the iceberg experiments. Of the receptor-mediated effects the E-CALUX and the AhR-CAFLUX did not show responses in AWRP1, which was unexpected

but suggested that neither of them was suitable for the iceberg experiments. Both assays were active in the samples from AWRP2 and AWRP3 at expected levels. The algal bioassay indicative of herbicides was active in all AWRPs and a substantial number of herbicides was detected throughout the treatment trains. Therefore, this assay was selected as an indicator bioassay for receptor-mediated mode of action.

For reactive toxicity, the umuC assay was active in all plants but at much higher enrichments than the AREc32 for oxidative stress response, therefore, AREc32 was selected for the iceberg experiments.

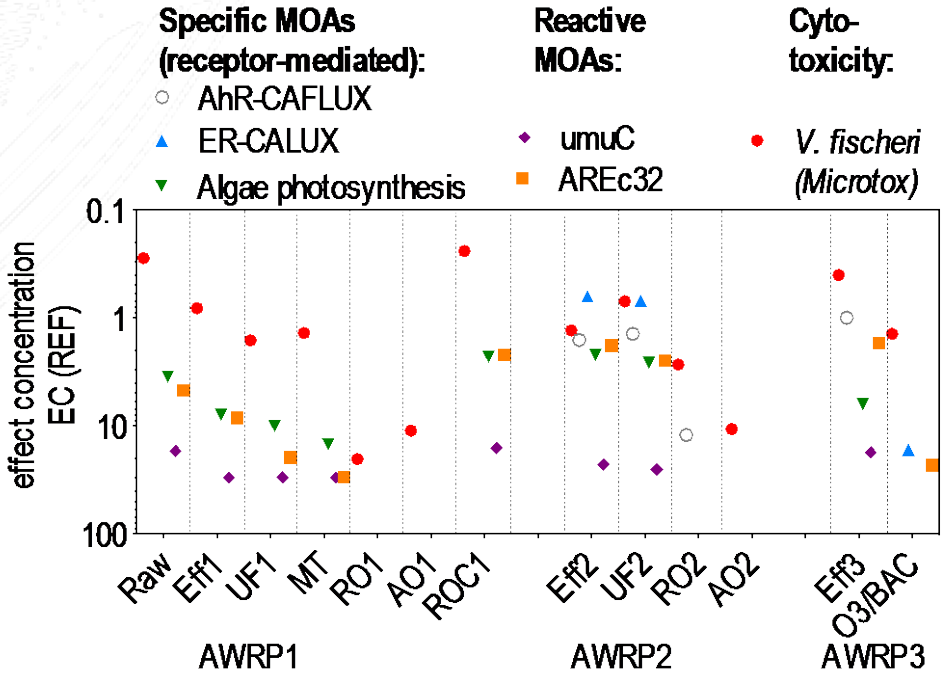


Figure 14. Effect concentrations for all samples in the six indicator bioassays.

3.6 Comparison of the effects of the detected chemicals (iceberg mixtures) with the water samples

For a comparison between the iceberg mixtures and the authentic water samples, we expressed the effects as bioanalytical equivalent concentrations (BEQ), which were defined as the concentration of a reference compound that had the same effect as the sample with a mixture of chemicals. A very detailed discussion is given in Appendices A to D for the different bioassays and all AWRPs, here we summarise only essential information relevant for all three AWRPs and compare the different AWRPs, which had been treated separately in Appendices A to D. Important to note here is that the comparison between the authentic water samples and the iceberg mixtures must be based on bioanalytical equivalents (BEQ) as effects cannot be compared but only effect concentrations and for simplicity the effect concentrations were converted to BEQs.

For the cytotoxicity assay, Microtox, there was no evident reference chemical because all chemicals contribute to the mixture effect and we therefore had earlier defined a virtual baseline toxicant and associated baseline toxicity equivalent concentrations (baseline-TEQ) (Escher *et al.*, 2008). The baseline-TEQ of the iceberg mixtures explained less than 3% of the baseline-TEQs of the extracted water samples (Figure 15) and the two to three orders of magnitude difference was consistent between all plants and sample types (Appendix A and D (Tang *et al.*, 2013; Tang *et al.*, 2014)). This finding suggests that there are many more chemicals (including transformation products but possibly also naturally occurring chemicals) present in authentic water samples, contributing to mixture cytotoxicity but which are not detected by routine chemical monitoring.

In contrast, for the diuron equivalent concentrations (DEQ) reporting, the effects in the algal photosynthesis inhibition assay, there was a very good agreement between the DEQ of the authentic water samples and the DEQ of the iceberg mixture (Figure 15, Appendices C and D (Tang and Escher, 2014)). Discrepancies could be explained by the fact that the chemical analysis was corrected for SPE recovery, while for the bioassays the SPE recovery is unknown as there is a complex unknown mixture of chemicals in the samples, thus possibly more herbicides were in the iceberg mixtures than in the extracted water samples.

Finally, for the oxidative stress response, an even larger fraction of effect could not be explained by the iceberg mixtures (Appendix B, (Escher *et al.*, 2013)). The t-butyl hydroquinone equivalent concentrations (tBHQ-EQ) of the iceberg mixtures explained less than 1% of the tBHQ-EQ in authentic water samples. A large number of chemicals active in this pathway may not be included in typical chemical monitoring, which is not surprising as of all the adaptive stress response pathways, the oxidative stress response, was expected to be responsive to the largest number of chemicals (Martin *et al.*, 2010).

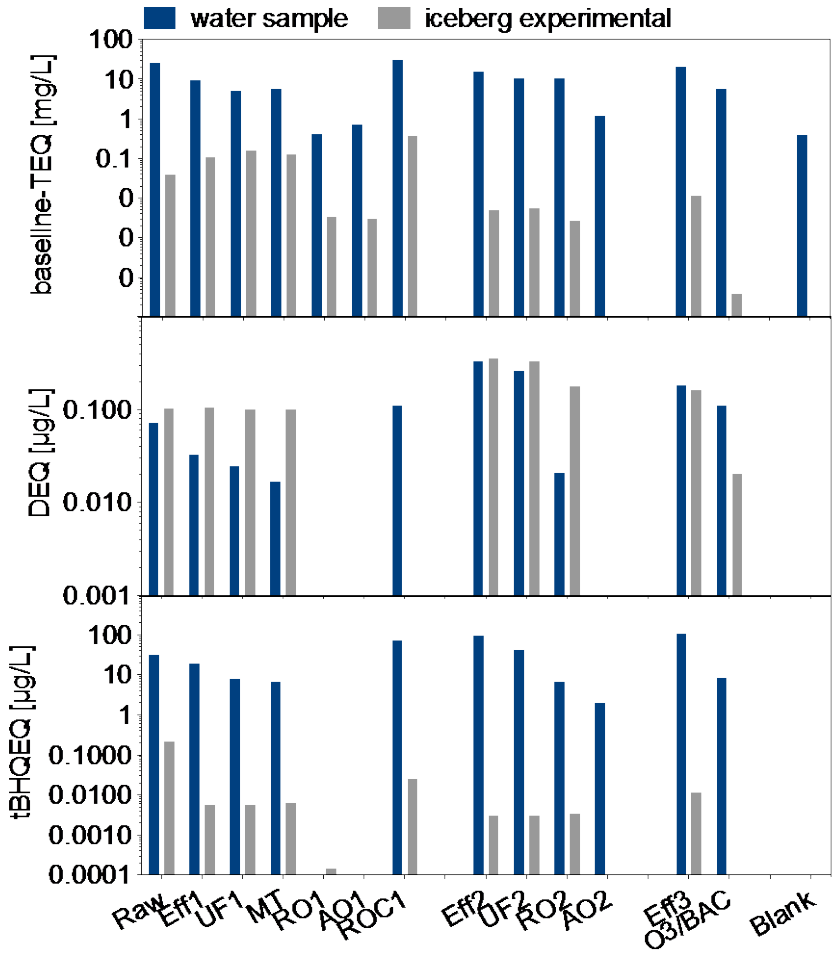


Figure 15. Comparison of the BEQ of the water samples compared with the BEQ of the iceberg mixtures. The sample code is given in Figure 11.

We also evaluated which chemical groups contribute to the mixture effects of the iceberg mixtures. The detailed results are given in Appendix D (Figure 4 of Appendix D). Pesticides and pharmaceuticals had an equal share but dominated the baseline-TEQ. As expected the pesticides dominated the DEQ. The

tBQ-EQ of the iceberg mixtures was made of 60% of pesticides, 30% pharmaceuticals and 10% other groups.

3.7 Conclusions

The results of this study have shown that there is no clear answer to the question of how much of the BEQ can be explained by known chemicals but that it depends on the type of effect. For receptor-mediated modes of action, the majority of the responsive chemicals have been identified, in this study for herbicides and in a previous work for estrogenic chemicals (Escher *et al.*, 2011). For more integrative endpoints, such as the oxidative stress response and cytotoxicity, there remain many unknowns and bioassays are clearly needed to get a full picture of the effects of micropollutants.

4 Do transformation products of micropollutants formed during relevant water recycling processes contribute to mixture toxicity?

4.1 Introduction

4.1.1 Motivation

Currently, transformation products (TPs) are of major interest in terms of recycled-water quality assessment because they represent a knowledge gap (Escher and Fenner, 2011). It is not known how many TPs are formed, in what quantities, and what the level of risk to being exposed to them is. Transformation products can result from a variety of pathways and can be formed in the environment as well as in engineered systems. Pharmaceuticals are extensively metabolised in humans and animals and they are typically not excreted in the same form as they were ingested, but rather as a variety of metabolites (Lienert *et al.*, 2007). So-called pro-drugs are only transformed to the pharmacologically active form in the body and this form may also be more potent than the parent with respect to its intended or unintended (possibly adverse) effects. Additionally, pesticides and other micropollutants can undergo biotic and abiotic transformation reactions in the environment. In surface water, exposure to sunlight can cause direct photodegradation or indirect oxidation of micropollutants via formation of reactive species.

Biodegradation is particularly intensive during biological wastewater treatment, however, full mineralisation to carbon dioxide and water is incomplete for most chemicals and formation of biotransformation products can result. Micropollutants in water can be transformed during advanced oxidation and disinfection processes. Furthermore, natural organic matter (NOM) can also act as precursors, reacting with oxidants and forming disinfection by-products (DBPs).

While most TPs are less persistent, less bioaccumulative, and less toxic compared to their parent compounds (Boxall *et al.*, 2004), there are a number of known exceptions (Escher and Fenner, 2011). One example is nonylphenol, which is a degradation product of the industrial surfactant nonylphenolpolyethoxylate (NPE). Nonylphenol is highly persistent, bioaccumulative and in addition to being more toxic than NPE, also exhibits weak estrogenic effects (Fenner *et al.*, 2002). Radjenovic et al. (2011) also demonstrated the formation of more potent TPs during electrochemical oxidation of reverse osmosis reject in an advanced water treatment plant. Some TPs are more persistent than the original parent compound and are thus found in higher concentrations in the environment.

Escher and Fenner (2011) previously proposed a tiered scheme on how to assess the risk of transformation products in relation to their parent compounds (Figure 16) This novel tiered approach for screening organic micropollutants for their potential to form toxicologically relevant TPs combines bioanalytical assessment tools and advanced analytical identification and quantification of TPs.

4.1.2 Goals

This project focuses on recycled water, thus the focus is on engineered treatment systems. For water recycling, tertiary treatment is mostly focused on membrane processes and/or oxidative treatments. During oxidative treatments of recycled water, transformation products (TPs) and oxidation by-products are formed—only a few of which are regulated. The traditional process of identifying, isolating and quantifying hazardous TPs and oxidation by-products is costly and time consuming. The goals of this research were to use a battery of bioassays to assess the potential toxicity of mixtures of TPs formed during tertiary treatment, and to identify the TPs (first stage in Figure 16). The challenge was to determine if mixture effects of TP are relevant. Once relevant TPs are identified, it would be important to synthesise them chemically, so that controlled experiments could be performed, allowing for a more

fundamental understanding of transformation pathways and kinetics as well as effects of individual TPs, which would better inform future recommendations for a comprehensive assessment of risk.

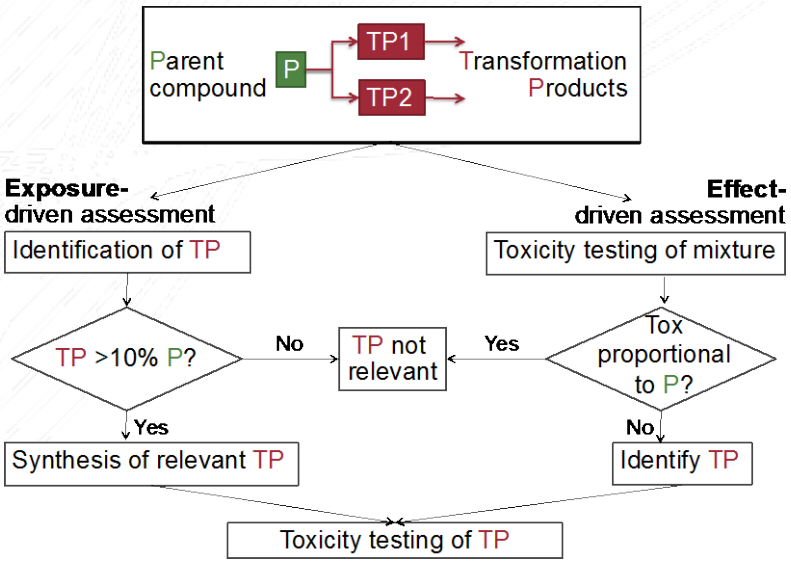


Figure 16. Tiered approach for screening organic micropollutants for their potential to form toxicologically relevant TPs (adapted from Escher and Fenner (2011)).

4.1.3 Approach

Firstly, a literature review on the formation of TPs from micropollutants was conducted, with a particular focus on ozone oxidation. Eight relevant compounds were then selected for further experimentation. The selection is discussed in Section 4.1.4 and the state-of-the-art knowledge on TPs for the selected micropollutants is summarised in each individual chapter.

Lab-scale experiments were conducted on the selected eight parent compounds (P) using analytical-grade chemicals spiked into a relevant matrix (reverse osmosis permeate collected from an Advanced Water Recycling Plant (sample RO from AWRP1, Figure 11). The selected parent compounds were added to a final concentration of 10 to 100 µM in RO water, a range of O_3 doses were individually added and the decrease of parent compound concentrations was measured (Figure 17). Once the experimental conditions were optimised to obtain more than 50% degradation, the experiments were repeated and the effects of the mixtures were assessed (Figure 17). If any of the bioassays showed effects caused by TPs, then the TP structures were identified by non-target analysis (Figure 17).

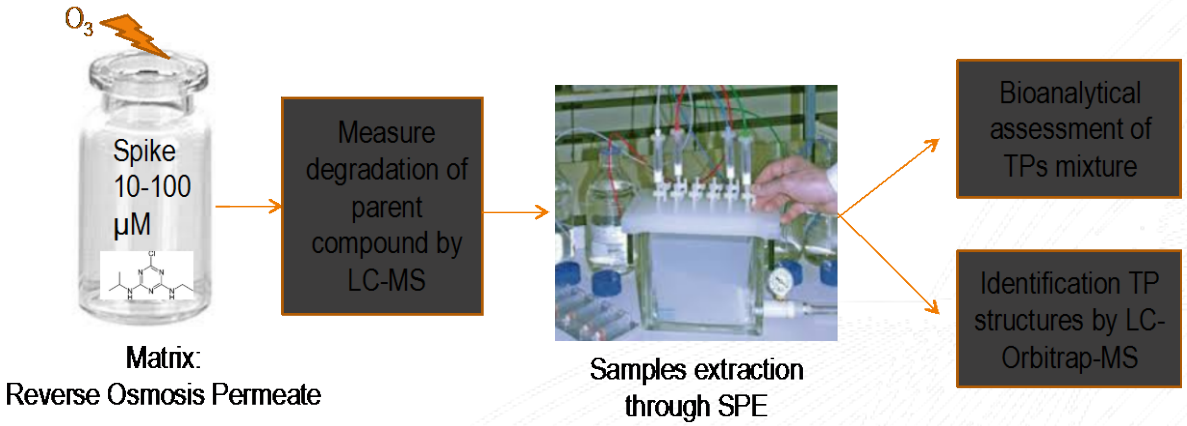


Figure 17. Experimental approach.

4.1.4 Choice of test chemicals

Eight test chemicals (atrazine, bisphenol A, carbamazepine, diclofenac, haloxyfop, hexazinone, iopromide sulfamethoxazole,) were selected for degradation experiments. The selection criteria for choosing a set of micropollutants for the investigation in this study were:

- Inclusion in the Australian Guidelines for Water Recycling (AGWR).
- Presence in reverse osmosis permeate (RO) or at least in WWTP effluent, which was the feed water into the AWRP.
- Literature evidence of the ability to form potentially toxic TPs.

Bisphenol A was present in RO. Haloxyfop and hexazinone were selected because they were detected in sample MF in AWRP1 ((Tang *et al.*, 2014), Appendix D).

Atrazine, diclofenac, carbamazepine, were selected because the analysis of the literature identified a number of TPs from the parent compounds. We chose iopromide, which is not toxic on its own but has the potential to form toxic I-DBPs during ozonation (Duirk *et al.*, 2011). Sulfamethoxazole was selected because there was literature evidence (e.g. (Abellan *et al.*, 2008)) of the potential formation of toxic TPs.

4.1.5 Choice of the bioassays

A single bioassay is not sufficient to assess if the formed TPs pose a toxicological hazard. Given the large number of samples obtained during degradation studies, it is also not feasible to run a large test battery of tests on each sample. We therefore focused on two or three bioassays for each ozonation experiment. The three bioassays were chosen that they covered the non-specific toxicity (cytotoxicity), reactive toxicity and specific toxicity (associated to the primary mode of action of the P) ((Figure 18).

Firstly, the non-specific toxicity was targeted by an integrative endpoint, which measures general cellular health. We used the Microtox assay to measure a relative decrease in light output from naturally bioluminescent marine bacteria, *Vibrio fischeri*, in exposed ozonation extracts. A decreased light output indicates interference with energy metabolism and reduced overall cellular health. This assay responds non-specifically to all compounds presented in the extracts and therefore it was suitable as an initial screening (Tang *et al.*, 2013). Transformation products can potentially add to the mixture effect as shown in Chapter 2. Therefore the Microtox assay was applied in all ozonation experiments (Figure 18).

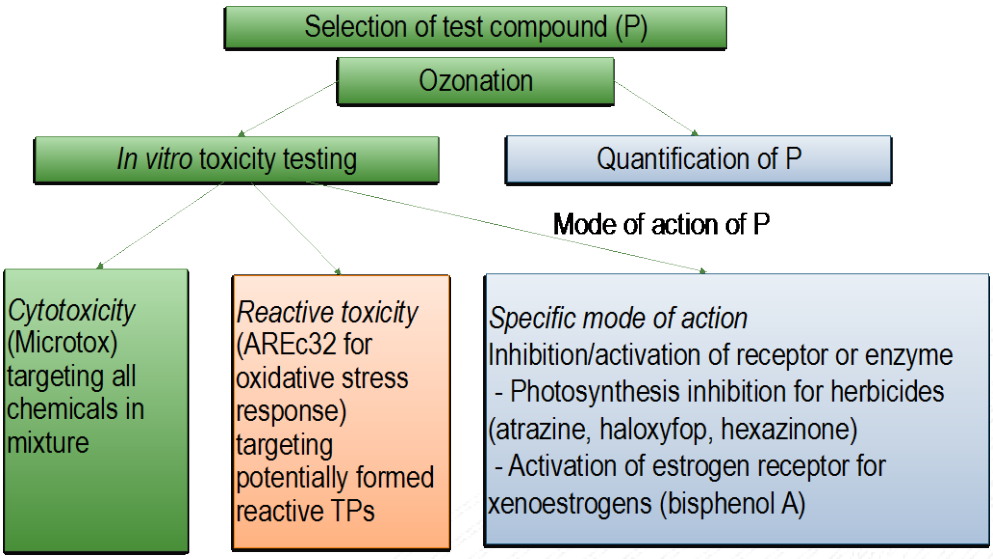


Figure 18. Selection of bioassays.

Secondly, reactive toxicity might be relevant because some TPs formed might have reactive properties. For reactive toxicity we selected the oxidative stress response because many TPs formed are likely be electrophilic (Figure 18). We used the AREc32 assay to detect any oxidative stress response in the reaction mixtures.

Finally, for chemicals that act according to a specific mode of action, we matched a bioassay that was indicative of the target mode of action of the parent compound. For the herbicides atrazine, haloxyfop and hexazinone we used photosynthesis inhibition in green algae. Photosynthesis inhibition is a key target mode of action for herbicides, which disrupts the photosynthetic electron transport chain (Moreland, 1980). The excitation energy is re-emitted as fluorescence rather than driving the photochemical processes. Algae are sensitive to herbicides, so we used a pulse-amplitude modulated fluorometry assay (IPAM) to quantify the amount of herbicidal activity in the samples (Figure 18).

Bisphenol A has low estrogenic potency and a bioassay for estrogenicity would be appropriate for this mode of action. Since a large number of studies have shown that the estrogenic effect is rapidly decreased by ozonation (as reviewed by Umar et al. (2013)), the E-CALUX was not applied in the present study. We recognise that this is a shortcoming of the present study but it was not possible to include this endpoint due to the limited time of the project. In future work we recommend the inclusion of the E-CALUX.

None of the other parent compounds exhibited a specific mode of action and for those only the Microtox assay and AREc32 was applied.

4.1.6 Interpretation of results

Three bioassays targeting different modes of action were chosen ((Figure 18). All results of the bioassays were plotted in Chapter 4 as shown in *Figure 19*, where both the effect and the parent compound concentration was plotted as a function of the ozone concentrations. Three different cases can be differentiated:

a) the mixture effect change proportionally to the concentration of the parent compound,
b) the mixture effect remains unchanged, or
c) the mixture effect is higher than the effect of the parent compound

In case (a), the TPs have no contribution to the mixture effects of their parent compound, and no identification of TPs would be required, as they are not toxicologically relevant. If case (b) applies, the TPs are equally as toxic as the parent and from the overall risk assessment perspective, no specific risk assessment of TPs is needed. In this case the risk assessment of the parent would be sufficient. Case (c) is the most environmentally relevant because it means that TPs are more toxic than the parent compound. In this case identification of TPs and a comprehensive risk assessment is imperative.

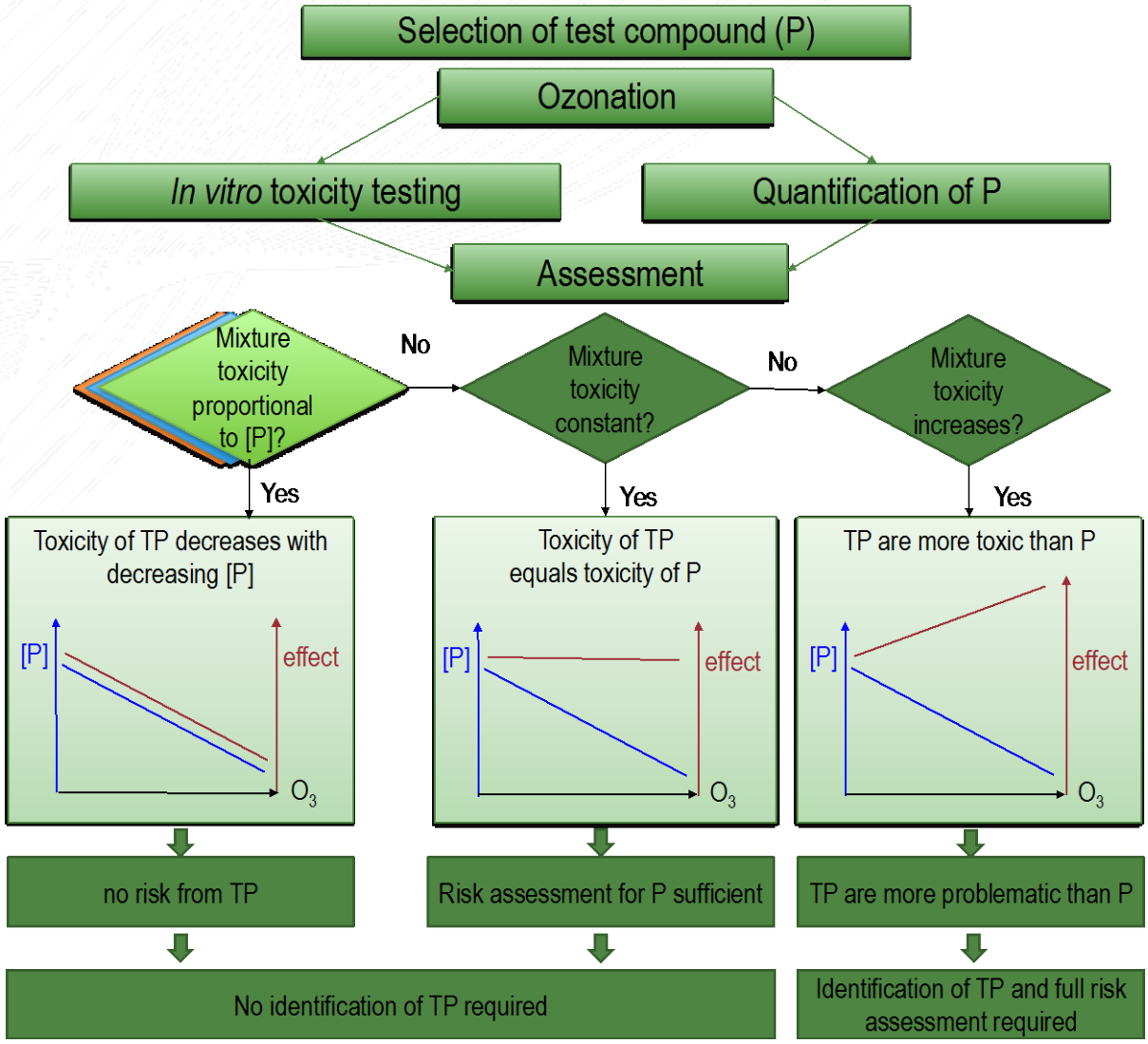


Figure 19. Flow chart for the bioassay selection and bioassay data evaluation.

4.2 Materials and Methods

4.2.1 Degradation experiments

In a first stage, ozone was used as oxidant since it is commonly used as tertiary treatment for the removal of micropollutants. Experiments were carried out with post-RO water (sample RO in Figure 11) collected from Beenyup AWRP in February 2013 (see Table 2 for water quality parameter). Each chemical (parent compound) was dissolved in post-RO water to a final concentration of 10 to 100 µM. Five different ozone concentrations were applied, typically ranging from 5 µM to 200 µM (concentrations in all Figures are reported in units of µM and can be converted into g/L by the molecular weight of ozone of 48 g/mol). The reaction mixture was analysed by direct injection liquid chromatography tandem mass spectrometry (LC-MS/MS) to assess the concentrations of the parent compounds.

The samples were extracted by SPE on HLB and coconut charcoal cartridges according to Tang et al. (2014) and the extracts were sent to Entox for toxicity testing. Depending upon the results of these toxicological tests (i.e., TPs being more effective than the parent compound) the reaction mixture was re-analysed by liquid chromatography high-resolution mass spectrometry (Orbitrap) and TPs were identified.

In a second stage, micropollutants showing similar or increasing toxicity or effect potency upon degradation of the parent compound, other disinfection processes were investigated. During ozonation, hydroxyl radicals (OH radical) are formed as by-products. OH radicals are generally highly reactive and they may assist in the formation of TPs. In order to better understand the reactivity of ozone alone, tert-butanol (t-BuOH) was added to the samples prior to the addition of ozone to scavenge the OH radical formed as secondary oxidant. Preliminary experiments were also carried out with UV (commonly used after RO membrane to inactivate pathogens) and using the advance oxidation process UV/H_2O_2 to further investigate the mechanisms involved in these oxidation processes. The advanced oxidation process UV/H_2O_2 is used for compounds recalcitrant to ozone oxidation because H_2O_2 produces OH radical when irradiated by UV. UV alone was also studied for two reasons. Firstly because when applying UV/H_2O_2 the compound might be degraded through a photolysis pathway and one might want to know if the reactivity is coming from OH radical or/and UV photolysis. Secondly, because in the water reuse scheme of Perth, UV is used as tertiary treatment. These samples were also analysed with bioassays. Each experiment was run at least in duplicate.

Table 2. Water quality parameters for post RO water collected from Beenyup AWRP in February 2013.

pH	Conductivity mS/M	DOC mg_C/L	Alkalinity mg/L as $CaCO_3$	Total N mg/L	Br^- mg/L	I^- mg/L	Cl^- mg/L	Ca^{2+} mg/L	Na^+ mg/L
5.6	3.6	<1	5	1.2	<0.02	<0.02	4	<0.1	6

4.2.2 Experimental set up for ozonation

Ozone was produced with the ozone generator depicted in Figure 20. The ozone concentration of the stock solution was standardized by measurement of the UV absorbance (absorbance at 258nm = 3000 $M^{-1}cm^{-1}$) using an UVmini-1240 spectrophotometer (Shimadzu) and was approximately 1mM (48 mg/L). The concentrations of dissolved ozone in the experiments were determined by the indigo method. For ozone dose experiments, aliquots of ozone stock solution were individually added to the water samples containing selected micropollutants at a concentration of 10 µM or 100 µM in 1mM phosphate buffer (pH 7.5) to reach the desired initial concentration (0 – 200 µM), and upon ozone addition, the solutions were mixed for 10 seconds. A contact time of 24h in all experimentswas used to ensure complete depletion of the oxidant. The solutions were subsequently sampled for analysis; no quenching agent was added. For the experiment with O_3 alone, the same experimental procedure was used except that the samples were added with 10 – 50 mM t-BuOH.

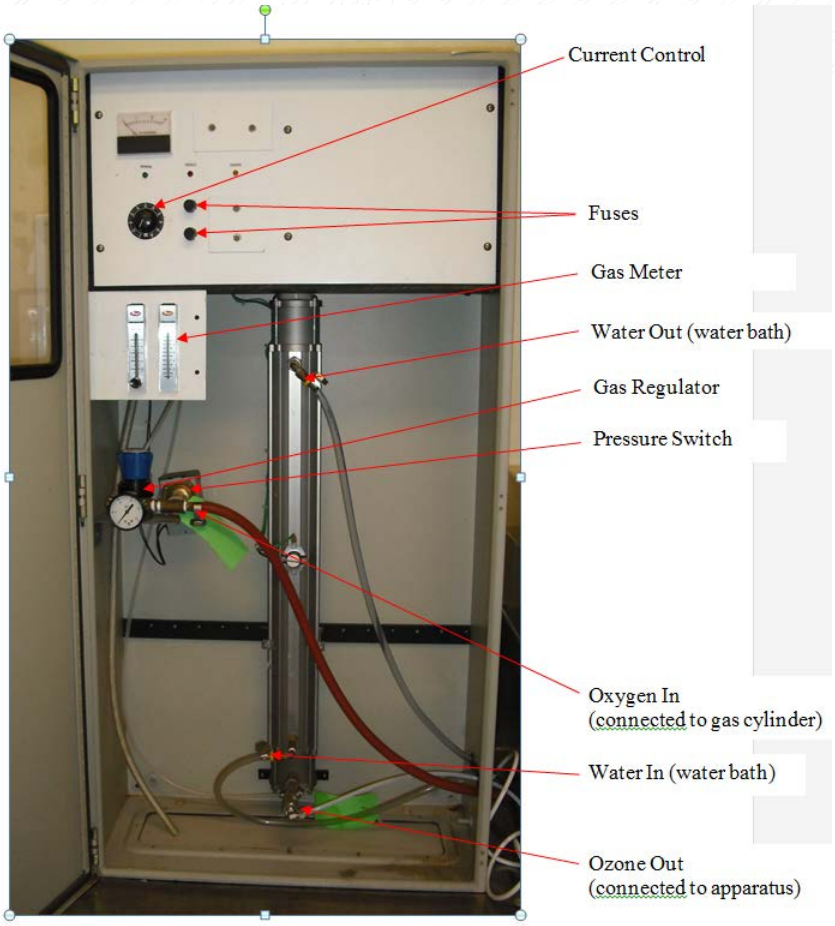


Figure 20. Ozone generator used for the AOP experiments.

4.2.3 Experimental set up for UV and UV/H_2O_2 experiments

The UV experiments were carried out with a low-pressure mercury (LP Hg) lamp Heraeus Noblelight model TNN 15/32 (nominal power 15W). Fluence rate values were determined by chemical actinometry using H_2O_2. A disinfection dose (fluence) of 4000 J m^{-2}, corresponding to 10 times the recommended disinfection dose (Canonica *et al.*, 2008), was used for the UV experiments. For the UV/H_2O_2 experiment a similar set up was used but 1mM H_2O_2 was spiked to the samples prior to irradiation.

4.2.4 Sample preparation

After completion of the degradation experiments, aqueous samples were processed through SPE on charcoal and HLB SPE material as described in Chapter 3. SPE cartridges were eluted at the CWQRC and shipped to Entox. The SPE extracts were tested in a range of bioassays at Entox while chemical analyses were conducted by CWQRC staff.

Sample preparation for analysis by Liquid Chromatography – High Resolution Mass Spectrometry: glassware was washed with HPLC grade methanol, rinsed with ultrapure water and then annealed at 530 °C overnight. The samples were transferred using disposable Pasteur pipettes into 1 mL brown vials, and then diluted 1:10 or 1:100 in MeOH:H_2O=50:50 (v:v) containing 0.1% of formic acid. Samples were either infused in the mass spectrometer using a syringe pump at 3-5 µL/min or injected into the LC column using the Accela 600 LC system.

4.2.5 Bioassays

Bioassays were performed as described in WP2. Three bioassays targeting different modes of action were used: namely the non-specific cytotoxicity assay (Microtox (Tang *et al.*, 2013)) oxidative stress response with AREc32 (Escher *et al.*, 2013) and photosynthesis inhibition assay (Escher *et al.*, 2008).

Unfortunately, the first two sets of experiments had unacceptable positive responses of the negative controls in most bioassays, possibly due to contamination of solvents used during SPE. Therefore these samples could not be used for data evaluation. The results reported in this report are thus based on one duplicate set of ozonation experiments performed independently on separate days.

4.2.6 Analysis of reaction mixtures for parent compounds degradation studies

Reaction mixtures were analysed using a LC-MS/MS system consisting of an 1100 Agilent (Palo Alto, CA, USA) LC system and a Micromass (Manchester, UK) Quattro Ultima Triple Quadrupole Mass spectrometer fitted with an electrospray ion source (ESI) operated in positive ionisation mode. ESI and MS setting were as following: capillary 3.25 KV; cone 25 V; hexapole1 0.0 V; aperture 0.2 V; hexapole2 0.2 V; desolvation temperature and source temperature were 325 ºC and 135 ºC, respectively. Cryogenic liquid nitrogen gas (BOC Gases, Perth, Australia) was used as desolvation and nebulizer gas; cone gas flow was set to 30 L/h, while the desolvation gas flow was set to 750 L/h. High purity Argon (99.997% purity) (BOC Gases) was used as collision gas (pressure = 2.1×10^{-4} kPa). Both quadrupoles (Q1 and Q3) were set at unit mass resolution; ion energy on Q1 and Q3 was set to 1.0 (arbitrary units), while the multiplier was set at 750 V. Chromatographic separation was achieved using a X-bridge C18 LC-MS column (50 mm × 2.1 mm, 3 µm, 100 Å) from Waters at a flow rate of 250 µL/min. The mobile phase was methanol (MeOH) (A) and ultrapure water (B) both containing 0.1% of formic acid. Chromatographic runs began with 30% (A) for 3 min, followed by a 10 min linear gradient to 95% (A). The mobile phase remained at 95% (A) for 10 min to elute analytes from the column. Afterwards, the initial conditions were re-established within 1 min and the column re-equilibrated for 10 min before injecting the next sample. To minimise potential carryover, before and after each injection, the needle of the injector was rinsed for 30 seconds in the injection port with MeOH. The injected volume was 10 µL.

Analytes were analysed in MRM or SRM mode using the following transitions (m/z):

- atrazine 216.3→ 174.0;
- carbamazepine 237.4→192, 194;
- diclofenac 296.1→ 215, 250;
- haloxyfop 361.7→ 316.2;
- hexazinone 253.3→ 171.1;
- iopromide 792.3→774.3;
- sulfamethoxazole 254.2→108, 156.

Bisphenol A was analysed by LC-UV @ 225 nm with a Luna C18 column (150 mm × 4.6 mm, 5 µm, 100 Å) from Phenomenex. A mobile phase constituted of 60% MeOH / 40% H_2O in isocratic, with a flow rate of 1 mL/min was used for elution.

4.2.7 Screening and identification of transformation products

Reaction mixtures were analysed for the presence of TPs using a liquid chromatography (LC) high resolution mass spectrometer (LC-HRMS) consisting of an Thermo Accela 600 LC system and a LTQ XL (Ion Trap) and an Orbitrap XL mass spectrometers fitted with an electrospray ion source (ESI) operated in positive ionization mode. ESI settings and $HRMS^n$ settings are reported in Table 3.

Table 3. Summary of ESI and HRMS^n parameters used for analysis of TPs in the reaction mixtures.

Parameter	Settings
Source Voltage (kV)	4
Capillary Temp (°C)	275
Sheath Gas Flow (Arb)	20
Aux Gas Flow (Arb)	0
Sweep Gas Flow(Arb)	0
Capillary Voltage (V)	45
Tube Lens (V)	70-110
Ion Trap MSn AGC Target	1E4
FTMS Full AGC Target	1E6
FTMS MSn AGC Target	5E4
Ion Trap and FT Micro Scans	3
Ion Trap MSn Max Ion Time (ms)	100
FTMS Full Max Ion Time (ms)	200
FTMS MSn Max Ion Time (ms)	200
Injection Waveform	Off

Full calibration of the Ion Trap and the Orbitrap in the 150-2000 m/z range was conducted weekly with the positive/negative ion calibration solution provided by Thermo Scientific. Optical lenses were optimised with a standard solution of caffeine ($[M+H]^+$ =195.19 m/z), prior each measurement. Samples previously analysed for degradation of parent compound (P) were analysed for structure and identity of TPs. Samples showing degradation of P> 40% were analysed in MS full scan to identify the m/z of the main TPs (i.e. $[M1+H]^+$, $[M2+H]^+$ etc). This screening analysis was initially conducted operating the Orbitrap mass spectrometer in full-scan mode from 70-1000 m/z with a mass resolution of 30.000 (@ 400 m/z). To proceed with the structural elucidation of TPs and fragments, samples were also analysed in high resolution MS^2 and MS^3 (multiple fragmentation stages) with a mass resolution of 30000 (@ 400 m/z). For substance identification, the deviation of the measured mass was compared against the theoretical (< 3ppm) and, where possible, the measured isotope pattern (i.e. fragmentation pattern) was compared with the theoretical fragmentation pattern. The agreement of MS^2 and MS^3 spectra between the sample and the standard was checked for parent compounds.

For chromatographic separation of TPs, a X-bridge C18 LC-MS column (50mm × 2.1 mm, 3 µm, 100 Å) from Waters at a flow rate of 250 µL/min was used. The mobile phase was methanol (MeOH) (A) and ultrapure water (B) both containing 0.1% of formic acid. Chromatographic runs began with 30% (A) for 3 min, followed by a 10 min linear gradient to 95% (A). The mobile phase remained at 95% (A) for 10 min to elute analytes from the column. Afterwards, the initial conditions were re-established within 1 min and the column re-equilibrated for 10 min before injecting the next sample. To minimise potential carryover, before and after each injection, the needle of the injector was rinsed for 30 seconds in the injection port with pure MeOH.

Reaction mixtures were also analysed in MS Scan, Daughter scan and Parent scan modes using a Quattro Ultima Triple quadrupole system from Micromass fitted with an electrospray ion source (ESI)

operated in positive ionisation mode. ESI and MS setting were as following: capillary 2.85 KV; cone 25V; hexapole1 0.0 V; aperture 0.1 V; hexapole2 0.1 V; desolvation temperature and source temperature were 325 ºC and 135 ºC, respectively. Chromatographic conditions were the same as those adopted for the HR analyses.

Data was processed using the Xcalibur QualBrowser 2.0.7 SP1 software.

4.2.8 Data evaluation

The degradation of parent compounds as a function of the ozone concentration, i.e., the fraction of concentration of remaining P was calculated with equation 5.

$$\text{fraction } P(C_{ozone}) = \frac{P(C_{ozone})}{P_0} \quad (5)$$

Where $P(C_{ozone})$ is the concentration of the parent compound in the reaction mixture undergone ozonation at a given ozone concentration and P_0 is the initial concentration of parent compound before ozonation.

The relative mixture effect was expressed as a function of the ratio of the effect concentration (EC) of the parent compound (EC_P, converted to units of relative enrichment factors using the initial parent concentration) over the EC of the mixture at a given ozone concentration ($EC_{mixture}(C_{ozone})$). A value of the relative mixture effect of 1 refers to unchanged effect after oz. If the relative mixture effect is >1, the effect of the mixture is higher than that of the initial concentration of P and if the relative mixture effect is <1, then the mixture effect is decreasing with ozone concentration.

$$\text{relative mixture effect}(C_{ozone}) = \frac{EC_P}{EC_{mixture}(C_{ozone})} \quad (6)$$

4.3 Results and Discussion of Preliminary Experiments

Preliminary transformation experiments were carried out to optimise the experimental conditions and to determine the highest experimental ozone concentration that caused at least 50% degradation of the parent compound. To follow the production of TPs during the oxidation process we evaluated the highest and 3-4 lower ozone concentrations. For most of the compounds (bisphenol A, diclofenac, haloxyfop, sulfamethoxazole, hexazinone and iopromide) an ozone dose ranging up to 200 µM was found suitable to achieve this goal with a concentration of 100 µM for the parent compound. However, for atrazine and carbamazepine, because of their low solubility, an ozone dose of 20 µM was used for an initial concentration of 10 µM. It was not possible to dissolve the compounds in an organic solvent since it affects the reactivity of ozone/OH radical and can lead to either an increase or a decrease of the rate and yield of reaction.

As reported in the progress report of September 2013, all initial experiments could not be evaluated and had to repeat because the blank samples (ozonated post RO water) were showing high toxicity and this background toxicity masked the effects of the spiked chemicals. We could not identify the cause of contamination; it could be a contamination of the solvents used for elution of the SPE.

Sulfamethoxazole, iopromide and bisphenol A were more than 50% oxidised by an O_3 concentration of 200 µM. The oxidation of the parent compound was positively correlated to the ozone concentration applied (Figure 21). As is described in more detail in the following sections, the measured effects in all bioassays were decreasing proportionally to the decay of the parent compounds, indicating that the TP did not contribute to the mixture effects. In these cases the identification of TPs was not further pursued. Ozonation is a good option to treat these pollutants since it appears that the transformation products are not significantly toxic.

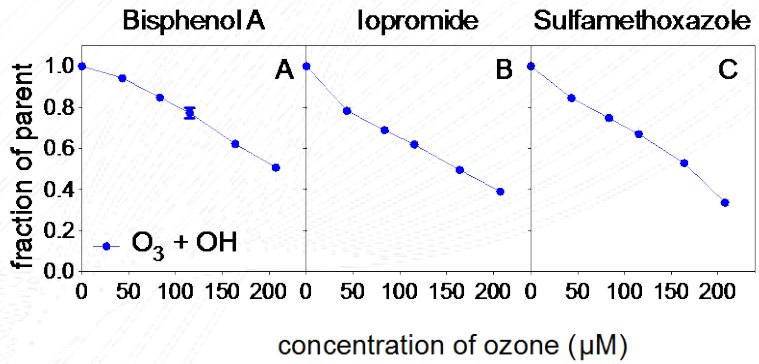


Figure 21. Fraction of parent remaining after ozonation (without suppression of hydroxy radicals) for (A) bisphenol A, (B) iopromide, and (C) sulfamethoxazole.

Haloxyfop and hexazinone required similarly high ozone concentrations for 50% degradation by oxidation, while 50% degradation was achieved for carbamazepine and atrazine with an ozone concentration of 10 µM since the concentration of the parent compound was 20 µM (Figure 22). Some of the bioassays revealed that the effects were either constant or even increasing with the degradation of the parent compound (see following sections). For these compounds, TPs were identified (see the following sections). Furthermore, to understand whether or not the toxic TPs were formed from reaction with ozone itself or due to oxidation by OH radicals, experiments were also conducted with the OH radicals suppressed by the addition of t-BuOH, so that ozone was the only reactive species.

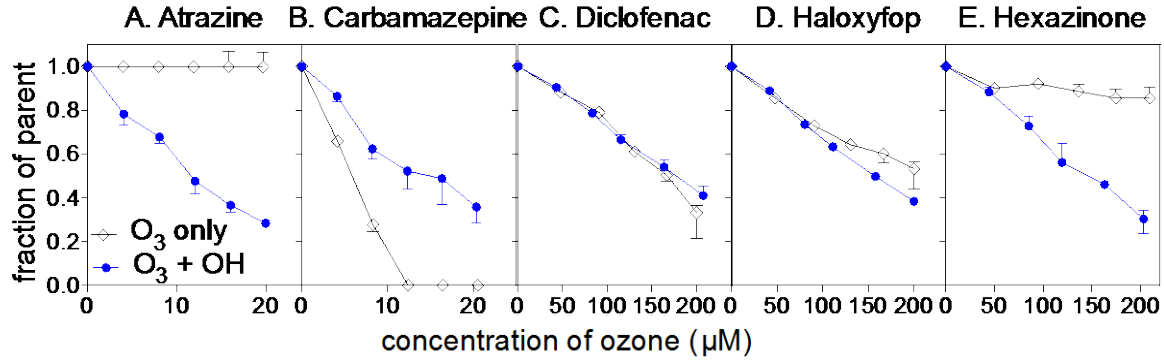


Figure 22. Fraction of parent remaining after ozonation (blue dots: without suppression of hydroxyl radicals, diamonds: O_3 alone, hydroxyl radicals quenched with t-BuOH for (A) atrazine, (B) carbamazepine, (C) diclofenac, (D) haloxyfop, (E) hexazinone.

Three different scenarios were observed. For haloxyfop (Figure 22D) and diclofenac (Figure 22C) little difference was observed between the experiments with and without OH radicals. The main oxidant in this case is O_3 and the reactivity of OH radicals is negligible.

Contrasting behaviour was observed for atrazine (Figure 22A) and hexazinone (Figure 22E) where a change was observed when OH radials were quenched. For the experiments with ozone alone atrazine and hexazinone were not degraded or poorly degraded. This means that only OH radicals are oxidising these chemicals.

For carbamazepine (Figure 22B), a greater degradation was observed for the experiment with ozone alone when hydroxyl radicals were quenched with t-BuOH. This might be explained by the fact that the presence of t-BuOH not only quenched the OH radicals but may also stabilise the ozone and as a consequence more ozone would be available to oxidise carbamazepine. To further investigate the mechanisms involved in these oxidation processes, preliminary experiments were carried out with UV and UV/H_2O_2. The advanced oxidation process UV/H_2O_2 is used for compounds recalcitrant to ozone

oxidation because H_2O_2 produces OH radicals when irradiated by UV. UV alone was also studied for two reasons. Firstly because when applying UV/H_2O_2 the compound might be degraded through a photolysis pathway and it would be interesting to know if the reactivity is coming from OH radical or/and UV photolysis. Secondly, because in the water reuse scheme of Perth, UV is used as tertiary treatment.

In Figure 23, the percentage of oxidised parent compound after application of the highest ozone dose (with and without t-BuOH) are compared to samples exposed to a UV irradiation of 4000 J m^{-2} and a similar UV dose with 1 mM H_2O_2 for the UV/H_2O_2 process.

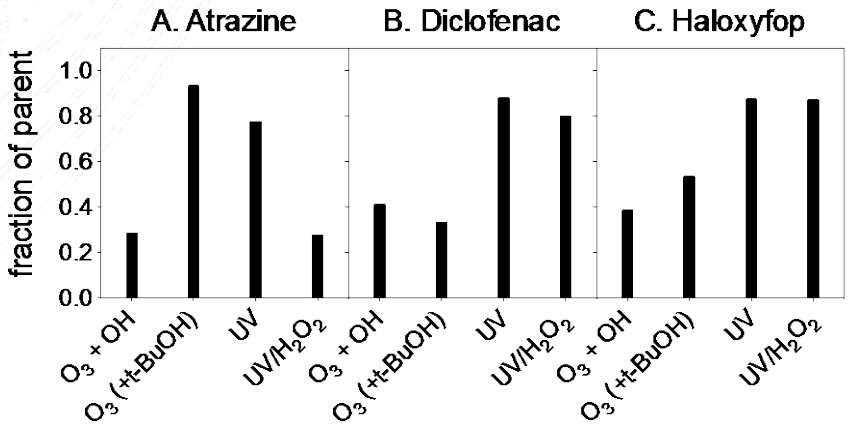


Figure 23. Fraction of parent remaining after treatment: (A) atrazine (O_3 concentration of 20 µM, UV at 4000 J m^{-2} and UV/H_2O_2 at 4000 J m^{-2} and 1 mM H_2O_2), (B) diclofenac (O_3 concentration of 200 µM, UV at 4000 J m^{-2} and UV/H_2O_2 at 4000 J m^{-2} and 1 mM H_2O_2) and (C) haloxyfop (O_3 concentration of 200 µM, UV at 4000 J m^{-2} and UV/H_2O_2 at 4000 J m^{-2} and 1 mM H_2O_2).

Figure 23 shows that the primary mechanism for haloxyfop oxidation is the reaction with O_3. Since the UV and UV/H_2O_2 experiments gave the same results it is conceivable that OH radical does not play a significant role in haloxyfop oxidation and that low reactivity is coming only from the UV exposure alone and not from hydroxyl radicals.

In contrast, for atrazine, a high reactivity is observed for both the ozone + OH and the UV/H_2O_2 experiments. The reactivity is mainly coming from OH radicals, which was confirmed by the low reactivity when quenching the hydroxyl radicals in ozone with t-BuOH and UV alone.

Diclofenac was mostly reactive with ozone. However, OH radical and UV photolysis are also slightly degrading diclofenac and cannot be neglected.

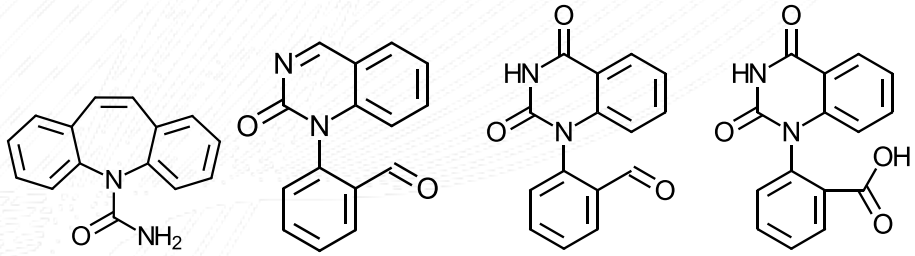
In the following sections, the ozonation experiments are discussed in more detail and compared with the mixture effect assessment. In those cases, where the mixture effects were substantial, the identity of the TPs was determined by high-resolution mass spectrometry.

4.4 Carbamazepine

4.4.1 Literature review of ozonation of carbamazepine

Carbamazepine is a drug commonly prescribed for the treatment of epilepsy. It is frequently found in wastewater and has been shown to pass sewage treatment without drastic change in concentration, as such, for example it has been found in the German aquatic environment at concentrations of 250 ng/L (Ternes, 1998). Carbamazepine has been also found in Western Australian treatment facilities at a median concentration of 0.940 µg/L in secondary effluent (97% detection frequency, n=29 samples) and below detection post–RO treatment (0% detection frequency, n=29 samples) (Van Buynder *et al.*,

2009). The oxidation of carbamazepine by ozone (Huber *et al.*, 2003) and the formation of the major transformation products (Figure 24) has been studied by McDowell (2005).



Carbamazepine TPs

Figure 24. Carbamazepine and TPs of carbamazepine identified by (McDowell et al., 2005).

4.4.2 Degradation of carbamazepine by ozonation and mixture effects

As shown in Figure 25, carbamazepine was oxidised through ozonation treatment (ozone + OH radical). The concentration of parent compound decreases linearly with ozone dose applied. For 20 µM of ozone, 65% of carbamazepine was oxidised. Non-specific toxicity (Microtox, Figure 25A) and oxidative stress response (AREc32, Figure 25B) were increased following a 4 µM ozone dose. The non-specific effects increased to ~5 fold at 20 µM and the oxidative stress response increased between 2-4 fold at 20 µM for two individual ozonation experiments.

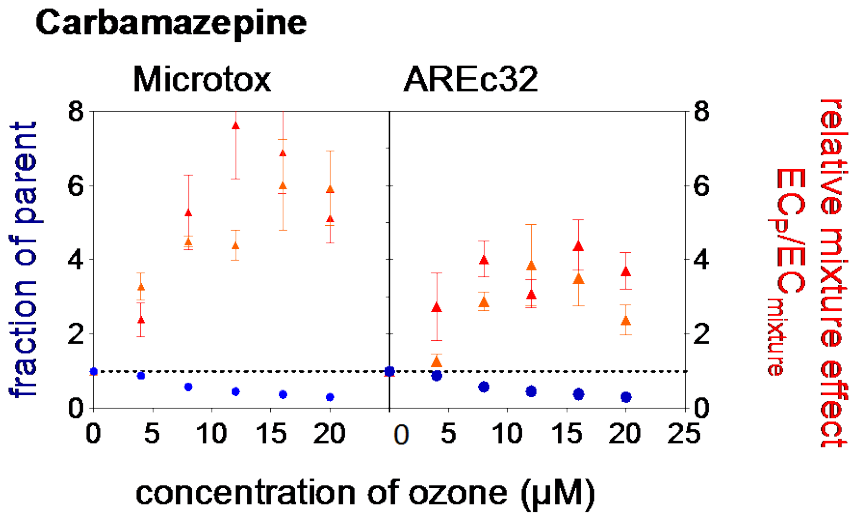


Figure 25 Comparison of the degradation of the carbamzepine with the change in the effects in the reaction mixture, (A) Microtox assay, (B) AREc32 (different shades in colour of the same symbol indicate results from independently repeated experiments).

4.4.3 Identification of TPs of carbamazepine

The following paragraphs (4.4.3 – 4.4.3.4) describe the details of the methodology adopted for the identification of the TPs of carbamazepine. The same methodological approach was used to identify TPs for the other compounds tested (i.e., diclofenac, atrazine, hexaxinone and haloxyfop-P).

A sample of 10 µM of carbamazepine treated with 20 µM of O_3 was run in low resolution MS scan mode to identify the m/z values of the molecular ions ($[M+H]^+$) of the parent compound carbamazepine and of the TPs (Figure 26). A sample of phosphate buffer treated with 20 µM of O_3 (blank) was also run for comparison and subtracted.

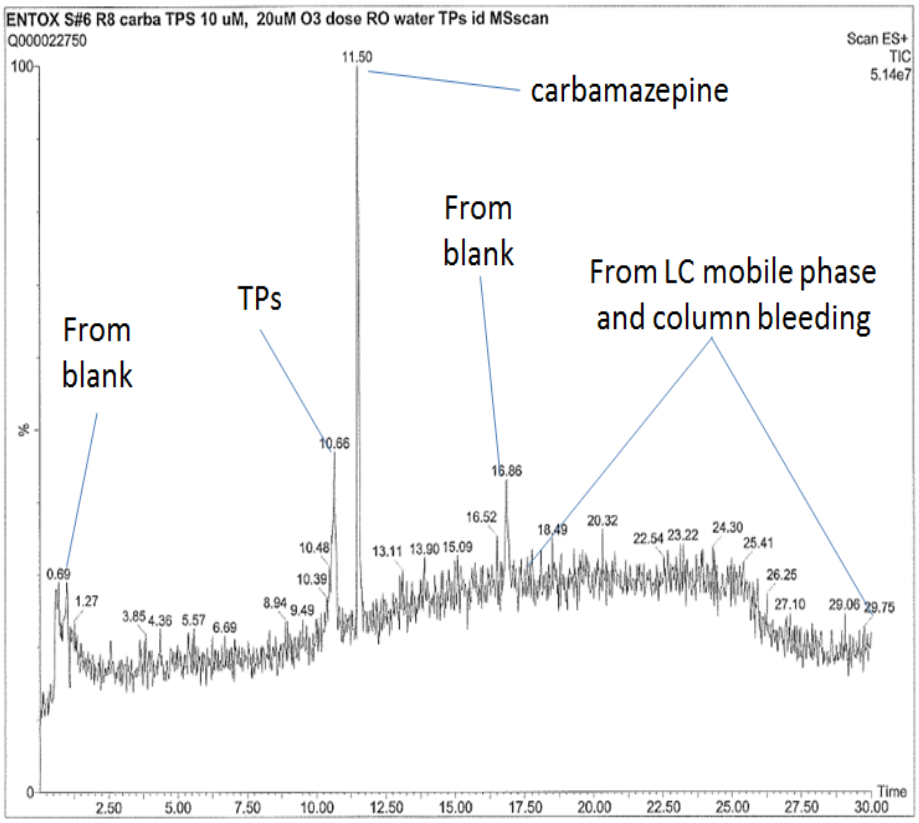


Figure 26. LC-MS chromatogram acquired in low resolution MS scan showing total ion current (TIC) in the 70-350 m/z range.

The extracted ion currents for carbamazepine and TPs (Figure 27) show the presence of main peaks:

RT (10.55 min): m/z= 267

RT (10.66 min): m/z=251 and m/z=283

RT (11.52 min): m/z=239

The first three LC-MS peaks in Figure 27 are TPs (namely TP251, TP283, TP267) while the fourth LC-MS peak is the parent compound carbamazepine.

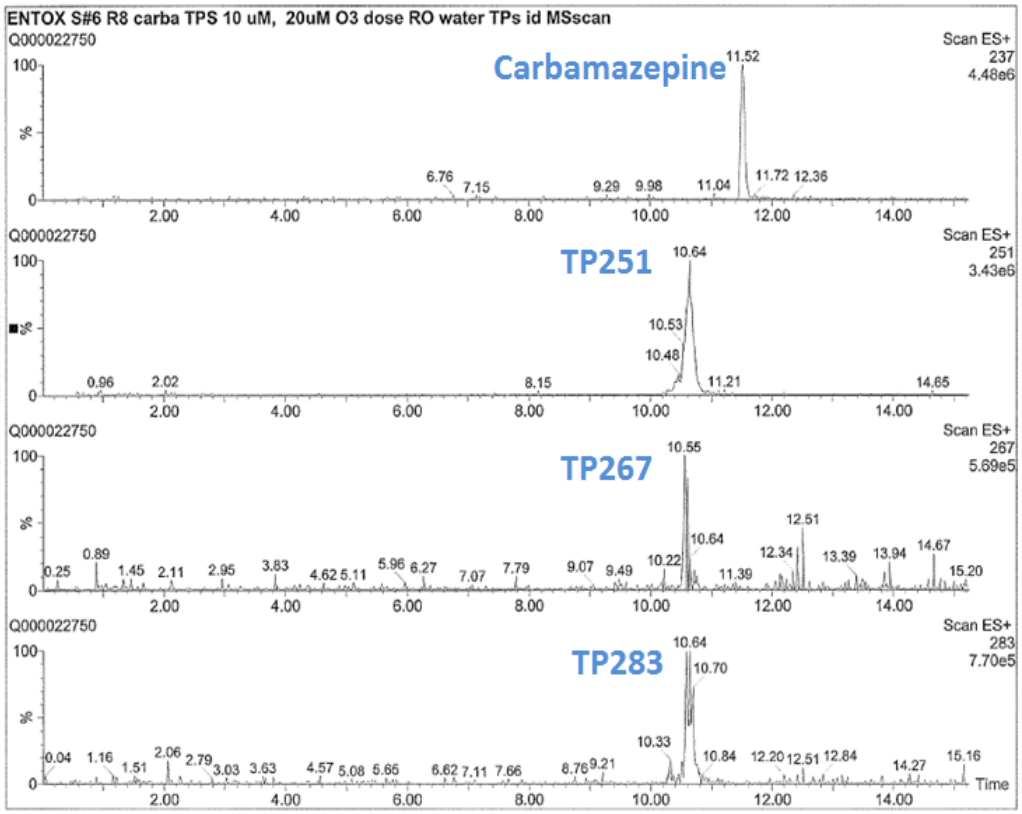


Figure 27. LC-MS chromatogram showing extracted ion currents of 283, 267, 251, and 237 m/z.

The high-resolution MS^2 spectra of the parent compound carbamazepine (i.e.,isolation of 237.10→fragmentation→HRMS scan) was run to identify fragmentation pattern and verify mass accuracy of the Orbitrap (Figure 28).

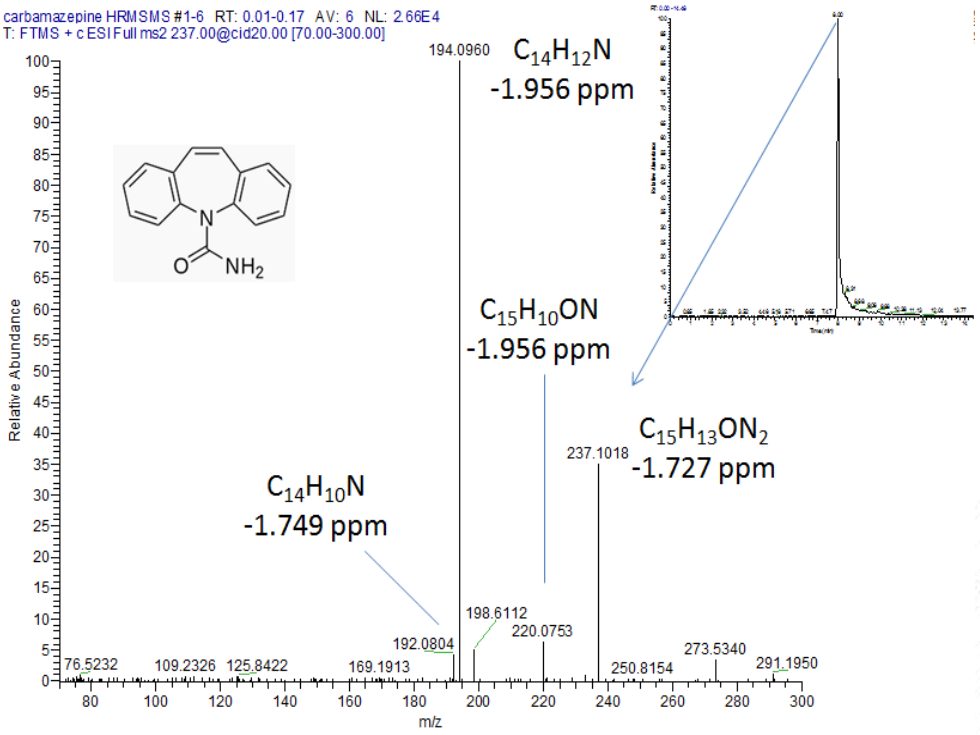


Figure 28. High resolution MS^2 of carbamazepine (237m/z) and proposed identity of fragments.

The fragmentation pattern showed:

- 237.1018→220.0753 (loss of NH_2)
- 220.0753 →194.0960; 192.0804 (loss of COH_2 and rearrangement)

The experimental value of the m/z of parent ion carbamazepine and fragments were found to be congruent with m/z theoretical value of 237.1022 $[M+H]^+$. Mass accuracy relative error was below 2 ppm indicating a good agreement between experimental and theoretical data. Following this, the same sample (10µM of carbamazepine treated with 20 µM of O_3) was scanned in $HRMS^2$ to record the fragmentation spectra of each TP.

4.4.3.1 Structural identification of TP267

A sample of 10µM of carbamazepine treated with 20 µM of O_3 was run in $HRMS^2$ (i.e. isolation of 267.88→fragmentation→HRMS scan). This returned only 1 chromatographic peaks corresponding to 1 TP. Figure 29 shows the $HRMS^2$ spectra of m/z=267 and proposed identity of fragments.

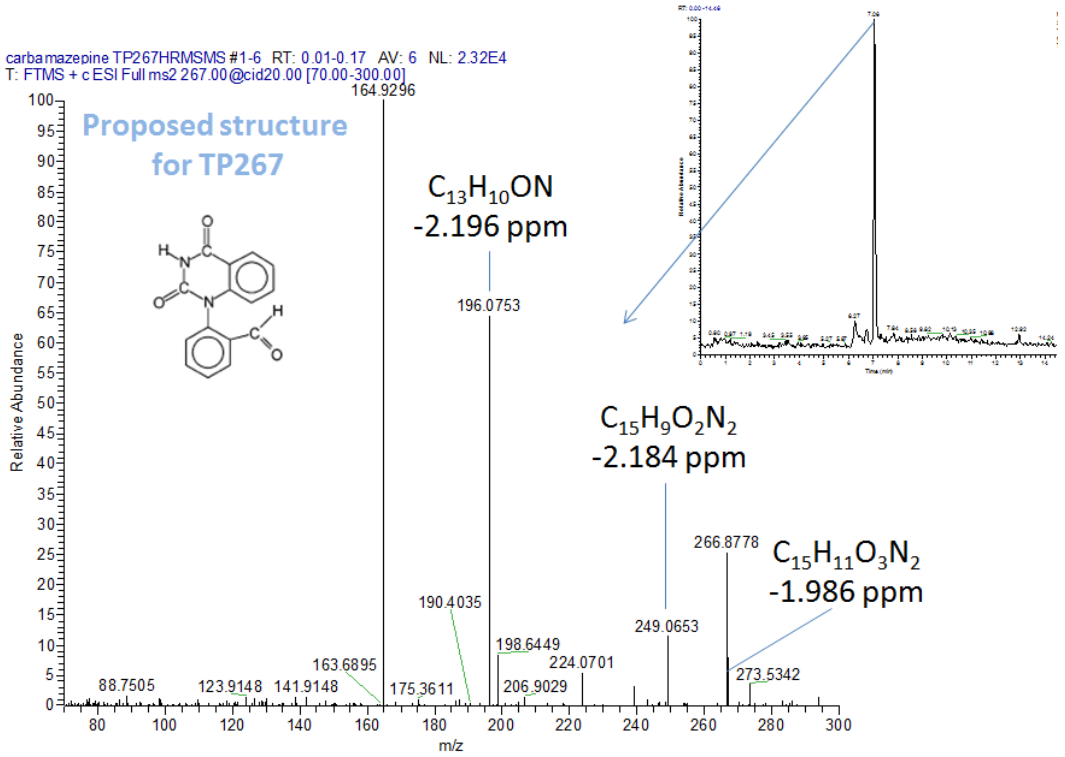


Figure 29. High resolution MS^2 of TP267 (m/z=267) and proposed identity of fragments.

The fragmentation pattern shows:

- 266.8778→249.0653 (loss of H_2O)
- 249.0653→224.0701 (loss of NH=C=O)
- 249.0653 →196.0753 (loss of C_2ONH)

The fragmentation pattern is congruent with proposed structure. Elemental formula of TP267 ($C_{15}H_{11}O_3N_2$) and fragments ($C_{15}H_9O_2N_2$ and $C_{13}H_{10}ON$) are <3ppm from theoretical values confirming the identity of TP267. The formation of TP267 from ozonation/OH has been reported previously and the fragmentation pattern also previous published in literature (McDowell *et al.*, 2005).

4.4.3.2 Structural identification of TP251

A sample of 10µM of carbamazepine treated with 20 µM of O_3 was run in $HRMS^2$ (i.e. isolation of 251.08→fragmentation→HRMS scan). This returned only 1 chromatographic peak corresponding to 1 TPs. Figure 30 shows the $HRMS^2$ spectra of m/z=251 and proposed identity of fragments.

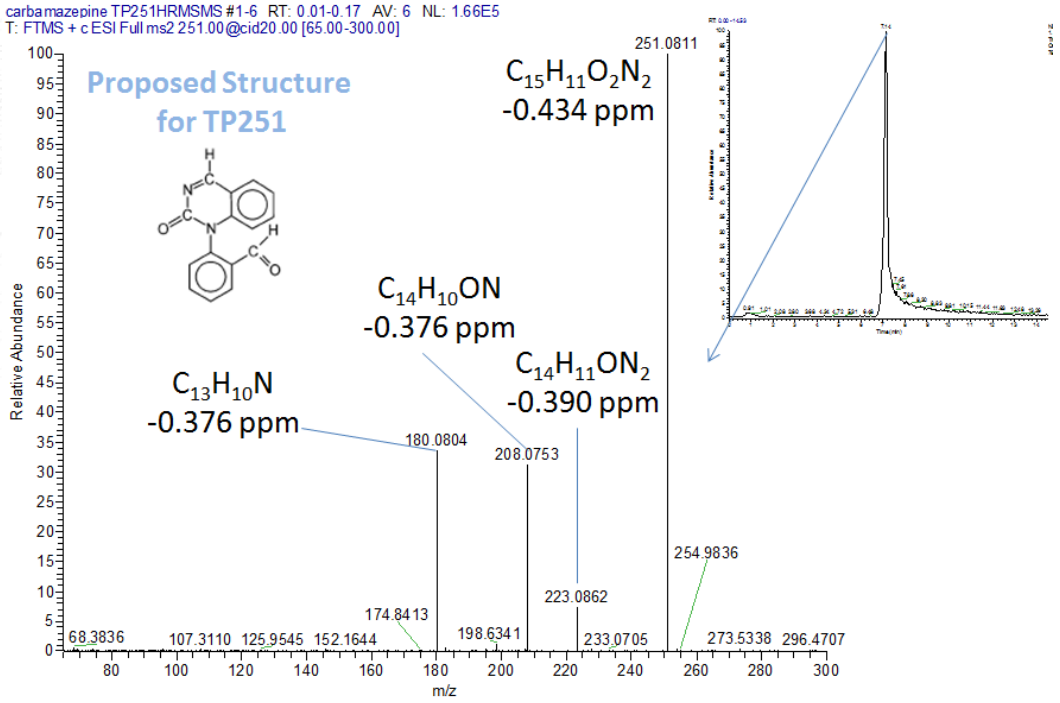


Figure 30. High resolution MS^2 of TP253 (m/z=253) and proposed identity of fragments.

The fragmentation pattern was:

- 251.0811→223.0862 (loss of CO)
- 223.0862 →208.0753 (loss of NH)
- 208.0753→180.0804 (loss of CO)

The fragmentation pattern is congruent with proposed structure. Elemental formula of TP267 ($C_{15}H_{11}O_2N_2$) and fragments ($C_{14}H_{11}ON_2$,$C_{14}H_{10}ON$ and $C_{13}H_{10}N$) are <1ppm from theoretical values confirming the of TP251. The presence of TP251 has been reported previously (McDowell *et al.*, 2005).

4.4.3.3 Structural identification of TP283

A sample of 10µM of carbamazepine treated with 20 µM of O_3 was run in $HRMS^2$ (i.e. isolation of 283.26→fragmentation→HRMS scan). This returned only 1 chromatographic peaks corresponding to 1 TP. Figure 31 shows the $HRMS^2$ spectra of m/z=283 and proposed identity of fragments.

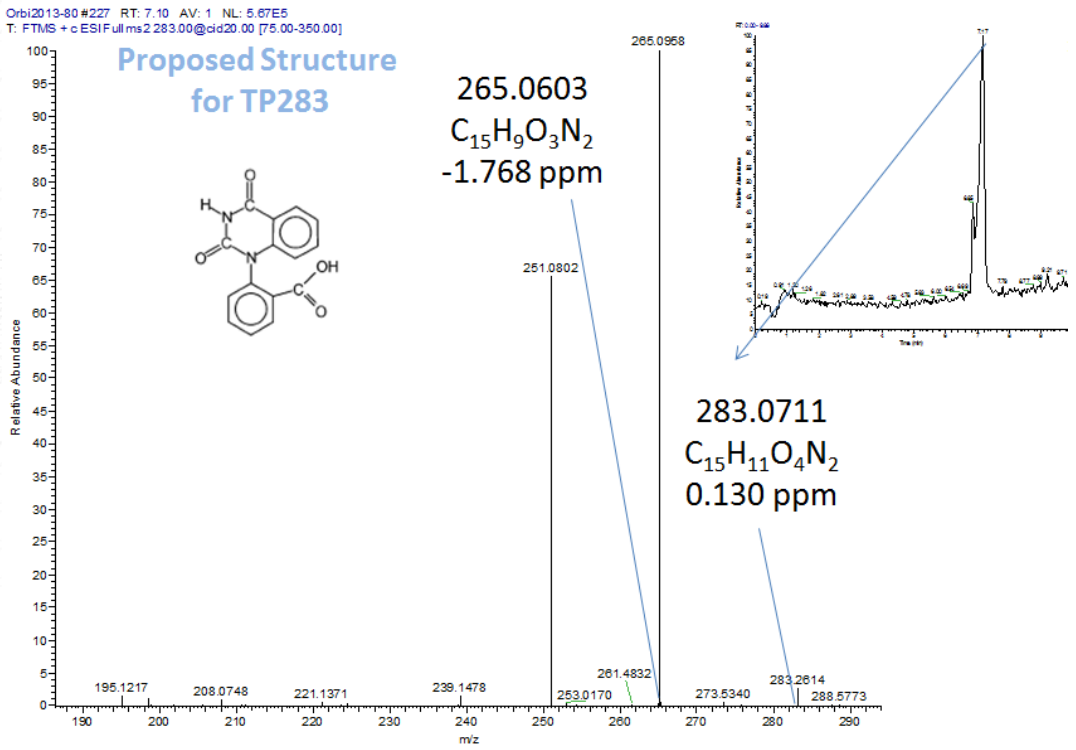


Figure 31. High resolution MS² of TP283 (m/z=283) and proposed identity of fragments.

The fragmentation pattern shows:

283.2627→265.0967 (loss of H_2O)

4.4.3.4 Summary of identification of TPs for carbamazepine

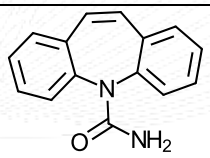
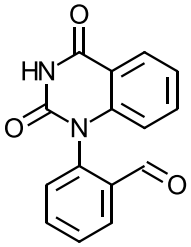
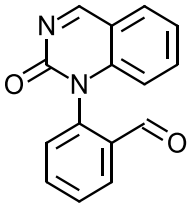
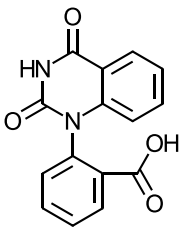
Ozonation of carbamazepine led to the formation of multiple TPs. Four main peaks emerged from MS scan chromatogram. These were m/z=237 (carbamazepine, intact parent compound) and m/z=251 (TP251), m/z=267 (TP267), m/z=283 (TP283).

Through low and high-resolution MS/MS and MS^n spectra, structures of all TPs were elucidated. The chromatographic retention times of TPs were found to be consistent with proposed structures (increased polarity of TP, lower retention time compared to P). The results also agree with previously published literature (McDowell *et al.*, 2005).

Table 4 summarises the TPs found from treatment of carbamazepine with ozonation/OH radical.

Table 4. Mass to charge ratios (m/z) observed from analysis of 10 µM of carbamazepine treated with 20 µM of O_3. Retention time (RT, min), proposed chemical structure, elemental formula, identification of fragments, experimental and theoretical m/z values as well as relative error (part per million, ppm) are also reported.

Species	RT (min)	Proposed Structure	Elemental formula	Loss of	Experi-mental value (m/z)	Theore-tical value (m/z)	Relative error (ppm)
Carbamazepine	11.52		$C_{15}H_{13}ON_2$	…	237.1018	237.1022	-1.727
Frag#1	…		$C_{15}H_{10}ON$	NH_3	220.0753	220.0757	-1.956

Species	RT (min)	Proposed Structure	Elemental formula	Loss of	Experi-mental value (m/z)	Theore-tical value (m/z)	Relative error (ppm)
Frag#2	…		$C_{14}H_{12}N$	NH-C=O	194.0960	194.0964	-1.749
Frag#3	…		$C_{14}H_{10}N$	NH_3=CO	192.0804	192.0808	-1.749
TRANSFORMATION PRODUCTS							
TP267	10.55		$C_{15}H_{11}O_3N_2$	…	267.0759	267.0764	-1.986
Frag#1	…		$C_{15}H_9O_2N_2$	H_2O	249.0653	249.0659	-2.184
Frag#2	…		$C_{14}H_{10}O_2N$	NH-C=O	224.0701	224.0706	-1.897
Frag#3	…		$C_{13}H_{10}ON$	NH-$(C=O)_2$	196.0753	196.0757	-2.196
TP251	10.66		$C_{15}H_{11}O_2N_2$	…	251.0811	251.0815	-0.434
Frag#1	…		$C_{14}H_{11}ON_2$	C=O	223.0862	223.0866	-0.390
Frag#2	…		$C_{14}H_{10}ON$	NH-C=O	208.0753	208.0757	-0.376
Frag#3	…		$C_{13}H_{10}N$	NH-$(C=O)_2$	180.0804	180.0808	-0.376
TP283	10.66		$C_{15}H_{11}O_4N_2$	…	283.0711	283.0712	-0.130
Frag#1	…		$C_{15}H_9O_3N_2$	H_2O	265.0603	265.0608	-1.768
Frag#2	…		…	…	…	…	…
Frag#3	…		…	…	…	…	…

4.4.4 Discussion

Carbamazepine showed a high reactivity towards ozone due to the high electron density of its olefinic C-C double bond. As demonstrated, in our case, (i.e. experiments performed with post RO water) the presence of OH radical reduces the yield of carbamazepine degradation, probably by reacting with the ozone itself, thus consuming the ozone. Ozonation of carbamazepine leads to the formation of three main TPs. Four main peaks emerged from MS scan chromatograms, these were m/z=237 (carbamazepine, intact parent compound), m/z=267 (TP267), m/z=251 (TP251) and m/z=283 (TP283). From $HRMS^n$ spectra, TP267 was identified as 1-(2-benzaldehyde)-(1H,3H)-quinazoline-2,4-dione, TP251 was identified as 1-(2-benzaldehyde)-4-hydro—(1H,3H)-quinazoline-2-one and TP283 was identified as 1-(2-benzoic acid)-(1H,3H)-quinazoline-2,4-dione. Chromatographic retention times of all TPs were found to be consistent with proposed structures (increased polarity of TPs, lower retention time compared to P). The identified TPs were consistent with previously published literature. The nature and the mechanism of formation of these TPs have also been reported previously (McDowell *et al.*, 2005).

Mixture effects increased dramatically in both the non-specific toxicity (Microtox) and oxidative stress response (AREc32), indicating that the TPs identified were more toxic than the parent compound and also exhibit a high reactive toxicity.

4.5 Atrazine

4.5.1 Literature review on ozonation of atrazine

Atrazine, a triazine herbicide, is one of the most widely used herbicides in Australian agriculture as well as in Europe and North America and as such it has been frequently detected in drinking water (at concentrations up to several μg/L (Nélieu *et al.*, 2000)). In Australia the drinking water guideline is relatively high (20 μg/L), compared to European drinking water directive 80/778, which sets a maximum contaminant level of 0.1 μg/L for a single pesticide and 0.5 μg/L for the sum of all pesticides. Previously, atrazine was detected in some Western Australian treatment facilities at a median concentration of 0.100 μg/L in secondary effluent (30% detection frequency, n=32 samples) and below detection in post–RO treated effluent (0% detection frequency, n=32 samples) (Van Buynder *et al.*, 2009). In France, AOPs are not used in drinking water treatment facilities because an increase of toxicity of waters has been observed when atrazine is present in solution. The ozonation of triazine herbicides led to wide range of TPs (Adams and Randtke, 1992; Nélieu *et al.*, 2000). Parent compounds as well as major TPs are presented in Figure 32.

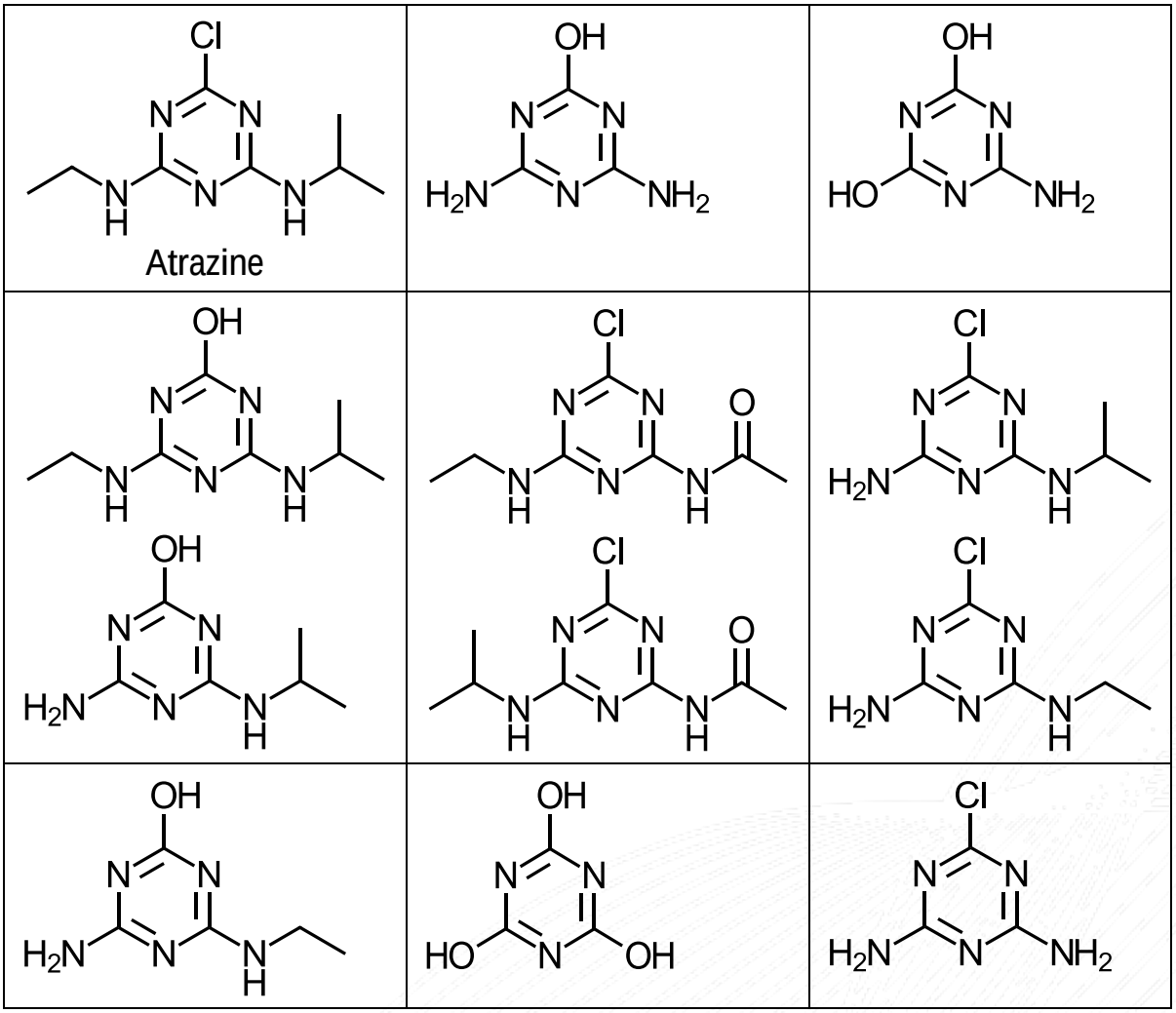


Figure 32 Previously identified TPs of atrazine (Adams and Randtke, 1992; Nélieu et al., 2000).

4.5.2 Degradation of atrazine by ozonation and mixture effects

Atrazine can be oxidised through ozonation treatment (ozone + OH radical) (Figure 33). The concentration of parent compound decreases linearly with the ozone dose applied. For 20 μM of ozone, 75% of the atrazine was oxidised. For the specific toxicity of photosynthesis inhibition, the effect decreased along with increasing ozone concentrations and the decrease in effect was proportional to the amount of fraction of parent retained (Figure 33A). In contrast, for non-specific toxicity (Microtox, Figure 33B) and oxidative stress response (AREc32, Figure 33C), the effects remained constant with increasing ozone concentrations despite having the parent compound oxidised in the mixture. This shows that the atrazine TPs formed are as equally potent as the parent compound in terms of non-specific toxicity and oxidative stress response.

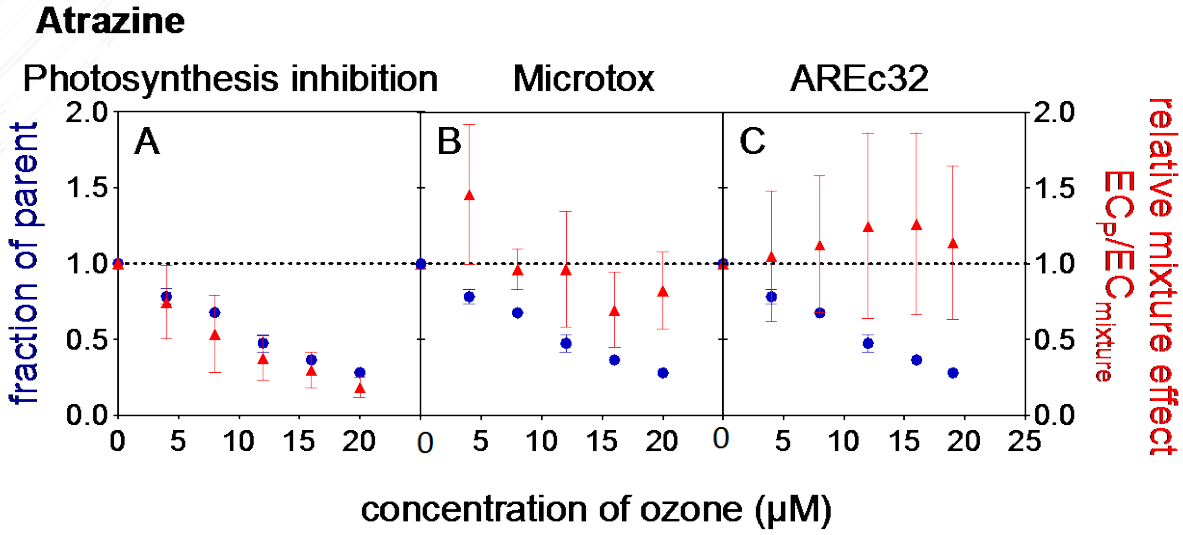


Figure 33. Comparison of the degradation of the parent compound with the change in the effects in the reaction mixture for atrazine.

4.5.3 Comparison of different oxidation agents and mixture effects of atrazine and TPs

In order to understand whether or not degradation was induced by ozone itself or by an OH radical oxidation process, experiments with ozone alone (i.e. addition of t-BuOH) were carried out. Results showed that atrazine is not oxidised by ozone even for the highest concentration of 20 μM (Figure 34). Only OH radicals are oxidising atrazine while O_3 alone is not active—Figure 34 compares results corresponding to the highest ozone dose (with and without t-BuOH) to samples exposed to a UV irradiation of 4000 J m^{-2}as well as to a similar UV dose with 1 mM H_2O_2 for the UV/H_2O_2 process. While UV is clearly degrading atrazine, a much higher reactivity was observed for the experiments with UV/H_2O_2, highlighting the fact that most of the reactivity was coming from the OH radical pathway.

As discussed in Section 4.3, only OH radicals were oxidising atrazine, while O_3 alone was not active. This was confirmed by bioassays, which showed the same effects as the parent for O_3 alone and UV alone. Ozonation without quenching lead to a substantial degradation (70%), while mixture effects did not change. Thus, the TPs formed from hydroxyl radicals are likely to be as potent as the parent compound. If UV/H_2O_2 was applied, there were even more potent TPs formed despite the overall percentage of oxidised atrazine being the same. By comparing with UV one can see that UV is not a strong oxidant, however, the formed TPs lead to rather active mixtures, in particular for the oxidative stress response. Recently Choi et al. (2013) compared atrazine degradation between UV and UV/H_2O_2 and found that *Daphnia magna* toxicity occurred in UV treatment but not in UV/H_2O_2 treatment.

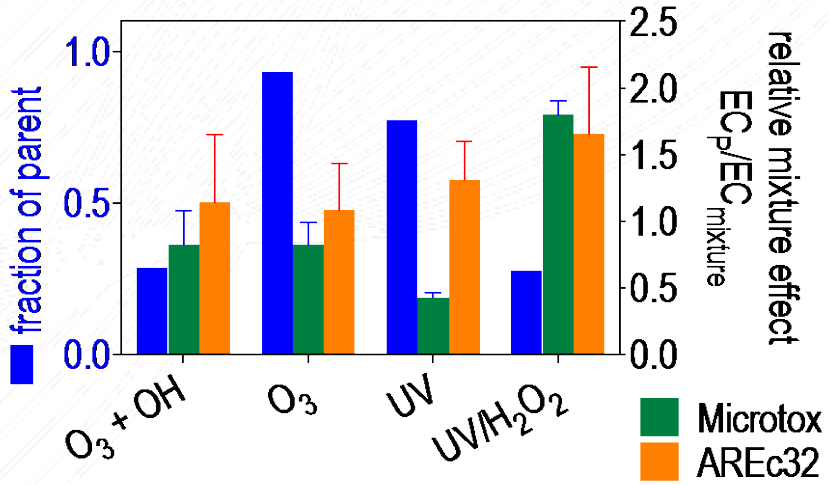


Figure 34 Comparison of different degradation reactions and their impact on the mixture effect.

4.5.4 Identification of transformation products of atrazine

A sample of 10 µM of atrazine treated with 20 µM of O_3 was selected to identify TPs. Ozonation of atrazine resulted in the formation of multiple TPs. These were TP174 (1 compound), TP146 (1 compound), TP188 (2 compounds), TPs230 (2 compounds), TP212 (1 compound), TP202 (1 compound), TP170 (1 compound) and TP232 (4 compounds).

Using both low and high resolution mass spectrometry (MS^2 and MS^3) the structures of most TPs were confirmed, with exception of TP232c and TP232d, for which the software could not return an elemental formula compatible with the parent compound atrazine within a 5ppm mass accuracy window.

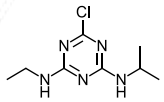
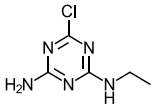
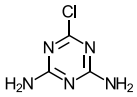
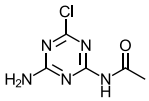
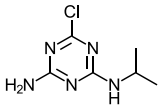
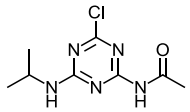
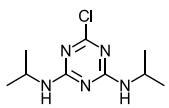
Elemental formulas obtained through $HRMS^n$ were all within 3 ppm error and Δm shift (observed shift of $[M+H]^+$ and fragments from m/z theoretical values) were consistent over base peaks and fragments. The chromatographic retention times of the TPs were consistent with the polarity of proposed structures.

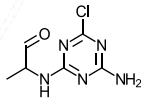
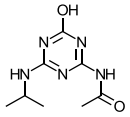
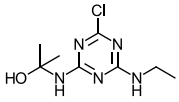
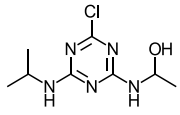
Results of TPs are also consistent with previously published literature (e.g. (Adams and Randtke, 1992; Nélieu *et al.*, 2000) for atrazine, with the exception of TP232c and TP232d. For these compounds, elemental formulas and fragmentation patterns could not be reconciled with chemical structures previously proposed (Nélieu *et al.*, 2000). Table 5 summarises the TPs found from treatment of atrazine with O_3 /OH radicals.

4.5.5 Discussion

Atrazine was not degraded by ozone but only eliminated by OH radicals as confirmed by the UV/H_2O_2 experiment. Ozonation of atrazine led to the formation of multiple TPs. These were TP174 (1 compound), TP146 (1 compound), TP188 (2 compounds), TPs230 (2 compounds), TP212 (1 compound), TP202 (1 compound), TP170 (1 compound), TP232 (4 compounds). Chromatographic retention times of all TPs were found to be consistent with proposed structures (increased polarity of TPs and lower retention time compared to P). The TPs identified through HR MS^2 and MS^3 were consistent with previously published literature. The nature and mechanism of formation of these TPs have been also reported previously (Nélieu *et al.*, 2000). What is new and remarkable is the observation that the specific effect of the parent compound atrazine, the inhibition of photosynthesis, disappeared proportionally with the decrease of atrazine concentration but cytotoxicity and oxidative stress response did not change, indicating identified TPs (Table 5) have equal potency as their parent atrazine in these endpoints.

Table 5 Mass to charge ratios (m/z) observed from analysis of 10µM of atrazine treated with 20 µM of O_3. Retention time (RT, min), proposed chemical structure, elemental formula, identification of fragments, experimental and theoretical m/z values as well as relative error (part per million, ppm) are also reported.

Species	RT (min)	Proposed Structure	Elemental formula	Loss of	Experimental value (m/z)	Theoretical value (m/z)	Relative error (ppm)
Atrazine	11.92		$C_8H_{15}N_5Cl$	…	216.1011	216.1010	-0.325
Frag#1	…		$C_5H_9N_5Cl$	$(CH_3)_2$-CH	174.0540	174.0541	-0.342
Frag#2	…		$C_3H_5N_5Cl$	CH_3CH_2	146.0227	146.0228	-0.750
TP174	2.86		$C_5H_9N_5Cl$	…	174.0540	174.0541	-0.630
Frag#1	…		$C_3H_5N_5Cl$	CH_3CH_2	146.0227	146.0228	-0.887
TP146	1.36		$C_3H_5N_5Cl$	…	146.0226	146.0228	-1.297
Frag#1	…		$C_3H_4N_5$	HCl	110.0459	110.0461	-1.742
TP188a	1.10		$C_5H_7ON_5Cl$	…	188.0331	188.0334	-1.298
Frag#1	…		$C_3H_5N_5Cl$	$CH_3C=O$	146.0226	146.0228	-1.366
TP188b	4.83		$C_6H_{11}N_5Cl$	…	188.0697	188.0697	-0.264
Frag#1	…		$C_3H_5N_5Cl$	$(CH_3)_2$-CH	146.0227	146.0228	-0.750
TP230a	6.80		$C_8H_{13}ON_5Cl$	…	230.0801	230.0803	-0.758
Frag#1	…		$C_6H_{11}N_5Cl$	CH_3-C=O	188.0696	188.0697	-0.758
Frag#2	…		$C_3H_5N_5Cl$	$(CH_3)_2$-CH	146.0226	146.0228	-1.777
TP230b	9.34		$C_9H_{17}N_5Cl$	…	230.1165	230.1167	-0.781
Frag#1	…		$C_6H_{11}N_5Cl$	$(CH_3)_2$-CH	188.0696	188.0697	-0.796
Frag#2	…		$C_3H_5N_5Cl$	$(CH_3)_2$-CH	146.0226	146.0228	-1.160
TP170	1.16		$C_6H_{12}ON_5$	…	170.1034	170.1036	-1.156

Species	RT (min)	Proposed Structure	Elemental formula	Loss of	Experimental value (m/z)	Theoretical value (m/z)	Relative error (ppm)
Frag#1	…		$C_3H_6ON_5$	$(CH_3)_2$-CH	128.0565	128.0567	-1.221
TP202	1.71		$C_6H_9ON_5Cl$	…	202.0488	202.0490	-1.208
Frag#1	…		$C_6H_7N_5Cl$	H_2O	184.0382	184.0384	-1.138
Frag#2	…		$C_3H_5N_5Cl$	CH_3CH-CHO	146.0226	146.0228	-1.229
TP212	1.51		$C_8H_{14}O_2N_5$	…	212.1140	212.1142	-0.901
Frag#1	…		$C_6H_{12}ON_5$	CH_3C=O	170.1035	170.1036	-1.038
Frag#2	…		C3H6ON5	$(CH_3)_2$ CH	128.0565	128.0567	-1.377
TP232a	5.93		$C_8H_{15}ON_5Cl$	…	232.0958		-0.579
Frag#1	…		$C_8H_{13}N_5Cl$	H_2O	214.0853		-0.699
Frag#2	…		$C_7H_{11}N_5Cl$	CH_2	200.0696		-0.598
Frag#3	…		$C_5H_9N_5Cl$	C_2H_2	174.0540		-0.744
TP232b	6.27		$C_8H_{15}ON_5Cl$	…	232.0958		-0.579
Frag#1	…		$C_8H_{13}N_5Cl$	H_2O	214.0852		-0.852
Frag#2	…		$C_5H_7N_5Cl$	$(CH_3)_2$ CH	172.0383		-1.043
TP232c	6.80	NOT AVAILABLE	…	…	232.1119	…	…
Frag#1	…		…	…	190.0666	…	…
TP232d	9.34	NOT AVAILABLE	…	…	232.1120	…	…
Frag#1	…		…	…	190.0666	…	…
Frag#2	…		…	…	148.0197	…	…

4.6 Bisphenol A

4.6.1 Literature review on ozonation of bisphenol A

Bisphenol A (BPA) is frequently used in plastics as an additive and antioxidant. Previously, BPA was identified in Western Australian treatment facilities at a median concentration of 0.012 µg/L in secondary effluent (17% detection frequency, n=20 samples) and at a concentration of 0.010 µg/L post-RO treatment (17% detection frequency, n=19 samples) (Van Buynder *et al.*, 2009). Bisphenol A is

a non steroidal endocrine-disrupting compound and was associated with disorders in women by epidemiological studies (Vandenberg *et al.*, 2007). Additionally, BPA has a mild estrogenic effect in the aquatic environment (Silva *et al.*, 2011; Zhang *et al.*, 2011). A bioanalytical study regarding this compound showed that the estrogenic activity decreased proportional with the concentration of the parent compound during ozonation (Tobias, 2009), indicating that the TPs have no estrogenic activity. The oxidation of bisphenol A has already been studied and the main oxidation products identified (Deborde *et al.*, 2008). In our study, bisphenol A has been detected in post-RO water, therefore it was selected for further investigation.

4.6.2 Ozonation of bisphenol A and mixture effects

The degradation of bisphenol A was linearly correlated with the ozone concentration (Figure 35). The cytotoxicity decreased slightly but not significantly with increasing O_3 concentration (One-Way ANOVA, Tukey's multiple comparisons test, alpha 0.05, no difference compared to 0 µM ozone). Bisphenol A is a weak estrogen agonist but a larger number of studies have shown that the estrogenic effect was rapidly decreased by ozonation (as reviewed by Umar et al. (2013)) therefore no estrogenicity assay was performed. There was a slight but not statistically significant increase in oxidative stress response (One Way ANOVA, Tukey's multiple comparisons test, alpha 0.05 (no difference compared to 0 µM ozone)) indicating the formation of reactive TPs although the cytotoxicity remained constant. These results indicate that the TPs have the same cytotoxic potency as the P.

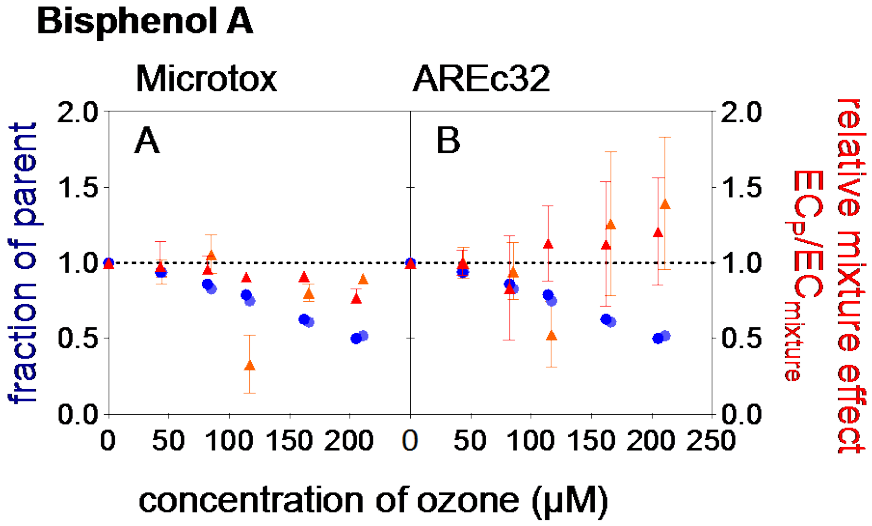


Figure 35. Comparison of the degradation of the parent compound with the change in the effects in the reaction mixture for bisphenol A, (A) Microtox assay, (B) AREc32 (different shades in colour of the same symbol indicate results from independently repeated experiments).

4.6.3 Discussion

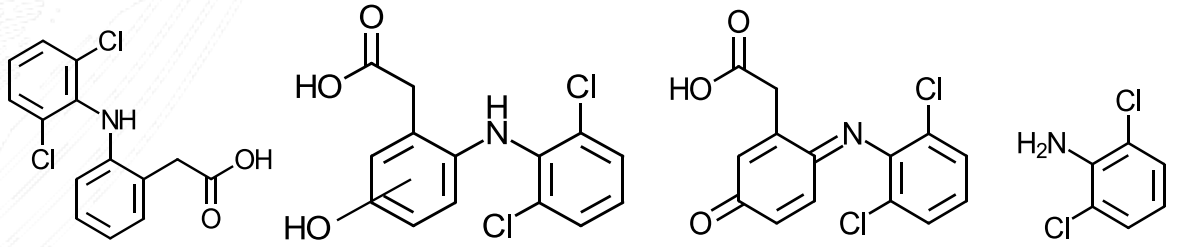
Bioassay results showed that mixture effects did not increase with the degradation of bisphenol A. The effects of the reaction mixture stayed constant despite substantial loss of parent compound. A constant effect means that the TP have similar effect potency with respect to cytotoxicity and induction of the oxidative stress response. This is a novel result and contrasts previous findings for estrogenicity (Tobias, 2009), which was lost with the ozonation of the parent compound.

4.7 Diclofenac

4.7.1 Literature review

Diclofenac is one of the most commonly used anti-inflammatory pharmaceuticals. It is poorly eliminated by biological wastewater treatment, therefore could be found at relatively high concentrations effluents (Ternes, 1998). Diclofenac has been found at Western Australian treatment facilities at a median concentration of 0.362 µg/L in secondary effluent (100% detection frequency, n=26 samples) and below detection post RO treatment (0% detection frequency, n=26 samples) (Van Buynder *et al.*, 2009).

The main TPs are 5-hydroxy-diclofenac, diclofenac-2,5-iminoquinone and 2,6-dichloroaniline (Vogna *et al.*, 2004) (Figure 36). It was also mentioned in the literature that TPs of diclofenac were detected during toxicity studies (Miyamoto *et al.*, 1997; Shen *et al.*, 1999).



diclofenac 5-hydroxy- diclofenac diclofenac-2,5-iminoquinone 2,6-dichloroaniline

Figure 36 Structure of diclofenac and TPs of diclofenac proposed in literature (Vogna et al., 2004).

4.7.2 Degradation of diclofenac by ozonation

Figure 37 shows diclofenac being oxidised through ozonation treatment (ozone + OH radical). The concentration of parent compound decreases linearly with ozone dose applied. For 200 µM of ozone 60% of the diclofenac was oxidised.

The cytotoxicity of the reaction mixture of diclofenac oxidation increased by approximately 50% (Figure 37). Similarly, Coelho et al. (2009; 2010) found that the non-specific toxicity (Microtox, 15 minute test) remained constant after ozonation treatment.

The oxidative stress response increased even more, the effect quadrupled (Figure 37). Both results indicate that the TPs were more toxic than the parent compound and mixture effects were substantial.

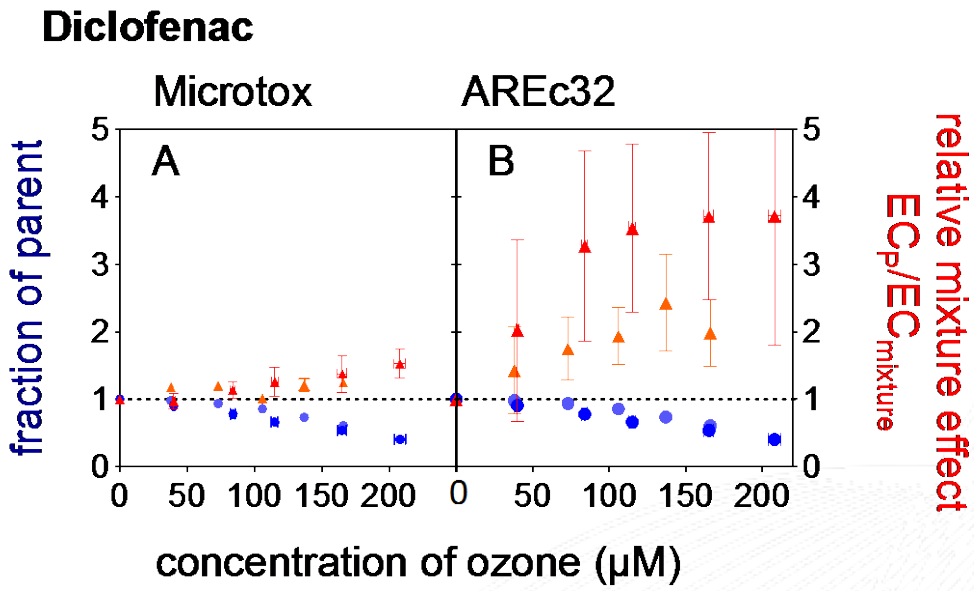


Figure 37. Comparison of the degradation of diclofenac with the change in the effects in the reaction mixture for (A) Microtox assay, (B) AREc32 (different shades in colour of the same symbol indicate results from independently repeated experiments).

4.7.3 Results of the chemical identification of the TPs for diclofenac

A sample of 100 µM of diclofenac treated with 160 µM of O_3 was selected to identify TPs. Ozonation of diclofenac lead to the formation of multiple TPs, these were TP310 (1 compound), TP312 (4 compounds), TP282 (1 compound), TP294 (3 compounds), TP162 (1 compound, very low concentration).

Through LR MS/MS and HR MS^n spectra, structures of most TPs were confirmed—with some exceptions [an interference at m/z 293.9898 (TP294a) and its water adduct at m/z 312.0003 (TP312a) could not be identified]. This unknown compound is unlikely to be a TP of diclofenac, however, the Xcalibur QualBrowser software used for spectra evaluation could not return an elemental formula compatible with the parent compound diclofenac within a 5ppm mass accuracy window.

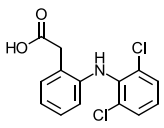
The chromatographic retention times of all were found to be consistent with proposed structures (increased polarity of TP, lower retention time compared to P). Table 6 summarises the TPs found from treatment of diclofenac with ozonation/OH radical.

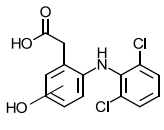
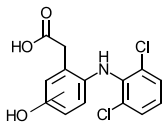
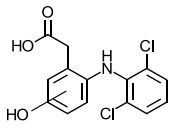
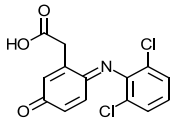
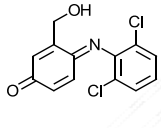
4.7.4 Discussion

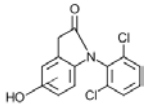
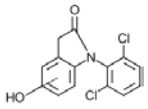
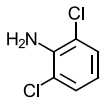
Diclofenac is mainly oxidised by ozone and reacts poorly with OH radicals and UV. Ozonation of diclofenac leads mainly to the formation of two TPs. Three main peaks emerged from MS scan chromatograms. These were m/z=296 (diclofenac, intact parent compound), m/z=310 (TP310) and m/z=312 (TP312). From HR MS^n spectra, TP312 was identified as 5-OH diclofenac, while TP310 was identified as diclofenac-2,5-iminoquinone. Chromatographic retention times of TP310 and TP312 were found to be consistent with proposed structures (increased polarity of TP, lower retention time compared to P). The identified TPs were consistent with previously published literature (Miyamoto *et al.*, 1997; Shen *et al.*, 1999; Vogna *et al.*, 2004; Sein *et al.*, 2008; Coelho *et al.*, 2010).

The bioassay results of for ozonation of diclofenac demonstrated that TPs were equally or more toxic than the parent compound. Given that both identified TPs were more hydrophilic than the parent, they must exhibit a high reactive toxicity. The iminoquinone group is clearly a structural alert for reactive toxicity. Future work should include isolating or synthesising TPs, to allow for a quantitative assessment of formation pathways for relevant TPs, something which cannot be achieved in the present study without quantification standards.

Table 6. Mass to charge ratios (m/z) observed from analysis of 100 µM of diclofenac treated with 160 µM of O_3. Retention Time (min), proposed chemical structure, elemental formula, identification of fragments, experimental and theoretical m/z values as well as relative error (part per million, ppm) are also reported.

Species	RT (min)	Proposed Structure	Elemental formula	Loss of	Experi-mental value (m/z)	Theore-tical value (m/z)	Relative error (ppm)
Diclofenac	10.71		$C_{14}H_{12}O_2NCl_2$	…	296.0247	296.0240	2.363
Frag#1	…		$C_{14}H_{10}ONCl_2$	H_2O	278.0141	278.0134	2.425
Frag#2	…		$C_{13}H_{10}NCl_2$	C=O	250.0191	250.0185	2.315
Frag#3	…		$C_{13}H_{10}NCl$	Cl	215.0501	215.0496	2.341
TRANSFORMATION PRODUCTS							

Species	RT (min)	Proposed Structure	Elemental formula	Loss of	Experi-mental value (m/z)	Theore-tical value (m/z)	Relative error (ppm)
TP312a	8.82	NOT AVAILABLE, water adduct of TP294a	…	…	312.0003	…	…
Frag#1	…		…	…	293.9898	…	…
Frag#2	…		…	…	268.0105	…	…
Frag#3	…		…	…	…	…	…
TP312b	8.84		$C_{14}H_{12}O_3NCl_2$	…	312.0191	312.0189	0.336
Frag#1	…		$C_{14}H_{10}O_2NCl_2$	H_2O	294.0085	294.0083	0.168
Frag#2	…		$C_{13}H_{10}ONCl_2$	C=O	266.0135	266.0134	0.241
Frag#3	…		$C_{13}H_{10}ONCl$	Cl	231.0447	231.0445	-0.014
TP312c	9.29		$C_{14}H_{12}O_3NCl_2$	…	312.0189	312.0189	0.144
Frag#1	…		$C_{14}H_{10}O_2NCl_2$	H_2O	294.0085	294.0083	0.577
Frag#2	…		$C_{13}H_{10}ONCl_2$	C=O	266.0135	266.0134	0.467
Frag#3	…		$C_{13}H_{10}ONCl$	Cl	231.0447	231.0445	0.506
TP312d	9.44		$C_{14}H_{12}O_3NCl_2$	…	312.0189	312.0189	0.048
Frag#1	…		$C_{14}H_{10}O_2NCl_2$	H_2O	294.0084	294.0083	0.372
Frag#2	…		$C_{13}H_{10}ONCl_2$	C=O	266.0135	266.0134	0.241
Frag#3	…		$C_{13}H_{10}ONCl$	Cl	231.0446	231.0445	0.246
TP310	8.87		$C_{14}H_{10}O_3NCl_2$	…	310.0032	310.0032	-0.081
Frag#1	…		$C_{14}H_8O_2NCl_2$	H_2O	219.9926	291.9927	-0.173
Frag#2	…		$C_{13}H_{10}ONCl_2$	C=O + 2H	266.0133	266.0134	-0.210
TP282	3.05		$C_{13}H_{10}O_2NCl_2$	…	282.0086	282.0083	1.701
Frag#1	…		$C_{13}H_8ONCl_2$	H_2O	263.9980	263.9927	0.660
Frag#2	…		$C_{13}H_9O_2NCl$	HCl	246.0319	246.0316	0.924

Species	RT (min)	Proposed Structure	Elemental formula	Loss of	Experi-mental value (m/z)	Theore-tical value (m/z)	Relative error (ppm)
Frag#2	…		C_7H_6ON	$C_6H_4OCl_2$	120.0444	120.0444	0.413
TP294a	8.87	NOT AVAILABLE	n.a.	n.a.	293.9898	n.a.	n.a.
Frag#1			n.a.	n.a.	265.9949	n.a.	n.a.
Frag#2			n.a.	n.a.	237.9999	n.a.	n.a.
Frag#3			n.a.	n.a.	194.0601	n.a.	n.a.
TP294b	9.25		$C_{14}H_{10}O_2NCl_2$	…	294.0084	294.0083	0.168
Frag#1	…		$C_{13}H_{10}ONCl_2$	C=O	266.0135	266.0134	0.918
Frag#2	…		$C_{13}H_{10}ONCl$	Cl	231.0446	231.0445	0.852
TP294c	9.41		$C_{14}H_{10}O_2NCl_2$	…	294.0084	294.0083	0.168
Frag#1	…		$C_{13}H_{10}ONCl_2$	C=O	266.0135	266.0134	0.918
Frag#2	…		$C_{13}H_{10}ONCl$	Cl	231.0446	231.0445	0.852
TP162	4.65		$C_6H_6NCl_2$	…	191.9872		-0.007

4.8 Haloxyfop

4.8.1 Literature review

Haloxyfop is a used as pre- and post-emergence selective herbicide. It is absorbed into plants and inhibits growth. It is classified as a moderately hazardous chemical in the WHO classification of pesticides (2009). Haloxyfop has not been analysed in previous monitoring programs in Western Australia and therefore little information is available on its occurrence in secondary wastewater and post-RO treatment. However, haloxyfop has been detected for the first time in our study at a concentration of 0.01 µg/L in secondary wastewater during the chemical screening conducted in WP 2. To our knowledge there is no literature on the oxidation and formation of transformation products of haloxyfop.

4.8.2 Degradation of haloxyfop by ozonation and mixture effects

Results for haloxyfop in the assay for inhibition of photosynthesis were not clear, the photosystem inhibition was not stable and the results in one experimental run could not be used as the effect did not reach 50%. Valid results were based on extrapolations and still fluctuated. Photosynthesis inhibition is not a target mode of action of haloxyfop, which acts to inhibit acetyl CoA carboxylase—a plant-specific

mode of action and thus algae were not target organisms. The effect observed must be a secondary effect at higher concentrations.

Cytotoxicity was virtually constant, however oxidative stress response increased substantially (Figure 38).

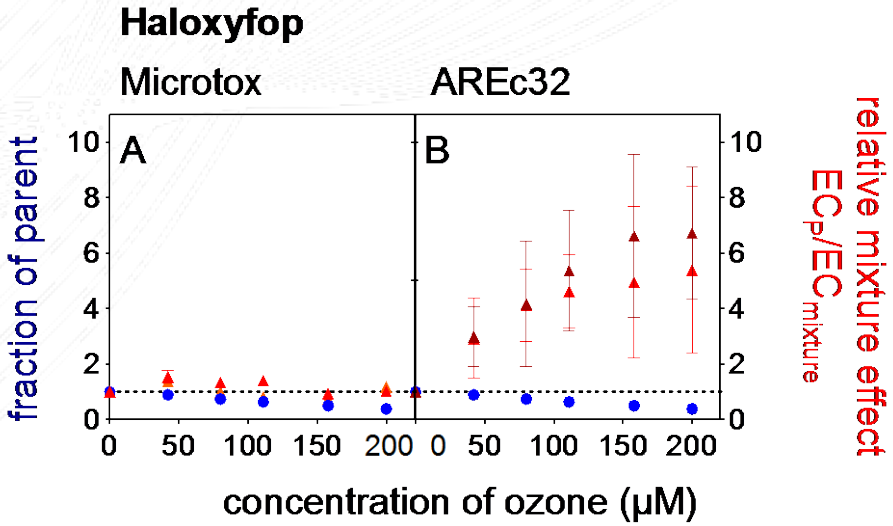


Figure 38. Comparison of the degradation of haloxyfop with the change in the effects in the reaction mixture (different shades in colour of the same symbol indicate results from independently repeated experiments).

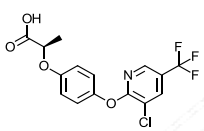
4.8.3 Results of the identification of TPs of haloxyfop

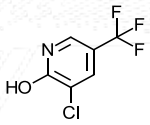
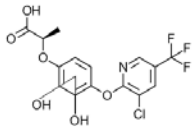
A sample of 100 µM of haloxyfop-P treated with 160 µM of O_3 was selected to identify TPs. Ozonation of haloxyfop led to the formation of two main TPs, these were TP198 (1 compound), TP394 (3 compounds).

Through low and high resolution MS^2 and MS^3, structures of all TPs detected were confirmed. Elemental formulas obtained through $HRMS^n$ were all within 5ppm error and Δm shifts were consistent over base peaks and fragments

The chromatographic retention times of TP198 and TPs394 were found to be consistent with the proposed structures (increased polarity of TP, lower retention time compared to P). Table 7 summarises TPs found after ozonation of haloxyfop with O_3/OH radicals.

Table 7. Mass to charge ratios (m/z) observed from analysis of 100µM of haloxyfop-P treated with 160 µM of O_3. Retention Time (min), proposed chemical structure, elemental formula, identification of fragments, experimental and theoretical m/z values as well as relative error (part per million, ppm) are also reported.

Species	RT (min)	Proposed Structure	Elemental formula	Loss of	Experimen-tal value (m/z)	Theore-tical value (m/z)	Relativ e error (ppm)
Haloxy-fop	10.76		$C_{15}H_{12}O_4NClF_3$	…	362.0393	362.0401	-2.2
Frag#1	…		$C_{14}H_{10}O_2NClF_3$	HCOOH	316.0336	316.0336	-3.4
Frag#2	…		$C_{13}H_{10}ONClF_3$	$C_3O_2H_5$	288.0389	288.0398	-3.0

Species	RT (min)	Proposed Structure	Elemental formula	Loss of	Experimen-tal value (m/z)	Theore-tical value (m/z)	Relativ e error (ppm)
Frag#3	…		$C_6H_4ONClF_3$	$C_9O_3H_9$	197.9921	197.9928	-3.5
TP198	6.72		$C_6H_4ONClF_3$	…	197.9922	197.9928	-3.1
Frag#1	…		$C_6H_2NClF_3$	H_2O	179.9832	179.9822	-5.1
TPs394	9.02-11.16		$C_{15}H_{12}O_6NClF_3$	…	394.0291	394.0300	-3.5
Frag#1	…		$C_{15}H_{10}O_5NClF_3$	H_2O	376.0182	376.0194	-2.4
Frag#2	…		$C_6H_4ONClF_3$	$C_9O_3H_9$	197.9921	197.9928	-3.7

4.8.4 Discussion

Haloxyfop is mainly eliminated by reaction with ozone and slightly through UV. Ozonation of haloxyfop led mainly to the formation of two TPs. Three main peaks emerged from MS scan chromatograms, these were: m/z=362 (haloxyfop-P, intact parent compound), m/z=198 (TP198), and m/z=394 (TPs394). From MS^2 and MS^3 spectra, TP198 was identified as the cleavage product of the bond between the 2 aromatic rings. TP394 was found to be a product of addition from two OH groups to the phenoxy-ring. Chromatographic retention times of TP198 (RT=6.72 min) and TP394 (RT=9.02-10.15 min) were found to be consistent with proposed structures of TPs, which shows increased polarity and consequently lower retention time compared to the parent compound (haloxyfop). To the best of our knowledge, haloxyfop TPs identified in this work have not been reported previously. Two TPs were identified but unfortunately other TPs resulting from the oxidation/breakdown of the molecule were missing and/or could not be identified by LC-MS.

With respect to mixture toxicity of the parent compound with TPs, our results remained inconclusive for photosynthesis inhibition and cytotoxicity. The oxidative stress response increased up to sixfold, suggesting the TPs formed were reactive.

4.9 Hexazinone

4.9.1 Literature review on ozonation of hexazinone

Hexazinone is a broad-spectrum herbicide. It is used for weed control in agriculture, on highways and on industrial plant sites. Hexazinone is highly soluble in water (33 g/L at 25 ˚C) and therefore has great potential for leaching into groundwater. Hexazinone has been found in Western Australia at treatment facilities in secondary effluent (0.11 µg/L) but was below detection limit post–RO treatment (0% detection frequency, n=43 samples in (Van Buynder *et al.*, 2009) and (Tang *et al.*, 2014)). Even though some work has been done on aerobic biodegradation (Kubilius and Bushway, 1998) and photocatalytic oxidation (Mei *et al.*, 2012) of hexazinone, to our knowledge there is no literature on the ozonation of hexazinone and the formation of TPs.

4.9.2 Degradation of hexazinone by ozonation and mixture effects

With 200 µM of ozone, 75% of hexazinone was oxidised (Figure 39). Hexazinone is a herbicide and therefore was further evaluated for its target mode of action, the inhibition of photosynthesis. The TPs of hexazinone clearly have no ability to inhibit photosynthesis (Figure 39). Cytotoxicity stayed virtually constant for both herbicides but oxidative stress response increased substantially (Figure 39).

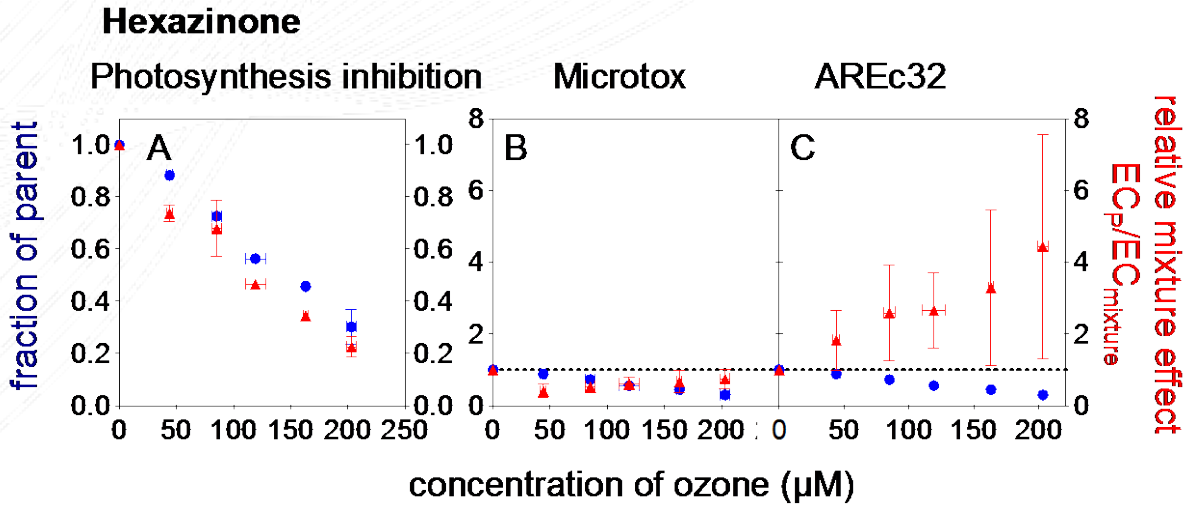


Figure 39. Comparison of the degradation of the parent compound with the change in the effects in the reaction mixture for hexazinone (different shades in colour of the same symbol indicate results from independently repeated experiments).

4.9.3 Results of the chemical identification of TPs of hexazinone

A sample of 10 µM of hexazinone treated with 20 µM of O_3 was selected to identify TPs. Ozonation of hexazinone lead to the formation of multiple TPs, these were TP239 (1 compound), TP253 (3 compounds), TP267 (3 compounds), and TPs269 (4 compounds).

Through low and high resolution MS^2 and MS^3, the structures of most TPs were confirmed with exception of TPs267 and TP253. The elemental formulas obtained through $HRMS^n$ were all within 5ppm error and Δm shift were consistent over base peaks and fragments.

The structure depicted in Figure 40 was proposed previously for TP267 from photocatalytic degradation of hexazinone (Mei *et al.*, 2012).

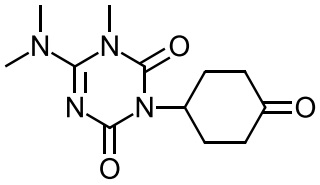


Figure 40. Proposed photocatalytic TP of hexazinone.

While elemental formula and HR fragmentation seem to be congruent with the proposed structure, chromatographic retention times of TPs267 are very low (0.79-3.12 min), indicative of ionic species (i.e. similar to TPs269). Isolation through prep-LC followed by ^{1}HNMR studies would be required to confirm the structure of TP267. A possible structure for TPs253 is depicted in Figure 41.

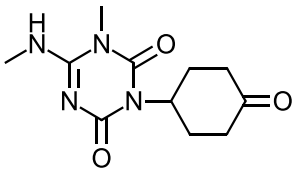


Figure 41. Proposed structure of TPs253, a TP of hexazinone.

While elemental formula and HR fragmentation seem to be congruent with the proposed structure, chromatographic retention times of TPs253 are very low (1.20-1.80 min), again indicating ionic species (i.e. similar to TPs269). Isolation through prep-LC followed by ^{1}HNMR studies would be required to confirm the structure of TP253. Table 8 summarises the TPs found after treatment of hexazinone with O_3/OH radical.

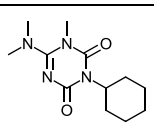
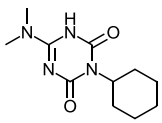
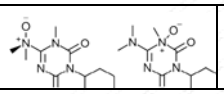
4.9.4 Discussion

Hexazinone was mainly oxidised by OH radicals. OH radicals being a non-specific oxidant, resulted in a variety of TPs being found, including: TP239 (1 compound), TP253 (3 compounds), TP267 (3 compounds), and TPs269 (4 compounds). From HR MS^2 and MS^3 spectra, TP239 was identified as a product of substitution of $-CH_3$ with -H on the tertiary amine. TP269 was found to be a product of addition of oxygen to form N^+-O^- ionic species, characterised by low retention on the LC column. The elemental formula of TPs267 ($C_{12}H_{19}O_3N_4$) suggested the addition of oxygen on the C_6H_{10} ring to form a cyclic ketone (see Figure 40). The fragmentation spectra were also congruent with this hypothesis, given the observed loss of C_6H_8O. However, the structure proposed previously was not confirmed on the base of chromatographic retention times, which instead suggested an ionic species similar to TP269.

Similarly, the elemental formula of TPs253 ($C_{11}H_{17}O_3N_4$) suggests the addition of oxygen on the C_6H_{10} ring to form a cyclic ketone (see Figure 41) and the substitution of $-CH_3$ with -H on the tertiary amine (as observed previously for TP239). The fragmentation spectra were also consistent with this hypothesis, given the observed loss of C_6H_8O. However, the structure proposed previously was not confirmed on the base of the chromatographic retention times, which instead suggested an ionic species similar to TP269. We speculate that TP253 and TP267 structures were similar to TPs formed during photocatalytic oxidation, given they were also primary oxidation products derived from OH radical reactions.

Hexazinone has clearly lost its ability to inhibit photosynthesis after ozonation but the TPs appeared to be highly reactive (despite being stable enough to be enriched by SPE) and caused a five-fold increase in the oxidative stress response.

Table 8. Mass to charge ratios (m/z) observed from analysis of 10µM of hexazinone treated with 20 µM of O_3. Retention Time (min), proposed chemical structure, elemental formula, identification of fragments, experimental and theoretical m/z values as well as relative error (part per million, ppm) are also reported.

Species	RT (min)	Proposed Structure	Elemental formula	Loss of	Experimental value (m/z)	Theoretical value (m/z)	Relative error (ppm)
Hexazinone	10.70		$C_{12}H_{21}O_2N_4$	…	253.1653	253.1659	-2.5
Frag#1	…		$C_6H_{11}O_2N_4$	C_6H_{10}	171.0872	171.0877	-2.8
TP239	6.80; 7.78		$C_{11}H_{19}O_2N_4$	…	239.1496	239.1503	-3.3
Frag#1	…		$C_5H_9O_2N_4$	C_6H_{10}	157.0715	157.0720	-3.1
TPs269	1.16-3.01		$C_{12}H_{21}O_3N_4$	…	269.1599	269.1608	-3.4

Species	RT (min)	Proposed Structure	Elemental formula	Loss of	Experimen-tal value (m/z)	Theore-tical value (m/z)	Relative error (ppm)
Frag#1	…		$C_{12}H_{19}O_2N_4$	H_2O	251.1493	251.1503	-3.5
Frag#2	…		$C_6H_{11}O_2N_4$	C_6H_8	171.0870	171.0877	-3.9
Frag#3	…		$C_5H_{10}ON_3$	$C_7H_{11}O_2N$	128.0813	128.0818	-3.9
TPs253	1.20-1.80	NOT AVAILABLE	$C_{11}H_{17}O_3N_4$	…	253.1287	253.1295	-3.3
Frag#1	…		$C_{11}H_{15}O_2N_4$	H_2O	235.1180	235.1190	-4.2
Frag#2	…		$C_5H_9O_2N_4$	C_6H_8	157.0714	157.0720	-3.6
TPs267	0.79-3.12	NOT AVAILABLE	$C_{12}H_{19}O_3N_4$	…	267.1444	267.1452	-2.8
Frag#1	…		$C_{12}H_{17}O_2N_4$	H_2O	249.1338	249.1346	-3.0
Frag#2	…		$C_6H_{11}O_2N_4$	C_6H_8	171.0871	171.0877	-3.3
Frag#3	…		$C_5H_{10}ON_3$	$C_6H_9O_2N$	128.0814	128.0818	-3.3

4.10 Iopromide

4.10.1 Literature review

Iopromide is an iodinated X-ray contrast media widely used to enable medical imaging. Iopromide has been found in Western Australian treatment facilities at a median concentration of 1.2 µg/L in secondary effluent (84% detection frequency, n=25 samples) and below detection post-RO treatment (0% detection frequency, n=25 samples) (Van Buynder *et al.*, 2009). Even though iodinated X-ray contrast media are partially removed during wastewater treatment and are also non-toxic; they are of interest because their degradation products can lead to the formation of potentially toxic iodinated organic compounds (Duirk *et al.*, 2011). Iopromide is recalcitrant to direct ozone oxidation but is reactive with OH radicals (Huber *et al.*, 2003).

4.10.2 Degradation of iopromide by ozonation and mixture effects

Iopromide was degraded during ozonation, with up to 60% removal for an ozone dose of 200 µM. Iopromide was only toxic at very high concentrations and consequently the variability was very high and no clear conclusions could be drawn (Figure 42).

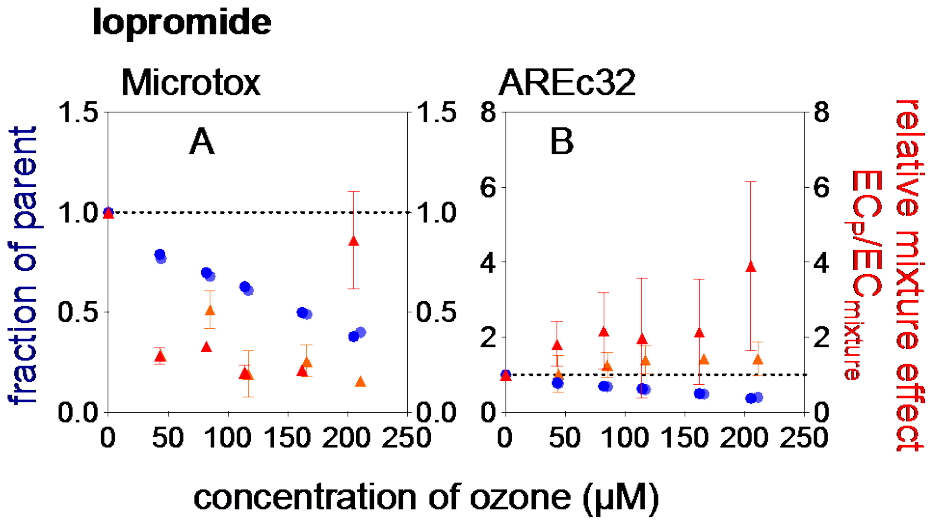


Figure 42. Comparison of the degradation of the parent compound with the change in the effects in the reaction mixture for iopromide (different shades in colour of the same symbol indicate results from independently repeated experiments).

4.10.3 Discussion

Iopromide was degraded during ozonation treatment. According to Huber et al. (2003), ozone is not reactive and OH radical is responsible of the oxidation. Although iopromide by itself is not toxic, a positive response was found for the AREc32 test at high ozone doses. However, the toxicity test did not lead to a definitive conclusion. Iopromide may form toxic iodinated organic compounds through a different pathway such as by direct oxidation of iopromide and formation of iodinated disinfection by-products. Another possible pathway is via an indirect reaction of the oxidised moieties (coming from iopromide) with the water matrix. Unintended consequences could arise if a post disinfection step, such as chlorination, were implemented after ozonation. In this case, the transformation products formed during the ozonation step might react with chlorine and form some toxic iodinated compounds.

4.11 Sulfamethoxazole

4.11.1 Literature review

Sulfamethoxazole is an antibiotic drug commonly found in hospital effluents, wastewater effluent and also in the aquatic environment. Sulfamethoxazole has been found in Western Australia treatment facilities at a median concentration of 0.54 µg/L in secondary effluent (100% detection frequency, n=29 samples) and below detection post-RO treatment (0% detection frequency, n=25 samples) (Van Buynder *et al.*, 2009). Sulfamethoxazole is efficiently degraded during ozone treatment (Dodd *et al.*, 2006) and leads to the formation of TPs. It was observed that these TPs are potentially toxic (Abellán *et al.*, 2008).

4.11.2 Degradation of sulfamethoxazole by ozonation and mixture effects

Sulfamethoxazole did not show a clear effect pattern (Figure 43). In two repeats of the cytotoxicity assay, results showed either a parallel decrease of cytotoxicity with decreasing parent concentrations or unchanged effects. The same results occurred for the oxidative stress response, however here, it looks like the mixture effect was unchanged from the parent compound effect.

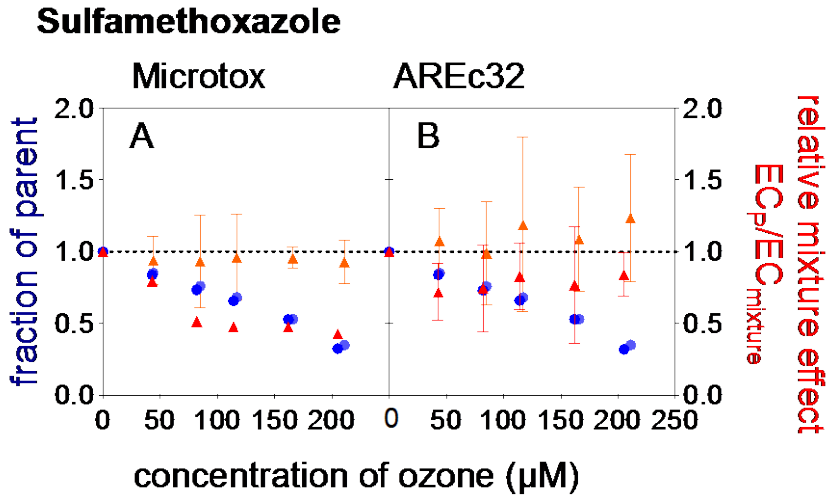


Figure 43. Comparison of the degradation of the parent compound with the change in the effects in the reaction mixture for sulfamethoxazole (different shades in colour of the same symbol indicate results from independently repeated experiments).

4.11.3 Discussion

Results from the bioassays showed that toxicity (i.e. Microtox and AREc32) was decreasing and correlated to a decay of the parent compounds. Therefore, ozonation is a good option for treating sulfamethoxazole since it appears that the TPs formed during ozonation treatment are not significantly toxic. Gomez-Ramos *et al.* (2011) found an increase in toxicity in *Daphna magna* immobilization test and *Psedokirchneriella subcapitata* growth inhibition test at 20 and 50 µM ozone concentrations but the effect decreased with increasing ozone concentrations, possibly relating to TP intermediates. Abellán *et al.* (2008) also found that the toxicity of *Daphna magna* increased after 5 min of ozonation but the effect diminished with increasing time and the toxicity of the reaction mixture remained constant between 10 and 20 min of ozonation. Abellán *et al.* (2008) postulated that toxic TPs formed at low ozone concentrations were TP intermediates.

4.12 Conclusions

All eight micropollutants investigated were well removed by ozonation but the TPs were numerous and in many cases also toxicologically relevant. Table 9 summarises the mixture toxicity observed after ozonation. Some general conclusions can be drawn:

As target effects we assessed photosynthesis inhibition of the two photosynthesis-inhibiting herbicides atrazine and hexazinone. For atrazine and hexazinone the effects decreased substantially, which shows that TPs have lost (or very much reduced) the specific activity.

In contrast, TPs all contributed to mixture toxicity in the cytotoxicity assay. Non-specific toxicity often stayed virtually constant, even if substantial fractions of the parent compound disappeared. The TPs appeared to be typically more hydrophilic than the corresponding parent compound. If the effect remained constant, it suggests that TPs have a similar potency as the parent compound. This appears to be the case because the identified TPs all have similar structures and are only oxidised at one or two functional groups. Remarkable was the substantial increase of the cytotoxicity of the reaction mixture of carbamazepine.

The oxidative stress response was never investigated on ozonated samples but from theoretical consideration one can expect that ozonation produces reactive intermediates. This hypothesis was confirmed for a number of parent compounds, including carbamazepine, diclofenac and hexazinone, where mixture effects were higher than effects of the parent compound. For all other compounds mixture effects were as similar as the effect of the parent compound, so overall TPs should not be neglected when assessing the risk of chemicals that are treated with oxidative tertiary processes.

Table 9 Summary of the bioassay results obtained for reaction mixtures undergoing ozonation

	Bioassay (target mode of action)		
Chemical	Microtox (cytotoxicity)	AREc32 (oxidative stress response)	IPAM (photosynthesis inhibition)
Atrazine	effect (TP) = effect (P)	effect (TP) = effect (P)	effect (TP) << effect (P)
Bisphenol A	effect (TP) = effect (P)	effect (TP) = effect (P)	n/a
Carbamazepine	6 fold increase of effect of TP mixture	3 fold increase of effect of TP mixture	n/a
Diclofenac	50% increase of effect of TP mixture	2-4 fold increase of effect of TP mixture (large variations)	n/a
Haloxyfop	effect (TP) = effect (P)	2-8 fold increase of effect of TP mixture (large variations)	n/a (herbicide but target mode of action is not photosynthesis inhibition)
Hexazinone	effect (TP) = effect (P)	~4 fold increase increase of effect of TP mixture	70% decrease of effect of TP mixture
Iopromide	effect (TP) = effect (P) large variation	effect (TP) = effect (P) large variation	n/a

	Bioassay (target mode of action)		
Chemical	Microtox (cytotoxicity)	AREc32 (oxidative stress response)	IPAM (photosynthesis inhibition)
Sulfamethoxazole	effect (TP) = effect (P)	effect (TP) = effect (P)	n/a

n/a: endpoint not applicable because P does not have MOA of photosynthesis inhibition.

A range of TPs were detected and identified through LR MS/MS and HR MS^2 and MS^3 analyses (Table 10). Elemental formulas obtained through $HRMS^n$ were all below the 5 ppm error and Δm shifts were also found to be consistent over the $[M+H]^+$ peaks and fragments. The chromatographic retention times of all TPs were found to be lower than the chromatographic retention time of the corresponding parent compounds, which is consistent with the increased polarity of TPs resulting from reactions of oxidation of the parent compounds. Not all TPs could be identified. Reasons for not being able to identify TPs included: 1) the m/z ratio of $[M+H^+]$ and fragments did not return an elemental formula congruent with the initial elemental formula of the parent compounds; or 2) the RT of the TPs were found to be not congruent with the structures previously proposed. Overall, the TPs identified in this work through HRMS were found to be consistent with previously published literature, thus substantiating the LC-HRMS procedure adopted here was capable of detecting and identifying TPs in reaction mixtures undergoing ozonation.

Table 10 Summary of the results obtained for the identification of TPs in reaction mixtures undergoing ozonation.

Chemical	Number of TPs detected	TPs identified	TPs not identified
Atrazine	13	TP174 (1 compound) TP146 (1 compound) TPs188 (2 compounds) TPs230 (2 compounds) TP212 (1 compound) TP202 (1 compound) TP170 (1 compound) TPs232 (2 compounds)	TP232c-d (2 compounds)
Bisphenol A	n.a.	n.a.	n.a.
Carbamazepine	3	TP251 (1 compound) TP267 (1 compound) TP283 (1 compound)	none
Diclofenac	10	TP310 (1 compound) TPs312 (4 compounds) TP282 (1 compound) TPs294 (2 compounds) TP162 (1 compound)	TP294a (1 compound)
Haloxyfop	4	TP198 (1 compound)	none

		TPs394 (3 compounds)	
Hexazinone	11	TP239 (1 compound) TPs269 (4 compounds)	TPs253a-c (3 compounds) TPs267a-c (3 compounds)
Iopromide	n.a.	n.a.	n.a.
Sulfamethoxazole	n.a.	n.a.	n.a.

Now that TPs have been identified and their toxicological relevance as mixtures have begun to be assessed, it would be interesting for future studies to synthesize/purchase TPs as standards to: (a) quantify the concentrations of formed TPs and (b) measure TP effects and relative effect potency in relation to the parent compound. While ozone was chosen because this is the oxidant most regularly applied, additional oxidants, including UV/H_2O_2 should be evaluated in the future, in analogous experiments as we have done for ozonation. This would help to evaluate which reaction mechanism(s) is/are underlying the oxidation process and determine if different oxidants lead to different TPs and variable mixture effects.

Despite the limits of the presented results and need for further research, this study has clearly demonstrated that the mixture effects of TPs from ozonation cannot be neglected. Previous work focused often on target effects of parents but this study clearly showed that TPs are relevant.

5 How much do we really know and are we currently monitoring right chemicals/chemical classes?

We have set out to answer four questions and results obtained in this project allowed us to answer all questions with confidence:

1. Do mixtures matter? How do the numerous chemicals present at low concentrations (below levels where they show any individual toxicity) act together in mixtures?

In this study we demonstrated, for the samples tested, that in all practicality mixture effects in cell-based bioassays of chemicals present in wastewater treated effluents and other water samples followed the concept of concentration addition. Thus we could predict the effects of mixtures provided all components were known (which is not necessarily the case as Chapter 3 has shown).

We have further demonstrated that the concept of bioanalytical equivalent concentrations is applicable not only for endpoints where it has been used for many decades, namely receptor-mediated modes of action such as dioxin-like response or more recently estrogenic and other hormone effects, but also for two examples relating to adaptive stress response and cytotoxicity.

The research focused on three biological endpoints—representative for receptor-mediated effects, effects related to induction of transcription factors (i.e., adaptive stress response) and general cytotoxicity. Thus it is conceivable that findings can be generalised, in particularly as the findings of this study, where focus was for the first time on mixture effects of chemicals in WWTP effluents, are consistent with theoretical expectations and a wealth of previous mixture toxicity studies on different types of endpoints and chemicals (Kortenkamp *et al.*, 2009).

2. How much of the iceberg do we see? How much do chemicals that are regulated in the Australian Guidelines for Water Recycling (AGWR) contribute to the overall toxicity of mixtures of organic micropollutants?

In Australia, there are guideline values for a large number of chemicals in recycled water. 349 organic chemicals are included in the Australian Guidelines for Water Recycling (NRMMC & EPHC & NHMRC, 2008). Many of those are present in wastewater treatment plant effluent and thus have the potential to occur in recycled water. After tertiary treatment, neither chemical occurrence nor most mode of action results were above analytical or bioassay limits of detection, respectively.

Depending on the step in the toxicity pathway and the mode of action, the regulated chemicals may or may not cover all effects found in water on AWRPs (Figure 44). For chemicals that act according to receptor-mediated modes of action, we generally know the high-potency, high-affinity compounds and they have been included in the AGWR. The regulated and regularly monitored chemicals account for the majority of biological effects in these modes of action. We have demonstrated good agreement amongst bioanalytical tools when comparing between iceberg mixtures and authentic water samples in this study for the inhibition of photosynthesis in this study and for estrogenicity and calculated mixture effects in previous work (Escher *et al.*, 2011).

However, more general endpoints, such as the oxidative stress response and cytotoxicity (Figure 44) are responsive to many more chemicals with a wide range of intrinsic potency. The detected chemicals from the AGWR were able to rationalise less than 3 % of the bioanalytical equivalents for cytotoxicity and less than 0.7 % of BEQ of the oxidative stress response. By analogy, the unknown chemicals are submerged and thus form the invisible part of the iceberg—and that part may be very large. With the work undertaken, we can begin to say how big this part is for various bioassays but we cannot say, what all the unknowns are. Attempting to measure an almost infinite number of compounds at

increasingly lower and lower concentrations cannot be justified, given the limitation of precious resources. Instead this project provides evidence to support the inclusion of bioassays, as a measure to more efficiently characterise unknown mixtures.

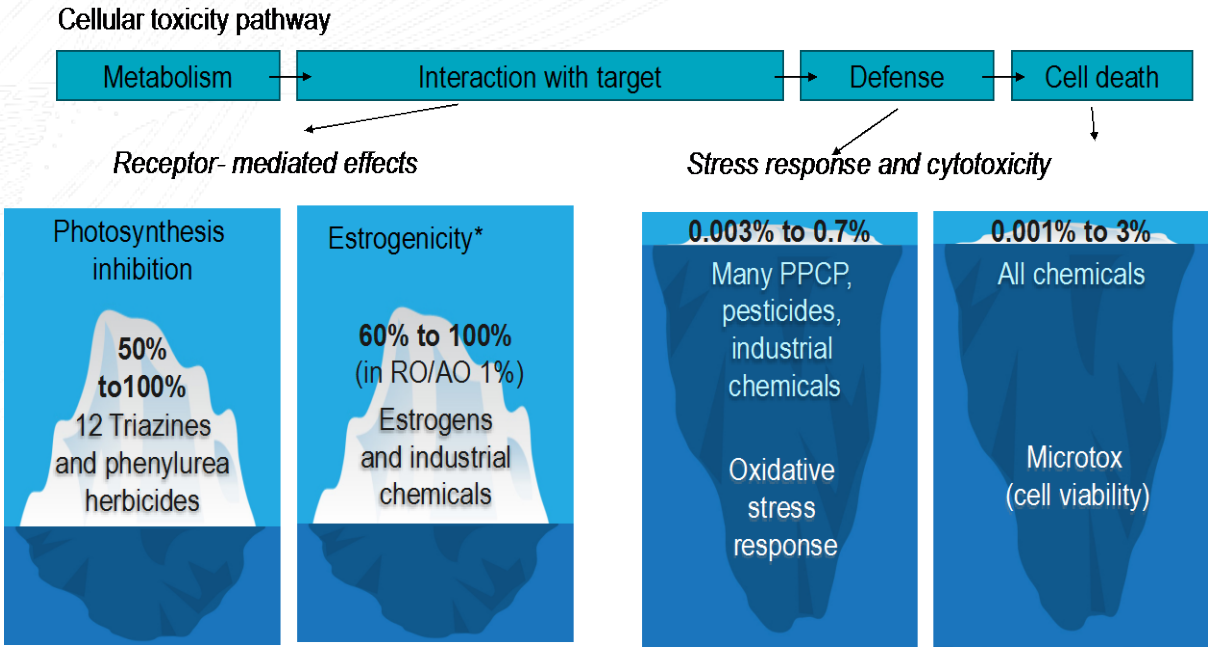


*Figure 44. Summary of results: the fraction of explained chemicals depends strongly on the step in the toxicity pathway or mode of toxic action. Data from Chapter 2 and *from previous work (Escher, B.I., Lawrence, M., Macova, M., Mueller, J.F., Poussade, Y., Robillot, C., Roux, A.,Gernjak, W. 2011. Environ. Sci. Technol., 45: 5387-5394).*

Calls to regulate more chemicals, without plausible evidence of potential adverse effects, does little to move us towards improved risk management of recycled waters. Given that transformation products appear to play a substantial role for the mixture effects, they need to be studied carefully. Therefore our proposal is to complement chemical monitoring by introducing a small set of informative indicator bioassays.

The research was focusing on three biological endpoints that were representative for different steps of the toxicity pathway and also had given positive responses in samples at the AWRPs (but not in the final recycled water). These three assays therefore serve as good indicator assays to assess the quality of recycled water and treatment efficacy of AWRPs.

3. Do transformation products of micropollutants formed during relevant water recycling processes contribute to mixture toxicity?

The degradation experiments of eight micropollutants that occur in WWTP effluent (and have the potential to break through into recycled water) showed that the mixture effects did not necessarily decrease despite degradation of the parent compound. In contrast, in some cases (carbamazepine, diclofenac, haloxyfop, hexazinone), the mixture effects, especially in the oxidative stress response, even increased several fold suggesting that not only the TPs contributed to mixture effects but their intrinsic potency must be higher than that of the associated parent compounds. Thus, loss of parent compound is not the only measure for assessing treatment efficiency but some sum parameter indicative of mixture effect should complement the measurement of loss of parent compound. Cell-based bioassays can provide such a sum parameter.

It is intriguing that the bioassay that had the largest fraction of unknown in the iceberg experiments, the induction of the oxidative stress response, also was most responsive to the formation of transformation products. This finding calls again for supplementing chemical analysis with bioanalytical tools, in particular for more general endpoints such as adaptive stress responses.

Since the identification of TPs, in the way it was undertaken in the present study, is very costly and time consuming, it is possibly a better choice to apply bioassays for monitoring, to assure that no unusual and highly potent TPs are formed during biodegradation or oxidative treatment. In addition, identification of TPs alone is not sufficient to assess their risk. For a comprehensive risk assessment the concentrations of TPs must be evaluated and this can only be done with precision if quantification standards are available. The synthesis of quantification standards was beyond the scope of the present project but it would be the imperative next step in a follow-up project because we found so many potent TPs of interest. The results obtained in this study are a significant contribution to research but more research is required to include TPs into water quality management.

4. Are we currently monitoring and regulating the right chemicals/chemical classes?

There is no clear answer to this question from the scientific perspective but the results obtained from this project can give some input to stakeholder discussions. This question was also discussed at the final workshop of the project in Perth, which took place on 29 January 2014.

As discussed throughout this final report, the AGWR includes 349 organic chemicals. The AGWR is a Commonwealth document and is not binding for any of the States, however, each state has to adapt, modify or reject the proposal by the AGWR. We focus the discussion on the two States where the AWRPs are situated that were used for the case studies of this project.

The State of Queensland has adopted the AGWR relatively unchanged into the Public Health Regulation (PHR), Schedule 3B Standards for quality of recycled water supplied to augment a supply of drinking water, revisions in Subordinate Legislation 2008 No. 218 (Queensland Government, 2005). The Act requires Recycled Water Management Plans (RWMP) that must be approved by the regulator before recycled water is used to augment drinking water supplies. It is further prescribed that only indirect potable reuse, no direct potable reuse is permitted: “the recycled water must be supplied into an aquifer, lake, watercourse or wetlands, or a dam on a watercourse, and stored under conditions that allow for sufficient management of any risk to the health of the public from the recycled water quality” (Queensland Government, 2005).

The RWMP for one of the plants investigated in this study includes frequent monitoring for water quality parameters. Due to the abundant rain since 2011, supplementation of a drinking water supply with recycled water never came into operation in Queensland to date (August 2014) but between 2008 and 2013 more than 400 samples were taken and tested for all parameters included in the PHR (www.seqwater.com.au), among them the organic micropollutants that are the focus of the present project.

The second AWRP investigated in this study is overseen by the Department of Health of Western Australia. Here, regulation is based on 292 Recycled Water Quality Parameters (RWQP) from which 18 Recycled Water Quality Indicators (RWQI) have been derived. The RWQI are defined as “chemicals or pathogens that best represent a larger group of chemicals or microbiological hazards“ (Water Corporation, 2013). Organic micropollutants included in the list of RWQI (excluding disinfection by-products) are 1,4-dioxane, fluorene, 1,4-dichlorobenzene, 2,4,6-trichlorobenzene, carbamazepine, estrone, trifluralin, diclofenac and octadioxin. These RWQI are representative of organic micropollutants and are regularly monitored. In addition, all RWQP are measured annually. Discussions at the final workshop of the project indicated that the bioanalytical monitoring as applied in this project could give valuable additional information and improved certainty if added during the annual monitoring.

With these two approaches in mind, the project team designed a flow chart proposing how bioanalytical assessment could be implemented in an overall screening/monitoring strategy (Figure 45). This strategy is generic and not a proposal to be implemented by either State and comprises just one of the many possibilities to include bioanalytical assessment into chemical monitoring.

In a first screening stage, a defined number of indicator chemicals and indicator bioassays should be tested and compared to set criteria, guideline values (GV) for chemicals and effect-based triggers (EBT) for bioassays (Figure 45). If neither GV nor EBT were exceeded, there would be no further action needed. If either of them is exceeded a larger number of chemicals (e.g., the entire AGWR list) would need to be monitored. It must be kept in mind that the likelihood of an exceedance is very low as operation of AWRP is to such a quality level that it normally meets the GV by far, which was also confirmed by the comparison of actual data with proposed EBT (Appendix A to C).

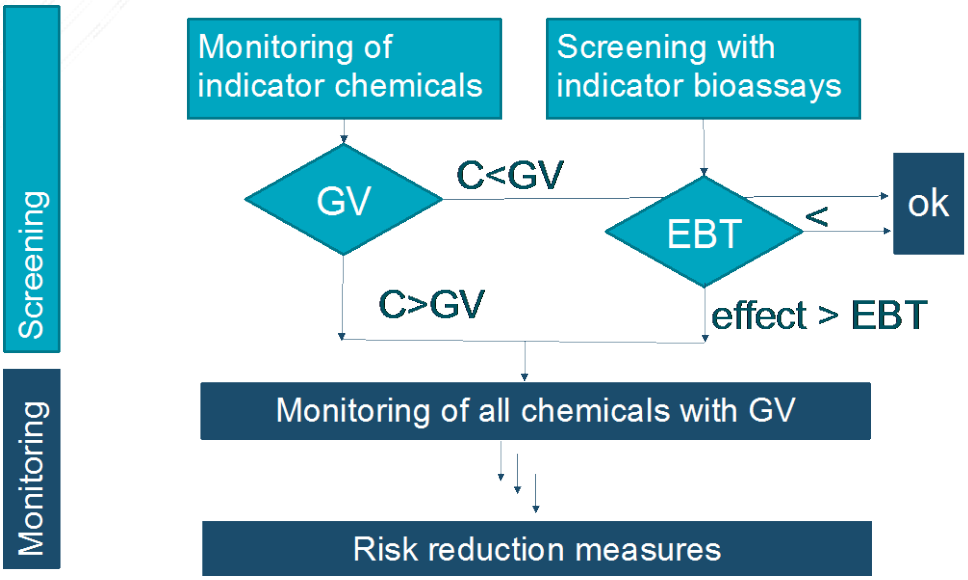


Figure 45. Flow chart for comprehensive assessment of micropollutants with chemical and bioanalytical monitoring. C: concentrations quantified analytically, GV: guideline value, EBT: effect-based trigger.

During the course of the project, we added on a fifth question that was not asked in the proposal:

5. What do bioassay results mean?

This is a question that is often asked, especially after fine-tuning the bioassays and their detection limits to a level that even in very clean water we can quantify effects. Thus it is important to provide regulators with some input on what could be effect-based trigger values that, if exceeded, call for further action. We have therefore proposed an approach and algorithms to derive EBTs (appendix A, B and (Escher *et al.*, 2014b)). Our proposal is based on a translation of existing chemical GVs into EBTs. This is a unique approach as it allows one to adapt EBTs to any regulation and thus has a wide applicability and is not restricted to Australia. It allows one to match any existing chemical guideline values with any indicator bioassay.

We have to differentiate between two cases, though, due to the findings in Chapter 3 that the iceberg mixture could explain either very much or very little of the effect in water samples. For chemicals that act according to receptor-mediated modes of action, such as estrogenicity, activation of the aryl hydrocarbon receptor or inhibition of photosynthesis, only a relatively small and well-defined number of chemicals act according to these modes of action. The good match between the iceberg mixture and water samples for such endpoints as shown in Chapter 3 for photosynthesis inhibition and in previous work for estrogenicity, confirmed that these active chemicals are well known. Thus we can postulate that if a guideline value is safe for a single compound, it should also be safe for the effect of a mixture

with the same mode of toxic action because as all those chemicals act concentration-additive. Thus we can translate all GV for effective chemicals in a given bioassay into the associated BEQ and from a distribution of these BEQs derive the fifth percentile as a precautionary EBT-BEQ (Appendix C and (Escher *et al.*, 2014b)). In earlier work we have proposed a series of such EBT-BEQs for various biological endpoints (e.g., the EBT-DEQ for the photosynthesis inhibition endpoint is derived in Appendix C) and all of the recycled water samples of the present study would have been compliant and not exceeded the EBT-BEQs (Escher *et al.*, 2014b).

A different approach has to be chosen for chemicals with non-specific modes of toxic action, cytotoxicity, and activation of adaptive stress responses. Here, a large number of chemicals are active and known chemicals could explain less than 3% of the effect in a water samples. Thus we have to explicitly account for the mixture effects and cannot use the BEQ concept but have to define EBT-effect concentrations for mixtures. Appendix A and B present an approach how to derive such EBT-effect concentrations (EBT-EC) for the Microtox and AREc32, respectively.

Not only were all recycled water samples compliant with the proposed EBT-BEQs and EBT-ECs, but they also provided a good discrimination between “untreated” and “recycled water” because often before treatment, the EBTs would have been exceeded. While it was not the focus to derive EBTs in the present study, it is a natural next step to give the regulators and stakeholders a way to help interpret the obtained bioanalytical results and put them into context.

In conclusion, mixtures and transformation products matter and cannot be overlooked for the risk assessment of organic micropollutants during water recycling. In response to our overarching question: “how much do we really know and are we currently monitoring the right chemicals/chemical classes?” we can respond that we know the relevant micropollutants responsible for receptor-mediated effects but that there are many unknowns for less specific modes of action, where a larger number of chemicals may be active. Thus the chemicals typically monitored, i.e. the chemicals from the AGWR, are clearly priority chemicals but they comprise not all of the mixture effect organic micropollutants in water may have, especially at low levels where individual chemicals may fall below the limit of detection without being zero.

Thus we highlight the value of a risk management approach that makes use of bioanalytical tools in a complementary manner to chemical analysis in regulatory monitoring, which incorporates mixture effects, unknown micropollutants and transformation products in the monitoring strategy.

6 Appendix

6.1 Peer-reviewed Publications

The following publications were prepared as a part of this project.

Tang, J.Y.M., McCarty, S., Glenn, E., Neale, P.A., Warne, M.S. and Escher, B.I. (2013). Mixture effects of organic micropollutants present in water: towards the development of effect-based water quality trigger values for baseline toxicity. *Water Research* 47(10): 3300-3314.

Escher, B.I., van Daele, C., Dutt, M., Tang, J.Y.M. and Altenburger, R. (2013). Most oxidative stress response in water samples comes from unknown chemicals: the need for effect-based water quality trigger values. *Environmental Science & Technology* 47(13): 7002-7011.

Tang, J.Y.M. and Escher, B.I. (2014). Realistic environmental mixtures of micropollutants in wastewater, recycled water and surface water: herbicides dominate the mixture toxicity towards algae. *Environmental Toxicology and Chemistry* 33(6): 1427-1436.

Tang, J.Y.M., Busetti, F., Charrois, J. and Escher, B. (2014). Which chemicals drive biological effects in wastewater and recycled water? *Water Research* 60: 289-299.

The following paper contains partial contributions by this project:

Escher, B.I., Allinson, M., Altenburger, R., Bain, P., Balaguer, P., Busch, W., Crago, J., Humpage, A., Denslow, N.D., Dopp, E., Hilscherova, K., Kumar, A., Grimaldi, M., Jayasinghe, B.S., Jarosova, B., Jia, A., Makarov, S., Maruya, K.A., Medvedev, A., Mehinto, A.C., Mendez, J.E., Poulsen, A., Prochazka, E., Richard, J., Schifferli, A., Schlenk, D., Scholz, S., Shiraishi, F., Snyder, S., Su, G., Tang, J., Burg, B.v.d., Linden, S.v.d., Werner, I., Westerheide, S.D., Wong, C.K.C., Yang, M., Yeung, B., Zhang, X. and Leusch, F.D.L. (2014). Benchmarking organic micropollutants in wastewater, recycled water and drinking water with in vitro bioassays. *Environmental Science & Technology*, 48: 1940-1956.

6.2 Conference presentations

The following conference abstracts were prepared as a part of this project.

6.2.1 Oral presentation at the SETAC Europe Annual Meeting 2012, Berlin Germany

Can bioanalytical tools help us ensure that our water is safe?

Beate I. Escher[1], Mriga Dutt[1], Eva Glenn[1], Frederic Leusch[2], Miroslava Macova[1], Erin Maylin[1], Ben Mewburn[1], Peta Neale[1], Anita Poulsen[1], Janet Tang[1]

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Chemical monitoring provides a quantitative assessment of single organic contaminants in a water sample but cannot account for the presence of non-target compounds such as unidentified transformation products and interactions between chemicals. Bioanalytical monitoring is complementary to chemical analysis and provides information on all bioactive micropollutants in a sample according to potency, *i.e.*, chemicals of higher toxicity will be weighted higher than less toxic chemicals. Cell-based bioassays provide measures of the cumulative effects of chemicals that exhibit the same mode of toxic action, for which the selected bioassays are indicative, and they can give a measure of the cytotoxicity of all chemicals acting together in a water sample. Improved detection of the presence of chemicals in water enhances risk assessment and informs water management options, among them water recycling from impaired sources such as sewage, or stormwater harvesting and reuse. In this presentation the design of a modular battery of bioassays based on toxicological principles will be presented. This bioanalytical test battery was used for monitoring organic micropollutants across an indirect potable reuse scheme testing sites encompassing the complete water cycle from sewage to drinking water to assess the efficacy of different treatment barriers, including source control, wastewater treatment plant, microfiltration, reverse osmosis, advanced oxidation, natural environment in a reservoir and drinking water treatment plant. The results of the various studies presented here indicate that bioanalytical tools provide valuable additional information to chemical analysis and should be implemented in the future as a monitoring tool.

6.2.2 Oral presentation at the SETAC Australasia Annual Meeting 2012, Brisbane, Australia

Predicting adverse health effects of transformation products formed from organic micropollutants during water treatment

Marcella L. Card and Beate I. Escher

National Research Centre for Environmental Toxicology, The University of Queensland, Coopers Plains, Qld, Australia

Abstract: In water treatment plants (WTPs), micropollutants are transformed via biotic and abiotic processes, resulting in transformation products that may be as toxic as or more toxic than the parent compound. This presents a significant uncertainty for risk assessment where water is recycled for human consumption or where such use is pending implementation. With more than 100,000 chemicals in daily use, there is a need for an efficient, reliable way to identify chemicals of concern, which may lead to toxic transformation products in WTPs. Therefore, we have developed a scheme to predict parent compounds, which may be transformed into toxic transformation products and the predictions will be validated by quantifying the toxicity of predicted parent compounds and transformation products using *in vitro* bioassays. Known toxicophores (functional groups which cause reactive or specific toxicity) were identified from the literature for each of several modes of toxic action relevant to human health. Moieties, which may be transformed into toxicophores, were then predicted based on

microbially-mediated transformations which occur in activated sludge and/or abiotic transformations which occur during UV or advanced oxidation treatments. Micropollutants carrying the putative precursor moieties were then identified from among those listed in the Australian Guidelines for Water Recycling and the U.S. Environmental Protection Agency Candidate Contaminant Lists. Micropollutants and predicted transformation products were removed from consideration when calculated physicochemical properties (e.g., hydrophobicity and volatility) indicated low biological relevance. To validate the predictive scheme, identified micropollutants of concern will be subjected to bench-scale activated sludge and/or advanced oxidation treatments. As the transformations progress, toxicity of the parent-product mixtures will be quantified using bioassays. If, as predicted, the transformation products are more toxic than the parent compounds, then the measured toxicity will not decrease relative to decreases in the ratio of parent concentration to product concentration.

6.2.3 Oral presentation at "RecycleWater2013", a national water recycling and technology conference, 30-31 May 2013, Melbourne, Australia.

Bioanalytical tools for assessment of chemicals, transformation products and their mixtures in recycled water

Beate I. Escher

The University of Queensland, National Research Centre for Environmental Toxicology (Entox), Brisbane QLD 4108, Australia

- Bioanalytical tools complement chemical analysis for cost-efficient water quality monitoring
- Bioanalytical tools are recognized as valuable research tool
- Bioanalytical tools give information on the mixture effects of chemicals and included unknowns and transformation products
- Bioanalytical tools give information on the mode of action and type of effect of the chemicals in a water sample
- Bioanalytical tools have a wide applicability across the water cycle and may serve for the assessment of treatment efficiency in water recycling schemes

Chemical pollution is an increasing threat to our waterways, oceans, and drinking water sources. The impact of chemical pollution will be amplified by population growth and, possibly, by some of the effects of climate change. However, conventional chemical monitoring programs have been criticised on the basis that they cannot include the full range of chemical pollutants that could occur in water sources, and they do not account for the combined effects of mixtures of chemicals. Bioanalytical tools may therefore complement chemical analysis for cost-efficient water quality monitoring.

Bioanalytical tools are cell-based bioassays that target specific mechanisms of toxicity and give a measure of the toxicity of mixtures of known and unknown chemicals, such as pesticides, industrial chemicals, pharmaceuticals and their transformation products. Bioanalytical tools provide measures of the cumulative effects of chemicals that exhibit the same mode of toxic action, for which the selected bioassays are indicative plus they can give a measure of the cytotoxicity of all chemicals acting together in a water sample. Improved detection of the presence of chemicals in water enhances risk assessment and informs water management options, among them water recycling from impaired sources such as sewage, coal seam gas water, or stormwater harvesting and reuse.

In this presentation the design of a modular battery of bioassays will be presented and some illustrative examples from recent applications in South East Queensland, Australia. The bioassays were selected from the three main categories of modes of action, namely non-specific, receptor-mediated specific and reactive toxicity. This bioanalytical test battery was used for monitoring organic micropollutants and disinfection by-products across an indirect potable reuse scheme testing sites across the complete water cycle from sewage to drinking water to assess the efficacy of different treatment barriers,

including source control, wastewater treatment plant, microfiltration, reverse osmosis, advance oxidation, natural environment in a reservoir and drinking water treatment plant.

6.2.4 Poster presentation at the SETAC Europe Annual Meeting 2013 in Glasgow, UK.

The University Of Queensland
AUSTRALIA

Effect-Based Water Quality Trigger Values Accounting for Mixture Effects of Organic Micropollutants in Water

Janet Y.M. Tang[1], Mriga Dutt[1], Eva Glenn[1], Shane McCarty[1], Peta Neale[1], Charlotte van Daele[1], Michael St. J. Warne[2], Rolf Altenburger[3], and Beate I. Escher[1*]
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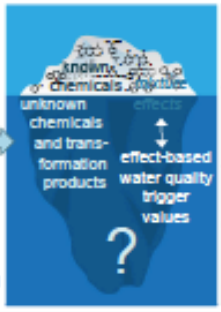
Motivation

- *In vitro* bioassays are frequently applied for water quality monitoring
- Water quality guideline values are available only for individual chemicals, no effect-based trigger (EBT) values exist
- For many modes of action only a fraction of the effects observed in water samples can be explained by known chemicals

Approach

- On the example of three bioassays for non-specific, specific and reactive modes of action, we propose an algorithm to develop EBTs
- We illustrate the approach on the example of recycled water but the algorithm is applicable for any guideline



Mode of action class	Mode of action	Bioassay	Fraction of effect concentration explained by quantified chemicals in samples from sewage to drinking water	
Non-specific	Baseline toxicity	Bioluminescence inhibition in *V. fischeri*	269 PPCP, pesticides, industrial chemicals	0.001 to 0.5 %
Specific	Photosynthesis inhibition	I-PAM (Phytotox)	12 Triazines and phenylureas	77 to 92 %
Reactive	Oxidative stress response	Induction of Nrf2 in *AREc32*	269 PPCP, pesticides, industrial chemicals	0.003 to 0.05 %

Guidelines for recycled water

- Australian guidelines for water recycling: managing health and environmental risks (phase 1) National Water Quality Management Strategy (NWQMS), Natural Resource Management Ministerial Council, Environment Protection and Heritage Council and National Health and Medical Research Council, Canberra, Australia, 2006, www.ephc.gov.au/taxonomy/term/39
- 349 pesticides, pharmaceuticals and personal care products (PPCP), industrial chemicals and consumer products regulated

n regulated chemicals
- chemical a
- chemical b.....
- .....chemical n

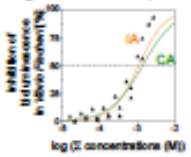
$$\sum_{i=1}^{m} \text{guideline value}_i$$

m chemicals considered at f % of guideline value

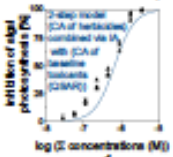
Mixture effects of regulated chemicals

- Up to 56 regulated chemicals were mixed in three types of mixtures: (a) equipotent concentration ratios, (b) ratios of the guideline values, and (c) ratios of concentrations detected in recycled water sample
- All experimental mixture effects could be satisfactorily described by the mixture toxicity model of concentration addition (CA), for the combined algae assay by a two-step model of CA combined with independent action; a few experimental examples are:

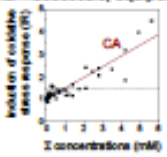
Baseline toxicity (20 chemicals at ratios of guideline values)



Inhibition of photosynthesis (6 herbicides and 34 others at ratios found in water)



Induction of oxidative stress response in *AREc32* (10 pharmaceuticals, equipotent)



mixture model for concentration addition

$$EC_{50(CA)} = \frac{1}{\sum_{i=1}^{n} \frac{p_i}{EC_{50i}}}$$

Effect-based trigger values

- EBT provide the opportunity to integrate mixtures into water quality assessment
- EBT are effect concentrations in units of relative enrichment factor (REF) for each mode of toxic action

$$EBT - EC = \frac{EC_{mixture}}{\frac{f \cdot m}{n} \sum_{i=1}^{n} \text{guideline value}_i}$$

- The EBT-EC can either be EC_{50} values derived from a log-logistic concentration-effect curve (Microtox and algae) or $EC_{IR1.5}$ for the induction ratio IR derived from a linear concentration-effect curve (AREc32)
- EBT-EC were calculated from existing chemical-based water quality guideline values
  - using sum of all concentrations (in molar units) for the n chemicals in a guideline
  - $EC_{mixture}$ were predicted for the n-component mixture
    - with the combined QSAR and CA model (Microtox)
    - with a 2-step CA/IA model (combined algae test)
    - with extrapolations of a larger number of experimental CA mixtures (AREc32)
  - 1000 Chemicals considered at 5% of their "mean" guideline value, i.e., m=1000, f =0.05

Proposed effect-based trigger value			Measured effect in recycled water (in units of REF)	
Mode of action	Bioassay	EBT in units of REF	After treatment with reverse osmosis and UV/H_2O_2	After treatment with ozonation and biologically activated carbon filtration
Baseline toxicity	*Vibrio fischeri*	EBT-EC_{50} = 2.8	EC_{50} = 51	EC_{50} = 10
PSII inhibition	I-PAM Phytotox	EBT-EC_{50} = 0.03	EC_{50} > 112	EC_{50} > 112
Oxidative stress response	*AREc32*	EBT-$EC_{IR1.5}$ = 6	$EC_{IR1.5}$ = 94	$EC_{IR1.5}$ = 22

Conclusion

All tested recycled water samples complied with the EBT in all evaluated bioassays, (i.e., EBT-EC_{50}<EBT measured)

More information

Tang, J.Y.M., McCarty, S., Glenn, E., Neale, P.A., Warne, M.S. and Escher, B.I. (2013) *Water Res.* 47, 3300-3314
Escher, B.I., van Daele, C., Dutt, M., Tang, J.Y.M. and Altenburger, R. (2013) *Environ. Sci. & Technol.* in press, http://dx.doi.org/10.1021/es304793h
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entox
HELMHOLTZ CENTRE FOR ENVIRONMENTAL RESEARCH - UFZ

Funding by the Australian Water Recycling Centre of Excellence (set up under the Commonwealth Government's Water for the Future Program), the WateReuse Research Foundation (WRF 10-07), and the Australian Research Council (FT100100894) with additional support from Seqwater, Water Corporation and Veolia

6.2.5 Keynote at the 8th Micropol and Ecohazard conference, International Water Association, Zürich, Switzerland, June 2013.

What's in our water? Bioanalytical tools for assessment of micropollutants, mixtures and transformation products

Beate I. Escher[1], Rolf Altenburger[2], Marcella Card[1], Mriga Dutt[1], Eva Glenn[1], Shane McCarty[1], Peta Neale[1], Daniel Stalter[1], Janet Y.M. Tang[1], Charlotte van Daele[1] and Michael St. J. Warne[1,3]

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Introduction. Chemical pollution is an increasing threat to our waterways, oceans, and drinking water sources. The impact of chemical pollution will be amplified by population growth and, possibly, by some of the effects of climate change. However, conventional chemical monitoring programs have been criticised on the basis that they cannot include the full range of chemical pollutants that could occur in water sources, and they do not account for the combined effects of mixtures of chemicals. Bioanalytical tools may therefore complement chemical analysis for cost-efficient water quality monitoring.(Escher and Leusch, 2012)

Bioanalytical tools are cell-based bioassays that target specific mechanisms of toxicity and give a measure of the toxicity of mixtures of known and unknown chemicals, such as pesticides, industrial chemicals, pharmaceuticals and their transformation products. Bioanalytical tools provide measures of the cumulative effects of chemicals that exhibit the same mode of toxic action, for which the selected bioassays are indicative plus they can give a measure of the cytotoxicity of all chemicals acting together in a water sample. Improved detection of the presence of chemicals in water enhances risk assessment and informs water management options, among them water recycling from impaired sources such as sewage, coal seam gas water, or stormwater harvesting and reuse.

Design of a bioanalytical test battery. In this presentation the design of a modular battery of bioassays will be presented that is based on an understanding of the cellular toxicity pathways of chemicals. The bioassays were selected from the three main categories of modes of action, non-specific, receptor-mediated specific and reactive toxicity. In addition bioassays that are indicative of the induction of the xenobiotic metabolism pathways and adaptive stress response can be exploited as indicators of the exposure to chemicals (Figure 1).

for damage but also early repsonse such as induction of xenobiotic mechanisms and adaptive stress response.

Benchmarking water quality and assessing treatment efficiency. This bioanalytical test battery was used for monitoring and benchmarking organic micropollutants and disinfection by-products across an indirect potable reuse scheme. We evaluated the efficacy of different treatment barriers, including wastewater treatment, advanced water treatment (microfiltration, reverse osmosis, advance oxidation), natural attenuation in a reservoir and drinking water treatment.(Macova *et al.*, 2011) Also results of a large study will be shown, where 19 laboratories all over the globe analysed ten water samples with a wide range of biological endpoints.

How do chemicals in real samples act together as mixtures? In the last decade the field of mixture toxicity assessment has progressed significantly and we have a reasonable understanding of the theoretical basis of mixture toxicity of defined mixtures with small number of components; it is has been demonstrated that even single chemicals present below concentrations causing a visible effect, they may contribute to the mixture effect.(Kortenkamp *et al.*, 2009) Ample experimental evidence showed that the mixture toxicity concept of concentration addition gives robust predictions for multicomponent mixtures of chemicals acting according to the same mode of toxic action and a number of excellent

studies were undertaken with the Microtox assay and algal toxicity assays. However, very little work has been done on the chemicals that actually occur in water samples. Therefore we evaluated the mixture effects of up to 56 chemicals in concentrations ratios equivalent to those detected in water samples, in equipotent concentrations and and in the concentrations of their recycled water guideline values. Overall, the established mixture toxicity concepts could explain the experimental effects satisfactorily, even for endpoints such as the oxidative stress response that has previously never been evaluated for mixture effects.

What is hidden underneath the iceberg? We further analysed 270 micropollutants in various water samples and mixed the chemicals in the concentrations detected, assessed the designed mixtures with the bioassays and compared with the entire water sample. In the bioassays for nonspecific toxicity and oxidative stress, less than 1% of the effect could be explained by the known chemicals, suggesting the presence of a wide array of unknown micropollutants (and transformation products) in environmental samples, possibly too many to ever be quantified and therefore bioassays should always accompany chemical analysis in water quality monitoring.

What about transformation products? With a focus on reactive toxicity, this test battery can also be adapted to target disinfection by-products formed during drinking water treatment.(Neale *et al.*, 2012)

Towards effect-based trigger values. Motivated by the experience with the mixtures we propose an algorithm to derive effect based trigger values for diverse types of water. The trigger values are based on existing individual chemical's water quality guideline values and if the numerical values exceed the set values for each type of water, then further investigations using other endpoints or chemical analysis are prompted. These proposed trigger values account for mixture effects and large numbers of chemicals in a water sample. There is not a single trigger value but for each mode of action and associated endpoint, a trigger value must be derived.

This research was funded by the Urban Water Security Research Alliance, the Australian Water Recycling Centre of Excellence (set up under the Commonwealth Government's Water for the Future Program), the WateReuse Research Foundation, the Australian Research Council, various industry partners (WQRA, Seqwater, Veolia) and the University of Queensland.

6.2.6 Oral presentation at the SETAC Australasia Annual Meeting 2013 in Melbourne, Australia, 1-3 Oct 2013

Combining chemical analysis and bioanalytical tools for a comprehensive assessment of organic compounds in recycled water

Janet Y.M. Tang[1*] Francesco Busetti[2], Jeffrey W.A. Charrois[2] and Beate I. Escher[1]

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Abstract: Compliance monitoring of drinking water and recycled water in Australia is predominantly based on chemical assessments using instrumental analysis. Bioanalytical tools have the potential to assess the mixture effects according to the mode of toxic action and can complement chemical analytical monitoring. In this study, grab samples were collected from an Australian Wastewater Treatment Plant (WWTP) with secondary treatment processes, including activated sludge treatment, followed by treatment in a trial Advanced Water Recycling Plant (AWRP). Within the AWRP, water treatment included ultrafiltration, chloramination, reverse osmosis (RO) and UV disinfection. Analysis of 278 compounds including pesticides, herbicides, pharmaceuticals, endocrine disrupting compounds and X-ray contrast media was undertaken at different points along the treatment train. Treatment efficiently removed organic compounds. Only very low levels of target analytes were detected in post-RO water, including: an anticorrosive compound, tolyltriazole; a plasticizer, bisphenol A; a pharmaceutical, triclosan; and the pesticides MCPA and 3,4-dichloroaniline. The positive low-level

detections of these compounds in post-RO water were found to be consistent with previous monitoring programs except for the pesticides, which were not detected before. Complete removal of all compounds targeted was observed in the post-UV water. In parallel to the chemical screening, a battery of cell-based bioassays covering a wide range of modes of toxic action were used to evaluate the samples. In laboratory studies, the identified chemicals were mixed in the concentration ratios they were detected at within the AWRP, and then dosed into the bioassays. The effects caused by these designed mixtures were compared to the effects of the corresponding entire samples. For receptor-mediated biological endpoints such as photosynthesis inhibition, where a small number of well-defined chemicals are known to be active, the majority of effects could be explained by the presence of identified compounds. For non-specific bioassays such as cytotoxicity or oxidative stress response, where all or many compounds contribute to the mixture effects, the detected chemicals could explain less than 1% of the measured effect, meaning that non-target chemicals and transformation products contribute to the mixture effects. Nevertheless, the levels of organic compounds and effects are of no concern post-RO, as was demonstrated by comparison with the Australian Guidelines for Water Recycling (AGWR). We translated the established chemical guideline values into tentative effect-based trigger values (EBT) and none of the recycled water samples exceeded these thresholds.

6.2.7 Oral presentation at the SETAC Australasia Annual Meeting 2013 in Melbourne, Australia, 1-3 Oct 2013

Effect-Based Water Quality Trigger Values Accounting for Mixture Effects of Organic Micropollutants in Recycled Water

Beate I. Escher[1], Rolf Altenburger[2], Mriga Dutt[1], Eva Glenn[1], Shane McCarty[1], Peta Neale[1], Janet Y.M. Tang[1], Charlotte van Daele[1] and Michael St. J. Warne[1,3]

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In vitro bioassays are frequently used for water quality assessment. The routine monitoring application is still hampered by the lack of appropriate interpretation guidelines. Here, we propose a systematic approach to derive effect-based water quality trigger values for three different types of toxic action: a) bioluminescence inhibition of *Vibrio fischeri* (Microtox), b) induction of an adaptive stress response pathway in the Nrf-2 mediated oxidative stress response (AREc32), c) inhibition of photosystem-II herbicides in the combined algae test. The trigger values can be derived by reading across from existing guideline values and accounting for mixture effects using the established mixture toxicity model of concentration addition. The derivation of the effect-based trigger values is illustrated on the example of recycled water and was applied to several different treatment schemes. All tested recycled water samples complied with the effect-based trigger values in all bioassays. This study was financially supported by the Australian Water Recycling Centre of Excellence.

6.2.8 Poster presentation at the SETAC Australasia Annual Meeting 2013 in Melbourne, Australia, 1-3 Oct 2013

Do transformation products contribute to mixture toxicity?

Janet Y.M. Tang[1*], Birgitte S. Cordua[1,3], Sebastien Allard[2], Francesco Busetti[2], Nina Cedergreen[3], Jeffrey W. A. Charrois[2], and Beate I. Escher[1]

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[3]University of Copenhagen, Department of Plant and Environmental Sciences, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark

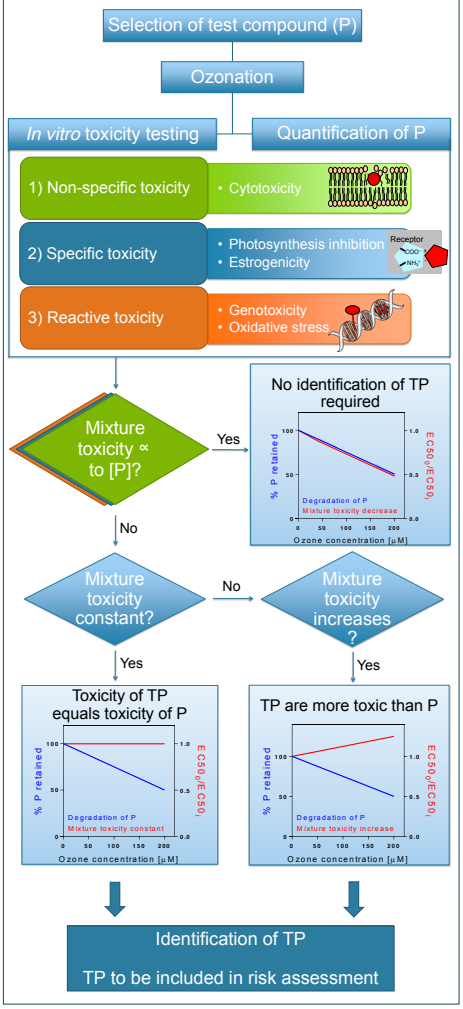
Background

- Oxidative treatment of wastewater may create transformation products (TPs) from micropollutants (parent, P) present in the water
- To date, regulation of TPs in water guidelines are limited
- Traditional processes of identifying, isolating and quantifying TPs are costly and time-consuming

Research Goals

- To develop a tiered approach for screening organic micropollutants by a combination of *in-vitro* bioanalytical assessment and univocal analytical identification of TPs via liquid chromatography HR mass spectrometry
- To quantify biological effect of mixture toxicity of TPs in combination with P

Approach



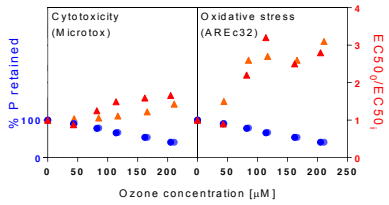
Selection of test compounds, ozonation experiments, bioassays

- 8 chemicals has been chosen based on literature reviews showing potential to form transformation products from oxidative treatment and previous detection of the compounds in secondary wastewater
  - pesticides: atrazine, hexazinone and haloxyfop
  - endocrine disrupting compound: bisphenol A
  - X-ray contrast media: iopromide
  - pharmaceuticals: carbamazepine and diclofenac
  - antibiotic: sulfamethoxazole

- Test compounds spiked into RO water, final concentration 10 - 100 µM
- Ozone dosing goal: Reach 40 - 50 % degradation of the spiked chemical
- Spiked with 5 different ozone doses in the range of 10 – 200 µM

- Samples were extracted using a sandwich set-up comprised of 0.5g Oasis® HLB cartridges and 2g Supelclean Coconut Charcoal cartridges
- Toxicity tested using bioassay test battery
- Bioassays: Bioluminescence inhibition of *V. fischeri* (Microtox); IPAM and E-CALUX; UmuC and AREc32

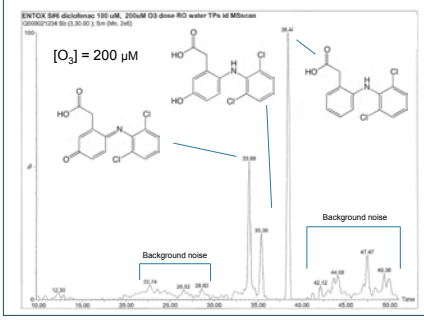
Results for diclofenac



- [P]↓ with [O_3]↑
- Cytotoxicity↑ with [O_3]↑
- Induction of oxidative stress↑ with [O_3]↑

→ TP(s) are more toxic than P

→ Mixture toxicity is relevant



- Chemical analysis using LC-IT/HRMS (Orbitrap) revealed formation of two TPs: 5-OH diclofenac and diclofenac-2,5 –iminoquinone

Next step

- Quantification of TP
- Effect assessment of TP

Conclusion and Outlook

Preliminary results indicate that

- TP(s) contributed to mixture toxicity for diclofenac
- TP contribute to additive mixture toxicity for hexazinone, haloxyfop, bisphenol-A, sulfamethoxazole and iopromide
- Additional experiments are needed to support the preliminary findings

Acknowledgements

This research was funded by the Australian Water Recycling Centre of Excellence (set up under the Commonwealth Government's Water for the Future Program), *Water Corporation* of *Western Australia* and the Australian Research Council (FT100100694). We also thank Marcy Card for helpful discussion.

• NHMRC, NRMMC (2011) *Australian Drinking Water Guidelines Paper 6 National Water Quality Management Strategy. National Health* and Medical Research Council, National Resource Management Ministerial Council, Commonwealth of Australia, Canberra.
• Council Directive 98/83/EC) of 3 November 1998 on the quality of water intended for human consumption
• U.S. EPA (2011): Finalization of Guidance on Incorporation of Water Treatment Effects on Pesticide Removal and Transformations in Drinking Water Exposure Assessments

6.2.9 Oral Presentation at the 5th EUCheMS Chemistry Conference, 31 Aug - 4 Sept. 2014, Istanbul, Turkey.

Can A Large Suite Of Target Micropollutants Explain The Biological Effects Observed In Wastewater And In Recycled Water?

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Removal of 299 organic micropollutants from a Western Australia advanced water recycling plant (AWRP) treating secondary wastewater (WW) through ultrafiltration (UF), reverse osmosis (RO) and UV disinfection was investigated by complementary chemical analysis and *in-vitro* cell-based bioassays. Chemical analyses were accomplished through gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometryLC-MS based analytical methods and targeted a wide range of micropollutants including pesticides, pharmaceuticals and personal care products, hormones, industrial chemicals, household chemicals. Four *in-vitro* cell-based bioassays were used targeting non-specific toxicity (i.e., Microtox assay for cytotoxicity), specific toxicity (i.e., IPAM assay for photosynthesis inhibition) and reactive toxicity (umuC assay for genotoxicity; AREc32 assay for oxidative stress response). Although low levels of the anticorrosive compound tolutriazole, the plasticizer bisphenol A, the pharmaceutical triclosan and the pesticides 2-methyl-4-chlorophenoxyacetic acid (MCPA) and 3,4-dichloraniline were detected in the water samples post RO treatment, concentrations of detected micropollutants were well below the Australian Guidelines for Water Recycling implying that detected chemicals were considered to pose negligible health risk. No micropollutants were detected post UV treatment, demonstrating a high degree of safety for the re-use of RO/UV-treated WW for groundwater replenishment. In order to quantitatively link the results from chemical analysis and bioassays, the detected chemicals were mixed in the concentration ratios detected and their effects assessed. The effects caused by these designed mixtures were compared to the effects of the corresponding water samples along the treatment train of the AWRP to assess, which of the detected chemicals drive the biological effect and which fraction of effect remains unexplained by detected chemicals. The pesticides detected in secondary treated WW explained all observed effects on photosynthesis inhibition. In contrast, mixture toxicity experiments with designed mixtures containing all detected chemicals at their detected concentrations demonstrated that the known chemicals explained less than 3% of the cytotoxicity and less than 1% of the oxidative stress response. Pesticides followed by pharmaceuticals and personal care products were found to dominate the observed mixture effects. The detected chemicals could not be related to the observed genotoxicity. In conclusion, given the large proportion of unknown toxicity observed in the water samples (i.e., non-specific toxicity and oxidative stress), routine effect monitoring through in-vitro cell-based bioassays should be used in conjunction to chemical monitoring to ensure the safety of recycled water.

6.2.10 Oral Presentation at the 5th EUCheMS Chemistry Conference, 31 Aug - 4 Sept. 2014, Istanbul, Turkey.

Degradation Of Atrazine During Advanced Oxidation Processes And Formation Of Toxic Transformation Products

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Advanced oxidation processes are designed to efficiently remove micropollutants refractory to usual disinfection treatment in water. However, full mineralisation to carbon dioxide is not realistic and transformation products (TPs) are formed. To date, little is known on the identity of the TPs and their relative toxicity.

In this study, post reverse osmosis water was spiked with atrazine and treated by UV/H_2O_2 as well as other common tertiary treatments (UV, ozone and ozone with t-BuOH to quench the OH radical for comparison). The main TPs were identified by liquid chromatography high-resolution mass spectrometry (Orbitrap) and the degradation of atrazine quantified in parallel with a bioanalytical assessment using a battery of toxicity tests. The bioassays applied were the Microtox assay for cytotoxicity, the chlorophyll fluorescence assay (IPAM) for photosynthesis inhibition and the AREc32 assay for oxidative stress response.

Results showed that atrazine was efficiently degraded by OH radicals as confirmed by the UV/H_2O_2 and ozone experiments (Figure 1). Degradation of atrazine led to the formation of multiple TPs. They were identified through high resolution MS2 and MS3 and molecular structure were proposed. As target effect for herbicides, the photosynthesis inhibition was assessed and as expected the effect decreased with the degradation of the atrazine. However, the cytotoxicity and the oxidative stress response remained constant or even increased in the case of UV/H_2O_2 despite disappearance of atrazine (Figure 1), indicating that the transformation products mixture has equal or higher toxicity compared to atrazine. The identified TPs have similar structure as atrazine, therefore the mixture effect on the toxicity results are easily justified. This study clearly shows that the formation of TPs has to be assessed and cannot be neglected since it is demonstrated that the TPs have a similar potency as the target chemical. The same approach was used with other micropollutants, i.e. haxazinone, diclofenac and carbamazepine and the formation of toxic TPs was also demonstrated.

6.2.11 Oral presentation at the SETAC Asia-Pacific Biannual and SETAC Australasia Annual Meeting 2014 in Adelaide, Australia, 14-17 Sept. 2014.

Micropollutants, mixtures and transformation products in recycled water: how much do we really know?

Beate I. Escher[1,3], Sebastian Allard[2], Francesco Busetti[2], Janet Y.M. Tang[1], Jeffrey W.A. Charrois[2]

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In this presentation, we give an overview on outcomes of the collaborative research project "Micropollutants, mixtures and transformation products in recycled water: how much do we really know?" which was undertaken jointly by the University of Queensland and Curtin University for the Australian Water Recycling of Excellence with contribution by Water Quality Research Australia, Watersecure, Water Corporation, Veolia Environnement, Department of Environment and Resource Management, Queensland Health, Melbourne Water, Seqwater. While a small number of individual chemicals are typically being monitored in recycled water, we do not know how many micropollutants are actually present and if the toxicological hazard can be assessed by the regulated chemicals alone. Mixture toxicity experiments, where we mixed the detected chemicals in wastewater treatment plant effluent and recycled water, indicated that there are many unknowns. For bioassays indicative of estrogenic effects or photosynthesis inhibition, the known chemicals can explain all biological effect in a typical water sample but for cytotoxicity and adaptive stress responses, there remain many unknowns. These unknowns not only include chemicals introduced by human activity into the wastewater stream but also those that are formed during water treatment such as biotransformation, ozonation and other oxidation processes as this study was able to demonstrate. In conclusion, we recommend that in the future chemical monitoring should be complemented by a small suite of indicator bioassays to account for the mixture effects of known and unknown micropollutants and their transformation products.

6.2.12 Poster presented at the STEAC Asia-Pacific Biannual and SETAC Australasia Annual Meeting 2014 in Adelaide, Australia, 14-17 Sept. 2014.

Can target chemicals explain biological effects from wastewater to recycled water?

Janet Y.M. Tang[1], Francesco Busetti[2], Jeffrey W.A. Charrois[2] and Beate I. Escher[1,3]

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A combination of chemical analysis and bioanalytical tools has been applied for the monitoring of water quality increasingly in recent years, however, so far the results had not been linked in a quantitative manner and traced back to individual chemical groups. In this study the removal of organic micropollutants from secondary wastewater treatment followed by ultrafiltration, chloramination, reverse osmosis and UV disinfection was evaluated by a battery of cell-based *in vitro* bioassays and chemical analysis of 299 organic micropollutants. Low levels of the anticorrosive compound tolutriazole, the plasticizer bisphenol A, the pharmaceutical triclosan and the pesticides MCPA and 3,4-dichloraniline were detected in the water samples post reverse osmosis. The concentrations detected after reverse osmosis were below the Australian Guidelines for Water Recycling. A complete removal of all compounds targeted was observed in the post UV water. In order to quantitatively linking the results from chemical analysis and bioassays, the detected chemicals were mixed in the concentration ratios detected and their effects assessed. The effects caused by these designed mixtures were compared to the effects of the corresponding water samples along the treatment train of the advanced water recycling plant. For the biological endpoint that targets the specific mode of action, in this case the photosynthesis inhibition, the pesticides in secondary treated wastewater effluent could explain all the observed effects. In contrast, for non-specific toxicity and oxidative stress response, less than 3% and 1% of the effects could be explained by the designed mixtures. The designed mixtures were broken down into individual classes and we found that pesticides and pharmaceuticals contributed substantially to the observed biological effects. Given that a large proportion of non-specific toxicity and oxidative stress could not be explained by targeted chemicals, bioanalytical tools should be used routinely to complement chemical analysis for water quality monitoring.

6.3 Supporting Information and Detailed Results

6.3.1 Appendix A

Tang, J.Y.M., McCarty, S., Glenn, E., Neale, P.A., Warne, M.S. and Escher, B.I. (2013). Mixture effects of organic micropollutants present in water: towards the development of effect-based water quality trigger values for baseline toxicity. *Water Research* 47(10): 3300-3314.

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6.3.2 Appendix B

Escher, B.I., van Daele, C., Dutt, M., Tang, J.Y.M. and Altenburger, R. (2013). Most oxidative stress response in water samples comes from unknown chemicals: the need for effect-based water quality trigger values. *Environmental Science & Technology* 47(13): 7002-7011.

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6.3.3 Appendix C

Tang, J.Y.M. and Escher, B.I. (2014). Realistic environmental mixtures of micropollutants in wastewater, recycled water and surface water: herbicides dominate the mixture toxicity towards algae. *Environmental Toxicology and Chemistry* 33(6): 1427-1436.

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6.3.4 Appendix D

Tang, J.Y.M., Busetti, F., Charrois, J. and Escher, B. (2014). Which chemicals drive biological effects in wastewater and recycled water? *Water Research 60: 289-299.*

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Mixture effects of organic micropollutants present in water: Towards the development of effect-based water quality trigger values for baseline toxicity

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ABSTRACT

In this study we propose for the first time an approach for the tentative derivation of effect-based water quality trigger values for an apical endpoint, the cytotoxicity measured by the bioluminescence inhibition in *Vibrio fischeri*. The trigger values were derived for the Australian Drinking Water Guideline and the Australian Guideline for Water Recycling as examples, but the algorithm can be adapted to any other set of guideline values. In the first step, a Quantitative Structure–Activity Relationship (QSAR) describing the 50% effect concentrations, EC_{50}, was established using chemicals known to act according to the nonspecific mode of action of baseline toxicity. This QSAR described the effect of most of the chemicals in these guidelines satisfactorily, with the exception of antibiotics, which were more potent than predicted by the baseline toxicity QSAR. The mixture effect of 10–56 guideline chemicals mixed at various fixed concentration ratios (equipotent mixture ratios and ratios of the guideline values) was adequately described by concentration addition model of mixture toxicity. Ten water samples were then analysed and 5–64 regulated chemicals were detected (from a target list of over 200 chemicals). These detected chemicals were mixed in the ratios of concentrations detected and their mixture effect was predicted by concentration addition. Comparing the effect of these designed mixtures with the effect of the water samples, it became evident that less than 1% of effect could be explained by known chemicals, making it imperative to derive effect-based trigger values. The effect-based water quality trigger value, EBT-EC_{50}, was calculated from the mixture effect concentration predicted for concentration-additive mixture effects of all chemicals in a given guideline divided by the sum of the guideline concentrations for individual components, and dividing by an extrapolation factor that accounts for the number of chemicals contained in the guidelines and for model uncertainties. While this concept was established using the example of Australian recycled water, it can be easily adapted to any other set of water quality guidelines for organic micropollutants. The cytotoxicity based trigger value cannot be used in isolation, it must be applied in conjunction with effect-based trigger values targeting critical specific modes of action such as estrogenicity or photosynthesis inhibition.

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1. Introduction

Organic micropollutants are omnipresent in our sewage, aquatic ecosystems and drinking water (Schwarzenbach et al., 2006). Although organic micropollutants occur typically at very low concentrations, they are numerous and can be transformed by biotic and abiotic transformation processes (Escher and Fenner, 2011), creating complex mixtures of unknown composition. There are regulations and water quality guidelines for individual chemicals in different water types available in many countries (for an overview see Escher and Leusch, 2011) and there is some guidance for including mixtures into Fresh and Marine Water Quality (ANZECC/ARMCANZ, 2000) and for the risk assessment of chemicals (USEPA, 2002; EU Council, 2009). Nevertheless there exist no effect-based water quality trigger values relating to simple screening type bioassays for cytotoxicity.

The field of mixture toxicity assessment has matured over the last decade (as reviewed by Kortenkamp et al. (2009)). From designed mixture toxicity studies, we have learnt that even if single chemicals are present below concentrations that cause a visible effect, they may contribute to the mixture effect (Silva et al., 2002; Kortenkamp et al., 2009). There is also ample experimental evidence that the mixture toxicity model of concentration addition (CA), which is strictly only valid for chemicals that have the same mode of toxic action, gives robust and accurate predictions for many multicomponent mixtures. The alternative concept of independent action (IA) holds for chemicals with dissimilar modes of action. For multicomponent mixtures the two mixture models of CA and IA often give fairly similar predicted effects although the subtle differences can be used as a diagnostic tool for mode-of-action analysis (Backhaus et al., 2000, Kortenkamp et al., 2009). Further, mixture effects of chemicals combined in ratios as they were found in environmental samples could be satisfactorily predicted by IA and CA (Altenburger et al., 2004; Junghans et al., 2006). Therefore it has been proposed to apply CA as a precautionary first tier in environmental risk assessment of mixtures (Posthuma et al., 2008; Backhaus and Faust, 2012).

In vitro cell-based bioassays have been widely and successfully applied for water quality monitoring, benchmarking of water quality and assessment of treatment technologies in a research context (Escher and Leusch, 2011) but they have not been used for regulatory purposes. The bioluminescence inhibition assay with *Vibrio fischeri* and other related bioluminescent bacterial assays have been used for many years to assess water quality (Johnson, 2005; ISO11348-1 2007) due to their ease of operation, rapidity and high sensitivity to organic chemicals and because their effect concentrations are highly correlated to other aquatic toxicity endpoints (e.g., Kaiser, 1993; 1998). The bioluminescence inhibition assay with *V. fischeri* has also been widely used to test mixture toxicity hypotheses (Altenburger et al., 2000; Backhaus et al., 2000) and to develop Quantitative Structure–Activity Relationships (QSARs) for the prediction of effect concentrations of untested chemicals using the octanol–water partition coefficient of the chemicals (selected examples are (Cronin and Schultz, 1997; Zhao et al., 1998; Vighi et al., 2009)).

Effect-based trigger values provide the opportunity to integrate mixtures into water quality assessment. Trigger values are numerical values that indicate an acceptable risk to the environment or human health provided they are not exceeded. The classical approach to setting effect-based trigger values would relate the outcomes of *in vitro* bioassays directly to adverse health outcomes but *in vitro* to *in-vivo* extrapolations have many limitations. Therefore we propose as an alternative approach to translate existing individual chemical based water quality guideline values directly to effect-based trigger values (Fig. 1).

In a first step we tested if chemicals typically encountered in water samples will fit QSAR models developed with known baseline toxicants (Section 3.1) and if the mixture effect of large numbers of chemicals commonly occurring in water, mixed in equipotent concentration ratios (Section 3.2) and in water quality guideline concentrations ratios (Section 3.3), can be predicted by the CA model of mixture toxicity. From these models we computed tentative effect-based trigger values (Section 3.4). We then validated the proposed approach using a diverse set of water samples, where we assessed both the effect with the bioluminescence inhibition assay with *V. fischeri* and quantified 269 chemicals analytically (Section 3.5). We mixed the detected chemicals in their encountered concentration ratios and called them "iceberg mixtures" (strictly speaking they should be called "tip-of-the-iceberg mixtures") as they constitute the known chemicals (tip of the iceberg) among the unknown complex mixture of chemicals in environmental samples (immersed part of the iceberg) together causing the observed mixture effect in an environmental water sample. The iceberg mixtures were tested for compliance with mixture toxicity predictions (Section 3.6) and it was independently assessed how much of the measured effect can be explained by the analytically quantified chemicals (Section 3.7).

As a case study we used water quality data and guideline values from Australia but the concepts are generic and can be

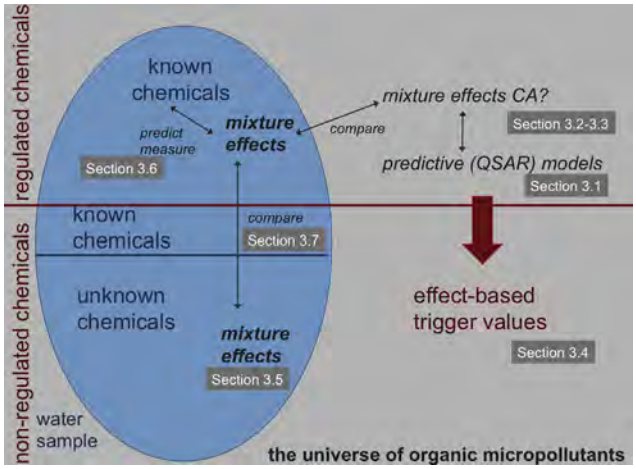

Fig. 1 – Approach taken in this paper to evaluate the contribution of known and unknown chemicals in a water sample and to derive effect-based trigger values, with paper sections where the different points will be addressed.

adapted to different types of water, and guideline documents and legislation with the equations developed here. In Australia 181 organic micropollutants have health-based guideline values in drinking water as described in the Australian Drinking Water Guidelines (ADWG) (NHMRC and NRMMC, 2011). The Australian Guidelines for Water Recycling: Augmentation of Drinking Water Supplies (AGWR), which were developed to support the augmentation of drinking water supplies with reclaimed water (NRMMC, EPHC and NHMRC, 2008) contain 349 health-based values for organic chemicals, many of which overlap with the ADWG. Jointly the two guidance documents contain 381 organic chemicals. In samples of wastewater, recycled water, surface water and drinking water from various sites in South East Queensland, we detected as many as 64 of the regulated chemicals out of an analytical test set of 269 organic chemicals. This set of detected chemicals, which was mainly comprised of pharmaceuticals, pesticides and some consumer product chemicals, was taken as the base list of chemicals for this study.

2. Material and methods

2.1. Chemicals

Phenol was used as a positive control and for quality assurance and quality control (QA/QC). The reference baseline toxicants were 2-butoxyethanol, 3-nitroaniline, 2-nitrotoluene, 4-n-pentylphenol, 2-phenylphenol, and 2,4,5-trichloroaniline. The 64 chemicals used in the mixture experiments are listed in Table 1, with the additional chemicals tested as single compounds listed in the Supplementary Information, Table SI-1. The chemical manufacturers and purity of the compounds are listed in the Table SI-2.

2.2. Water samples

Ten grab water samples were collected in December 2011 and January 2012 from various sites in South East Queensland. They constitute a subset of samples that were previously investigated with a large battery of bioassays (Macova et al., 2011). One sample (Eff-1) is a secondary treated sewage effluent plant that serves as the influent to an Advanced Water Treatment Plant (Escher et al., 2011b). Three samples were taken in this plant, after microfiltration (MF), reverse osmosis (RO) and advanced oxidation (AO). The second plant investigated was an Enhanced Water Treatment Plant, where secondary treated sewage effluent (Eff-2) is ozonated followed by biologically activated carbon filtration (O_3/BAC) (Reungoat et al., 2010, 2011, 2012). River and drinking water (DW) samples were the influent and effluent of a drinking water treatment plant (Neale et al., 2012). Stormwater (SW) samples were taken from a stormwater collection site in the northern Brisbane suburb of Fitzgibbon after a rain event. The laboratory blank was ultrapure water run through the same solid phase extraction process as the samples. All samples were extracted as described previously (Macova et al., 2011; NWC, 2011), for details see Supplementary Information, Section SI-1.

2.3. Chemical analysis of the water samples

269 chemicals were quantified using the standard GC–MS and LC–MS methods by Queensland Health Forensic and Scientific Services (QHFSS), which is a commercial analytical laboratory running NATA (National Association of Testing Authorities, Australia) accredited analytical methods. Target chemicals were pharmaceuticals, pesticides, endocrine disruptors and some consumer products. Information on the analysed chemicals, the analytical methods and their detection limits are given in the Supplementary Information, Section SI-2 and Table SI-3.

2.4. Bioluminescence inhibition test with V. fischeri

This bioassay is also commercially available under the trade name Microtox (Johnson, 2005). *V. fischeri* were cultured according to a protocol modified from the European Union Waste Ringtest 2006/2007 by Becker et al. (2005). The assay was performed according to the ISO standard method 11348-1 (ISO11348-1, 2007) modified to a 96 well plate format according to Escher et al. (2008) and details are given in the Supplementary Information, Section SI-3.

The inhibition of bioluminescence was calculated as described in ISO standard method 11348-3 (ISO, 1998) and the median effect concentrations EC_{50} were calculated with Equation (1), where s is the slope of the concentration effect curve.

$$\text{inhibition}[\%] = \frac{100\%}{1 + 10^{s\cdot(\log EC_{50} - \log \text{concentration})}} \quad (1)$$

The EC_{50} values of the individual chemicals are given in units of mol/L because the mixture toxicity concept cannot be applied in a simple way with mass-based concentrations (μg/L). The EC_{50} values of the designed mixtures with known composition are given as the sum of concentrations in the mixture (mol/L). The EC_{50} values of the environmental samples are given in units of relative enrichment factors (REF). A REF of 10 relates to a 10 times enriched sample in the bioassay and a REF of 0.1 to a 10 times diluted sample in the bioassay and a REF of 1 is equivalent to the original water sample. Effects in the environmental samples were also expressed as baseline toxicity equivalents (baseline-TEQ) (Escher et al., 2008).

2.5. QSAR

The baseline toxicity QSAR has the form given in Equation (2). Typically the hydrophobicity descriptor chosen would be the octanol–water partition coefficient, K_{ow}, but it has been demonstrated that the liposome-water partition coefficient K_{lipw} is a better descriptor as it allows for the development of a common QSAR for polar and nonpolar baseline toxicants (Vaes et al., 1998). Since some of the investigated chemicals are acids or bases that are charged at pH 7, we replaced the K_{lipw} with the liposome–water distribution ratio at pH 7, D_{lipw}(pH7), when applying the QSAR.

$$\log(1/EC_{50}(\text{M})) = \text{slope} \log K_{lipw} (\text{or} \log D_{lipw}(\text{pH7})) + \text{intercept} \quad (2)$$

Table 1 – Chemicals used in the mixture experiments, their physicochemical descriptors, experimental and modelled EC_{50} values and fractions in the 56-component equipotent mixture.

Chemical	CAS number	Molar weight (g/mol)	ADWG guideline value (μg/L)[a]	AGWR guideline value (μg/L)[b]	log K_{ow}	log K_{lipw} (L/kg)	log D_{lipw} (pH7) (L/kg)	log 1/EC_{50} (M) QSAR	EC_{50} (g/L) QSAR	Measured log 1/EC_{50} (M)	Std. dev	Fraction f_i in equipotent mixture
17β-Estradiol	50-28-2	272.39		0.175	4.01	4.16	4.16	3.86	0.04			1.06E-04
2-Methyl-4-chlorophenoxyacetic acid (MCPA)	94-74-6	200.62	40	2	3.25	3.39	2.39	2.62	0.48	3.81	0.28	1.81E-03
2,4-Dichlorophenoxyacetic acid (2,4-D)	94-75-7	221.04	30	30	2.81	2.94	1.94	2.31	1.08			3.74E-03
4-Nonylphenol (4NP)	104-40-5	220.36		0.5	5.76	5.95	5.95	5.10	0.002			5.95E-06
4-Tert Octylphenol	140-66-9	206.33		0.05	5.28	5.46	5.46	4.76	0.004			1.32E-05
6-Acetyl-1,1, 2,4,4, 7-hexamethyltetraline (AHTN, Tonalide)	21145-77-7	258.41		4	5.70	5.88	5.88	5.06	0.002			7.95E-06
Acetylsalicylic acid (Aspirin)	50-78-2	180.16		29	1.19	1.28	0.29	1.16	12.52			5.30E-02
Atenolol	29122-68-7	266.34		25	0.16	0.23	−0.73	0.45	95.14	1.42	0.56	2.72E-01
Atorvastatin	134523-00-5	558.66		5	6.36	6.56	5.56	4.84	0.01			1.13E-05
Atrazine	1912-24-9	215.69	20[c]	40	2.61	2.73	2.73	2.86	0.29	2.61	0.39	1.04E-03
Bisphenol A	80-05-7	228.29		0.2	3.32	3.46	3.46	3.37	0.10			3.28E-04
Caffeine	58-08-2	194.19		0.35	−0.07	0.00	0.00	0.96	21.39	2.11	0.51	8.40E-02
Carbamazepine	298-46-4	236.28		100	2.45	2.57	2.57	2.75	0.42			1.62E-03
Cephalexin	15686-71-2	347.39		35	0.65	0.73	0.32	1.18	22.92			5.03E-02
Chlorpyrifos	2921-88-2	350.59	10	10	4.96	5.13	5.13	4.54	0.01			2.23E-05
Citalopram	59729-33-8	324.4		4	3.74	3.88	2.89	2.98	0.34	2.96	0.23	8.11E-04
Codeine	76-57-3	299.37		50	1.19	1.28	1.28	1.85	4.19			1.28E-02
Cyclophosphamide	50-18-0	261.09		3.5	0.63	0.71	0.71	1.46	9.14	1.94	0.14	2.64E-02
Desmethylcitalopram	144025-14-9[d]	310.37[e]		4	3.53	3.67	2.67	2.82	0.47			1.38E-03
Desmethyldiazepam (Nordazepam)	1088-11-5	270.72		3	2.93	3.06	3.06	3.09	0.22			7.41E-04
Diazepam (Valium)	439-14-5	284.75		2.5	2.82	2.95	2.95	3.01	0.28			8.87E-04
Diazinon	333-41-5	304.35	4	3	3.81	3.96	3.96	3.72	0.06			1.46E-04
Dicamba	1918-00-9	221.04	100	100	2.21	2.32	1.32	1.88	2.90	2.73	0.32	1.00E-02
Diclofenac	15307-86-5	296.15		1.8	4.51	4.67	3.68	3.52	0.09	3.48	0.28	2.32E-04
Diuron	330-54-1	233.1	20	30	2.68	2.80	2.80	2.91	0.28			9.31E-04
Doxycycline	564-25-0	444.44		10.5	−0.02	0.05	0.01	0.97	48.06	4.63	0.07	–
Erythromycin	114-07-8	733.95		17.5	3.06	3.19	2.39	2.62	1.75			–
Fipronil	120068-37-3	437.15	0.7		4.00	4.15	4.15	3.85	0.06			1.07E-04
Fluoxetine (Prozac)	2-84-9	309.33		10	4.05	4.20	3.21	3.20	0.20	3.91	0.21	1.06E-04
Furosemide or Frusemide	54-31-9	330.74		10	2.03	2.14	1.14	1.75	5.82			1.34E-02
Galaxolide (1,3,4,6,7, 8-Hexahydro-4,6,6,7,8, 8-hexamethylcyclopenta [g]-2-benzopyran, HHCB)	1222-05-5	258.41		1800	5.90	6.09	6.09	5.20	0.00			5.71E-06
Gemfibrozil	25812-30-0	250.34		600	4.77	4.94	3.95	3.71	0.05	2.99	0.40	1.48E-04
Hexazinone	51235-04-2	252.32	400	300	1.85	1.96	1.00	1.66	5.56	2.65	0.46	3.59E-03

(continued on next page)

Table 1 – (*continued*)

Chemical	CAS number	Molar weight (g/mol)	ADWG guideline value (μg/L)[a]	AGWR guideline value (μg/L)[b]	log K_{ow}	log K_{lipw} (L/kg)	log D_{lipw} (pH7) (L/kg)	log 1/EC_{50} (M) QSAR	EC_{50} (g/L) QSAR	Measured log 1/EC_{50} (M)	Std. dev	Fraction f_i in equipotent mixture
Hydrochlorthiazide	58-93-5	297.73		12.5	−0.07	0.00	−0.02	0.95	33.52			8.43E-02
Ibuprofen	15687-27-1	206.29		400	3.97	4.12	3.13	3.14	0.15	3.07	0.24	1.14E-04
Indomethacin	53-86-1	357.8		25	4.27	4.43	3.44	3.36	0.16	3.74	0.06	6.81E-05
Lincomycin	154-21-2	406.54		3500	0.20	0.27	−0.46	0.64	93.83	1.81	0.17	–
Metolachlor	51218-45-2	283.8	300	300	3.13	3.26	3.26	3.23	0.17			4.47E-04
Metoprolol	37350-58-6	267.37		25	1.88	1.99	1.02	1.67	5.73	2.25	0.53	1.96E-02
Naproxen	22204-53-1	230.27		220	3.18	3.31	2.33	2.58	0.60	2.81	0.34	2.41E-03
Norflaxin	70458-96-7	319.34		400	−1.03	−0.98	−1.00	0.26	175.27			–
Oxazepam	604-75-1	286.72		15	2.24	2.35	2.35	2.60	0.72			2.29E-03
Oxycodone	76-42-6	315.37		10	0.66	0.74	−0.09	0.89	40.23			–
Oxytetracycline	79-57-2	460.44		105	−0.90	−0.85	−1.76	−0.27	853.4	3.20[f]	0.14	–
Paracetamol (acetaminophen)	103-90-2	151.17		175	0.46	0.54	0.54	1.33	6.99	1.49		3.50E-02
Picloram	1918-02-1	241.46	300	300	1.90	2.01	1.01	1.66	5.26			1.66E-02
Praziquantel	55268-74-1	312.42		70	2.42	2.54	2.54	2.73	0.58	2.66	0.78	1.45E-03
Propoxur	114-26-1	209.25		70	1.52	1.62	1.62	2.09	1.71	3.64	0.34	6.19E-03
Propranolol	525-66-6	259.35		40	3.48	3.62	2.66	2.81	0.40	2.56	0.89	1.19E-03
Ranitidine	66357-35-5	314.41		26	0.27	0.35	−0.61	0.53	92.34	0.67	0.31	2.24E-01
Roxithromycin	80214-83-1	837.07		150	2.75	2.88	2.29	2.56	2.32	Not	Active	–
Simazine	122-34-9	201.66	20	20	2.18	2.29	2.29	2.56	0.56			2.53E-03
Sulfadiazine	68-35-9	250.28		35	−0.09	−0.02	−0.87	0.35	111.07			–
Sulfamethoxazole	723-46-6	253.28		35	0.89	0.98	0.98	1.64	5.79	2.06	0.10	1.74E-02
Sulfasalazine	599-79-1	398.4		500	3.81	3.96	2.96	3.02	0.38	3.50	0.29	7.30E-04
Temazepam	846-50-4	300.75		5	2.19	2.30	2.30	2.57	0.82			2.49E-03
Tributylphosphate	126-73-8	266.32		0.5	4.00	4.15	4.15	3.85	0.04			1.09E-04
Triclopyr	55335-06-3	256.47	20	10	2.53	2.65	1.65	2.11	1.99			5.89E-03
Triclosan	3380-34-5	289.55		0.35	4.76	4.93	4.89	4.37	0.01			3.26E-05
Trimethoprim	738-70-5	290.32		70	0.91	1.00	0.88	1.57	7.81			2.05E-02
Tris(2-chloroethyl) phosphate (TCEP)	115-96-8	285.49		1	1.44	1.54	1.54	2.03	2.65			7.06E-03
Venlafaxine	93413-69-5	277.41		75	3.28	3.42	2.42	2.65	0.63			1.72E-03
DEET (N,N-diethyltoluamide (N,N-diethyl-3-methylbenzamide))	134-62-3	191.28		2500	2.18	2.29	2.29	2.56	0.53			2.53E-03
5-Methyl-1H-benzotriazole (tolutriazole)	136-85-6	133.15		0.007	1.71	1.81	1.81	2.22	0.80			5.39E-03

a (NHMRC & NRMMC, 2011).
b (NRMMC & EPHC & NHMRC, 2008).
c Total, including metabolites.
d Hydrochloride.
e Free acid.
f Experimental data for tetracycline.

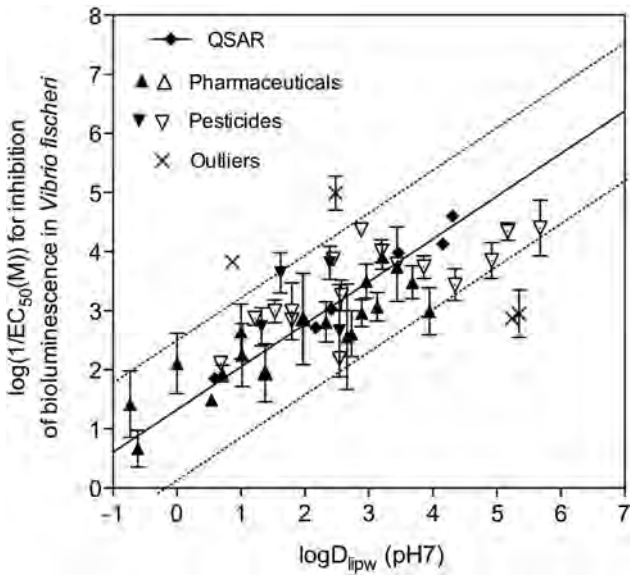

Fig. 2 – Median effect concentration (EC_{50}) values (Table 1) of pharmaceuticals (triangles facing up, filled: pharmaceuticals detected in water samples, empty: additional pharmaceuticals) and pesticides (triangles facing down, filled: pesticides detected in water samples, empty: additional pesticides). Four outliers are marked with an ×.

The K_{lipw} values of the reference baseline toxicants were measured values (Vaes et al., 1997), and the D_{lipw}(pH7) values of the pesticides and pharmaceuticals were estimated from the K_{ow} values (retrieved from databases, e.g., EPA, 2009, as described in Hawker et al., 2011) using the QSAR equation developed by Endo et al. (2011).

The D_{lipw}(pH7) is the sum of the products of the fraction of a given chemical species j and the K_{lipw} of this species (Equation (3)) and was estimated with Equation (4) (Escher et al., 2011a).

$$D_{lipw}(pH7) = \sum_j f_j \cdot K_{lipw,j} \quad (3)$$

$$D_{lipw}(pH7) = f_{neutral} \cdot K_{lipw,neutral} + \sum_j f_{j,charged} \cdot \frac{K_{lipw,neutral}}{10} \quad (4)$$

The fraction of neutral chemical species, $f_{neutral}$, was calculated from the acidity constants, which were calculated with SPARC (Hilal et al., 2005).

A measure of the specificity of the effect of a compound is the toxic ratio TR_i (Verhaar et al., 1996; Maeder et al., 2004), which is the quotient of the EC_{50} predicted with the baseline toxicity QSAR $EC_{50\ baseline\text{-}QSAR,i}$ and the experimental $EC_{50\ experimental,i}$ (Equation (5)). If the TR_i exceeds 10, then a chemical is considered to exhibit a specific mode of toxic action (Verhaar et al., 1996; Maeder et al., 2004).

$$TR_i = \frac{EC_{50\ baseline-QSAR,i}}{EC_{50\ experimental,i}} \quad (5)$$

2.6. Mixture experiments

All mixture experiments were conducted at a fixed concentration ratio and full concentration effect curves were measured. Three different concentration ratios were used to create the mixtures assessed in this study. The first type of concentration ratio was equipotent, i.e., the concentrations were normalized to their effect concentrations so that each chemical should have the same contribution to the mixture effect (Supplementary Information, Table SI-5). The second concentration ratio was the ratio of the guideline values found in the ADWG and AGWRs (abbreviated as ADWG mixture from here on, Supplementary Information, Table SI-6). If there were different guideline values for a given chemical in the two guidelines, the higher concentration was chosen for calculating the ratios in the ADWG mixture experiments. The third concentration ratio was the ratio of the chemical concentrations detected in the environmental samples (iceberg mixtures, Supplementary Information, Table SI-7).

The experimental mixture EC_{50} values ($EC_{50,mixture}$) were compared with predictions for mixture effects according to the IA and CA models. For a mixture with n components i, the

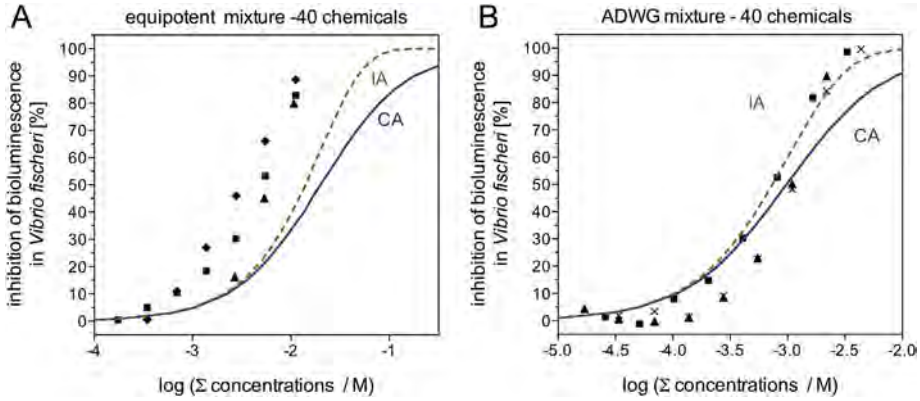

Fig. 3 – Concentration-effect curves of A. the equipotent mixture of 40 chemicals, and B. the mixture of 40 chemicals in concentration ratios of the drinking water/recycled water guidelines (ADWG mixture). The different symbols represent independent experiments. The predictions for concentration addition (CA) are the blue solid lines, the predictions for independent action (IA) are the green broken lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2 – Experimental median effect concentration EC_{50} values of the mixtures and comparison with prediction for concentration addition (CA) and independent action (IA).

	Prediction for concentration addition		Prediction for independent Action		Experimental		# Components	cTEI
	log EC_{50} (CA)	EC_{50} (CA)	log EC_{50} (IA)	EC_{50} (IA)	log EC_{50} (M)	EC_{50} (M)		
Equipotent mixtures								
EP1	−1.36	$4.35\cdot10^{-2}$	−1.51	$3.12\cdot10^{-2}$	−2.03	$9.39 \pm 1.19\cdot10^{-3}$	10	3.63
EP2	−2.15	$7.13\cdot10^{-3}$	−2.29	$5.12\cdot10^{-3}$	−2.64	$2.28 \pm 0.47\cdot10^{-3}$	10	2.12
EP3	−1.64	$2.29\cdot10^{-2}$	−1.78	$1.64\cdot10^{-2}$	−2.24	$5.77 \pm 1.10\cdot10^{-3}$	10	2.96
EP4	−2.19	$6.45\cdot10^{-3}$	−2.33	$4.63\cdot10^{-3}$	−2.97	$1.07 \pm 0.24\cdot10^{-3}$	10	5.02
EP5	−1.70	$2.00\cdot10^{-2}$	−1.85	$1.40\cdot10^{-2}$	−2.35	$4.50 \pm 1.38\cdot10^{-3}$	40	3.44
EP6	−1.61	$2.45\cdot10^{-2}$	−1.77	$1.72\cdot10^{-2}$	−2.06	$8.72 \pm 5.01\cdot10^{-3}$	30	1.81
EP7	−1.92	$1.21\cdot10^{-2}$	−2.07	$8.55\cdot10^{-3}$	−2.47	$3.42 \pm 0.25\cdot10^{-3}$	30	2.55
EP8	−1.61	$2.43\cdot10^{-2}$	−1.77	$1.70\cdot10^{-2}$	−2.23	$5.86 \pm 2.41\cdot10^{-3}$	30	3.14
EP9	−1.72	$1.90\cdot10^{-2}$	−1.88	$1.33\cdot10^{-2}$	−2.31	$4.86 \pm 1.55\cdot10^{-3}$	30	2.92
EP10	−1.60	$2.53\cdot10^{-2}$	−1.75	$1.79\cdot10^{-2}$	−1.93	$1.16 \pm 0.20\cdot10^{-2}$	20	1.17
EP11	−2.17	$6.79\cdot10^{-3}$	−2.25	$5.64\cdot10^{-3}$	−2.78	$1.65 \pm 0.54\cdot10^{-3}$	20	3.12
EP12	−1.83	$1.47\cdot10^{-2}$	−1.98	$1.04\cdot10^{-2}$	−2.49	$3.23 \pm 0.50\cdot10^{-3}$	20	3.53
EP13	−1.63	$2.36\cdot10^{-2}$	−1.79	$1.64\cdot10^{-2}$	−2.34	$4.56 \pm 1.01\cdot10^{-3}$	56	4.17
ADWG mixtures								
ADWG1	−3.51	$3.07\cdot10^{-4}$	−3.61	$2.46\cdot10^{-4}$	−3.04	$9.17 \pm 2.49\cdot10^{-4}$	10	−1.99
ADWG2	−2.64	$2.31\cdot10^{-3}$	−2.70	$2.01\cdot10^{-3}$	−2.92	$1.21 \pm 0.10\cdot10^{-3}$	10	0.91
ADWG3	−3.22	$6.02\cdot10^{-4}$	−3.30	$4.98\cdot10^{-4}$	−2.94	$1.15 \pm 0.24\cdot10^{-3}$	10	−0.90
ADWG4	−2.50	$3.18\cdot10^{-3}$	−2.62	$2.40\cdot10^{-3}$	−2.92	$1.21 \pm 0.20\cdot10^{-3}$	10	1.63
ADWG5	−3.01	$9.81\cdot10^{-4}$	−3.15	$7.10\cdot10^{-4}$	−3.04	$9.12 \pm 1.96\cdot10^{-4}$	40	0.07
ADWG6	−3.02	$9.51\cdot10^{-4}$	−3.14	$7.17\cdot10^{-3}$	−3.03	$9.23 \pm 2.17\cdot10^{-4}$	30	0.03
ADWG7	−2.97	$1.07\cdot10^{-3}$	−3.08	$8.38\cdot10^{-3}$	−3.01	$9.66 \pm 0.87\cdot10^{-4}$	30	0.10
ADWG8	−3.22	$6.03\cdot10^{-4}$	−3.32	$4.74\cdot10^{-3}$	−3.14	$7.31 \pm 2.82\cdot10^{-4}$	30	−0.21
ADWG9	−2.77	$1.69\cdot10^{-3}$	−2.89	$1.30\cdot10^{-3}$	−2.90	$1.25 \pm 0.44\cdot10^{-3}$	30	0.35
ADWG10	−2.79	$1.63\cdot10^{-3}$	−2.89	$1.28\cdot10^{-3}$	−2.92	$1.21 \pm 0.25\cdot10^{-3}$	20	0.34
ADWG11	−2.63	$2.36\cdot10^{-3}$	−2.67	$2.13\cdot10^{-3}$	−2.87	$1.36 \pm 0.36\cdot10^{-3}$	20	0.73
ADWG12	−2.85	$1.42\cdot10^{-3}$	−2.85	$1.42\cdot10^{-3}$	−3.02	$9.46 \pm 0.23\cdot10^{-4}$	20	0.50
Iceberg mixtures								
Iceberg Eff-1	−4.25	$5.61\cdot10^{-5}$	−4.29	$5.18\cdot10^{-5}$	−3.28	$5.3 \pm 2.0\cdot10^{-4}$	40	−8.44
Iceberg MF	−4.26	$5.45\cdot10^{-5}$	−4.26	$5.45\cdot10^{-5}$	−3.30	$5.0 \pm 1.9\cdot10^{-4}$	39	−8.14
Iceberg RO	−2.47	$3.35\cdot10^{-3}$	−2.71	$1.97\cdot10^{-3}$	−3.70	$2.0 \pm 0.5\cdot10^{-4}$	6	15.75
Iceberg Eff-2	−3.65	$2.22\cdot10^{-4}$	−3.74	$1.82\cdot10^{-4}$	−3.27	$5.4 \pm 0.8\cdot10^{-4}$	48	−1.43
Iceberg O3/BAC	−2.39	$4.10\cdot10^{-3}$	−2.56	$2.73\cdot10^{-3}$	−2.54	$2.9 \pm 1.3\cdot10^{-3}$	6	0.43
Iceberg SW	−3.00	$1.00\cdot10^{-3}$	−3.07	$8.52\cdot10^{-4}$	−3.96	$1.1 \pm 0.2\cdot10^{-4}$	5	8.18

biological effect of the mixture ($effect_{IA}$) according to the IA model is

$$effect_{IA} = 1 - \prod_{i=1}^{n}(1 - effect_i) \quad (6)$$

where $effect_i$ is the fractional biological effect of component i at the concentration in the mixture and Π stands for the product (multiplication).

For a mixture of n components i, present in fractions p_i, the EC_{50} of the mixture, $EC_{50,CA}$, according to the CA model is:

$$EC_{50,CA} = \frac{1}{\sum_{i=1}^{n}\frac{p_i}{EC_{50,i}}} \quad (7)$$

A measure for the deviation from CA is the corrected toxicity enhancement index cTEI (Warne and Hawker, 1995), also called the index on prediction quality (Altenburger et al., 1996) or relative model deviation ratio or effect residual ratio (Wang et al., 2010). The cTEI is defined by Equations (8) and (9).

$$\text{If } EC_{50,CA} > EC_{50,mixture} \text{ then } cTEI = \frac{EC_{50,CA}}{EC_{50,mixture}} - 1 \quad (8)$$

$$\text{If } EC_{50,CA} < EC_{50,mixture} \text{ then } cTEI = 1 - \frac{EC_{50,mixture}}{EC_{50,CA}} \quad (9)$$

A ratio of two between CA prediction ($EC_{50,CA}$) and the experimental $EC_{50,mixture}$ yields a cTEI of −1 (if CA is more potent than the experiment) and +1 (if CA is less potent than the experiment), a ratio of 3 yields a cTEI of ±2, a ratio of 4 yields a cTEI of ±3 etc.

3. Results and discussion

3.1. QSAR for baseline toxicity in bioluminescence inhibition assay with V. fischeri

The baseline toxicity QSAR derived for the six known baseline toxicants (Equation (10)) was of similar sensitivity to the previously published QSAR for the 96 well plate assay and the

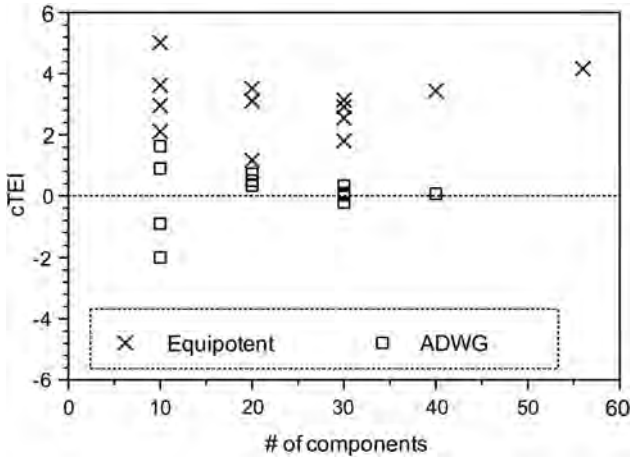

Fig. 4 – Deviation from the concentration addition (CA) prediction expressed as corrected toxicity enhancement index cTEI (Equations (8) and (9)) in relation to the number of components in the mixture.

classical cuvette version of the assay performed in various laboratories (Cronin and Schultz, 1997; Escher et al., 2008; Vighi et al., 2009; Aruoja et al., 2011). For more details see Supplementary Information, Section SI-4, Figs. SI-1 and SI-2 and Table SI-4.

$$\log(1/EC_{50}(M)) = (0.72 \pm 0.06)\log K_{lipw} + (1.32 \pm 0.18); \quad r^2 = 0.975, n = 6, F = 155 \tag{10}$$

Although pharmaceuticals and pesticides typically exhibit specific modes of action and should thus be more toxic than baseline toxicity, almost all fell within one order of magnitude of the baseline toxicity QSAR (Equation (10)), with TR-values below 10 (Fig. 2) and they can therefore be classified as acting as baseline toxicants in this assay. The two outliers with TRs of 200 and 180 were carbaryl and dimethoate, respectively, and the reason for the high TR, which classifies them as specifically acting or reactive toxicants, is unknown. Antibiotics are known to be specifically acting in bacteria (Backhaus and Grimme, 1999, see also Supplementary Information, Section SI-4 and Fig. SI-3) and were therefore omitted from the QSAR. The equipotent mixture was prepared without the antibiotics but they were included in the iceberg mixtures. There was a general trend for the more hydrophobic chemicals to level off slightly from the QSAR line, indicating a general experimental problem with more hydrophobic compounds, whose uptake kinetics might not come into steady state during the 30 min incubation time or they might be sorbed to the plastic of the 96-well plates. Ethion (log K_{ow} 5.7) and pendimethalin (log K_{ow} 5.2) had a TR of 0.02 and are examples of this effect.

3.2. *Equipotent mixtures of 10–56 organic micropollutants*

Ten to 40 chemicals were mixed in ratios of their predicted EC_{50} values (equipotent mixtures (EP), Supplementary Information, Table SI-5). In addition, a subset of 56 chemicals of the iceberg mixture chemicals was also mixed in equipotent concentration ratios (Table 1). The only antibiotic included in the 56-component EP mixture was sulfamethoxazole as its EC_{50} matched the baseline toxicity QSAR despite the fact that antibiotics otherwise showed a specific mode of action and higher toxicity than baseline in this bioassay. The concentration–effects curve for a 40-component mixture was compared with predictions for CA and IA in Fig. 3A and all other results are in the Supplementary Information, Fig. SI-4. The CA and IA models gave very similar predictions (Table 2 and Supplementary Information, Fig. 3A and SI-4), which is not unusual for mixtures with a large number of components (Backhaus et al., 2000; Dyer et al., 2000; Chevre et al., 2006; Junghans et al., 2006).

The experimental EP mixtures were more active than predicted by the CA model by a factor of two to six (Table 2). This deviation is within the uncertainty of the prediction method because the EC_{50} values of the individual chemicals were predicted from the baseline toxicity QSAR (Equation (10)) and the experimental data for the tested chemicals from the ADWG list differed by up to a factor of ten (corresponding to $0.1 < TR < 10$) from the QSAR prediction. Thus we can conclude that the deviations from the CA model are within the range to be expected. While the data do not support one model over the other (CA or IA), the baseline QSAR derived suggests a common mode of toxic action, i.e., baseline toxicity, and thus CA is likely to be valid for chemicals occurring in water.

In accordance with a common mode of action the cTEI of the EP ratio mixtures were in all cases lower than ±5 (Fig. 4) and there was no clear trend between cTEI and the number of components in a mixture.

3.3. *Mixtures with the concentration ratio of the water quality guideline values*

The mixtures developed using the concentration ratios of the water quality guideline values differed greatly from an equipotent mixture ratio (ADWG mixtures, Supplementary Information, Table SI-6) because the guideline values are not correlated to the EC_{50} values from the Microtox assay (Supplementary Information, Fig. SI-5). Nevertheless in all the ADWG mixtures the experimental EC_{50} values generally agreed very well with the predictions by CA (Fig. 3B for the 40-

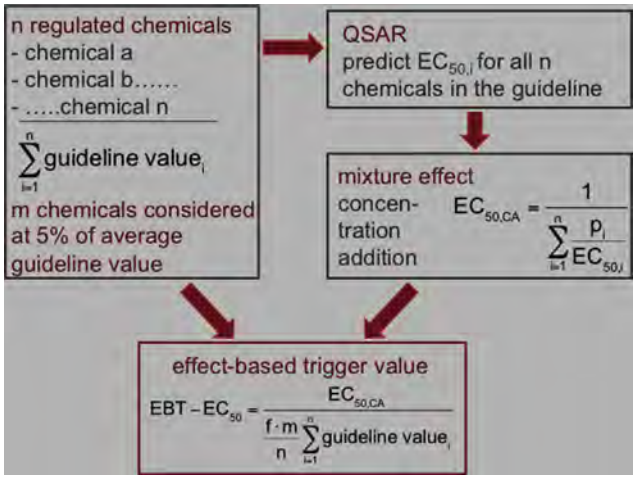

Fig. 5 – Derivation of the effect-based trigger value EBT-EC_{50} according to Equations (11) and (12).

Table 3 – Experimental median effect concentrations EC_{50} and baseline-TEQs of the environmental samples and the iceberg mixtures.

Sampling site	Secondary treated effluent (influent to MF)	After micro-filtration	After reverse osmosis	After advanced oxidation	Secondary effluent (influent to O_3/BAC)	After ozonation and biologically activated carbon filtration	Drinking water plant influent (river)	Drinking water plant outlet	Storm-water	Lab blank
Sample name	Eff-1	MF	RO	AO	Eff-2	O_3/BAC	River	DW	SW	Blank
EC_{50}(REF)	4.16	6.09	6.06	50.57	2.98	10.27	12.93	3.43	9.21	77.03
Standard deviation of mean	0.24	0.47	1.40	0.07	0.26	0.07	0.63	0.06	3.28	22.90
Baseline-TEQ (mg/L)	16.0	10.7	10.3	1.2	20.3	5.6	4.4	18.1	6.9	0.9
Standard deviation (error propagation)	1.9	1.4	2.8	0.1	3.0	0.7	0.6	2.0	2.7	0.3
# of chemicals detected	40	39	6	0	48	6	0	0	5	0
Sum of concentration of detected chemicals (M)	$4.11\cdot10^{-8}$	$4.27\cdot10^{-8}$	$2.68\cdot10^{-9}$	<LOR	$9.45\cdot10^{-8}$	$1.73\cdot10^{-9}$	<LOR	<LOR	$1.85\cdot10^{-9}$	<LOR
Assumed error of mix 5%	$2.05\cdot10^{-9}$	$2.14\cdot10^{-9}$	$1.34\cdot10^{-10}$		$4.72\cdot10^{-9}$	$8.64\cdot10^{-11}$			$9.27\cdot10^{-11}$	
EC_{50}(M) of iceberg mix	$5.30\cdot10^{-4}$	$4.98\cdot10^{-4}$	$6.68\cdot10^{-5}$		$5.40\cdot10^{-4}$	$2.87\cdot10^{-3}$			$1.09\cdot10^{-4}$	
Standard deviation of mean	$2.02\cdot10^{-4}$	$1.90\cdot10^{-4}$	$1.53\cdot10^{-5}$		$8.48\cdot10^{-5}$	$1.31\cdot10^{-3}$			$2.35\cdot10^{-5}$	
EC_{50}(REF) of iceberg mix	12,912	11,662	24,972		5716	1,657,321			58,760	
Standard deviation (error propagation)	4964	4478	5856		942	761,723			13,008	
Baseline-TEQ (mg/L) of iceberg mix (experimental)	$5.16\cdot10^{-3}$	$5.72\cdot10^{-3}$	$2.67\cdot10^{-3}$		$1.17\cdot10^{-2}$	$4.02\cdot10^{-5}$			$1.13\cdot10^{-3}$	
Standard deviation (error propagation)	$2.05\cdot10^{-3}$	$2.27\cdot10^{-3}$	$6.81\cdot10^{-4}$		$2.25\cdot10^{-3}$	$1.89\cdot10^{-5}$			$2.76\cdot10^{-4}$	
% Baseline-TEQ explained by detected chemicals (experimental)	0.032%	0.054%	0.026%		0.057%	0.001%			0.016%	
Baseline TEQ (mg/L) of iceberg mix (QSAR)	$4.88\cdot10^{-2}$	$5.23\cdot10^{-2}$	$5.32\cdot10^{-5}$		$2.84\cdot10^{-2}$	$2.81\cdot10^{-5}$			$1.24\cdot10^{-4}$	
% Baseline-TEQ explained by detected chemicals (QSAR)	0.304%	0.489%	0.001%		0.140%	0.001%			0.002%	

component mixture, all other results in Table 2 and Supplementary Information, Fig. SI-6). The 10 component mixtures had the highest deviation of $-2 < cTEI < 2$ but the mixtures with 20–40 components had an almost perfect agreement with the CA model with $-0.2 < cTEI < 0.7$.

The experimental EC_{50} of various combinations of ADWG mixtures that contained 10, 20, 30, and 40 chemicals were used to estimate the effect at the sum of the corresponding guideline value concentrations to base the extrapolation of effect-based trigger values on broader experimental evidence. The predicted effects at guideline value concentrations increased linearly with increasing number of components from $0.56 \pm 0.05\%$ inhibition for the 10-component mixtures to 2.8% inhibition for the 40-component mixture ($r^2 = 0.74$) (Supplementary Information, Fig. SI-7). If the concentrations were scaled up to the sum of all guideline values (while keeping the composition of the twelve different mixtures constant), effects would be constant and would come to around 10% (there were two outliers for the 10-component mixture, Supplementary Information, Fig. SI-7). Thus we can conclude that independent of the composition of the sample, we are likely to encounter similar effects for multicomponent mixtures and the approach of extrapolating from experimental mixtures of a lower number of compounds to predicted mixture with a large number of compounds is robust.

3.4. *Derivation of tentative effect-based trigger value*

Having confirmed that the baseline toxicity QSAR is adequate to describe the effect in the bioluminescence inhibition assay with *V. fischeri* for most compounds (Section 3.1) and that CA

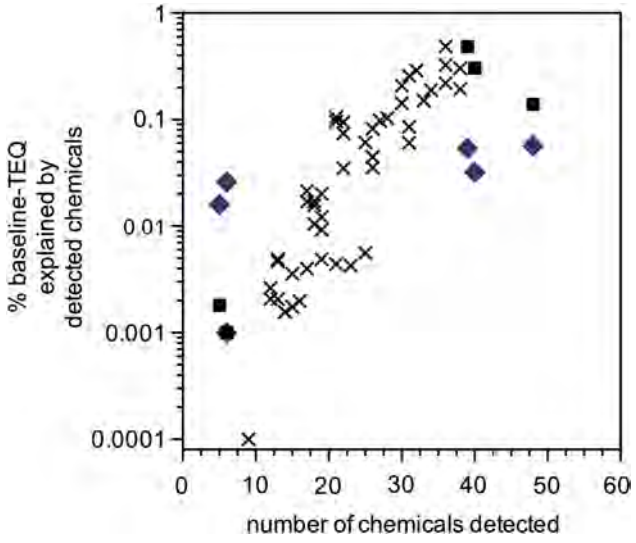

Fig. 6 – Relationship between the number of chemicals detected in environmental samples and the fraction of baseline-TEQ in the water samples explained by detected chemicals (from the iceberg mixtures). The diamonds refer to the experimental iceberg mixtures, the squares to the QSAR predictions of effects of the iceberg mixtures. For comparison, previously published data, partially from passive sampling experiments, is depicted with × (Reungoat et al., 2010; Escher et al., 2011b; Reungoat et al., 2011; Reungoat et al., 2012).

is a robust model for the mixture effect in this bioassay (Sections 3.2–3.3), we can now apply these models to derive tentative effect-based trigger values. As an example, we use the ADWG (NHMRC and NRMMC, 2011) and the AGWR (NRMMC, EPHC and NHMRC, 2008) but the principle can be applied to any set of water quality guidelines/criteria for any water type from wastewater to surface water.

The ADWG lists 181 unique organic chemicals. Sum parameters like total trihalomethanes and total trichlorobenzenes were omitted and for "pesticide plus metabolites" just the parent compound was used. In total, the sum of all guideline values comes to $1.79 \cdot 10^{-4}$ M. Using the liposome-water distribution ratios at pH 7 (D_{lipw}(pH7)) listed in Table 1 plus additional ones calculated using the same approach (Supplementary Information, Table SI-1), we can predict the EC_{50} for all individual chemicals with the QSAR of Equation (10). The resulting predicted EC_{50} values for individual chemicals ranged from $2 \cdot 10^{-8}$ to $4 \cdot 10^{-3}$ M. After computing the fraction p_i in a mixture of all regulated chemicals at guideline concentrations, we can use the CA model (Equation (7)) to predict $EC_{50,CA}$ of the total concentration of all 181 regulated chemicals, which is $1.47 \cdot 10^{-4}$ M. Thus if all chemicals were present at their guideline concentrations (resulting in a total concentration of $1.79 \cdot 10^{-4}$ M), the mixture would elicit 55% inhibition of bioluminescence in *V. fischeri*.

Applying the same approach to the 384 chemicals in the AGWR (NRMMC, EPHC and NHMRC, 2008) would result in an $EC_{50,CA}$ of $1.09 \cdot 10^{-4}$ M that corresponds to a 72% bioluminescence inhibition effect if all chemicals were present at their guideline concentrations (resulting in a total concentration of $2.75 \cdot 10^{-4}$ M). In this calculation stigmastanol (predicted log D_{lipw}(pH7) = 9.99, solubility 0.4 μg/L, guideline value 1000 μg/L) and cholesterol (predicted log D_{lipw}(pH7) = 8.98, solubility 3 μg/L, guideline value 7 μg/L) were omitted because as human endogenous compounds they have high guideline values but due to their exceedingly high hydrophobicity their toxicity cannot be predicted as they are well outside the validity range of the QSAR and they would also not be soluble enough in water.

The comparison of the AGWR mixture that contains 384 chemicals and the ADWG mixture that contains 181 chemicals demonstrates the limitation of this approach: if a guideline contains a larger number of chemicals, then invariably the mixture effect calculation will yield a higher effect level for the sum of the guideline values. Thus these mixture toxicity predictions for all regulated chemicals cannot be used directly for the derivation of effect-based water quality criteria, rather they need to be normalised to the number of chemicals that are contained in the given guideline document. If we normalised the acceptable effect level to the number of chemicals, we would actually base the trigger value on a single chemical with a quasi-average property of the mixture. This would be overprotective and ignores that there are many chemicals in a mixture. If, in contrast, we assume that many chemicals, e.g., more than 100 or 1000 are present at their guideline values, then the corresponding effect-based trigger value would be underprotective.

To account for this problem and to account for model uncertainties (Section 3.2), we propose that an extrapolation factor, EF, is included in the derivation of the effect-based

trigger value (Equation (11)). The EF should account for: the number of chemicals (m) that should be included in the derivation of the effect-based trigger value, model uncertainties, extrapolation from a few to many chemicals, and the fraction of chemical-based guideline values (f) that is acceptable if a large number of chemicals is included in the mixture calculation. For example if we account for 1000 chemicals, they cannot all be at their chemical-based trigger value but a lower fraction f, e.g., 5% of that value, should not be exceeded.

$$\mathrm{EF} = f \cdot m \tag{11}$$

The EF needs to be set to a number that is acceptable to the appropriate regulatory organisation and its choice is more a management decision than a scientific decision. For the purpose of demonstration of the principle we set the EF to 50. An EF of 50 corresponds to $m = 1000$ chemicals at $f = 0.05$, i.e., 5% of their guideline concentrations as the trigger threshold or, alternatively, 100 chemicals at 50% of their guideline concentrations or any combination thereof.

The effect-based trigger EC_{50}, EBT-EC_{50}, can then be calculated by Equation (12) and the derivation is also conceptualised in Fig. 5.

$$\mathrm{EBT-EC_{50}} = \frac{\mathrm{EC_{50,CA}}}{\mathrm{EF}}\left(\frac{1}{n}\sum_{i=1}^{n}\text{guideline value}_i\right)^{-1} \tag{12}$$

The sum of the guideline value refers to the sum of all concentrations for the n chemicals in a guideline and $EC_{50,CA}$ refers to the predicted mixture EC_{50} of the n-component mixture predicted by the QSAR (Equation (10)) and the CA model. The EBT-EC_{50} is an EC_{50} value and has the units of REF.

Insertion of EF 50 as an example into Equation (12) yields an EBT-EC_{50} for drinking water of 3 and for recycled water of 2.8. Thus if the EC_{50} of a drinking or recycled water sample is smaller than 3 or 2.8, respectively, it would trigger further higher tier investigation and chemical analytical identification of the chemicals in this sample.

This approach can be adapted to any type of water and associated set of water quality guideline values, including surface water, sewage, stormwater, product water from natural gas exploitation operations etc. It is the decision of the regulators to choose an appropriate extrapolation factor and to decide on the number of chemicals to be integrated in an effect-based trigger value. The resulting effect-based trigger value(s) can be very easily computed with the algorithm derived here (Equation (12)).

3.5. *Environmental samples*

Ten samples from wastewater to recycled water and drinking water were tested with the bioluminescence inhibition assay with *V. fischeri* and 269 chemicals were quantified using the standard GC–MS and LC–MS methods of a commercial analytical laboratory (QHFSS). 175 of those chemicals are also in the combined ADWG and AGWR list, and we focused on those included in the list of guideline values. The set of target analytes includes commonly used pesticides, pharmaceuticals and consumer products (Supplementary Information, Table SI-3). The analysed chemical were detected in 6 out of the 10 samples. Four samples (after advanced oxidation (AO), the samples taken at the drinking water treatment plant (river and DW), and the blank) were below the limit of reporting (LOR) for all targeted chemicals. In the secondary treated effluent samples (Eff-1 and Eff-2), 40 to 48 chemicals were detected, while the number of detections fell from 39 to 6 before and after reverse osmosis (RO) and from 48 to 6 before and after treatment with ozone and biologically activated carbon filtration (O_3/BAC). In the stormwater sample (SW), only 5 chemicals were above the LOR. The concentrations of chemicals detected in the samples (Supplementary Information, Table SI-7) were generally in agreement with previous work on the advanced and enhanced water treatment plants (Reungoat et al., 2010, 2011, 2012; Escher et al., 2011b).

The EC_{50} values were above the LOR in all samples (Table 3) and agreed reasonably well with previous work on the same sampling sites considering that these were grab samples taken in different seasons years apart (Supplementary Information, Fig. SI-8). The baseline-TEQs (Table 3) decreased in each treatment train, consistent with expectations and analytical data, and the increase in baseline-TEQ during drinking water treatment (from sample River to DW, Table 3) can be attributed to the formation of disinfection by-products (Neale et al., 2012).

The measured EC_{50} can now be compared to the EBT-EC_{50}. If the EC_{50} were lower than the EBT-EC_{50} (indicating higher toxicity) then further action should be triggered, if they are higher (indicating lower toxicity) the sample can be considered compliant. In Section 3.5, we derived an example EBT-EC_{50} of REF 3 for drinking water and 2.8 for recycled water. The recycled water samples (AO and O_3/BAC, Table 3) both had EC_{50} values clearly above the EBT-EC_{50} of REF 3, and therefore no further action is triggered.

The drinking water sample (DW) with an EC_{50} of 3.4 just complied with the EBT-EC_{50} of REF 2.8 for the ADWG. However, it must be noted that the guideline values are referring to micropollutants and only very few disinfection by-products are included. The increase in effect during drinking water treatment (from sample River to DW) is caused by disinfection by-products that are formed from the reaction of precursor organic matter and inorganic halide ions with disinfectants such as chlorine or chloramine (Neale et al., 2012). Thus we recommend using, in this case, the drinking water treatment plant influent to assess the micropollutants and to use the drinking water treatment plant outlet to assess the disinfection by-products. The influent sample (River) had an EC_{50} value well above the EBT-EC_{50} and thus no action is triggered. Effect-based trigger values for disinfection by-products could be derived with a similar approach but this topic is beyond the scope of the present study.

3.6. *Mixtures in the ratios of concentrations found in wastewater and recycled water (iceberg mixtures)*

All chemicals that were above the LOR were mixed in the concentration ratios as they were detected in the six environmental samples (Supplementary Information, Table SI-7). All concentration–effect curves of the iceberg mixtures are depicted in the Supplementary Information, Fig. SI-9, and the associated EC_{50} values for the experiments and the CA and IA predictions are given in Table 2.

For two of the iceberg mixtures (Eff-1 and MF) the CA predicted toxicity was one order of magnitude higher than the experimental value. This is because for these two mixtures, the most hydrophobic compounds dominated the mixture effect (Supplementary Information, Fig. SI-9, right column). For Eff-1 and MF these were the fragrance materials tonalide (log D_{lipw} 5.88, Table 1) and galaxolide (log D_{lipw} 6.09). Galaxolide was also the third most abundant chemical in the two samples with a concentration of 1.0 and 1.1 μg/L, while tonalide was found at 10 times lower concentrations but is the most hydrophobic chemical of the entire test set. The next largest contributors to the toxicity of these mixtures were 4-t-octylphenol (log D_{lipw} 5.46), carbamazepine (log D_{lipw} 2.57), venlafaxine (log D_{lipw} 2.42) and metolachlor (log D_{lipw} 3.26), which are of lower hydrophobicity and thus have higher EC_{50} values but they were also more abundant with carbamazepine leading the concentration ranking, followed by venlafaxine and metolachlor as fourth most abundant chemical.

As the mixture toxicity predictions were made based on the QSAR (Equation (10)) developed for chemicals with a log D_{lipw} up to 4.5 and the experimental EC_{50} values for the more hydrophobic test chemicals were higher (and therefore less toxic) than the QSAR predicted for the reasons discussed in Section 3.1, it can be expected that mixtures with a high abundance of very hydrophobic chemicals will show lower toxicity than predicted by the combined QSAR and CA model. This was the case for Eff-1 and MF. Nevertheless, despite all the shortcomings this model is still able to predict mixture effects within one order of magnitude from experimental results.

The other wastewater treatment plant effluent sample (Eff-2) showed a much better agreement between experimental and CA predicted toxicity. In Eff-2, eight chemicals were present at concentrations above 1 μg/L and five chemicals contributed substantially to the mixture effect (Supplementary Information, Fig. SI-9), and they had a much wider range of hydrophobicity, thus reducing the influence of individual outliers. The highest contribution to the mixture effect came from galaxolide with 1.6 μg/L because it has highest hydrophobicity and the second ranked one was chlorpyrifos, which was the most abundant chemical with 5.6 μg/L and is fairly hydrophobic (log D_{lipw} 5.13).

The three mixtures with only five or six components (i.e., the iceberg mixtures RO, O_3/BAC and SW) all showed higher toxicity than predicted with the combined QSAR and CA model. In the iceberg RO mixture, 5-methyl-1H-benzotriazole dominated the composition (88% of a molar basis) and explained also the majority of the mixture's effect. In the iceberg O_3/BAC mixture, five chemicals made similar contributions to the mixture's effect with only DEET having a negligible contribution to the mixture effect. The flame retardant tris(2-chloroethyl)phosphate had ten times higher concentration than the other components with 0.3 μg/L but due to its relatively low hydrophobicity it had the highest contribution to the mixture effect but not a dominant one.

Finally the iceberg SW mixture had an entirely different composition, with bisphenol A dominating both the mixture's effect and concentration, and DEET the second highest concentration, but having an order of magnitude lower contribution to the mixture's effect.

This analysis demonstrates how not only the absolute concentrations of the mixture components are important but also their contribution to the mixture effect, which is driven by their hydrophobicity as that drives the toxicity in the QSAR. Often but not always a few chemicals dominate the mixture toxicity entirely. Nevertheless, the concept of CA has proven to be successful in explaining how arbitrary mixtures of chemicals act together. This good agreement retrospectively confirms the validity of the TEQ approach for apical endpoints.

3.7. *How much of the effects measured in environmental water samples can be explained by known chemicals?*

As a next step, the experimental results of the iceberg mixtures were compared to the effects in the complex environmental samples they were derived from. As CA has been validated as a reasonable model of mixture toxicity in the present study, we cannot directly compare effect levels but the EC_{50} values were first converted to baseline-TEQ and then compared the environmental samples with the iceberg mixtures. Adding up baseline-TEQs is equivalent to applying the mixture toxicity concept of CA.

On average the iceberg mixtures could only explain 0.033% of the baseline-TEQ in the environmental water samples (Table 3). This finding is surprising on first view as 269 chemicals were targeted with the chemical analysis. However, there can be thousands and millions of different chemicals in our waterways and they can form even more complex mixtures of transformation products during treatment processes and by environmental degradation processes (Schwarzenbach et al., 2006; Escher and Fenner, 2011).

Interestingly, the more chemicals that are detected in a sample the higher the percentage of baseline-TEQ that can be explained by the quantified chemicals (Fig. 6). There is a difference between the experimental iceberg mixtures and the QSAR predictions but the agreement is very good in two samples (Eff-2 and O_3/BAC), while in two other samples (Eff-1 and MF) the QSAR predicts a 10 times higher fraction than was explained by the experimental iceberg mixture and in the remaining two samples (RO and SW) it is the other way round, indicating that the discrepancies are arbitrary and caused by the deficiencies of the QSAR model discussed above, not by any systematic aberrations.

We previously made similar comparisons but only using QSAR mixture predictions (Reungoat et al., 2010;, 2011, 2012; Escher et al., 2011b). These data are also plotted in Fig. 6. All literature data stem from the same advanced treatment plants that were also investigated in the present study (plus some additional ones). There were typically a lower number of chemicals targeted by chemical analysis in the previous studies but the mixture toxicity model included the number and concentrations of chemicals detected, thus it is possible to compare the data from the different studies.

The results obtained with the bioluminescence inhibition assay with *V. fischeri* as an indicator of the joint baseline effect of all chemicals is in contrast to previous findings with bioassays for specific modes of toxic action, where typically a larger fraction of effect can be explained by the chemicals quantified with chemical analysis. For estrogenic effects in

wastewater treatment plant effluents and surface waters the estradiol equivalent concentrations from bioassays and chemical analysis, EEQ_{bio} and EEQ_{chem}, often matched quite well (Rutishauser et al., 2004; Leusch et al., 2010). However, for samples with low levels of estrogenic chemicals, such as samples similar to the MF, RO and AO samples in the present study, only 0.1–1.1% of estradiol equivalents could be explained by chemical analysis (Escher et al., 2011b). This was explained by the lower detection limit of the bioassay E-SCREEN (approximately 0.01 ng/L EEQ) compared to that of the chemical analysis (in that study 1 ng/L for each quantified estrogenic compound).

Herbicides that cause photosynthesis inhibition can be captured with a very sensitive algal assay that fluorometrically quantifies the photosynthesis efficiency (Escher et al., 2008). The effects were translated into diuron equivalent concentrations DEQ. DEQ_{chem} could explain average 65% of the DEQ_{bio} in wastewater treatment plant effluents (Vermeirssen et al., 2010), even more in the study at the advanced water treatment plant (Escher et al., 2011b) as well as for surface water (Escher et al., 2006). This good agreement is consistent with the fact that we know the identity of typically applied herbicides very well and the detection limits of bioassays and chemical analysis are similar.

In contrast, all organic chemicals contribute to the mixture effect in the present study. In addition, it is conceivable that some low molecular fraction of natural organic matter is contributing to the overall mixture effect because usually 40–70% of overall dissolved organic carbon (not differentiating between organic micropollutants and natural organic matter) are extracted from a water sample by a similar SPE method (Neale and Escher, in press) to that used in the current study. However, as the natural organic matter in colloidal form is not bioavailable and the low molecular weight fractions are fairly hydrophilic they are not expected to contribute in a dominant way to the baseline toxicity.

4. Conclusion

The results of the mixture experiments demonstrate that chemicals in real water samples act together in mixtures. The study substantiated earlier recommendations that CA is a useful reference concept for predicting the toxicity of complex environmental mixtures. The good consistency between experimental data and predictions made using the CA model is a retrospective confirmation that the concept of toxic equivalent concentrations is appropriate not only for receptor mediated mechanisms but also for general cytotoxicity. In addition, apart from the antibiotics, all evaluated micropollutants acted as baseline toxicants in the 30-min bioluminescence inhibition test despite covering a large and diverse range of specific modes of toxic action. Thus the derivation of baseline-TEQ as was proposed earlier (Escher et al., 2008) is legitimate and a useful expansion of the TEQ concept.

Using the iceberg analogy, currently we “see” remarkably few of the chemicals in environmental samples, 99.99% remain “submersed” or “invisible” with standard analytical tools. This is not unexpected, given that all chemicals and possibly even low molecular weight natural organic matter contribute to the mixture baseline toxicity of the sample.

We do not know the *in vivo* toxicological implications of baseline toxicity and can therefore not derive any risk-based trigger value for this endpoint. However, we have linked the established water quality guideline values to the effects measured with the bioluminescence inhibition assay with *V. fischeri* and derived EBT-EC_{50} values for the Australian Drinking Water Guidelines and the Australian Guidelines for Water Recycling. The purpose of this exercise was to test the validity of the concept. We were able to demonstrate that all water samples analysed using this method were compliant. As newly formed disinfection by-products contributed substantially to toxicity but were not included in the EBT-EC_{50} derivation, we recommend using drinking water samples prior to the disinfection in a drinking water treatment plant and to develop specific EBT-EC_{50} for disinfected drinking water in the future.

In addition, the EBT-EC_{50} can only be as good as the sets of guideline values they are derived from. The algorithm proposed here does not question or take into account the validity of the existing guideline values. Before an EBT-EC_{50} is implemented into a regulatory framework it should be assured that the chemical-based guideline values are suitable for the proposed approach. Thus all chemicals included in a given set of guideline values should have risk-based guideline values, which should have been derived with a uniform method. This is not always the case in practise. In the AGWR that was used here as an illustrative case study, the guideline values for 21 out of the 384 organics (Table A6 in NRMMC, EPHC and NHMRC, 2008) were derived from Thresholds of Toxicological Concern (TTC), which are not based on toxicological data of individual chemicals but on the Cramer Classification rules and thus are typically very conservative estimates (Schriks et al., 2010). Nevertheless a sensitivity analysis of the EBT-EC_{50}, where the TTC values were replaced by NOAELs (personal communication, Janet Cummings, Queensland Health) indicated only a slight change of the EBT-EC_{50} from 2.8 to 2.7 REF.

The effect-based water quality trigger values for cytotoxicity derived here cannot be used alone but must be accompanied by a series of trigger values for specific modes of action such as estrogenicity or inhibition of photosynthesis. For receptor-mediated specific modes of action the toxic equivalency concept will be appropriate and fairly straightforward to derive as CA has been generally established for these endpoints and chemical acting according to a common mode of action.

Only such a battery-based approach will minimise false-negative results that could occur if specifically acting compounds are present, which would not be picked up by the bioluminescence inhibition assay with *V. fischeri*, or if there is only a small number of chemicals present but at exceedingly high concentrations that drive the mixture toxicity. The latter case is unlikely in recycled and drinking water unless there is an accidental spill. As previous work has shown, bioassays can potentially detect spills and extraordinary conditions (Vermeirssen et al., 2010). If the approach were expanded to wastewater effluent or industrial wastewater then it would be important include the possibility of individual chemicals dominating the mixture.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2013.03.011.

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Supplementary Information

Mixture Effects of Organic Micropollutants Present in Water: Towards the Development of Effect-Based Water Quality Trigger Values for Baseline Toxicity

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Table SI-1 Chemicals contained in the Australian Drinking Water Guidelines (ADWG) but not detected and therefore only used for the QSAR comparison and not in the mixtures, their physicochemical descriptors, experimental and modelled EC_{50} values.

Chemical	CAS Number	Molar weight (g/mol)	logK_{ow}	logK_{lipw} (L/kg)	logD_{lipw} (pH7) (L/kg)	Experimental log 1/ EC_{50} (M)	stdev	Predicted log 1/ EC_{50} (M) QSAR
Aldicarb	116-06-3	190.26	1.13	1.22	1.22	2.87	0.12	1.81
Amitraz	33089-61-1	293.42	5.5	5.68	5.68	4.40	0.47	4.92
Azinophos-methyl	86-50-0	317.32	2.75	2.88	2.88	4.37	0.05	2.96
Carbaryl	63-25-2	201.23	2.36	2.48	2.48	4.99	0.29	2.69
Carbofuran	1563-66-2	221.26	2.32	2.44	2.44	3.87	0.11	2.66
Dichlorprop	120-36-5	235.07	3.43	3.57	2.57	3.26	0.26	2.75
Dichlorvos	62-73-7	220.98	1.43	1.53	1.53	2.99	0.19	2.02
Dimethoate	60-51-5	229.25	0.78	0.87	0.87	3.82	0.11	1.56
Ethion	563-12-2	384.46	5.07	5.24	5.24	2.87	0.06	4.61
Fenitrothion	122-14-5	277.23	3.3	3.44	3.44	3.79	0.63	3.35
Fluometuron	2164-17-2	232.21	2.42	2.54	2.54	2.19	0.05	2.73
Methomyl	16752-77-5	162.21	0.6	0.68	0.68	2.11	0.07	1.43
Metribuzin	21087-64-9	214.29	1.7	1.80	1.80	2.99	0.48	2.22
Pendimethalin	40487-42-1	281.31	5.18	5.35	5.35	2.95	0.40	4.69
Piperonyl butoxide	51-03-6	338.45	4.75	4.92	4.92	3.85	0.30	4.39
Pirimicarb	23103-98-2	238.29	1.7	1.80	1.80	2.84	0.06	2.21
Pirimiphos-methyl	23505-41-1	333.39	4.2	4.35	4.35	3.44	0.27	3.99
Propanil	709-98-8	218.08	3.07	3.20	3.20	4.01	0.19	3.19
Propargite	2312-35-8	350.48	5	5.17	5.17	4.34	0.15	4.56
Propiconazole	60207-90-1	342.23	3.72	3.86	3.86	3.74	0.19	3.65
Salicylic acid	69-72-7	138.12	2.26	2.38	1.38	1.95	0.49	1.92
Warfarin	81-81-2	308.34	2.7	2.82	1.97	2.86	0.77	2.33

13 *Table SI-2 Chemicals used in the present study, their manufacturer, purity and chemical category/type.*

Chemical	CAS	Manufacturer	Catalogue number	Grade	Chemical Category
Reference chemicals					
2-Butoxyethanol	111-76-2	Sigma-Aldrich	537551-1L-A	≥99%	Reference
2-Nitrotoluene	88-72-2	Aldrich	438804-5mL	≥99%	Reference
3-Nitroaniline	99-09-2	Supelco	442392	Analytical Standard	Reference
2,4,5-Trichloraniline	636-30-6	Fluka	35828-1g	Pestanal	Reference
4-n-Pentylphenol	1438-35-3	Aldrich	77102-10g	≥98%	Reference
2-Phenylphenol	90-43-7	Fluka	45529-250mg	Pestanal	Reference
Phenol	108-95-2	Fluka	77610-250g	≥99.5%	Reference
Mixture chemicals					
17β-estradiol	50-28-2	Sigma	E8875-1g	≥98%	Pharmaceutical
2,4-Dichlorophenoxyacetic acid (2,4,D)	94-75-7	Fluka	31518-250mg	Pestanal	Herbicide
4-Nonylphenol (4NP)	104-40-5	Fluka	46405-100mg	Pestanal	Herbicide
4-Tert Octylphenol	140-66-9	Supelco	442858	Analytical Standard	Consumer/industrial chemical
Acetylsalicylic acid (Aspirin)	50-78-2	Aldrich	239631-1g	≥99%	Pharmaceutical
Atenolol	29122-68-7	Sigma	A7655-1g	≥98%	Pharmaceutical
Atorvastatin calcium	134523-03-8	Dr. Ehrenstorfer GmBh	C10318000	Ref Std	Pharmaceutical
Atrazine (total) including metabolites	1912-24-9	Fluka	45330-250mg-R	Pestanal	Herbicide
Bisphenol A	80-05-7	Aldrich	239658-50g	≥99 %	Consumer/industrial chemical
Caffeine	58-08-2	Sigma-Aldrich	C1778-1VL	Sigma Ref Std	Pharmaceutical
Carbamazepine	298-46-4	Sigma-Aldrich	49939-1g		
Cephalexin	15686-71-2	Fluka	33989-100mg-R	Vetranal	Antibiotics
Chlorpyrifos	2921-88-2	Fluka	45395-250mg	Pestanal	Organophosphate Insecticide
Citalopram hydrobromide	59729-32-7	USP	1134233	Ref Std	Pharmaceutical
Codeine	76-57-3	Cerilliant	C-006	Certified Reference Material	Pharmaceutical
Cyclophosphamide monohydrate	6055-19-2	Sigma	C7397-1g	Ref Std	Pharmaceutical
DEET (N,N-diethyltoluamide (NN-diethyl-3-methylbenzamide))	134-62-3	Fluka	36542-250mg	Pestanal	Consumer/industrial chemical
Desmethyl citalopram	144025-14-9	Cerilliant	D-047	Certified Reference Material	Pharmaceutical Metabolite
Desmethyl diazepam (Nordiazepam)	1088-11-5	Cerilliant	N-905	Certified Reference Material	Pharmaceutical Metabolite

Diazepam (Valium)	439-14-5	Sigma	D0899-100mg	Ref Std	Pharmaceutical
Diazinon	333-41-5	Fluka	45428-250mg	Pestanal	Organophosphate Insecticide
Dicamba	1918-00-9	Sigma-Aldrich	45430-250mg	Pestanal	Organochlorine Herbicide
Diclofenac acid	15307-86-5	Dr. Ehrenstorfer GmBh	C 12537000	Ref Std	Pharmaceutical
Diuron	330-54-1	Fluka	45463-250mg	Pestanal	
Doxycycline hyclate	24390-14-5	Fluka	33429-100mg-R	Vetranal	Antibiotics
Erythromycin	114-07-8	Fluka	16221-500mg	Pharmaceutical secondary standard	Antibiotics
Fipronil	120068-37-3	Fluka	46451-100mg	Pestanal	Insecticide
Fluoxetine hydrochloride (Prozac)	56296-78-7	Fluka	34012-10mg-R	Vetranal	Pharmaceutical
Furosemide	54-31-9	Fluka	09205-1g	Pharmaceutical Secondary Standard	Pharmaceutical
Galaxolide (1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[g]-2-benzopyran)	1222-05-5	Dr. Ehrenstorfer GmBh	C 1421300	Ref Std	Consumer/industrial chemical
Gemfibrozil	25812-30-0	Sigma	G9518-5g	Ref Std	Pharmaceutical
Hexazinone	51235-04-2	Fluka	36129-100mg	Pestanal	Herbicide
Hydrochlorthiazide	58-93-5	Fluka	08213-1g	Pharmaceutical secondary standard	Pharmaceutical
Ibuprofen 400	15687-27-1	Fluka	32424-100mg	Vetranal	Pharmaceutical
Indomethacin	53-86-1	Sigma	I8280-5g	USP Testing Spec	Pharmaceutical
Lincomycin hydrochloride monohydrate	7179-49-9	Fluka	31727-250mg	Vetranal	Antibiotics
MCPA (2-Methyl-4-chlorophenoxyacetic acid)	94-74-6	Fluka	45555-250mg	Pestanal	Herbicide
Metolachlor	51218-45-2	AccuStandard	P-158NB-250	96.4%	Herbicide
Metoprolol tartrate salt	56392-17-7	Fluka	77376-1g	Pharmaceutical Secondary c Standard	Pharmaceutical
Naproxen	22204-53-1	Fluka	36405-500mg	Pharm Sec Std	Pharmaceutical
Norfloxacin	70458-96-7	Fluka	33899-100mg-R	Vetranal	Antibiotics
Oxazepam	604-75-1	Cerilliant	O-902	Certified Reference Material	Pharmaceutical
Oxycodone	76-42-6	Cerilliant	O-002	Certified Reference	Pharmaceutical

				Material	
Oxytetracycline dihydrate(Terramycin)	6153-64-6	Sigma	O4636-10g	≥99%	Antibiotics
Paracetamol (acetaminophen)	103-90-2	Sigma-Aldrich	A3035-1VL	Analytical Standard	Pharmaceutical
Picloram	1918-02-1	Fluka	36774-250mg-R	Pestanal	Herbicide
Praziquantel	55268-74-1	Fluka	46648-250mg	Vetranal	Pharmaceutical
Propoxur	114-26-1	Fluka	45644-250mg	Pestanal	Carbamate Insecticide
Propranolol hydrocloride	318-98-9	Sigma	P0884-1g	≥99%	Pharmaceutical
Ranitidine hydrochloride	66357-59-3	Fluka	44404-500mg	Pharmaceutical Secondary c Standard	Pharmaceutical
Roxithromycin	80214-83-1	Sigma	R4393-1g	≥90%	Antibiotics
Simazine	122-34-9	Fluka	32059-250mg	Pestanal	Pharmaceutical
Sulfadiazine	68-35-9	Fluka	35033-100mg	Vetranal	Pharmaceutical
Sulfamethoxazole	723-46-6	Fluka	31737-250mg	Vetranal	Antibiotics
Sulfasalazine	599-79-1	Fluka	S0883-10g	≥98%	Pharmaceutical
Temazepam	846-50-4	Sigma-Aldrich	T-907	Certified Reference Material	Pharmaceutical
Tolutriazole (5-Methyl-1H-benzotriazole)	136-85-6	Aldrich	196304-10g	98%	Pharmaceutical
Tonalide (AHTN, 6-Acetyl-1,1,2,4,4,7-hexamethyltetraline)	21145-77-7	Aldrich	CDS009866-50mg	CPR	Musk
Tributylphosphate	126-73-8	Aldrich	240494-5mL	≥99%	Consumer/industrial chemical
Triclopyr	55335-06-3	Fluka	32016-250mg	Pestanal	Herbicide
Triclosan (Irgasan)	3380-34-5	Sigma	72779-5g-F	≥97%	Consumer/industrial chemical
Trimethoprim	738-70-5	Fluka	46984-250mg	Vetranal	Antibiotics
Tris(2-chloroethyl)phosphate (TCEP)	115-96-8	Aldrich	119660-25g	97%	Consumer/industrial chemical
Venlafaxine hydrochloride	99300-78-4	Sigma-	V7264-10mg	≥98%	Pharmaceutical

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Section SI-1 Additional information on sample preparation

All samples were acidified to pH 3. Samples containing chlorine were quenched with sodium thiosulphate (1 g/L), and filtered with a glass fibre filter (GF/A Whatman) before extraction. Samples were extracted by passing through two 6 cc solid phase cartridges in series, first an Oasis® HLB (500mg, Catalogue Number 186000115, Waters) followed by a Supelclean coconut charcoal cartridge (2g, Catalogue Number 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 methanol and 10 mL of 5 mM HCl in MilliQ water. One litre of water was extracted on each pair of HLB and coconut charcoal cartridges under vacuum. Cartridges were sealed individually and 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 and were evaporated under purified nitrogen gas before being solvent exchanged to methanol at a final volume of 1 mL.

Section SI-2 Additional information on the chemical analytical method

Chemical analysis was performed at a commercial NATA accredited analytical laboratory, Queensland Health Forensic and Scientific Services (QHFSS). Water samples underwent either SPE or liquid-liquid extraction before subject to GCMS or LCMS. Three standard analytical methods were used: QIS25391 Determination of endocrine disrupting compounds in effluent, river and recycled water, QIS27701 Phamaceuticals and Personal Care Products (PPCP) in water, preparation and analysis by SPE and LCMSMS, QIS16315 Organochlorine, organophosphorous and synthetic pyrethroid pesticides, urea and triazine herbicides and PCBs in water.

Table SI-3 Analysed chemicals (in alphabetical order of analyte name) with methods and limit of reporting (LOR).

Analytical method	Analyte	Units	LOR
Endocrine Disrupting Compounds by GC-MS	17-α-Ethynylestradiol	ng/L	5
Endocrine Disrupting Compounds by GC-MS	17-β-Estradiol	ng/L	5
GC-MS Screen	1H-Benzotriazole	µg/L	0.2
GC-MS Screen	1H-Benzotriazole, 1-methyl	µg/L	0.2
GC-MS Screen	1H-Benzotriazole, 5-methyl	µg/L	0.2
Pesticides by GC-MS	2,4-D	µg/L	0.1
Pesticides by GC-MS	2,4-DB	µg/L	0.1
Pesticides by GC-MS	2,4-DP (Dichlorprop)	µg/L	0.1
Pesticides by GC-MS	2,4,5-T	µg/L	0.1
GC-MS Screen	2,6-Di-t-butyl-p-cresol (BHT)	µg/L	0.5
GC-MS Screen	2,6-Di-t-butylphenol	µg/L	0.2
Pesticides by GC-MS	3-Hydroxycarbofuran	µg/L	0.1
Herbicides and Other Compounds by LC-MS	3,4-Dichloroaniline	µg/L	0.01
GC-MS Screen	4-Chloro-3,5-dimethylphenol	µg/L	0.1
Endocrine Disrupting Compounds by GC-MS	4-t-Octylphenol	ng/L	10
Pharmaceuticals by LC-MS	Acesulfame	µg/L	0.01
Pharmaceuticals by LC-MS	Acetylsalicylic acid	µg/L	0.01
Pesticides by GC-MS	Aldicarb	µg/L	0.1
Pesticides by GC-MS	Aldicarb sulfone (Aldoxycarb)	µg/L	0.1
Pesticides by GC-MS	Aldicarb sulfoxide	µg/L	0.1
Pesticides by GC-MS	Aldrin (HHDN)	µg/L	0.1
Pesticides by GC-MS	Allethrin	µg/L	0.1
Herbicides and Other Compounds by LC-MS	Ametryn	µg/L	0.01

Analytical method	Analyte	Units	LOR
Herbicides and Other Compounds by LC-MS	Amitraz	µg/L	0.1
Endocrine Disrupting Compounds by GC-MS	Androsterone	ng/L	5
Pharmaceuticals by LC-MS	Atenolol	µg/L	0.01
Pharmaceuticals by LC-MS	Atorvastatin	µg/L	0.01
Herbicides and Other Compounds by LC-MS	Atrazine	µg/L	0.01
Pesticides by GC-MS	Azinphos-ethyl	µg/L	0.1
Pesticides by GC-MS	Azinphos-methyl	µg/L	0.1
Pesticides by GC-MS	Benalaxyl	µg/L	0.1
Pesticides by GC-MS	Bendiocarb	µg/L	0.1
Pesticides by GC-MS	Bifenthrin	µg/L	0.1
Pesticides by GC-MS	Bioresmethrin	µg/L	0.1
GC-MS Screen	Bisphenol A	µg/L	0.1
Pesticides by GC-MS	Bitertanol	µg/L	0.1
Herbicides and Other Compounds by LC-MS	Bromacil	µg/L	0.01
Pesticides by GC-MS	Bromophos-ethyl	µg/L	0.1
Pesticides by GC-MS	Cadusafos	µg/L	0.1
Pharmaceuticals by LC-MS	Caffeine	µg/L	0.02
Pesticides by GC-MS	Captan	µg/L	0.2
Pharmaceuticals by LC-MS	Carbamazepine	µg/L	0.01
Pesticides by GC-MS	Carbaryl	µg/L	0.01
Pesticides by GC-MS	Carbofuran	µg/L	0.1
Pesticides by GC-MS	Carbophenothion	µg/L	0.1
Pharmaceuticals by LC-MS	Cephalexin	µg/L	0.01
Pharmaceuticals by LC-MS	Chloramphenicol	µg/L	0.1
Pesticides by GC-MS	Chlordene	µg/L	0.1
Pesticides by GC-MS	Chlordene Epoxide	µg/L	0.1
Pesticides by GC-MS	Chlordene-1-hydroxy	µg/L	0.1
Pesticides by GC-MS	Chlordene-1-hydroxy-2,3-epoxide	µg/L	0.1
Pesticides by GC-MS	Chlorfenvinphos	µg/L	0.1
Pesticides by GC-MS	Chlorpyrifos	µg/L	0.1
Pesticides by GC-MS	Chlorpyrifos oxon	µg/L	0.1
Pesticides by GC-MS	Chlorpyrifos-methyl	µg/L	0.1
Pharmaceuticals by LC-MS	Chlortetracycline	µg/L	0.2
Pharmaceuticals by LC-MS	Ciprofloxacin	µg/L	0.15
Pesticides by GC-MS	cis -Nonachlor	µg/L	0.1
Pesticides by GC-MS	cis-Chlordane	µg/L	0.1
Pharmaceuticals by LC-MS	Citalopram	µg/L	0.01
Pesticides by GC-MS	Clopyralid	µg/L	0.1

Analytical method	Analyte	Units	LOR
Pharmaceuticals by LC-MS	Codeine	µg/L	0.1
Pesticides by GC-MS	Coumaphos	µg/L	0.1
Pharmaceuticals by LC-MS	Cyclophosphamide	µg/L	0.01
Pesticides by GC-MS	Cyfluthrin	µg/L	0.1
Pesticides by GC-MS	Cyhalothrin	µg/L	0.2
Pesticides by GC-MS	Cypermethrin	µg/L	0.1
Herbicides and Other Compounds by LC-MS	Dalapon (2,2-DPA)	µg/L	0.05
Pharmaceuticals by LC-MS	Dapsone	µg/L	0.01
Pharmaceuticals by LC-MS	DEET	µg/L	0.01
Pesticides by GC-MS	DEET	µg/L	0.0
Pesticides by GC-MS	Deltamethrin	µg/L	0.1
Pesticides by GC-MS	Demeton-S-methyl	µg/L	0.1
Herbicides and Other Compounds by LC-MS	Desethyl Atrazine	µg/L	0.01
Herbicides and Other Compounds by LC-MS	Desisopropyl Atrazine	µg/L	0.01
Pharmaceuticals by LC-MS	Desmethyl Citalopram	µg/L	0.01
Pharmaceuticals by LC-MS	Desmethyl Diazepam	µg/L	0.01
Pharmaceuticals by LC-MS	Diazepam	µg/L	0.01
Herbicides and Other Compounds by LC-MS	Diazinon	µg/L	0.02
Pesticides by GC-MS	Dicamba	µg/L	0.1
Pesticides by GC-MS	Dichlofluanid	µg/L	0.1
Pesticides by GC-MS	Dichlorvos	µg/L	0.1
Pharmaceuticals by LC-MS	Diclofenac	µg/L	0.01
Pesticides by GC-MS	Diclofop-methyl	µg/L	0.1
Pesticides by GC-MS	Dicloran	µg/L	0.1
Pesticides by GC-MS	Dicofol	µg/L	3.0
Pesticides by GC-MS	Dieldrin (HEOD)	µg/L	0.05
Pesticides by GC-MS	Dimethoate	µg/L	0.1
Pesticides by GC-MS	Dimethomorph	µg/L	0.2
Pesticides by GC-MS	Dioxathion	µg/L	0.1
Pesticides by GC-MS	Disulfoton	µg/L	0.1
Herbicides and Other Compounds by LC-MS	Diuron	µg/L	0.01
Pharmaceuticals by LC-MS	Doxylamine	µg/L	0.01
Pesticides by GC-MS	Endosulfan Ether	µg/L	0.1
Pesticides by GC-MS	Endosulfan Lactone	µg/L	0.1
Pesticides by GC-MS	Endosulfan Sulfate	µg/L	0.05
Pesticides by GC-MS	Endrin	µg/L	0.1
Pesticides by GC-MS	Endrin aldehyde	µg/L	0.1
Pharmaceuticals by LC-MS	Enrofloxacin	µg/L	0.02

Analytical method	Analyte	Units	LOR
Pharmaceuticals by LC-MS	Erythromycin	µg/L	0.01
Pharmaceuticals by LC-MS	Erythromycin anhydrate	µg/L	0.01
Endocrine Disrupting Compounds by GC-MS	Estriol	ng/L	5
Endocrine Disrupting Compounds by GC-MS	Estrone	ng/L	5
Pesticides by GC-MS	Ethion	µg/L	0.1
Pesticides by GC-MS	Ethoprophos	µg/L	0.1
Endocrine Disrupting Compounds by GC-MS	Etiocholanolone	ng/L	5
Pesticides by GC-MS	Etrimphos	µg/L	0.1
Pesticides by GC-MS	Famphur	µg/L	0.1
Pesticides by GC-MS	Fenamiphos	µg/L	0.1
Pesticides by GC-MS	Fenchlorphos	µg/L	0.1
Pesticides by GC-MS	Fenitrothion	µg/L	0.1
Pesticides by GC-MS	Fenoprop (2,4,5-TP)	µg/L	0.1
Pesticides by GC-MS	Fenthion (methyl)	µg/L	0.1
Pesticides by GC-MS	Fenthion-ethyl	µg/L	0.1
Pesticides by GC-MS	Fenvalerate	µg/L	0.1
Pesticides by GC-MS	Fipronil	µg/L	0.1
Pesticides by GC-MS	Fluazifop-butyl	µg/L	0.1
Herbicides and Other Compounds by LC-MS	Fluometuron	µg/L	0.01
Pharmaceuticals by LC-MS	Fluoxetine	µg/L	0.01
Pesticides by GC-MS	Fluroxypyr	µg/L	0.1
Pesticides by GC-MS	Fluvalinate	µg/L	0.1
Pharmaceuticals by LC-MS	Fluvastatin	µg/L	0.01
Pharmaceuticals by LC-MS	Frusemide	µg/L	0.01
Pesticides by GC-MS	Furalaxyl	µg/L	0.1
Pharmaceuticals by LC-MS	Gabapentin	µg/L	0.05
FRAGRANCES by GC-MS	Galaxolide	µg/L	0.1
Pharmaceuticals by LC-MS	Gemfibrozol	µg/L	0.01
Herbicides and Other Compounds by LC-MS	Haloxyfop (acid)	µg/L	0.01
Herbicides and Other Compounds by LC-MS	Haloxyfop-2-etotyl	µg/L	0.01
Herbicides and Other Compounds by LC-MS	Haloxyfop-methyl	µg/L	0.01
Pesticides by GC-MS	HCB	µg/L	0.1
Pesticides by GC-MS	Heptachlor	µg/L	0.03
Pesticides by GC-MS	Heptachlor Epoxide	µg/L	0.03
Herbicides and Other Compounds by LC-MS	Hexazinone	µg/L	0.01
Pharmaceuticals by LC-MS	Hydrochlorthiazide	µg/L	0.01
Pharmaceuticals by LC-MS	Ibuprofen	µg/L	0.07
Pharmaceuticals by LC-MS	Ifosfamide	µg/L	0.01

Analytical method	Analyte	Units	LOR
Herbicides and Other Compounds by LC-MS	Imidacloprid	µg/L	0.01
Pharmaceuticals by LC-MS	Indomethacin	µg/L	0.01
Pharmaceuticals by LC-MS	Iopromide	µg/L	0.2
Pesticides by GC-MS	Isofenphos	µg/L	0.1
Pesticides by GC-MS	Lambda-cyhalothrin	µg/L	0.1
Pharmaceuticals by LC-MS	Lincomycin	µg/L	0.01
Pesticides by GC-MS	Lindane (γ-HCH)	µg/L	0.1
Pesticides by GC-MS	Malathion (Maldison)	µg/L	0.1
Pesticides by GC-MS	MCPA	µg/L	0.1
Pesticides by GC-MS	MCPB	µg/L	0.1
Pesticides by GC-MS	Mecoprop	µg/L	0.1
Pesticides by GC-MS	Metalaxyl	µg/L	0.1
Pesticides by GC-MS	Methidathion	µg/L	0.1
Pesticides by GC-MS	Methiocarb	µg/L	0.1
Pesticides by GC-MS	Methomyl	µg/L	0.1
Pesticides by GC-MS	Methomyl oxime	µg/L	0.5
Pesticides by GC-MS	Methoprene	µg/L	0.1
Pesticides by GC-MS	Methoxychlor	µg/L	0.1
Herbicides and Other Compounds by LC-MS	Metolachlor	µg/L	0.01
Pharmaceuticals by LC-MS	Metoprolol	µg/L	0.01
Pesticides by GC-MS	Metribuzin	µg/L	0.1
Pesticides by GC-MS	Mevinphos	µg/L	0.1
GC-MS Screen	Moclobemide	µg/L	0.5
Pesticides by GC-MS	Molinate	µg/L	0.1
Pesticides by GC-MS	Monocrotophos	µg/L	0.5
FRAGRANCES by GC-MS	Musk Ketone	µg/L	0.1
FRAGRANCES by GC-MS	Musk Xylene	µg/L	0.1
GC-MS Screen	N-Butyl benzenesulfonamide	µg/L	0.1
GC-MS Screen	N-Butyltoluenesulfonamide	µg/L	0.1
Pharmaceuticals by LC-MS	Naproxen	µg/L	0.1
Endocrine Disrupting Compounds by GC-MS	Nonylphenol	ng/L	100
Pharmaceuticals by LC-MS	Norfloxacin	µg/L	0.05
Endocrine Disrupting Compounds by GC-MS	Norgestrel	ng/L	10
Pesticides by GC-MS	o,p-DDD	µg/L	0.1
Pesticides by GC-MS	o,p-DDE	µg/L	0.1
Pesticides by GC-MS	o,p-DDT	µg/L	0.1
Pesticides by GC-MS	Omethoate	µg/L	0.5
Pesticides by GC-MS	Oxadiazon	µg/L	0.1

Analytical method	Analyte	Units	LOR
Pesticides by GC-MS	Oxamyl	µg/L	0.5
Pesticides by GC-MS	Oxamyl oxime	µg/L	0.5
Pharmaceuticals by LC-MS	Oxazepam	µg/L	0.01
Pesticides by GC-MS	Oxychlordane	µg/L	0.1
Pharmaceuticals by LC-MS	Oxycodone	µg/L	0.01
Pesticides by GC-MS	Oxydemeton-methyl	µg/L	0.1
Pesticides by GC-MS	Oxyfluorfen	µg/L	0.1
Pharmaceuticals by LC-MS	Oxytetracycline	µg/L	0.4
Pesticides by GC-MS	p,p-DDD	µg/L	0.1
Pesticides by GC-MS	p,p-DDE	µg/L	0.1
Pesticides by GC-MS	p,p-DDT	µg/L	0.1
Pharmaceuticals by LC-MS	Paracetamol	µg/L	0.02
Pesticides by GC-MS	Parathion (ethyl)	µg/L	0.1
Pesticides by GC-MS	Parathion-methyl	µg/L	0.1
Pesticides by GC-MS	Pendimethalin	µg/L	0.1
Pesticides by GC-MS	Permethrin	µg/L	0.1
Pesticides by GC-MS	Phenothrin	µg/L	0.1
Pharmaceutical by LC-MS	Phenytoin	µg/L	0.01
Pesticides by GC-MS	Phorate	µg/L	0.1
Pesticides by GC-MS	Phosmet	µg/L	0.1
Pesticides by GC-MS	Phosphamidon	µg/L	0.1
Pesticides by GC-MS	Picloram	µg/L	0.1
Pesticides by GC-MS	Piperonyl Butoxide	µg/L	0.1
Pesticides by GC-MS	Pirimicarb	µg/L	0.1
Pesticides by GC-MS	Pirimiphos-methyl	µg/L	0.1
Pharmaceuticals by LC-MS	Praziquantel	µg/L	0.01
Pharmaceuticals by LC-MS	Primidone	µg/L	0.01
Pesticides by GC-MS	Procymidone	µg/L	0.1
Pesticides by GC-MS	Profenofos	µg/L	0.1
Pesticides by GC-MS	Promecarb	µg/L	0.1
Herbicides and Other Compounds by LC-MS	Prometryn	µg/L	0.01
Pesticides by GC-MS	Propanil	µg/L	0.1
Pesticides by GC-MS	Propargite	µg/L	0.2
Pesticides by GC-MS	Propazine	µg/L	0.1
Pesticides by GC-MS	Propiconazole	µg/L	0.1
Pesticides by GC-MS	Propoxur	µg/L	0.01
Pesticides by GC-MS	Propoxur	µg/L	0.01
Pharmaceuticals by LC-MS	Propranolol	µg/L	0.01

Analytical method	Analyte	Units	LOR
Pesticides by GC-MS	Prothiophos	µg/L	0.1
Pesticides by GC-MS	Pyrazophos	µg/L	0.1
Pharmaceuticals by LC-MS	Ranitidine	µg/L	0.05
Pesticides by GC-MS	Rotenone	µg/L	0.1
Pharmaceuticals by LC-MS	Roxithromycin	µg/L	0.02
Pharmaceuticals by LC-MS	Salicylic acid	µg/L	0.1
Pharmaceuticals by LC-MS	Sertraline	µg/L	0.01
Herbicides and Other Compounds by LC-MS	Simazine	µg/L	0.01
Pharmaceuticals by LC-MS	Simvastatin	µg/L	0.1
Pharmaceuticals by LC-MS	Sulfasalazine	µg/L	0.01
Pharmaceuticals by LC-MS	Sulfsalazine	µg/L	0.01
Pharmaceuticals by LC-MS	Sulphadiazine	µg/L	0.01
Pharmaceuticals by LC-MS	Sulphamethoxazole	µg/L	0.01
Pharmaceuticals by LC-MS	Sulphathiazole	µg/L	0.01
Pesticides by GC-MS	Sulprofos	µg/L	0.1
Pesticides by GC-MS	Tebuconazole	µg/L	0.1
Herbicides and Other Compounds by LC-MS	Tebuthiuron	µg/L	0.01
Pharmaceuticals by LC-MS	Temazepam	µg/L	0.01
Pesticides by GC-MS	Terbufos	µg/L	0.1
Pesticides by GC-MS	Terbufos	µg/L	0.1
Pesticides by GC-MS	Terbuthylazine	µg/L	0.1
Herbicides and Other Compounds by LC-MS	Terbutryn	µg/L	0.01
Endocrine Disrupting Compounds by GC-MS	Testosterone	ng/L	10
Pesticides by GC-MS	Tetrachlorvinphos	µg/L	0.1
Pharmaceuticals by LC-MS	Tetracycline	µg/L	0.1
Pesticides by GC-MS	Tetradifon	µg/L	0.1
Pesticides by GC-MS	Tetramethrin	µg/L	0.1
Pesticides by GC-MS	Thiabendazole	µg/L	0.2
Pesticides by GC-MS	Thiodicarb	µg/L	0.1
FRAGRANCES by GC-MS	Tonalid	µg/L	0.1
Pharmaceuticals by LC-MS	Tramadol	µg/L	0.01
Pesticides by GC-MS	trans-Chlordane	µg/L	0.1
Pesticides by GC-MS	trans-Nonachlor	µg/L	0.1
Pesticides by GC-MS	Transfluthrin	µg/L	0.1
GC-MS Screen	Tri-n-butyl phosphate	µg/L	0.1
Pesticides by GC-MS	Triadimefon	µg/L	0.3
Pesticides by GC-MS	Triadimenol	µg/L	0.1
Pesticides by GC-MS	Triallate	µg/L	0.1

Analytical method	Analyte	Units	LOR
Pesticides by GC-MS	Triclopyr	µg/L	0.1
GC-MS Screen	Triclosan	µg/L	0.01
GC-MS Screen	Triclosan methyl ether	µg/L	0.1
GC-MS Screen	Triethyl phosphate	µg/L	0.1
Pesticides by GC-MS	Trifluralin	µg/L	0.1
Pharmaceuticals by LC-MS	Trimethoprim	µg/L	0.01
GC-MS Screen	Tris(chloroethyl) phosphate	µg/L	0.1
GC-MS Screen	Tris(chloropropyl) phosphate isomers	µg/L	0.1
GC-MS Screen	Tris(dichloropropyl) phosphate	µg/L	0.1
Pharmaceuticals by LC-MS	Tylosin	µg/L	0.05
Pharmaceuticals by LC-MS	Venlafaxine	µg/L	0.01
Pesticides by GC-MS	Vinclozolin	µg/L	0.1
Pharmaceuticals by LC-MS	Warfarin	µg/L	0.01
Pesticides by GC-MS	α-Endosulfan	µg/L	0.05
Pesticides by GC-MS	α-HCH (α-BHC)	µg/L	0.1
Pesticides by GC-MS	β-Endosulfan	µg/L	0.05
Pesticides by GC-MS	β-HCH (β-BHC)	µg/L	0.1
Pesticides by GC-MS	δ-HCH (δ-BHC)	µg/L	0.1

Section SI-3 Additional information on the bioluminescence inhibition test with *Vibrio fischeri*

The growth medium contained 513 mM NaCl, 44.2 mM NaH_2PO_4, 12.0 mM $K_2HPO_4 \cdot 3H_2O$, 0.83 mM $MgSO_4 \cdot 7H_20$, 3.78 mM $(NH_4)_2SO_4$, 41.0 mM glycerol, 5 g/L tryptone, and 0.50 g/L yeast extract. The cultures were allowed to grow at 20°C and 180 rpm until mid-exponential phase (22 hours) when they were diluted and frozen in liquid N_2. *V. fischeri* bacteria were stored at -80°C for up to 3 weeks prior to being used in the bioassay.

The assay was performed with autoclaved saline buffer containing 4 mM KCl, 10 mM $MgCl_2$, 10 mM MOPS (3-[N-morpholino] propanesulfonic acid), 342 mM NaCl with the pH adjusted to 7.0±0.2 with HCl/NaOH as the test medium. Briefly, the methanolic stock solutions of the reference compounds, the baseline toxicants or the extracts were either pipetted into a 96-well microtiter plate (Catalogue Number 655180, cell culture plate, 96 well, PS, F-Bottom (Chimney well), crystal-clear, sterile, Greiner Bio-One, Frickenhausen, Germany) and diluted with saline buffer (max 2% methanol in final bioassay) or the methanol was evaporated in a high recovery glass vial (Catalogue Number 5183-2030, high recovery screw vials, Agilent) and the residues were redissolved in saline buffer and transferred to the microtiter plate.

After a geometric dilution series in saline buffer the samples in 100 μL of saline buffer were then added to 50 μL of *V. fischeri* in growth medium in a white plate (Catalogue Number 655075, cell culture plate, 96 well, F-Bottom (Chimney well), medium binding, white, sterile, Greiner Bio-One, Frickenhausen, Germany). The luminescence output of the bacteria was measured prior to addition of sample and after 30-min incubation

(Luminescence mode, FluoStar Optima, BMG Labtech, Ortenberg, Germany). The relative light units (RLU) should be around 150,000 to 850,000 at 4095 gain prior to sample addition.

Section SI-4 Additional information on the QSAR for baseline toxicity in bioluminescence inhibition assay with *Vibrio fischeri*

We previously developed baseline toxicity QSAR for the 30-min bioluminescence inhibition assay (Escher et al. 2008) using the six test compounds listed in Table SI-4 but as demonstrated in Figure SI-1A, the sensitivity of the assay has decreased since the publication in 2008, with butoxyethanol being equally toxic but the other reference chemicals having up to 8 times higher EC_{50} values (Table SI-4). The QSAR thus had a similar slope to an earlier published QSAR using the same compounds (Escher et al. 2008) but differed in the intercept, which indicates that the overall sensitivity of the current assay (indicated by the y-intercept) was slightly lower while the relative sensitivity (indicated by the slope) remained the same. The difference is not due to the fact that the cells were grown in the laboratory and shock-frozen as the commercially obtained freeze-dried cells as well as freshly grown cells showed the same sensitivity (Figure SI-1A). The EC_{50} values after 24 h incubation for all the reference baseline toxicants were in the same order of magnitude but the results were much more variable after 24 h incubation than after 30 min and the QSAR equation was of lower quality (Supplementary Information, Figure SI-1B).

The 96 well plate assay was of similar sensitivity to the classical cuvette version of the assay performed in various laboratories (Cronin and Schultz 1997, Vighi et al. 2009, Aruoja et al. 2011) (Figure SI-2).

Table SI-4 Physicochemical properties and experimental median effect concentration EC_{50} of the reference baseline toxicants.

	log K_{ow}[a]	logK_{lipw}[a]	log(1/EC_{50}(M))
2-Butoxyethanol	0.83	0.595	1.85 ± 0.02
2-Nitrotoluene	2.3	2.41	3.03 ± 0.03
3-Nitroanilin	1.37	2.17	2.71 ± 0.03
2,4,5-Trichloranilin	3.69	4.16	4.13 ± 0.03
4-n-Pentylphenol	4.24	4.31	4.60 ± 0.01
2-Phenylphenol	3.09	3.46	3.98 ± 0.01

[a]Data from (Vaes et al. 1997).

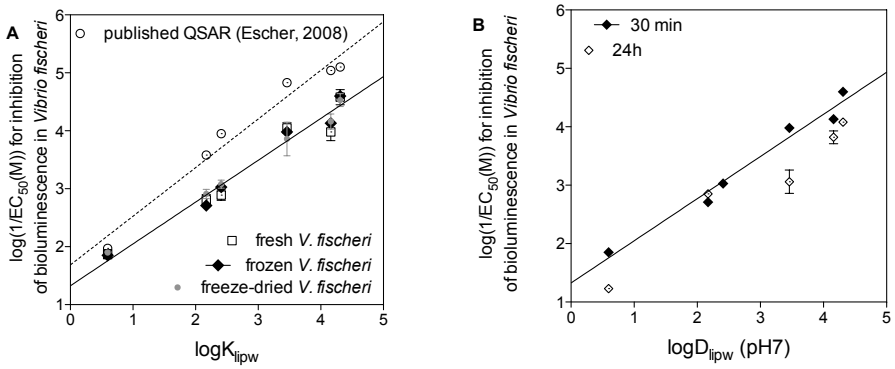


Figure SI-1A. QSAR for baseline toxicity established with 6 confirmed baseline toxicants. The empty circles and the dotted line describe the previously established QSAR $log(1/EC_{50}(M)) = (0.84 \pm 0.08) \cdot logK_{lipw} (1.69 \pm 0.24)$ (Escher et al., 2008). The black diamonds and solid line correspond to the new QSAR from the current project $log(1/EC_{50}(M)) = (0.72 \pm 0.06) \cdot logK_{lipw} + (1.32 \pm 0.18)$. For comparison the EC_{50} values for fresh (empty squares) and commercial freeze-dried (grey circles) Vibrio fischeri are also depicted. B. Comparison of the median effect concentrations (EC_{50}) of the reference baseline toxicants after 30 min and 24h of incubation.

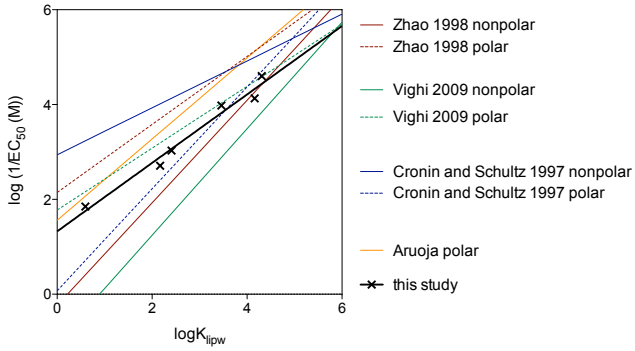


Figure SI-2. Comparison of the Quantitative Structure-Activity Relationship (QSAR) derived in the present study (in bold) with QSARs published in literature (Cronin and Schultz 1997, Zhao et al. 1998, Vighi et al. 2009), rescaled from K_{ow} to K_{lipw} (all chemicals are neutral so no pH correction to $D_{lipw}(pH7)$ was necessary.

According to literature, antibiotics often do not show any effects in the standard bioluminescence inhibition assay after 30 min of incubation, thus it has been recommended that the test should be extended to 24 h to capture the effect of antibiotics (Backhaus and Grimme 1999). In the present study the activity of the antibiotics after 30 min incubation was similar or higher than the QSAR predicted and after 24 h of incubation the antibiotics increased by three to six orders of magnitude in toxicity (Figure SI-3). Thus antibiotics have a specific effect on the bacteria *V. fischeri*. Antibiotics also pose a problem in the QSAR as many are very hydrophilic, multifunctional and/or zwitterionic, so the estimation of their $logD_{lipw}(pH7)$ is difficult and many would fall outside the validity range of the QSAR equation ($0.5 < logD_{lipw}(pH7) < 4.5$).

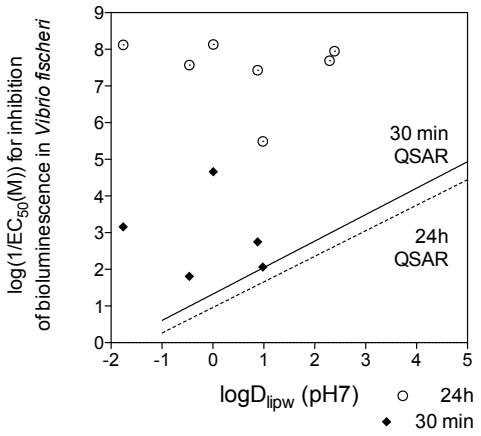


Figure SI-3. Comparison of the EC_{50} of the antibiotics after 30 min (diamond shape) and 24h (open circles) of incubation. The drawn line is the baseline toxicity QSAR for 30 min incubation, the dotted line is the baseline toxicity QSAR for 24h incubation.

125 *Table SI-5 Mixture ratios of the chemicals in the equipotent mixtures (EP).*

Mixture Composition	EP1	EP2	EP3	EP4	EP5	EP6	EP7	EP8	EP9	EP10	EP11	EP12
17β-estradiol	0.03%				0.02%	0.02%		0.02%	0.02%	0.03%		
2,4-Dichlorophenoxyacetic acid	1.13%				0.61%	0.67%		0.67%	0.86%	0.97%		
4-Nonylphenol	0.002%				0.001%	0.001%		0.001%	0.001%	0.002%		
4-Tert Octylphenol	0.004%				0.002%	0.002%		0.002%	0.003%	0.003%		
6-Acetyl-1, 1, 2, 4, 4, 7-hexamethyltetraline (AHTN, Tonalide)	0.002%				0.001%	0.001%		0.001%	0.002%	0.002%		
Acetylsalicylic acid	15.98%				8.69%	9.46%		9.55%	12.18%	13.73%		
Atenolol	82.13%				44.69%	48.61%		49.07%	62.60%	70.57%		
Atorvastatin	0.0033%				0.0018%	0.0020%		0.00%	0.00%	0.00%		
Atrazine	0.31%				0.17%	0.19%		0.19%	0.24%	0.27%		
Carbamazepine	0.41%				0.22%	0.24%		0.24%	0.31%	0.35%		
Bisphenol A		0.60%			0.05%	0.06%	0.12%		0.07%	0.08%	0.32%	
DEET		3.88%			0.35%	0.38%	0.76%		0.48%	0.55%	2.04%	
Chlorpyrifos		0.04%			0.00%	0.00%	0.01%		0.01%	0.01%	0.02%	
Cyclophosphamide		49.11%			4.38%	4.76%	9.61%		6.13%	6.91%	25.79%	
Diazinon		0.27%			0.02%	0.03%	0.05%		0.03%	0.04%	0.14%	
Dicamba		18.39%			1.64%	1.78%	3.60%		2.30%	2.59%	9.66%	
Diclofenac		0.42%			0.04%	0.04%	0.08%		0.05%	0.06%	0.22%	
Diuron		1.71%			0.15%	0.17%	0.33%		0.21%	0.24%	0.90%	
Fluoxetine		0.89%			0.08%	0.09%	0.17%		0.11%	0.13%	0.47%	
Furosemide		24.68%			2.20%	2.39%	4.83%		3.08%	3.48%	12.96%	
Gemfibrozil			0.09%		0.02%	0.03%	0.05%	0.03%				0.07%
Hexazinone			9.64%		2.76%	3.00%	6.05%	3.03%				7.52%
Hydrochlorthiazide			49.24%		14.08%	15.32%	30.90%	15.46%				38.41%
Ibuprofen			0.31%		0.09%	0.10%	0.20%	0.10%				0.25%
Indomethacin			0.19%		0.05%	0.06%	0.12%	0.06%				0.15%
Metolachlor			0.26%		0.07%	0.08%	0.16%	0.08%				0.20%
Metoprolol			9.38%		2.68%	2.92%	5.88%	2.94%				7.31%
Naproxen			1.15%		0.33%	0.36%	0.72%	0.36%				0.90%

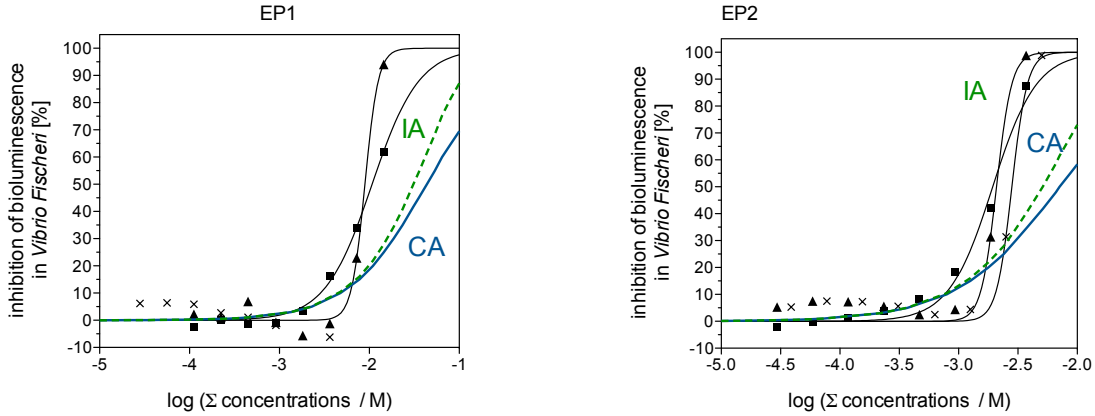
Praziquantel				2.90%	0.23%		0.51%	0.26%	0.33%		1.38%	0.64%
Propoxur				12.64%	1.02%		2.24%	1.12%	1.43%		6.00%	2.78%
Propranolol				2.39%	0.19%		0.42%	0.21%	0.27%		1.14%	0.53%
Simazine				4.29%	0.35%		0.76%	0.38%	0.48%		2.04%	0.94%
Tributylphosphate				0.22%	0.02%		0.04%	0.02%	0.02%		0.10%	0.05%
Triclopyr				12.03%	0.97%		2.13%	1.07%	1.36%		5.71%	2.65%
Triclosan				0.07%	0.01%		0.01%	0.01%	0.01%		0.03%	0.01%
Trimethoprim				41.71%	3.36%		7.38%	3.69%	4.71%		19.81%	9.18%
Tris(2-chloroethyl)phosphate (TCEP)				14.41%	1.16%		2.55%	1.28%	1.63%		6.84%	3.17%
5-Methyl-1H-benzotriazole				9.34%	0.75%		1.65%	0.83%	1.06%		4.44%	2.06%

126

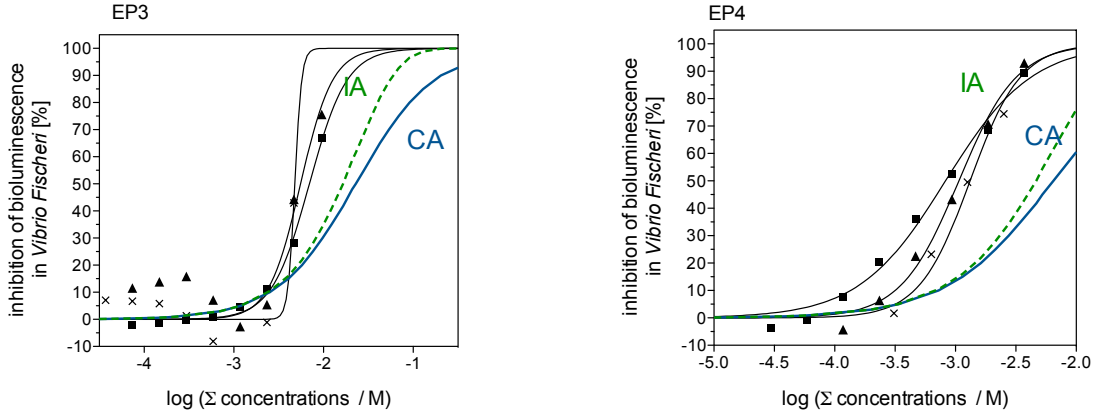
127 *Table SI-6 Mixture ratios of the Australian Drinking Water Guidelines (ADWG mixtures).*

Mixture Composition	ADWG1	ADWG2	ADWG3	ADWG4	ADWG5	ADWG6	ADWG7	ADWG8	ADWG9	ADWG10	ADWG11	ADWG12
17β-estradiol	0.07%				0.0024%	0.0025%		0.01%	0.00%	0.00%		
2,4-Dichlorophenoxyacetic acid	14.53%				0.52%	0.54%		1.08%	0.86%	0.93%		
4-Nonylphenol	0.24%				0.009%	0.009%		0.02%	0.01%	0.02%		
4-Tert Octylphenol	0.03%				0.0009%	0.0010%		0.00%	0.00%	0.00%		
6-Acetyl-1, 1, 2, 4, 4, 7-hexamethyltetraline (AHTN, Tonalide)	1.66%				0.06%	0.06%		0.12%	0.10%	0.11%		
Acetylsalicylic acid	17.23%				0.61%	0.64%		1.28%	1.02%	1.10%		
Atenolol	10.05%				0.36%	0.37%		0.74%	0.59%	0.64%		
Atorvastatin	0.96%				0.03%	0.04%		0.07%	0.06%	0.06%		
Atrazine	9.93%				0.35%	0.37%		0.74%	0.59%	0.63%		
Carbamazepine	45.31%				1.61%	1.68%		3.36%	2.68%	2.89%		
Bisphenol A		0.01%			0.0033%	0.0035%	0.0034%		0.0055%	0.0060%	0.0059%	
DEET		95.17%			49.61%	51.85%	51.44%		82.70%	89.11%	87.89%	
Chlorpyrifos		0.21%			0.11%	0.11%	0.11%		0.18%	0.19%	0.19%	
Cyclophosphamide		0.10%			0.05%	0.05%	0.05%		0.08%	0.09%	0.09%	
Diazinon		0.10%			0.05%	0.05%	0.05%		0.08%	0.09%	0.09%	
Dicamba		3.29%			1.72%	1.79%	1.78%		2.86%	3.08%	3.04%	
Diclofenac		0.04%			0.02%	0.02%	0.02%		0.04%	0.04%	0.04%	
Diuron		0.62%			0.33%	0.34%	0.34%		0.54%	0.58%	0.58%	
Fluoxetine		0.24%			0.12%	0.13%	0.13%		0.20%	0.22%	0.22%	

Furosemide		0.22%			0.11%	0.12%	0.12%		0.19%	0.21%	0.20%	
Gemfibrozil			22.74%		9.10%	9.51%	9.43%	19.01%				20.53%
Hexazinone			15.04%		6.02%	6.29%	6.24%	12.57%				13.58%
Hydrochlorthiazide			0.40%		0.16%	0.17%	0.17%	0.33%				0.36%
Ibuprofen			18.40%		7.36%	7.69%	7.63%	15.38%				16.61%
Indomethacin			0.66%		0.27%	0.28%	0.27%	0.55%				0.60%
Metolachlor			10.03%		4.01%	4.19%	4.16%	8.38%				9.05%
Metoprolol			0.89%		0.35%	0.37%	0.37%	0.74%				0.80%
Naproxen			9.07%		3.63%	3.79%	3.76%	7.58%				8.18%
Paracetamol			10.98%		4.39%	4.59%	4.56%	9.18%				9.91%
Picloram			11.79%		4.72%	4.93%	4.89%	9.85%				10.64%
Praziquantel				19.69%	0.85%		0.88%	1.78%	1.42%		1.51%	1.92%
Propoxur				29.40%	1.27%		1.32%	2.65%	2.12%		2.25%	2.86%
Propranolol				13.56%	0.59%		0.61%	1.22%	0.98%		1.04%	1.32%
Simazine				8.72%	0.38%		0.39%	0.79%	0.63%		0.67%	0.85%
Tributylphosphate				0.17%	0.01%		0.01%	0.01%	0.01%		0.01%	0.02%
Triclopyr				6.85%	0.30%		0.31%	0.62%	0.49%		0.52%	0.67%
Triclosan				0.11%	0.00%		0.005%	0.010%	0.008%		0.008%	0.010%
Trimethoprim				21.19%	0.92%		0.95%	1.91%	1.53%		1.62%	2.06%
Tris(2-chloroethyl)phosphate (TCEP)				0.31%	0.01%		0.01%	0.03%	0.02%		0.02%	0.03%
5-Methyl-1H-benzotriazole				0.0046%	0.0002%		0.0002%	0.0004%	0.0003%		0.0004%	0.0005%


EP1
EP2
inhibition of bioluminescence in Vibrio Fischeri [%]
log (Σ concentrations / M)
IA
CA

128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EP4
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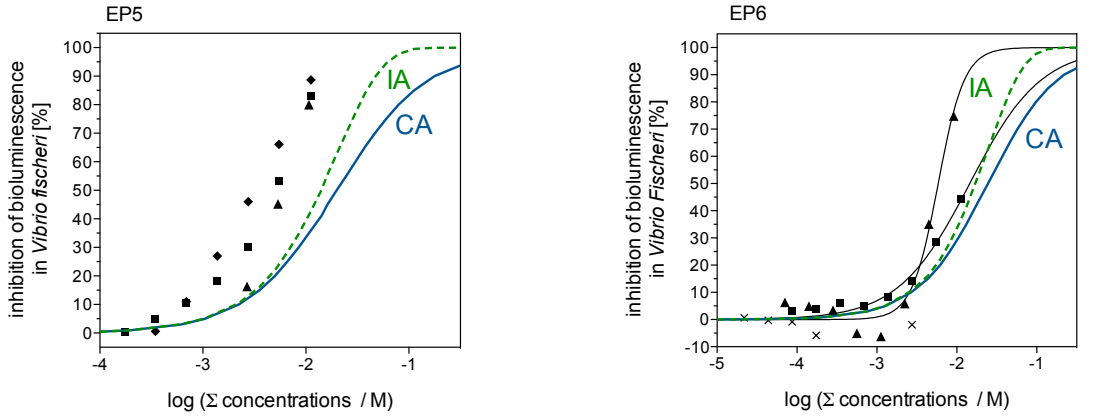
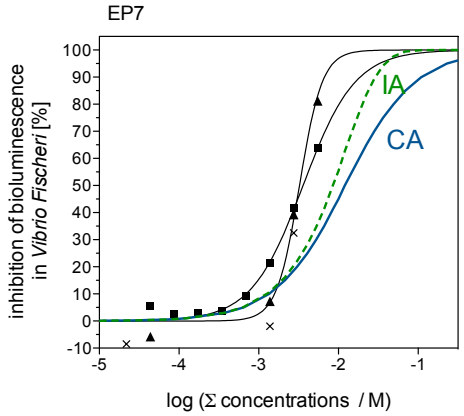
log (Σ concentrations / M)
IA
CA

129


EP5
EP6
inhibition of bioluminescence in Vibrio fischeri [%]
inhibition of bioluminescence in Vibrio Fischeri [%]
log (Σ concentrations / M)
IA
CA

130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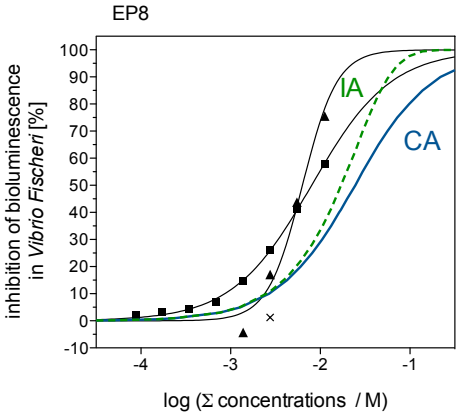
log (Σ concentrations / M)
IA
CA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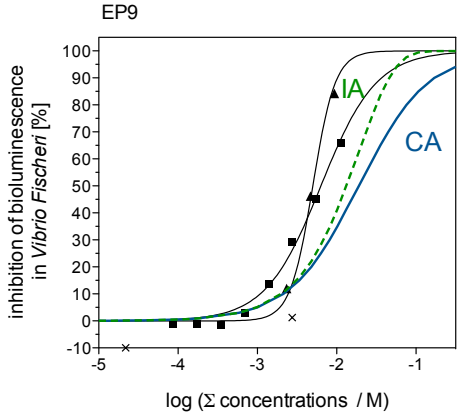
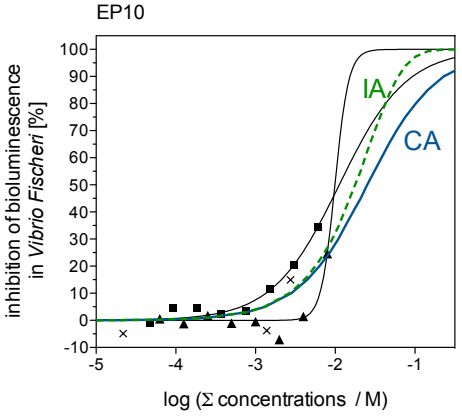
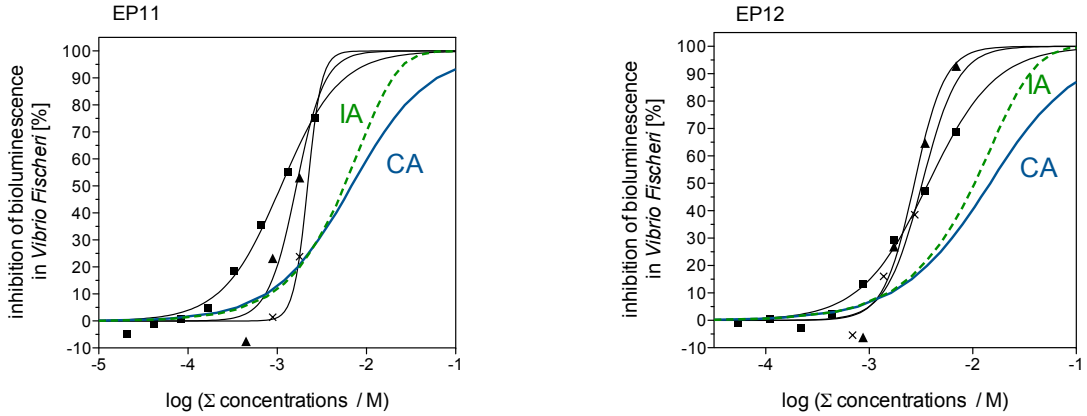
inhibition of bioluminescence in *Vibrio Fischeri* [%]
log (Σ concentrations / M)
IA
CA

131


EP9
inhibition of bioluminescence in *Vibrio Fischeri* [%]
log (Σ concentrations / M)
IA
CA


EP10
inhibition of bioluminescence in *Vibrio Fischeri* [%]
log (Σ concentrations / M)
IA
CA

132



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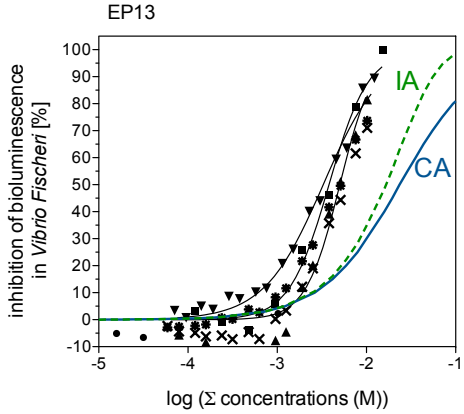

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135

136 *Figure SI-4 Concentration-effect curves of equipotent mixtures (EP).*

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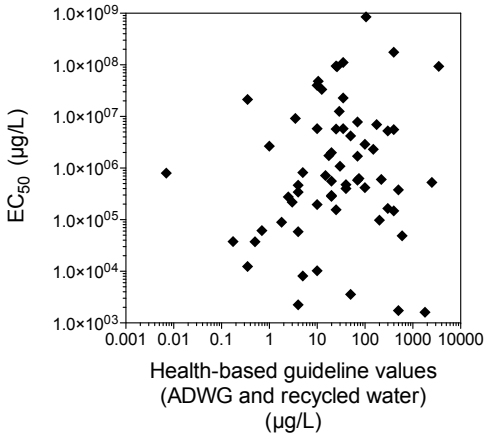
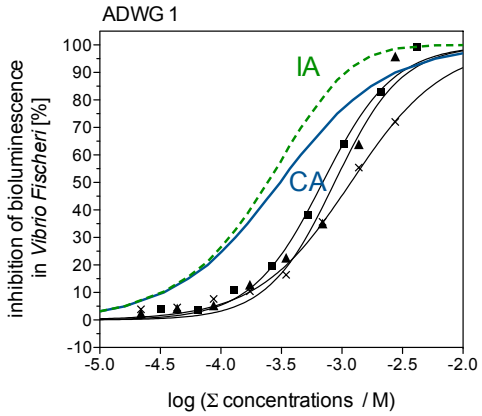


139

140 *Figure SI-5 The guideline values are not correlated to the EC_{50} values in the bioluminescence inhibition*
141 *assay with* Vibrio fischeri.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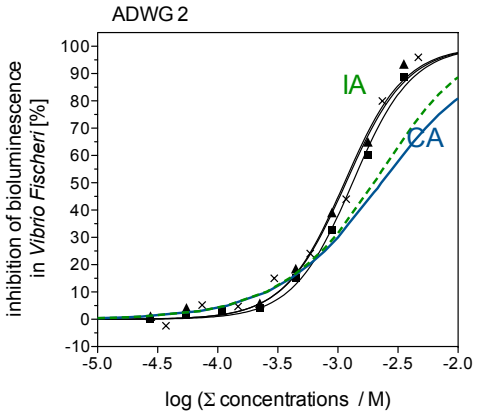
log (Σ concentrations / M)
IA
CA


ADWG 2
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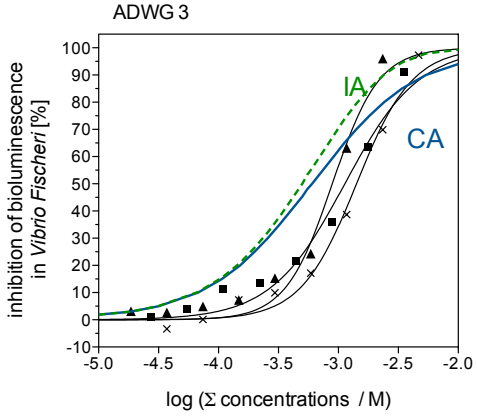
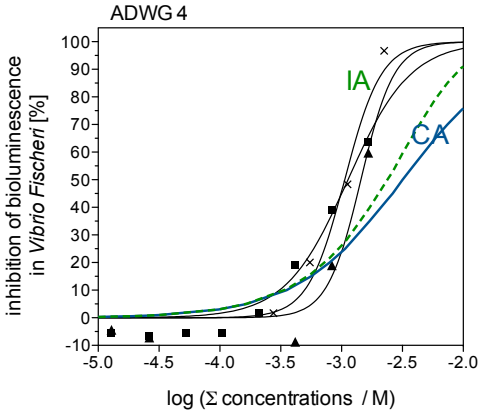
log (Σ concentrations / M)
IA
CA

142


ADWG 3
inhibition of bioluminescence in *Vibrio Fischeri* [%]
log (Σ concentrations / M)
IA
CA


ADWG 4
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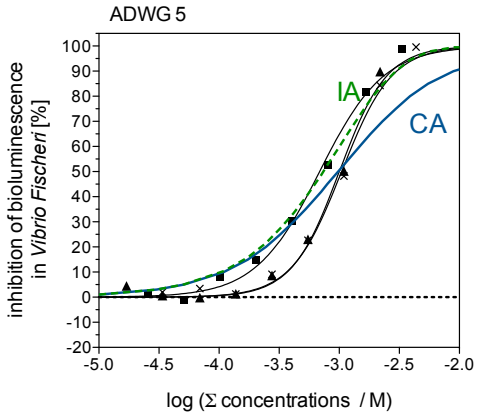
log (Σ concentrations / M)
IA
CA

143


ADWG 5
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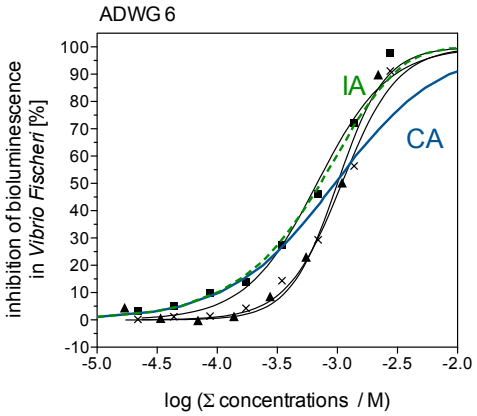
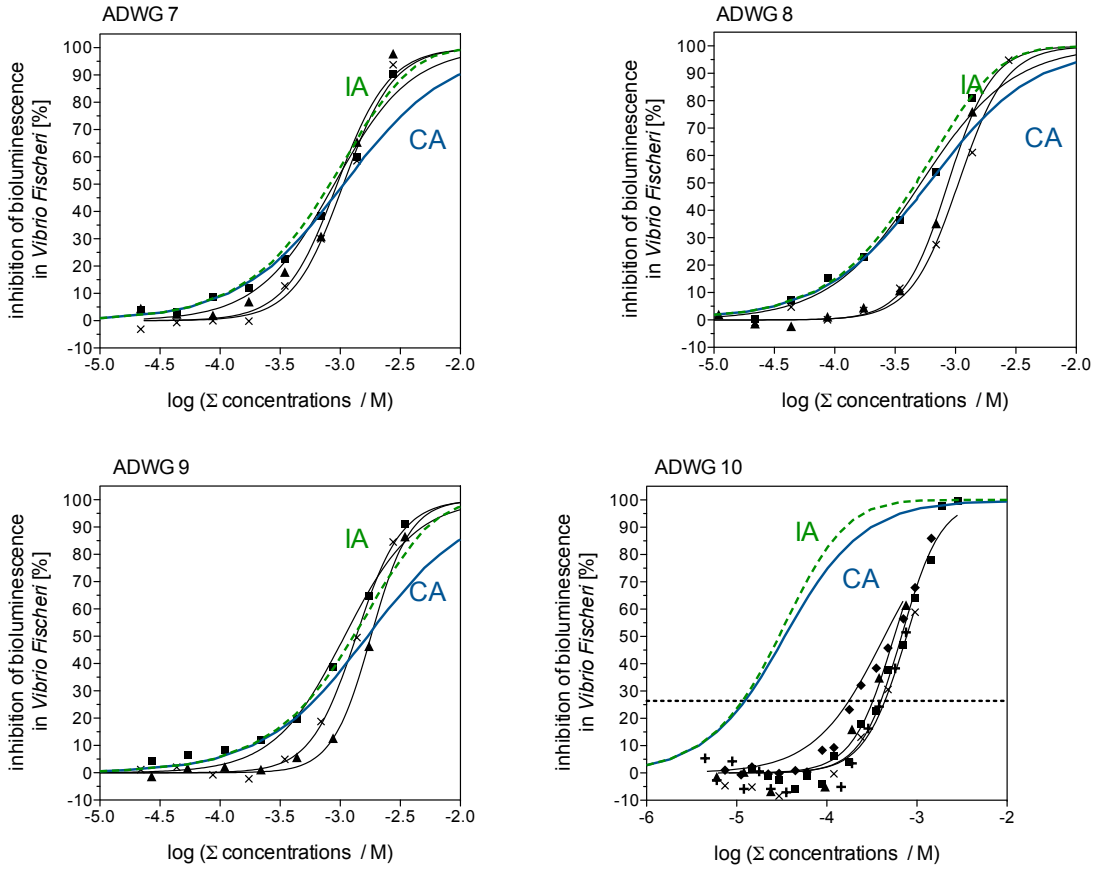
log (Σ concentrations / M)
IA
CA


ADWG 6
inhibition of bioluminescence in *Vibrio Fischeri* [%]
log (Σ concentrations / M)
IA
CA

144



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146

147 *Figure SI-6 Concentration-effect curves of mixtures (ADWG).*

148

149

150 *Table SI-7 Detected chemicals in the six environmental samples where chemicals were present at*
151 *concentrations about the limit of reporting (LOR).*

Analyte	**Units**	**LOR**	**Eff-1** (secondary treated effluent, influent to MF)	**MF** (after micro-filtration)	**RO** (after reverse osmosis)	**Eff-2** (secondary effluent (influent to O_3/BAC)	**O_3/BAC** (after ozonation and biologically activated carbon filtration)	**SW** (storm-water)
number of chemicals detected			40	39	6	48	6	5
17-β-Estradiol	µg/L	0.005				0.006		
Nonylphenol	µg/L	0.1				0.13		
4-*t*-Octylphenol	µg/L	0.1		0.017		0.11		
Tonalid	µg/L	0.1	0.1	0.1		0.1		
Atenolol	µg/L	0.01	0.10	0.10		0.94		
Atorvastatin	µg/L	0.01				0.04		
Atrazine	µg/L	0.01	0.35	0.39				
Bisphenol A	µg/L	0.01		0.018		0.13		0.20
Caffeine	µg/L	0.02	0.05	0.04		0.21		
Carbamazepine	µg/L	0.01	1.6	1.9	0.02	2.5		
Cephalexin	µg/L	0.01				0.12		
Chlorpyrifos	µg/L	0.1				5.6		
Citalopram	µg/L	0.01	0.13	0.10		0.27	0.02	
Codeine	µg/L	0.1				0.24		
Cyclophos-phamide	µg/L	0.01	0.01			0.04		
Desmethyl Citalopram	µg/L	0.01	0.14	0.10		0.24	0.01	
Desmethyl Diazepam	µg/L	0.01	0.03	0.03		0.05		
Diazepam	µg/L	0.01	0.01			0.01		
Diazinon	µg/L	0.1				0.16		
Diclofenac	µg/L	0.01	0.11	0.12		0.26		
Diuron	µg/L	0.01	0.16	0.14	0.03	0.07		0.04
Doxylamine	µg/L	0.01	0.24	0.18		0.44		
Erythromycin	µg/L	0.01	0.02	0.02		0.05		
Fipronil	µg/L	0.1	0.1	0.1				
Fluoxetine	µg/L	0.01	0.03	0.03		0.03		
Frusemide	µg/L	0.01	0.13	0.15		1.3		
Galaxolide	µg/L	0.1	1.0	1.1		1.6		
Gemfibrozol	µg/L	0.01	0.08	0.07		0.15		
Hexazinone	µg/L	0.01	0.02	0.02				
Hydrochlor-thiazide	µg/L	0.01	0.76	0.65	0.01	1.5		
Indomethacin	µg/L	0.01				0.08		
Lincomycin	µg/L	0.01						
Metolachlor	µg/L	0.01	0.82	0.73	0.01	0.01	0.01	
Metoprolol	µg/L	0.01	0.12	0.14		0.97		
Naproxen	µg/L	0.1				0.32		

Analyte	**Units**	**LOR**	**Eff-1** (secondary treated effluent, influent to MF)	**MF** (after micro-filtration)	**RO** (after reverse osmosis)	**Eff-2** (secondary effluent (influent to O_3/BAC)	**O_3/BAC** (after ozonation and biologically activated carbon filtration)	**SW** (storm-water)
Norfloxacin	µg/L	0.05	0.06			0.10		
Oxazepam	µg/L	0.01	0.60	0.57		1.1		
Oxycodone	µg/L	0.01	0.03	0.03		0.16		
Paracetamol	µg/L	0.02						0.02
Praziquantel	µg/L	0.01	0.01	0.01				
Propoxur	µg/L	0.01	0.03	0.03		0.05		
Propranolol	µg/L	0.01	0.01	0.02		0.14		
Ranitidine	µg/L	0.05				0.70		
Roxithromycin	µg/L	0.02	0.05	0.04		0.08		
Simazine	µg/L	0.01	0.18	0.23		0.17		0.02
Sulphadiazine	µg/L	0.01	0.03	0.03		0.13		
Sufamethoxazole	µg/L	0.01	0.15	0.07		0.21		
Temazepam	µg/L	0.01	0.47	0.50		0.65		
Triclosan	µg/L	0.01	0.02	0.02		0.05		
Trimethoprim	µg/L	0.01	0.07	0.05		0.23		
Tris(chloroethyl) phosphate	µg/L	0.1	0.4	0.4		0.4	0.3	
Venlafaxine	µg/L	0.01	1.6	1.9	0.01	2.4	0.10	
DEET	µg/L	0.01	0.11	0.10		0.18	0.03	0.11
1H-Benzotriazole, 5-methyl	µg/L	0.2	0.53	0.54	0.32	1.3		

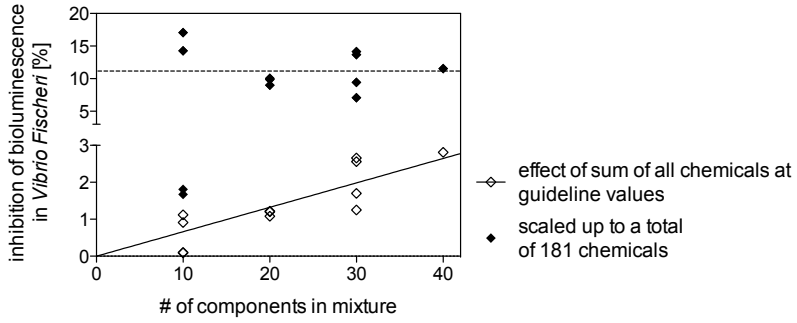
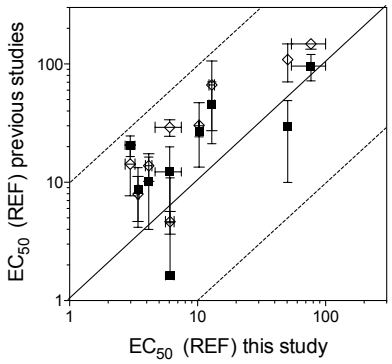


Figure SI-7 Validation of the proposed guideline value with the experimental ADWG mixtures (10 to 40 compounds).



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160 *Figure SI-8 Comparison of the median effect concentrations EC_{50} in the present study with previous work*
161 *at the same sampling sites (samplings in 2010 to 2012), black squares: data from (Macova et al. 2011),*
162 *empty black diamonds: data from (Escher et al. 2012).*

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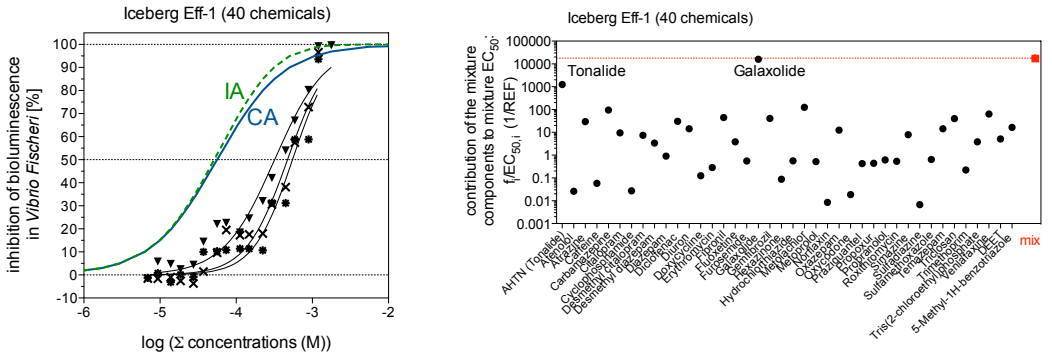

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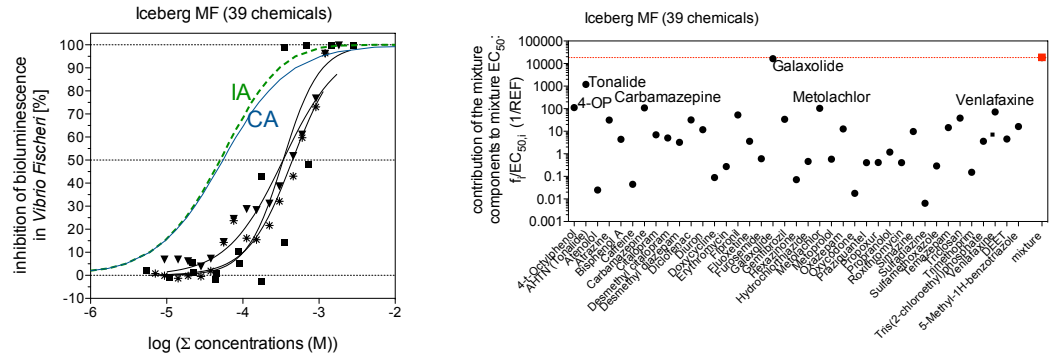

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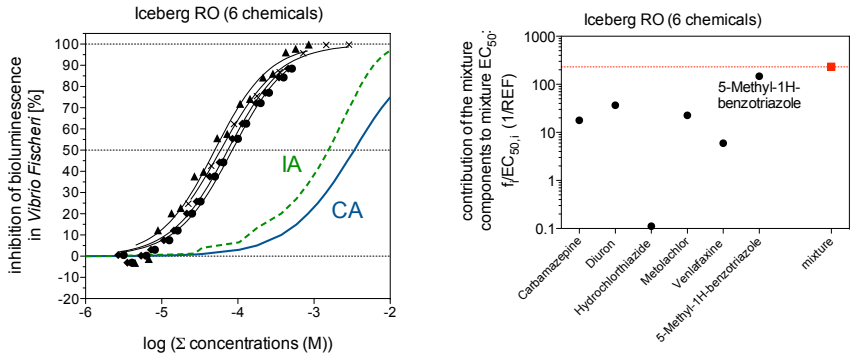

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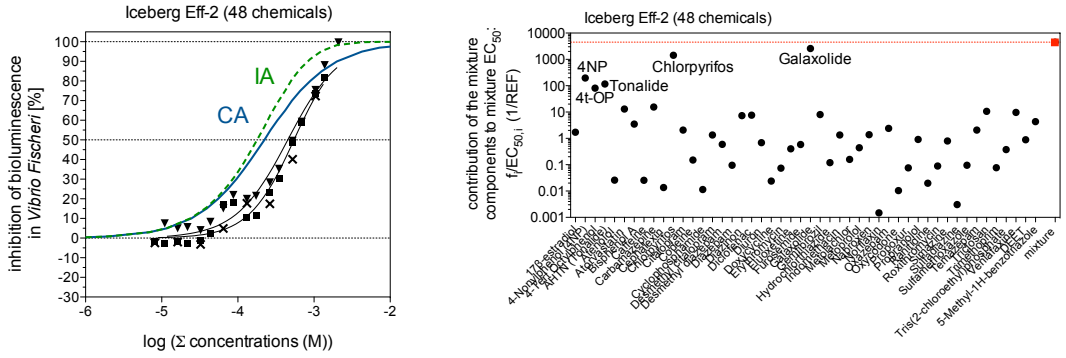

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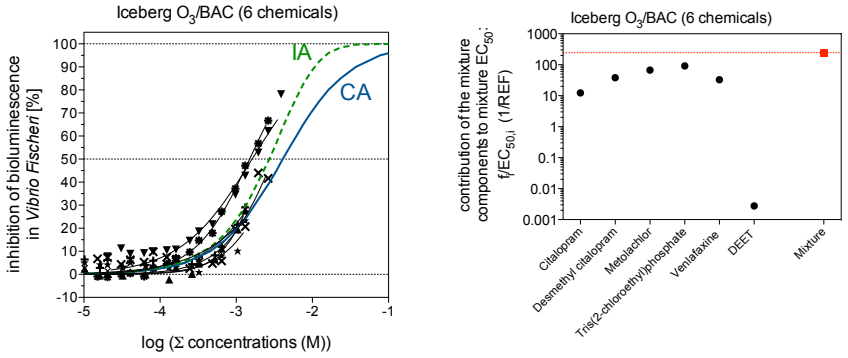

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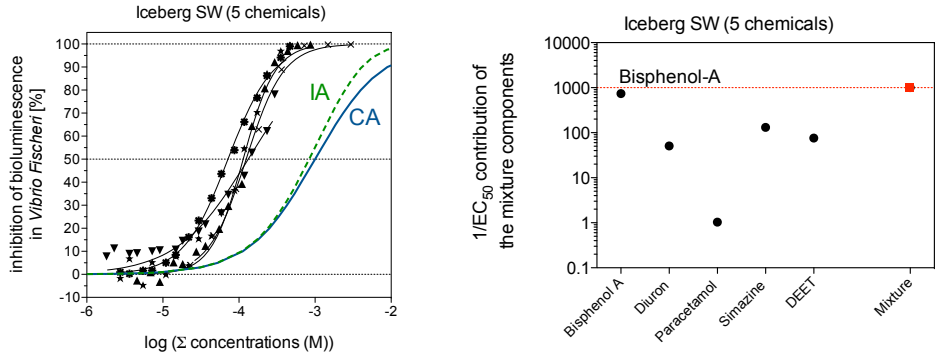

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172 *Figure SI-9 Concentration-effect curves of the iceberg mixture of chemicals in the environmental samples*
173 *(left) and contribution of the individual components to the EC_{50} of the mixture (total effect of mixture*
174 *indicated in red).*

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Supplementary Information

Mixture Effects of Organic Micropollutants Present in Water: Towards the Development of Effect-Based Water Quality Trigger Values for Baseline Toxicity

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Table of content

Table SI-1 Chemicals contained in the Australian Drinking Water Guidelines (ADWG) but not detected and therefore only used for the QSAR comparison and not in the mixtures, their physicochemical descriptors, experimental and modelled EC_{50} values.

Chemical	CAS Number	Molar weight (g/mol)	$logK_{ow}$	$logK_{lipw}$ (L/kg)	$logD_{lipw}$ (pH7) (L/kg)	Experimental log 1/ EC_{50} (M)	stdev	Predicted log 1/ EC_{50} (M) QSAR
Aldicarb	116-06-3	190.26	1.13	1.22	1.22	2.87	0.12	1.81
Amitraz	33089-61-1	293.42	5.5	5.68	5.68	4.40	0.47	4.92
Azinophos-methyl	86-50-0	317.32	2.75	2.88	2.88	4.37	0.05	2.96
Carbaryl	63-25-2	201.23	2.36	2.48	2.48	4.99	0.29	2.69
Carbofuran	1563-66-2	221.26	2.32	2.44	2.44	3.87	0.11	2.66
Dichlorprop	120-36-5	235.07	3.43	3.57	2.57	3.26	0.26	2.75
Dichlorvos	62-73-7	220.98	1.43	1.53	1.53	2.99	0.19	2.02
Dimethoate	60-51-5	229.25	0.78	0.87	0.87	3.82	0.11	1.56
Ethion	563-12-2	384.46	5.07	5.24	5.24	2.87	0.06	4.61
Fenitrothion	122-14-5	277.23	3.3	3.44	3.44	3.79	0.63	3.35
Fluometuron	2164-17-2	232.21	2.42	2.54	2.54	2.19	0.05	2.73
Methomyl	16752-77-5	162.21	0.6	0.68	0.68	2.11	0.07	1.43
Metribuzin	21087-64-9	214.29	1.7	1.80	1.80	2.99	0.48	2.22
Pendimethalin	40487-42-1	281.31	5.18	5.35	5.35	2.95	0.40	4.69
Piperonyl butoxide	51-03-6	338.45	4.75	4.92	4.92	3.85	0.30	4.39
Pirimicarb	23103-98-2	238.29	1.7	1.80	1.80	2.84	0.06	2.21
Pirimiphos-methyl	23505-41-1	333.39	4.2	4.35	4.35	3.44	0.27	3.99
Propanil	709-98-8	218.08	3.07	3.20	3.20	4.01	0.19	3.19
Propargite	2312-35-8	350.48	5	5.17	5.17	4.34	0.15	4.56
Propiconazole	60207-90-1	342.23	3.72	3.86	3.86	3.74	0.19	3.65
Salicylic acid	69-72-7	138.12	2.26	2.38	1.38	1.95	0.49	1.92
Warfarin	81-81-2	308.34	2.7	2.82	1.97	2.86	0.77	2.33

Table SI-2 Chemicals used in the present study, their manufacturer, purity and chemical category/type.

Chemical	CAS	Manufacturer	Catalogue number	Grade	Chemical Category
Reference chemicals					
2-Butoxyethanol	111-76-2	Sigma-Aldrich	537551-1L-A	≥99%	Reference
2-Nitrotoluene	88-72-2	Aldrich	438804-5mL	≥99%	Reference
3-Nitroaniline	99-09-2	Supelco	442392	Analytical Standard	Reference
2,4,5-Trichloraniline	636-30-6	Fluka	35828-1g	Pestanal	Reference
4-n-Pentylphenol	1438-35-3	Aldrich	77102-10g	≥98%	Reference
2-Phenylphenol	90-43-7	Fluka	45529-250mg	Pestanal	Reference
Phenol	108-95-2	Fluka	77610-250g	≥99.5%	Reference
Mixture chemicals					
17-estradiol	50-28-2	Sigma	E8875-1g	≥98%	Pharmaceutical
2,4-Dichlorophenoxyacetic acid (2,4,D)	94-75-7	Fluka	31518-250mg	Pestanal	Herbicide
4-Nonylphenol (4NP)	104-40-5	Fluka	46405-100mg	Pestanal	Herbicide
4-Tert Octylphenol	140-66-9	Supelco	442858	Analytical Standard	Consumer/industrial chemical
Acetylsalicylic acid (Aspirin)	50-78-2	Aldrich	239631-1g	≥99%	Pharmaceutical
Atenolol	29122-68-7	Sigma	A7655-1g	≥98%	Pharmaceutical
Atorvastatin calcium	134523-03-8	Dr. Ehrenstorfer GmBh	C10318000	Ref Std	Pharmaceutical
Atrazine (total) including metabolites	1912-24-9	Fluka	45330-250mg-R	Pestanal	Herbicide
Bisphenol A	80-05-7	Aldrich	239658-50g	≥99 %	Consumer/industrial chemical
Caffeine	58-08-2	Sigma-Aldrich	C1778-1VL	Sigma Ref Std	Pharmaceutical
Carbamazepine	298-46-4	Sigma-Aldrich	49939-1g		
Cephalexin	15686-71-2	Fluka	33989-100mg-R	Vetranal	Antibiotics
Chlorpyrifos	2921-88-2	Fluka	45395-250mg	Pestanal	Organophosphate Insecticide
Citalopram hydrobromide	59729-32-7	USP	1134233	Ref Std	Pharmaceutical
Codeine	76-57-3	Cerilliant	C-006	Certified Reference Material	Pharmaceutical
Cyclophosphamide	6055-19-2	Sigma	C7397-1g	Ref Std	Pharmaceutical

Chemical	CAS	Manufacturer	Catalogue number	Grade	Chemical Category
monohydrate					
DEET (N,N-diethyltoluamide (NN-diethyl-3-methylbenzamide))	134-62-3	Fluka	36542-250mg	Pestanal	Consumer/industrial chemical
Desmethyl citalopram	144025-14-9	Cerilliant	D-047	Certified Reference Material	Pharmaceutical Metabolite
Desmethyl diazepam (Nordiazepam)	1088-11-5	Cerilliant	N-905	Certified Reference Material	Pharmaceutical Metabolite
Diazepam (Valium)	439-14-5	Sigma	D0899-100mg	Ref Std	Pharmaceutical
Diazinon	333-41-5	Fluka	45428-250mg	Pestanal	Organophosphate Insecticide
Dicamba	1918-00-9	Sigma-Aldrich	45430-250mg	Pestanal	Organochlorine Herbicide
Diclofenac acid	15307-86-5	Dr. Ehrenstorfer GmBh	C 12537000	Ref Std	Pharmaceutical
Diuron	330-54-1	Fluka	45463-250mg	Pestanal	
Doxycycline hyclate	24390-14-5	Fluka	33429-100mg-R	Vetranal	Antibiotics
Erythromycin	114-07-8	Fluka	16221-500mg	Pharmaceutical secondary standard	Antibiotics
Fipronil	120068-37-3	Fluka	46451-100mg	Pestanal	Insecticide
Fluoxetine hydrochloride (Prozac)	56296-78-7	Fluka	34012-10mg-R	Vetranal	Pharmaceutical
Furosemide	54-31-9	Fluka	09205-1g	Pharmaceutical Secondary Standard	Pharmaceutical
Galaxolide (1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[g]-2-benzopyran)	1222-05-5	Dr. Ehrenstorfer GmBh	C 1421300	Ref Std	Consumer/industrial chemical
Gemfibrozil	25812-30-0	Sigma	G9518-5g	Ref Std	Pharmaceutical
Hexazinone	51235-04-2	Fluka	36129-100mg	Pestanal	Herbicide
Hydrochlorthiazide	58-93-5	Fluka	08213-1g	Pharmaceutical secondary standard	Pharmaceutical

Chemical	CAS	Manufacturer	Catalogue number	Grade	Chemical Category
Ibuprofen 400	15687-27-1	Fluka	32424-100mg	Vetranal	Pharmaceutical
Indomethacin	53-86-1	Sigma	I8280-5g	USP Testing Spec	Pharmaceutical
Lincomycin hydrochloride monohydrate	7179-49-9	Fluka	31727-250mg	Vetranal	Antibiotics
MCPA (2-Methyl-4-chlorophenoxyacetic acid)	94-74-6	Fluka	45555-250mg	Pestanal	Herbicide
Metolachlor	51218-45-2	AccuStandard	P-158NB-250	96.4%	Herbicide
Metoprolol tartrate salt	56392-17-7	Fluka	77376-1g	Pharmaceutical Secondary c Standard	Pharmaceutical
Naproxen	22204-53-1	Fluka	36405-500mg	Pharm Sec Std	Pharmaceutical
Norfloxacin	70458-96-7	Fluka	33899-100mg-R	Vetranal	Antibiotics
Oxazepam	604-75-1	Cerilliant	O-902	Certified Reference Material	Pharmaceutical
Oxycodone	76-42-6	Cerilliant	O-002	Certified Reference Material	Pharmaceutical
Oxytetracycline dihydrate(Terramycin)	6153-64-6	Sigma	O4636-10g	≥99%	Antibiotics
Paracetamol (acetaminophen)	103-90-2	Sigma-Aldrich	A3035-1VL	Analytical Standard	Pharmaceutical
Picloram	1918-02-1	Fluka	36774-250mg-R	Pestanal	Herbicide
Praziquantel	55268-74-1	Fluka	46648-250mg	Vetranal	Pharmaceutical
Propoxur	114-26-1	Fluka	45644-250mg	Pestanal	Carbamate Insecticide
Propranolol hydrocloride	318-98-9	Sigma	P0884-1g	≥99%	Pharmaceutical
Ranitidine hydrochloride	66357-59-3	Fluka	44404-500mg	Pharmaceutical Secondary c Standard	Pharmaceutical
Roxithromycin	80214-83-1	Sigma	R4393-1g	≥90%	Antibiotics
Simazine	122-34-9	Fluka	32059-250mg	Pestanal	Pharmaceutical
Sulfadiazine	68-35-9	Fluka	35033-100mg	Vetranal	Pharmaceutical
Sulfamethoxazole	723-46-6	Fluka	31737-250mg	Vetranal	Antibiotics
Sulfasalazine	599-79-1	Fluka	S0883-10g	≥98%	Pharmaceutical

Chemical	CAS	Manufacturer	Catalogue number	Grade	Chemical Category
Temazepam	846-50-4	Sigma-Aldrich	T-907	Certified Reference Material	Pharmaceutical
Tolutriazole (5-Methyl-1H-benzotriazole)	136-85-6	Aldrich	196304-10g	98%	Pharmaceutical
Tonalide (AHTN, 6-Acetyl-1,1,2,4,4,7-hexamethyltetraline)	21145-77-7	Aldrich	CDS009866-50mg	CPR	Musk
Tributylphosphate	126-73-8	Aldrich	240494-5mL	≥99%	Consumer/industrial chemical
Triclopyr	55335-06-3	Fluka	32016-250mg	Pestanal	Herbicide
Triclosan (Irgasan)	3380-34-5	Sigma	72779-5g-F	≥97%	Consumer/industrial chemical
Trimethoprim	738-70-5	Fluka	46984-250mg	Vetranal	Antibiotics
Tris(2-chloroethyl)phosphate (TCEP)	115-96-8	Aldrich	119660-25g	97%	Consumer/industrial chemical
Venlafaxine hydrochloride	99300-78-4	Sigma-	V7264-10mg	≥98%	Pharmaceutical

Section SI-1 Additional information on sample preparation

The SPE extraction was performed according to Macova et al. (2011) with the sorbent material validated in NWC (2011). All samples were acidified to pH 3. Samples containing chlorine were quenched with sodium thiosulphate (1 g/L), and filtered with a glass fibre filter (GF/A Whatman) before extraction. Samples were extracted by passing through two 6 cc solid phase cartridges in series, first an Oasis® HLB (500mg, Catalogue Number 186000115, Waters) followed by a Supelclean coconut charcoal cartridge (2g, Catalogue Number 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 methanol and 10 mL of 5 mM HCl in MilliQ water. One litre of water was extracted on each pair of HLB and coconut charcoal cartridges under vacuum. Cartridges were sealed individually and 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 and were evaporated under purified nitrogen gas before being solvent exchanged to methanol at a final volume of 1 mL.

Section SI-2 Additional information on the chemical analytical method

Chemical analysis was performed at a commercial NATA accredited analytical laboratory, Queensland Health Forensic and Scientific Services (QHFSS). Water samples underwent either SPE or liquid-liquid extraction before subject to GC-MS or LC-MS. Three standard analytical methods were used: QIS25391 Determination of endocrine disrupting compounds in effluent, river and recycled water, QIS27701 Pharmaceuticals and Personal Care Products (PPCP) in water, preparation and analysis by SPE and LCMSMS, QIS16315 Organochlorine, organophosphorous and synthetic pyrethroid pesticides, urea and triazine herbicides and PCBs in water.

Table SI-3 Analysed chemicals (in alphabetical order of analyte name) with methods and limit of reporting (LOR).

Analytical method	Analyte	Units	LOR
Endocrine Disrupting Compounds by GC-MS	17-α-Ethynylestradiol	ng/L	5
Endocrine Disrupting Compounds by GC-MS	17-β-Estradiol	ng/L	5
GC-MS Screen	1H-Benzotriazole	µg/L	0.2
GC-MS Screen	1H-Benzotriazole, 1-methyl	µg/L	0.2
GC-MS Screen	1H-Benzotriazole, 5-methyl	µg/L	0.2
Pesticides by GC-MS	2,4-D	µg/L	0.1
Pesticides by GC-MS	2,4-DB	µg/L	0.1
Pesticides by GC-MS	2,4-DP (Dichlorprop)	µg/L	0.1
Pesticides by GC-MS	2,4,5-T	µg/L	0.1

Analytical method	Analyte	Units	LOR
GC-MS Screen	2,6-Di-t-butyl-p-cresol (BHT)	µg/L	0.5
GC-MS Screen	2,6-Di-t-butylphenol	µg/L	0.2
Pesticides by GC-MS	3-Hydroxycarbofuran	µg/L	0.1
Herbicides and Other Compounds by LC-MS	3,4-Dichloroaniline	µg/L	0.01
GC-MS Screen	4-Chloro-3,5-dimethylphenol	µg/L	0.1
Endocrine Disrupting Compounds by GC-MS	4-t-Octylphenol	ng/L	10
Pharmaceuticals by LC-MS	Acesulfame	µg/L	0.01
Pharmaceuticals by LC-MS	Acetylsalicylic acid	µg/L	0.01
Pesticides by GC-MS	Aldicarb	µg/L	0.1
Pesticides by GC-MS	Aldicarb sulfone (Aldoxycarb)	µg/L	0.1
Pesticides by GC-MS	Aldicarb sulfoxide	µg/L	0.1
Pesticides by GC-MS	Aldrin (HHDN)	µg/L	0.1
Pesticides by GC-MS	Allethrin	µg/L	0.1
Herbicides and Other Compounds by LC-MS	Ametryn	µg/L	0.01
Herbicides and Other Compounds by LC-MS	Amitraz	µg/L	0.1
Endocrine Disrupting Compounds by GC-MS	Androsterone	ng/L	5
Pharmaceuticals by LC-MS	Atenolol	µg/L	0.01
Pharmaceuticals by LC-MS	Atorvastatin	µg/L	0.01
Herbicides and Other Compounds by LC-MS	Atrazine	µg/L	0.01
Pesticides by GC-MS	Azinphos-ethyl	µg/L	0.1
Pesticides by GC-MS	Azinphos-methyl	µg/L	0.1
Pesticides by GC-MS	Benalaxyl	µg/L	0.1
Pesticides by GC-MS	Bendiocarb	µg/L	0.1
Pesticides by GC-MS	Bifenthrin	µg/L	0.1
Pesticides by GC-MS	Bioresmethrin	µg/L	0.1
GC-MS Screen	Bisphenol A	µg/L	0.1
Pesticides by GC-MS	Bitertanol	µg/L	0.1
Herbicides and Other Compounds by LC-MS	Bromacil	µg/L	0.01
Pesticides by GC-MS	Bromophos-ethyl	µg/L	0.1
Pesticides by GC-MS	Cadusafos	µg/L	0.1
Pharmaceuticals by LC-MS	Caffeine	µg/L	0.02

Analytical method	Analyte	Units	LOR
Pesticides by GC-MS	Captan	g/L	0.2
Pharmaceuticals by LC-MS	Carbamazepine	g/L	0.01
Pesticides by GC-MS	Carbaryl	g/L	0.01
Pesticides by GC-MS	Carbofuran	g/L	0.1
Pesticides by GC-MS	Carbophenothion	g/L	0.1
Pharmaceuticals by LC-MS	Cephalexin	g/L	0.01
Pharmaceuticals by LC-MS	Chloramphenicol	g/L	0.1
Pesticides by GC-MS	Chlordene	g/L	0.1
Pesticides by GC-MS	Chlordene Epoxide	g/L	0.1
Pesticides by GC-MS	Chlordene-1-hydroxy	g/L	0.1
Pesticides by GC-MS	Chlordene-1-hydroxy-2,3-epoxide	g/L	0.1
Pesticides by GC-MS	Chlorfenvinphos	g/L	0.1
Pesticides by GC-MS	Chlorpyrifos	g/L	0.1
Pesticides by GC-MS	Chlorpyrifos oxon	g/L	0.1
Pesticides by GC-MS	Chlorpyrifos-methyl	g/L	0.1
Pharmaceuticals by LC-MS	Chlortetracycline	g/L	0.2
Pharmaceuticals by LC-MS	Ciprofloxacin	g/L	0.15
Pesticides by GC-MS	cis -Nonachlor	g/L	0.1
Pesticides by GC-MS	cis-Chlordane	g/L	0.1
Pharmaceuticals by LC-MS	Citalopram	g/L	0.01
Pesticides by GC-MS	Clopyralid	g/L	0.1
Pharmaceuticals by LC-MS	Codeine	g/L	0.1
Pesticides by GC-MS	Coumaphos	g/L	0.1
Pharmaceuticals by LC-MS	Cyclophosphamide	g/L	0.01
Pesticides by GC-MS	Cyfluthrin	g/L	0.1
Pesticides by GC-MS	Cyhalothrin	g/L	0.2
Pesticides by GC-MS	Cypermethrin	g/L	0.1
Herbicides and Other Compounds by LC-MS	Dalapon (2,2-DPA)	g/L	0.05
Pharmaceuticals by LC-MS	Dapsone	g/L	0.01
Pharmaceuticals by LC-MS	DEET	g/L	0.01
Pesticides by GC-MS	DEET	g/L	0.0

Analytical method	Analyte	Units	LOR
Pesticides by GC-MS	Deltamethrin	g/L	0.1
Pesticides by GC-MS	Demeton-S-methyl	g/L	0.1
Herbicides and Other Compounds by LC-MS	Desethyl Atrazine	g/L	0.01
Herbicides and Other Compounds by LC-MS	Desisopropyl Atrazine	g/L	0.01
Pharmaceuticals by LC-MS	Desmethyl Citalopram	g/L	0.01
Pharmaceuticals by LC-MS	Desmethyl Diazepam	g/L	0.01
Pharmaceuticals by LC-MS	Diazepam	g/L	0.01
Herbicides and Other Compounds by LC-MS	Diazinon	g/L	0.02
Pesticides by GC-MS	Dicamba	g/L	0.1
Pesticides by GC-MS	Dichlofluanid	g/L	0.1
Pesticides by GC-MS	Dichlorvos	g/L	0.1
Pharmaceuticals by LC-MS	Diclofenac	g/L	0.01
Pesticides by GC-MS	Diclofop-methyl	g/L	0.1
Pesticides by GC-MS	Dicloran	g/L	0.1
Pesticides by GC-MS	Dicofol	g/L	3.0
Pesticides by GC-MS	Dieldrin (HEOD)	g/L	0.05
Pesticides by GC-MS	Dimethoate	g/L	0.1
Pesticides by GC-MS	Dimethomorph	g/L	0.2
Pesticides by GC-MS	Dioxathion	g/L	0.1
Pesticides by GC-MS	Disulfoton	g/L	0.1
Herbicides and Other Compounds by LC-MS	Diuron	g/L	0.01
Pharmaceuticals by LC-MS	Doxylamine	g/L	0.01
Pesticides by GC-MS	Endosulfan Ether	g/L	0.1
Pesticides by GC-MS	Endosulfan Lactone	g/L	0.1
Pesticides by GC-MS	Endosulfan Sulfate	g/L	0.05
Pesticides by GC-MS	Endrin	g/L	0.1
Pesticides by GC-MS	Endrin aldehyde	g/L	0.1
Pharmaceuticals by LC-MS	Enrofloxacin	g/L	0.02
Pharmaceuticals by LC-MS	Erythromycin	g/L	0.01
Pharmaceuticals by LC-MS	Erythromycin anhydrate	g/L	0.01
Endocrine Disrupting Compounds by GC-MS	Estriol	ng/L	5

Analytical method	Analyte	Units	LOR
Endocrine Disrupting Compounds by GC-MS	Estrone	ng/L	5
Pesticides by GC-MS	Ethion	g/L	0.1
Pesticides by GC-MS	Ethoprophos	g/L	0.1
Endocrine Disrupting Compounds by GC-MS	Etiocholanolone	ng/L	5
Pesticides by GC-MS	Etrimphos	g/L	0.1
Pesticides by GC-MS	Famphur	g/L	0.1
Pesticides by GC-MS	Fenamiphos	g/L	0.1
Pesticides by GC-MS	Fenchlorphos	g/L	0.1
Pesticides by GC-MS	Fenitrothion	g/L	0.1
Pesticides by GC-MS	Fenoprop (2,4,5-TP)	g/L	0.1
Pesticides by GC-MS	Fenthion (methyl)	g/L	0.1
Pesticides by GC-MS	Fenthion-ethyl	g/L	0.1
Pesticides by GC-MS	Fenvalerate	g/L	0.1
Pesticides by GC-MS	Fipronil	g/L	0.1
Pesticides by GC-MS	Fluazifop-butyl	g/L	0.1
Herbicides and Other Compounds by LC-MS	Fluometuron	g/L	0.01
Pharmaceuticals by LC-MS	Fluoxetine	g/L	0.01
Pesticides by GC-MS	Fluroxypyr	g/L	0.1
Pesticides by GC-MS	Fluvalinate	g/L	0.1
Pharmaceuticals by LC-MS	Fluvastatin	g/L	0.01
Pharmaceuticals by LC-MS	Frusemide	g/L	0.01
Pesticides by GC-MS	Furalaxyl	g/L	0.1
Pharmaceuticals by LC-MS	Gabapentin	g/L	0.05
FRAGRANCES by GC-MS	Galaxolide	g/L	0.1
Pharmaceuticals by LC-MS	Gemfibrozol	g/L	0.01
Herbicides and Other Compounds by LC-MS	Haloxyfop (acid)	g/L	0.01
Herbicides and Other Compounds by LC-MS	Haloxyfop-2-etotyl	g/L	0.01
Herbicides and Other Compounds by LC-MS	Haloxyfop-methyl	g/L	0.01
Pesticides by GC-MS	HCB	g/L	0.1
Pesticides by GC-MS	Heptachlor	g/L	0.03
Pesticides by GC-MS	Heptachlor Epoxide	g/L	0.03

Analytical method	Analyte	Units	LOR
Herbicides and Other Compounds by LC-MS	Hexazinone	µg/L	0.01
Pharmaceuticals by LC-MS	Hydrochlorthiazide	µg/L	0.01
Pharmaceuticals by LC-MS	Ibuprofen	µg/L	0.07
Pharmaceuticals by LC-MS	Ifosfamide	µg/L	0.01
Herbicides and Other Compounds by LC-MS	Imidacloprid	µg/L	0.01
Pharmaceuticals by LC-MS	Indomethacin	µg/L	0.01
Pharmaceuticals by LC-MS	Iopromide	µg/L	0.2
Pesticides by GC-MS	Isofenphos	µg/L	0.1
Pesticides by GC-MS	Lambda-cyhalothrin	µg/L	0.1
Pharmaceuticals by LC-MS	Lincomycin	µg/L	0.01
Pesticides by GC-MS	Lindane (γ-HCH)	µg/L	0.1
Pesticides by GC-MS	Malathion (Maldison)	µg/L	0.1
Pesticides by GC-MS	MCPA	µg/L	0.1
Pesticides by GC-MS	MCPB	µg/L	0.1
Pesticides by GC-MS	Mecoprop	µg/L	0.1
Pesticides by GC-MS	Metalaxyl	µg/L	0.1
Pesticides by GC-MS	Methidathion	µg/L	0.1
Pesticides by GC-MS	Methiocarb	µg/L	0.1
Pesticides by GC-MS	Methomyl	µg/L	0.1
Pesticides by GC-MS	Methomyl oxime	µg/L	0.5
Pesticides by GC-MS	Methoprene	µg/L	0.1
Pesticides by GC-MS	Methoxychlor	µg/L	0.1
Herbicides and Other Compounds by LC-MS	Metolachlor	µg/L	0.01
Pharmaceuticals by LC-MS	Metoprolol	µg/L	0.01
Pesticides by GC-MS	Metribuzin	µg/L	0.1
Pesticides by GC-MS	Mevinphos	µg/L	0.1
GC-MS Screen	Moclobemide	µg/L	0.5
Pesticides by GC-MS	Molinate	µg/L	0.1
Pesticides by GC-MS	Monocrotophos	µg/L	0.5
FRAGRANCES by GC-MS	Musk Ketone	µg/L	0.1
FRAGRANCES by GC-MS	Musk Xylene	µg/L	0.1

Analytical method	Analyte	Units	LOR
GC-MS Screen	N-Butyl benzenesulfonamide	µg/L	0.1
GC-MS Screen	N-Butyltoluenesulfonamide	µg/L	0.1
Pharmaceuticals by LC-MS	Naproxen	µg/L	0.1
Endocrine Disrupting Compounds by GC-MS	Nonylphenol	ng/L	100
Pharmaceuticals by LC-MS	Norfloxacin	µg/L	0.05
Endocrine Disrupting Compounds by GC-MS	Norgestrel	ng/L	10
Pesticides by GC-MS	o,p-DDD	µg/L	0.1
Pesticides by GC-MS	o,p-DDE	µg/L	0.1
Pesticides by GC-MS	o,p-DDT	µg/L	0.1
Pesticides by GC-MS	Omethoate	µg/L	0.5
Pesticides by GC-MS	Oxadiazon	µg/L	0.1
Pesticides by GC-MS	Oxamyl	µg/L	0.5
Pesticides by GC-MS	Oxamyl oxime	µg/L	0.5
Pharmaceuticals by LC-MS	Oxazepam	µg/L	0.01
Pesticides by GC-MS	Oxychlordane	µg/L	0.1
Pharmaceuticals by LC-MS	Oxycodone	µg/L	0.01
Pesticides by GC-MS	Oxydemeton-methyl	µg/L	0.1
Pesticides by GC-MS	Oxyfluorfen	µg/L	0.1
Pharmaceuticals by LC-MS	Oxytetracycline	µg/L	0.4
Pesticides by GC-MS	p,p-DDD	µg/L	0.1
Pesticides by GC-MS	p,p-DDE	µg/L	0.1
Pesticides by GC-MS	p,p-DDT	µg/L	0.1
Pharmaceuticals by LC-MS	Paracetamol	µg/L	0.02
Pesticides by GC-MS	Parathion (ethyl)	µg/L	0.1
Pesticides by GC-MS	Parathion-methyl	µg/L	0.1
Pesticides by GC-MS	Pendimethalin	µg/L	0.1
Pesticides by GC-MS	Permethrin	µg/L	0.1
Pesticides by GC-MS	Phenothrin	µg/L	0.1
Pharmaceutical by LC-MS	Phenytoin	µg/L	0.01
Pesticides by GC-MS	Phorate	µg/L	0.1
Pesticides by GC-MS	Phosmet	µg/L	0.1

Analytical method	Analyte	Units	LOR
Pesticides by GC-MS	Phosphamidon	g/L	0.1
Pesticides by GC-MS	Picloram	g/L	0.1
Pesticides by GC-MS	Piperonyl Butoxide	g/L	0.1
Pesticides by GC-MS	Pirimicarb	g/L	0.1
Pesticides by GC-MS	Pirimiphos-methyl	g/L	0.1
Pharmaceuticals by LC-MS	Praziquantel	g/L	0.01
Pharmaceuticals by LC-MS	Primidone	g/L	0.01
Pesticides by GC-MS	Procymidone	g/L	0.1
Pesticides by GC-MS	Profenofos	g/L	0.1
Pesticides by GC-MS	Promecarb	g/L	0.1
Herbicides and Other Compounds by LC-MS	Prometryn	g/L	0.01
Pesticides by GC-MS	Propanil	g/L	0.1
Pesticides by GC-MS	Propargite	g/L	0.2
Pesticides by GC-MS	Propazine	g/L	0.1
Pesticides by GC-MS	Propiconazole	g/L	0.1
Pesticides by GC-MS	Propoxur	g/L	0.01
Pesticides by GC-MS	Propoxur	g/L	0.01
Pharmaceuticals by LC-MS	Propranolol	g/L	0.01
Pesticides by GC-MS	Prothiophos	g/L	0.1
Pesticides by GC-MS	Pyrazophos	g/L	0.1
Pharmaceuticals by LC-MS	Ranitidine	g/L	0.05
Pesticides by GC-MS	Rotenone	g/L	0.1
Pharmaceuticals by LC-MS	Roxithromycin	g/L	0.02
Pharmaceuticals by LC-MS	Salicylic acid	g/L	0.1
Pharmaceuticals by LC-MS	Sertraline	g/L	0.01
Herbicides and Other Compounds by LC-MS	Simazine	g/L	0.01
Pharmaceuticals by LC-MS	Simvastatin	g/L	0.1
Pharmaceuticals by LC-MS	Sulfasalazine	g/L	0.01
Pharmaceuticals by LC-MS	Sulfsalazine	g/L	0.01
Pharmaceuticals by LC-MS	Sulphadiazine	g/L	0.01
Pharmaceuticals by LC-MS	Sulphamethoxazole	g/L	0.01

Analytical method	Analyte	Units	LOR
Pharmaceuticals by LC-MS	Sulphathiazole	g/L	0.01
Pesticides by GC-MS	Sulprofos	g/L	0.1
Pesticides by GC-MS	Tebuconazole	g/L	0.1
Herbicides and Other Compounds by LC-MS	Tebuthiuron	g/L	0.01
Pharmaceuticals by LC-MS	Temazepam	g/L	0.01
Pesticides by GC-MS	Terbufos	g/L	0.1
Pesticides by GC-MS	Terbufos	g/L	0.1
Pesticides by GC-MS	Terbuthylazine	g/L	0.1
Herbicides and Other Compounds by LC-MS	Terbutryn	g/L	0.01
Endocrine Disrupting Compounds by GC-MS	Testosterone	ng/L	10
Pesticides by GC-MS	Tetrachlorvinphos	g/L	0.1
Pharmaceuticals by LC-MS	Tetracycline	g/L	0.1
Pesticides by GC-MS	Tetradifon	g/L	0.1
Pesticides by GC-MS	Tetramethrin	g/L	0.1
Pesticides by GC-MS	Thiabendazole	g/L	0.2
Pesticides by GC-MS	Thiodicarb	g/L	0.1
FRAGRANCES by GC-MS	Tonalid	g/L	0.1
Pharmaceuticals by LC-MS	Tramadol	g/L	0.01
Pesticides by GC-MS	trans-Chlordane	g/L	0.1
Pesticides by GC-MS	trans-Nonachlor	g/L	0.1
Pesticides by GC-MS	Transfluthrin	g/L	0.1
GC-MS Screen	Tri-n-butyl phosphate	g/L	0.1
Pesticides by GC-MS	Triadimefon	g/L	0.3
Pesticides by GC-MS	Triadimenol	g/L	0.1
Pesticides by GC-MS	Triallate	g/L	0.1
Pesticides by GC-MS	Triclopyr	g/L	0.1
GC-MS Screen	Triclosan	g/L	0.01
GC-MS Screen	Triclosan methyl ether	g/L	0.1
GC-MS Screen	Triethyl phosphate	g/L	0.1
Pesticides by GC-MS	Trifluralin	g/L	0.1
Pharmaceuticals by LC-MS	Trimethoprim	g/L	0.01

Analytical method	Analyte	Units	LOR
GC-MS Screen	Tris(chloroethyl) phosphate	µg/L	0.1
GC-MS Screen	Tris(chloropropyl) phosphate isomers	µg/L	0.1
GC-MS Screen	Tris(dichloropropyl) phosphate	µg/L	0.1
Pharmaceuticals by LC-MS	Tylosin	µg/L	0.05
Pharmaceuticals by LC-MS	Venlafaxine	µg/L	0.01
Pesticides by GC-MS	Vinclozolin	µg/L	0.1
Pharmaceuticals by LC-MS	Warfarin	µg/L	0.01
Pesticides by GC-MS	α-Endosulfan	µg/L	0.05
Pesticides by GC-MS	α-HCH (α-BHC)	µg/L	0.1
Pesticides by GC-MS	β-Endosulfan	µg/L	0.05
Pesticides by GC-MS	β-HCH (β-BHC)	µg/L	0.1
Pesticides by GC-MS	δ-HCH (δ-BHC)	µg/L	0.1

Section SI-3 Additional information on the bioluminescence inhibition test with *Vibrio fischeri*

The growth medium contained 513 mM NaCl, 44.2 mM NaH_2PO_4, 12.0 mM $K_2HPO_4 \cdot 3H_2O$, 0.83 mM $MgSO_4 \cdot 7H_20$, 3.78 mM $(NH_4)_2SO_4$, 41.0 mM glycerol, 5 g/L tryptone, and 0.50 g/L yeast extract. The cultures were allowed to grow at 20°C and 180 rpm until mid-exponential phase (22 hours) when they were diluted and frozen in liquid N_2. *V. fischeri* bacteria were stored at -80°C for up to 3 weeks prior to being used in the bioassay.

The assay was performed with autoclaved saline buffer containing 4 mM KCl, 10 mM $MgCl_2$, 10 mM MOPS (3-[N-morpholino] propanesulfonic acid), 342 mM NaCl with the pH adjusted to 7.0±0.2 with HCl/NaOH as the test medium. Briefly, the methanolic stock solutions of the reference compounds, the baseline toxicants or the extracts were either pipetted into a 96-well microtiter plate (Catalogue Number 655180, cell culture plate, 96 well, PS, F-Bottom (Chimney well), crystal-clear, sterile, Greiner Bio-One, Frickenhausen, Germany) and diluted with saline buffer (max 2% methanol in final bioassay) or the methanol was evaporated in a high recovery glass vial (Catalogue Number 5183-2030, high recovery screw vials, Agilent) and the residues were redissolved in saline buffer and transferred to the microtiter plate.

After a geometric dilution series in saline buffer the samples in 100 µL of saline buffer were then added to 50 µL of *V. fischeri* in growth medium in a white plate (Catalogue Number 655075, cell culture plate, 96 well, F-Bottom (Chimney well), medium binding, white, sterile, Greiner Bio-One, Frickenhausen, Germany). The luminescence output of the bacteria was measured prior to addition of sample and after 30-min incubation (Luminescence mode, FluoStar Optima, BMG Labtech, Ortenberg, Germany). The relative light units (RLU) should be around 150,000 to 850,000 at 4095 gain prior to sample addition.

Section SI-4 Additional information on the QSAR for baseline toxicity in bioluminescence inhibition assay with *Vibrio fischeri*

We previously developed baseline toxicity QSAR for the 30-min bioluminescence inhibition assay (Escher et al. 2008) using the six test compounds listed in Table SI-4 but as demonstrated in Figure SI-1A, the sensitivity of the assay has decreased since the publication in 2008, with butoxyethanol being equally toxic but the other reference chemicals having up to 8 times higher EC_{50} values (Table SI-4). The QSAR thus had a similar slope to an earlier published QSAR using the same compounds (Escher et al. 2008) but differed in the intercept, which indicates that the overall sensitivity of the current assay (indicated by the y-intercept) was slightly lower while the relative sensitivity (indicated by the slope) remained the same. The difference is not due to the fact that the cells were grown in the laboratory and shock-frozen as the commercially obtained freeze-dried cells as well as freshly grown cells showed the same sensitivity (Figure SI-1A). The EC_{50} values after 24 h incubation for all the reference baseline toxicants were in the same order of magnitude but the results were much more variable after 24 h incubation than after 30 min and the QSAR equation was of lower quality (Supplementary Information, Figure SI-1B).

The 96 well plate assay was of similar sensitivity to the classical cuvette version of the assay performed in various laboratories (Cronin and Schultz 1997, Vighi et al. 2009, Aruoja et al. 2011) (Figure SI-2).

Table SI-4 Physicochemical properties and experimental median effect concentration EC_{50} of the reference baseline toxicants.

	log K_{ow}[a]	logK_{lipw}[a]	log(1/EC_{50}(M))
2-Butoxyethanol	0.83	0.595	1.85 ± 0.02
2-Nitrotoluene	2.3	2.41	3.03 ± 0.03
3-Nitroanilin	1.37	2.17	2.71 ± 0.03
2,4,5-Trichloranilin	3.69	4.16	4.13 ± 0.03
4-n-Pentylphenol	4.24	4.31	4.60 ± 0.01
2-Phenylphenol	3.09	3.46	3.98 ± 0.01

[a] *Data from (Vaes et al. 1997).*

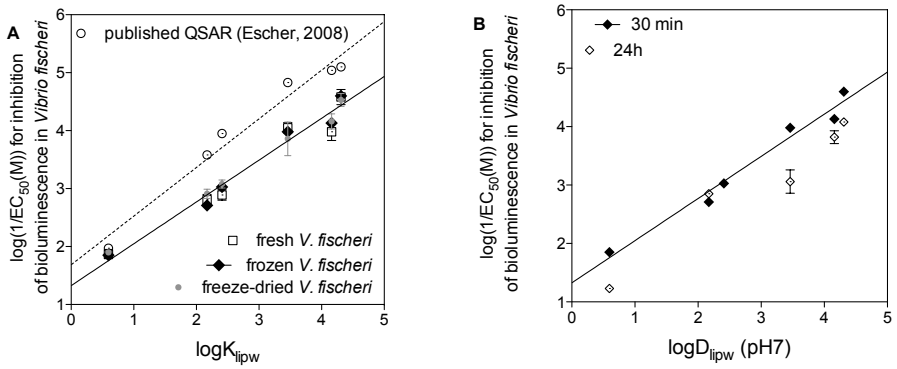


Figure SI-1A. QSAR for baseline toxicity established with 6 confirmed baseline toxicants. The empty circles and the dotted line describe the previously established QSAR log(1/EC_{50} (M)) = (0.84 ± 0.08)·logK_{lipw} (1.69 ± 0.24) (Escher et al., 2008). The black diamonds and solid line correspond to the new QSAR from the current project log(1/EC_{50} (M)) = (0.72 ± 0.06)·logK_{lipw} + (1.32 ± 0.18). For comparison the EC_{50} values for fresh (empty squares) and commercial freeze-dried (grey circles) Vibrio fischeri are also depicted. B. Comparison of the median effect concentrations (EC_{50}) of the reference baseline toxicants after 30 min and 24h of incubation.

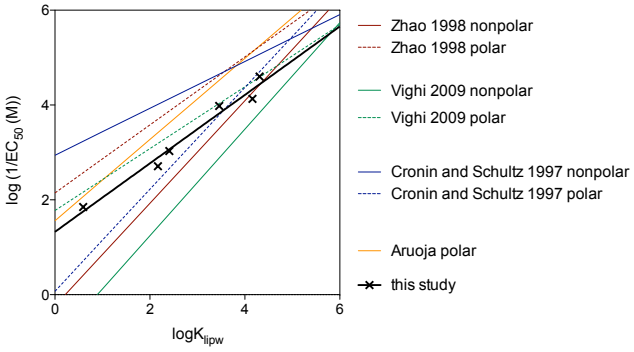


Figure SI-2. Comparison of the Quantitative Structure-Activity Relationship (QSAR) derived in the present study (in bold) with QSARs published in literature (Cronin and Schultz 1997, Zhao et al. 1998, Vighi et al. 2009), rescaled from K_{ow} to K_{lipw} (all chemicals are neutral so no pH correction to D_{lipw}(pH7) was necessary.

According to literature, antibiotics often do not show any effects in the standard bioluminescence inhibition assay after 30 min of incubation, thus it has been recommended that the test should be extended to 24 h to capture the effect of antibiotics (Backhaus and Grimme 1999). In the present study the activity of the antibiotics after 30 min incubation was similar or higher than the QSAR predicted and after 24 h of incubation the antibiotics increased by three to six orders of magnitude in toxicity (Figure SI-3). Thus antibiotics have a specific effect on the bacteria *V. fischeri.* Antibiotics also pose a problem in the QSAR as many are very hydrophilic, multifunctional and/or zwitterionic, so the estimation of their logD_{lipw}(pH7) is difficult and many would fall outside the validity range of the QSAR equation (0.5 < logD_{lipw}(pH7) < 4.5).

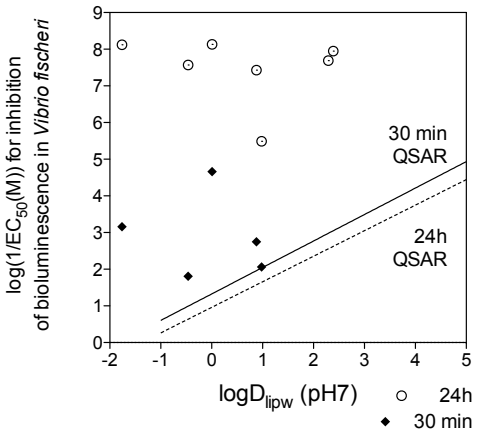


Figure SI-3. Comparison of the EC_{50} of the antibiotics after 30 min (diamond shape) and 24h (open circles) of incubation. The drawn line is the baseline toxicity QSAR for 30 min incubation, the dotted line is the baseline toxicity QSAR for 24h incubation.

Table SI-5 Mixture ratios of the chemicals in the equipotent mixtures (EP).

Mixture Composition	EP1	EP2	EP3	EP4	EP5	EP6	EP7	EP8	EP9	EP10	EP11	EP12
17-estradiol	0.03%				0.02%	0.02%		0.02%	0.02%	0.03%		
2,4-Dichlorophenoxyacetic acid	1.13%				0.61%	0.67%		0.67%	0.86%	0.97%		
4-Nonylphenol	0.002%				0.001%	0.001%		0.001%	0.001%	0.002%		
4-Tert Octylphenol	0.004%				0.002%	0.002%		0.002%	0.003%	0.003%		
6-Acetyl-1, 1, 2, 4, 4, 7-hexamethyltetraline (AHTN, Tonalide)	0.002%				0.001%	0.001%		0.001%	0.002%	0.002%		
Acetylsalicylic acid	15.98%				8.69%	9.46%		9.55%	12.18%	13.73%		
Atenolol	82.13%				44.69%	48.61%		49.07%	62.60%	70.57%		
Atorvastatin	0.0033%				0.0018%	0.0020%		0.00%	0.00%	0.00%		
Atrazine	0.31%				0.17%	0.19%		0.19%	0.24%	0.27%		
Carbamazepine	0.41%				0.22%	0.24%		0.24%	0.31%	0.35%		
Bisphenol A		0.60%			0.05%	0.06%	0.12%		0.07%	0.08%	0.32%	
DEET		3.88%			0.35%	0.38%	0.76%		0.48%	0.55%	2.04%	
Chlorpyrifos		0.04%			0.00%	0.00%	0.01%		0.01%	0.01%	0.02%	
Cyclophosphamide		49.11%			4.38%	4.76%	9.61%		6.13%	6.91%	25.79%	
Diazinon		0.27%			0.02%	0.03%	0.05%		0.03%	0.04%	0.14%	
Dicamba		18.39%			1.64%	1.78%	3.60%		2.30%	2.59%	9.66%	
Diclofenac		0.42%			0.04%	0.04%	0.08%		0.05%	0.06%	0.22%	
Diuron		1.71%			0.15%	0.17%	0.33%		0.21%	0.24%	0.90%	
Fluoxetine		0.89%			0.08%	0.09%	0.17%		0.11%	0.13%	0.47%	
Furosemide		24.68%			2.20%	2.39%	4.83%		3.08%	3.48%	12.96%	
Gemfibrozil			0.09%		0.02%	0.03%	0.05%	0.03%				0.07%
Hexazinone			9.64%		2.76%	3.00%	6.05%	3.03%				7.52%

Ibuprofen			0.31%		0.09%	0.10%	0.20%	0.10%				0.25%
Indomethacin			0.19%		0.05%	0.06%	0.12%	0.06%				0.15%
Metolachlor			0.26%		0.07%	0.08%	0.16%	0.08%				0.20%
Metoprolol			9.38%		2.68%	2.92%	5.88%	2.94%				7.31%
Naproxen			1.15%		0.33%	0.36%	0.72%	0.36%				0.90%
Paracetamol			20.23%		5.79%	6.29%	12.69%	6.35%				15.78%
Picloram			9.53%		2.72%	2.96%	5.98%	2.99%				7.43%
Praziquantel				2.90%	0.23%		0.51%	0.26%	0.33%		1.38%	0.64%
Propoxur				12.64%	1.02%		2.24%	1.12%	1.43%		6.00%	2.78%
Propranolol				2.39%	0.19%		0.42%	0.21%	0.27%		1.14%	0.53%
Simazine				4.29%	0.35%		0.76%	0.38%	0.48%		2.04%	0.94%
Tributylphosphate				0.22%	0.02%		0.04%	0.02%	0.02%		0.10%	0.05%
Triclopyr				12.03%	0.97%		2.13%	1.07%	1.36%		5.71%	2.65%
Triclosan				0.07%	0.01%		0.01%	0.01%	0.01%		0.03%	0.01%
Trimethoprim				41.71%	3.36%		7.38%	3.69%	4.71%		19.81%	9.18%
Tris(2-chloroethyl)phosphate (TCEP)				14.41%	1.16%		2.55%	1.28%	1.63%		6.84%	3.17%
5-Methyl-1H-benzotriazole				9.34%	0.75%		1.65%	0.83%	1.06%		4.44%	2.06%

Table SI-6 Mixture with concentration ratios according to the Australian Drinking Water Guidelines (ADWG mixtures).

Mixture Composition	ADWG1	ADWG2	ADWG3	ADWG4	ADWG5	ADWG6	ADWG7	ADWG8	ADWG9	ADWG10	ADWG11	ADWG12
17-estradiol	0.07%				0.0024%	0.0025%		0.01%	0.00%	0.00%		
2,4-Dichlorophenoxyacetic acid	14.53%				0.52%	0.54%		1.08%	0.86%	0.93%		
4-Nonylphenol	0.24%				0.009%	0.009%		0.02%	0.01%	0.02%		
4-Tert Octylphenol	0.03%				0.0009%	0.0010%		0.00%	0.00%	0.00%		
6-Acetyl-1, 1, 2, 4, 4, 7-hexamethyltetraline (AHTN, Tonalide)	1.66%				0.06%	0.06%		0.12%	0.10%	0.11%		

Acetylsalicylic acid	17.23%				0.61%	0.64%		1.28%	1.02%	1.10%		
Atenolol	10.05%				0.36%	0.37%		0.74%	0.59%	0.64%		
Atorvastatin	0.96%				0.03%	0.04%		0.07%	0.06%	0.06%		
Atrazine	9.93%				0.35%	0.37%		0.74%	0.59%	0.63%		
Carbamazepine	45.31%				1.61%	1.68%		3.36%	2.68%	2.89%		
Bisphenol A		0.01%			0.0033%	0.0035%	0.0034%		0.0055%	0.0060%	0.0059%	
DEET		95.17%			49.61%	51.85%	51.44%		82.70%	89.11%	87.89%	
Chlorpyrifos		0.21%			0.11%	0.11%	0.11%		0.18%	0.19%	0.19%	
Cyclophosphamide		0.10%			0.05%	0.05%	0.05%		0.08%	0.09%	0.09%	
Diazinon		0.10%			0.05%	0.05%	0.05%		0.08%	0.09%	0.09%	
Dicamba		3.29%			1.72%	1.79%	1.78%		2.86%	3.08%	3.04%	
Diclofenac		0.04%			0.02%	0.02%	0.02%		0.04%	0.04%	0.04%	
Diuron		0.62%			0.33%	0.34%	0.34%		0.54%	0.58%	0.58%	
Fluoxetine		0.24%			0.12%	0.13%	0.13%		0.20%	0.22%	0.22%	
Furosemide		0.22%			0.11%	0.12%	0.12%		0.19%	0.21%	0.20%	
Gemfibrozil			22.74%		9.10%	9.51%	9.43%	19.01%				20.53%
Hexazinone			15.04%		6.02%	6.29%	6.24%	12.57%				13.58%
Hydrochlorthiazide			0.40%		0.16%	0.17%	0.17%	0.33%				0.36%
Ibuprofen			18.40%		7.36%	7.69%	7.63%	15.38%				16.61%
Indomethacin			0.66%		0.27%	0.28%	0.27%	0.55%				0.60%
Metolachlor			10.03%		4.01%	4.19%	4.16%	8.38%				9.05%
Metoprolol			0.89%		0.35%	0.37%	0.37%	0.74%				0.80%
Naproxen			9.07%		3.63%	3.79%	3.76%	7.58%				8.18%
Paracetamol			10.98%		4.39%	4.59%	4.56%	9.18%				9.91%
Picloram			11.79%		4.72%	4.93%	4.89%	9.85%				10.64%
Praziquantel				19.69%	0.85%		0.88%	1.78%	1.42%		1.51%	1.92%

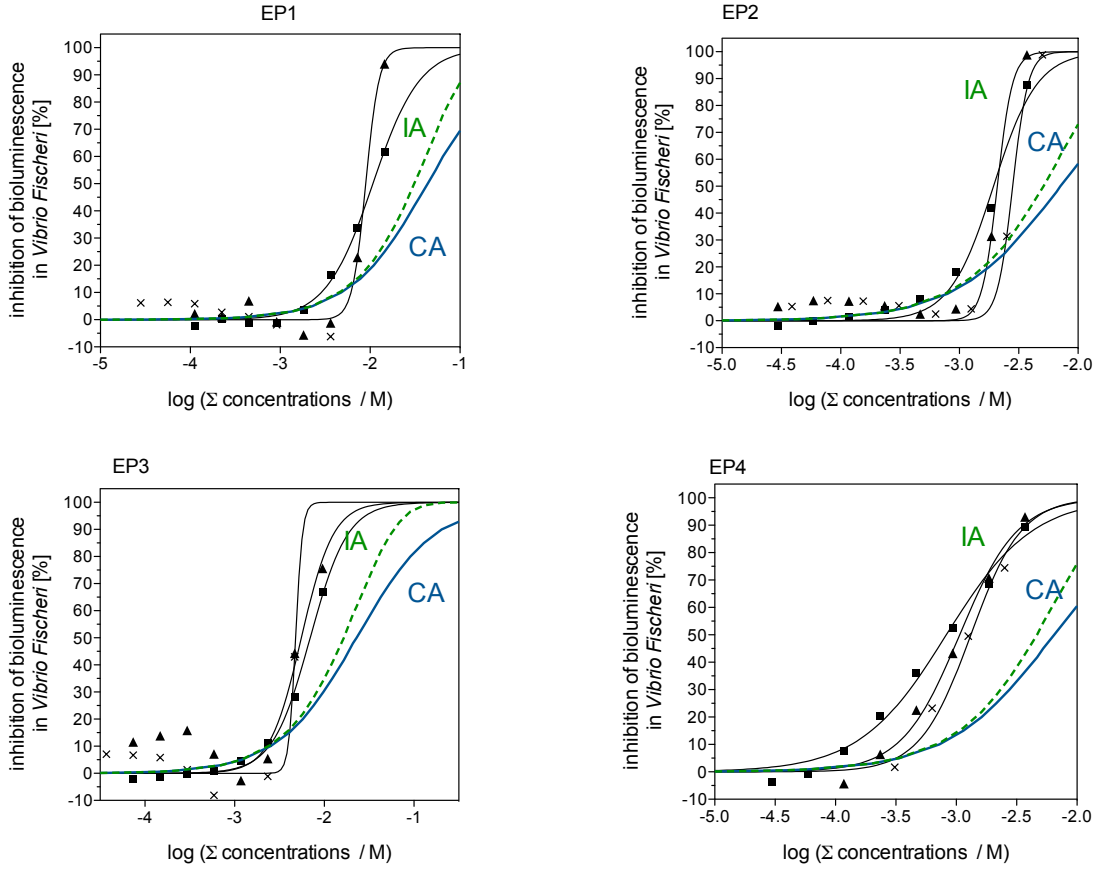
Propoxur				29.40%	1.27%		1.32%	2.65%	2.12%		2.25%	2.86%
Propranolol				13.56%	0.59%		0.61%	1.22%	0.98%		1.04%	1.32%
Simazine				8.72%	0.38%		0.39%	0.79%	0.63%		0.67%	0.85%
Tributylphosphate				0.17%	0.01%		0.01%	0.01%	0.01%		0.01%	0.02%
Triclopyr				6.85%	0.30%		0.31%	0.62%	0.49%		0.52%	0.67%
Triclosan				0.11%	0.00%		0.005%	0.010%	0.008%		0.008%	0.010%
Trimethoprim				21.19%	0.92%		0.95%	1.91%	1.53%		1.62%	2.06%
Tris(2-chloroethyl)phosphate (TCEP)				0.31%	0.01%		0.01%	0.03%	0.02%		0.02%	0.03%
5-Methyl-1H-benzotriazole				0.0046%	0.0002%		0.0002%	0.0004%	0.0003%		0.0004%	0.0005%

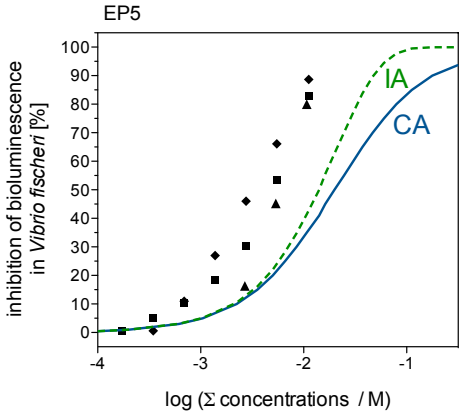
Table SI-7 Detected chemicals in the six environmental samples where chemicals were at concentrations about the limit of reporting (LOR).

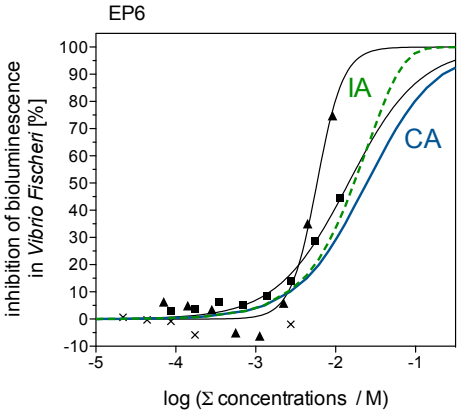
Analyte	**Units**	**LOR**	**Eff-1** (secondary treated effluent, influent to MF)	**MF** (after micro-filtration)	**RO** (after reverse osmosis)	**Eff-2** (secondary effluent (influent to O_3/BAC)	**O_3/BAC** (after ozonation and biologically activated carbon filtration)
number of chemicals detected			40	39	6	48	6
17-β-Estradiol	µg/L	0.005				0.006	
Nonylphenol	µg/L	0.1				0.13	
4-*t*-Octylphenol	µg/L	0.1		0.017		0.11	
Tonalid	µg/L	0.1	0.1	0.1		0.1	
Atenolol	µg/L	0.01	0.10	0.10		0.94	
Atorvastatin	µg/L	0.01				0.04	
Atrazine	µg/L	0.01	0.35	0.39			
Bisphenol A	µg/L	0.01		0.018		0.13	
Caffeine	µg/L	0.02	0.05	0.04		0.21	
Carbamazepine	µg/L	0.01	1.6	1.9	0.02	2.5	
Cephalexin	µg/L	0.01				0.12	
Chlorpyrifos	µg/L	0.1				5.6	
Citalopram	µg/L	0.01	0.13	0.10		0.27	0.02
Codeine	µg/L	0.1				0.24	
Cyclophos-phamide	µg/L	0.01	0.01			0.04	
Desmethyl Citalopram	µg/L	0.01	0.14	0.10		0.24	0.01
Desmethyl Diazepam	µg/L	0.01	0.03	0.03		0.05	
Diazepam	µg/L	0.01	0.01			0.01	
Diazinon	µg/L	0.1				0.16	
Diclofenac	µg/L	0.01	0.11	0.12		0.26	
Diuron	µg/L	0.01	0.16	0.14	0.03	0.07	
Doxylamine	µg/L	0.01	0.24	0.18		0.44	
Erythromycin	µg/L	0.01	0.02	0.02		0.05	

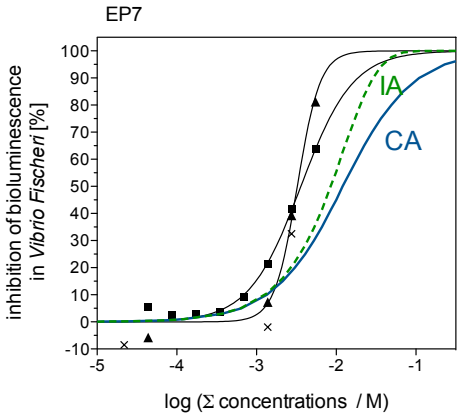
Analyte	**Units**	**LOR**	**Eff-1** (secondary treated effluent, influent to MF)	**MF** (after micro-filtration)	**RO** (after reverse osmosis)	**Eff-2** (secondary effluent (influent to O_3/BAC)	**O_3/BAC** (after ozonation and biologically activated carbon filtration)	**SW** (storm-water)
Fipronil	g/L	0.1	0.1	0.1				
Fluoxetine	g/L	0.01	0.03	0.03		0.03		
Frusemide	g/L	0.01	0.13	0.15		1.3		
Galaxolide	g/L	0.1	1.0	1.1		1.6		
Gemfibrozol	g/L	0.01	0.08	0.07		0.15		
Hexazinone	g/L	0.01	0.02	0.02				
Hydrochlor-thiazide	g/L	0.01	0.76	0.65	0.01	1.5		
Indomethacin	g/L	0.01				0.08		
Lincomycin	g/L	0.01						
Metolachlor	g/L	0.01	0.82	0.73	0.01	0.01	0.01	
Metoprolol	g/L	0.01	0.12	0.14		0.97		
Naproxen	g/L	0.1				0.32		
Norfloxacin	g/L	0.05	0.06			0.10		
Oxazepam	g/L	0.01	0.60	0.57		1.1		
Oxycodone	g/L	0.01	0.03	0.03		0.16		
Paracetamol	g/L	0.02						0.02
Praziquantel	g/L	0.01	0.01	0.01				
Propoxur	g/L	0.01	0.03	0.03		0.05		
Propranolol	g/L	0.01	0.01	0.02		0.14		
Ranitidine	g/L	0.05				0.70		
Roxithromycin	g/L	0.02	0.05	0.04		0.08		
Simazine	g/L	0.01	0.18	0.23		0.17		0.02
Sulphadiazine	g/L	0.01	0.03	0.03		0.13		
Sufamethoxazole	g/L	0.01	0.15	0.07		0.21		
Temazepam	g/L	0.01	0.47	0.50		0.65		
Triclosan	g/L	0.01	0.02	0.02		0.05		
Trimethoprim	g/L	0.01	0.07	0.05		0.23		
Tris(chloroethyl) phosphate	g/L	0.1	0.4	0.4		0.4	0.3	

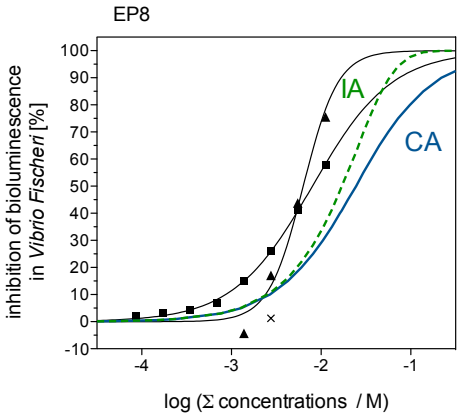
Analyte	**Units**	**LOR**	**Eff-1** (secondary treated effluent, influent to MF)	**MF** (after micro-filtration)	**RO** (after reverse osmosis)	**Eff-2** (secondary effluent (influent to O_3/BAC)	**O_3/BAC** (after ozonation and biologically activated carbon filtration)	**SW** (storm-water)
Venlafaxine	g/L	0.01	1.6	1.9	0.01	2.4	0.10	
DEET	g/L	0.01	0.11	0.10		0.18	0.03	0.11
1H-Benzotriazole, 5-methyl	g/L	0.2	0.53	0.54	0.32	1.3		

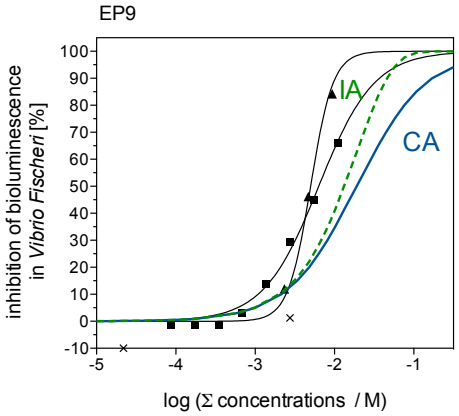


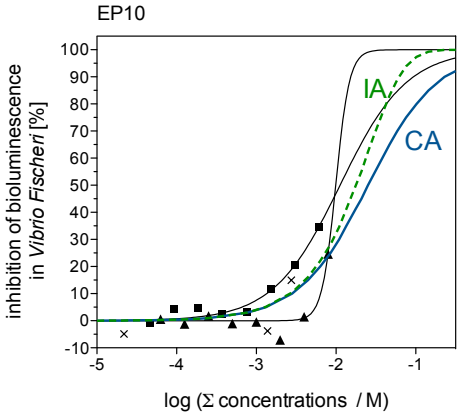

EP5
inhibition of bioluminescence in *Vibrio fischeri* [%]
log (Σ concentrations / M)
IA
CA


EP6
inhibition of bioluminescence in *Vibrio Fischeri* [%]
log (Σ concentrations / M)
IA
CA


EP7
inhibition of bioluminescence in *Vibrio Fischeri* [%]
log (Σ concentrations / M)
IA
CA


EP8
inhibition of bioluminescence in *Vibrio Fischeri* [%]
log (Σ concentrations / M)
IA
CA


EP9
inhibition of bioluminescence in *Vibrio Fischeri* [%]
log (Σ concentrations / M)
IA
CA


EP10
inhibition of bioluminescence in *Vibrio Fischeri* [%]
log (Σ concentrations / M)
IA
CA

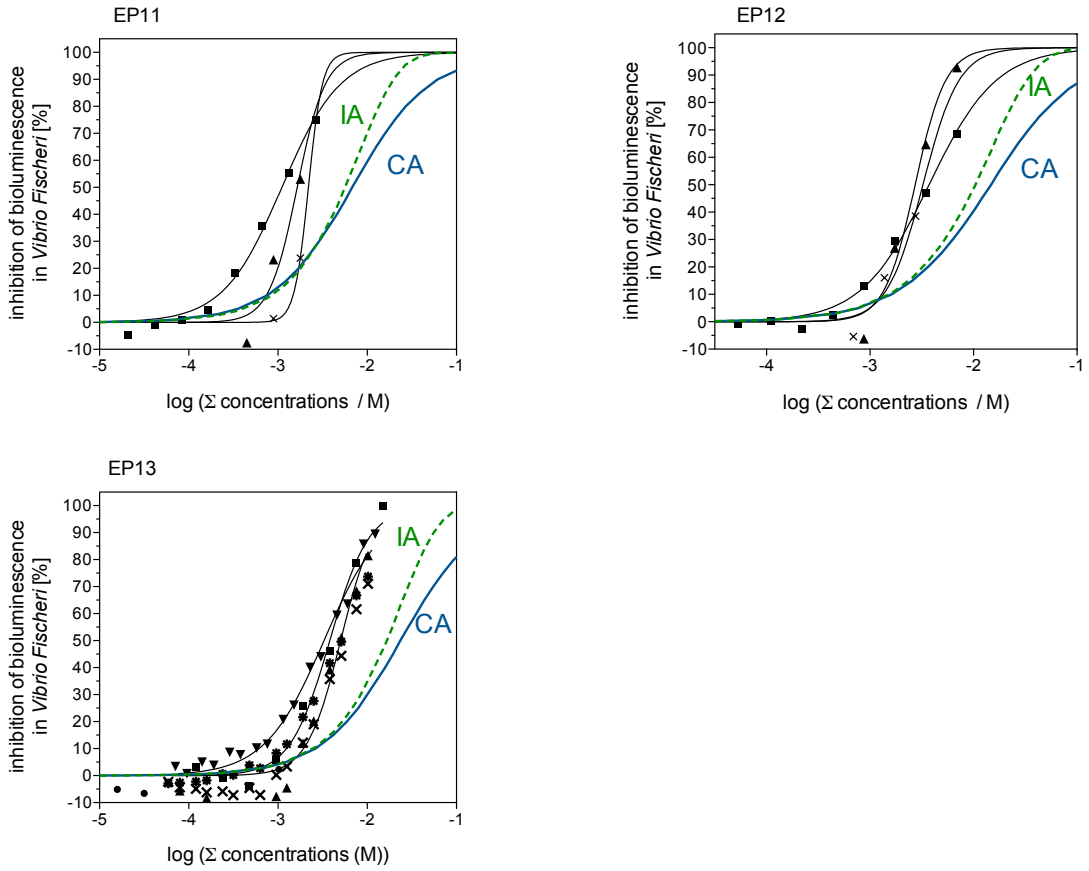


Figure SI-4 Concentration-effect curves of equipotent mixtures (EP).

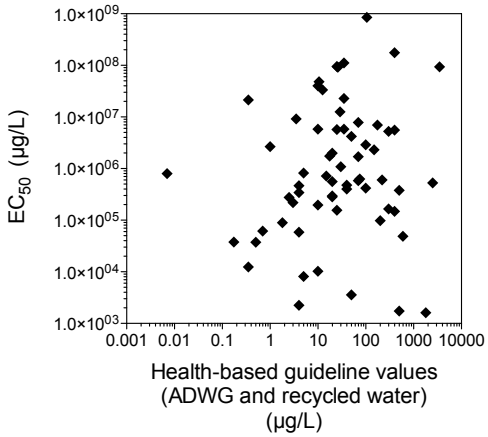
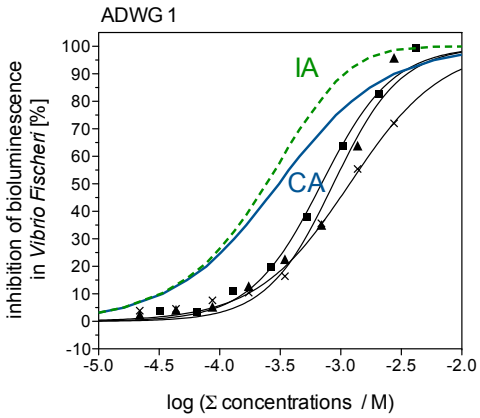
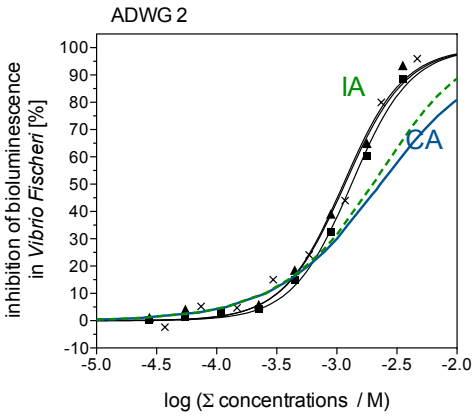
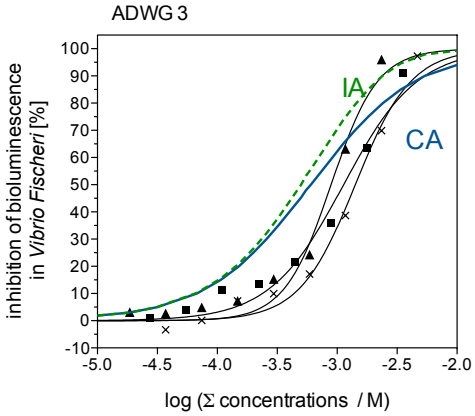
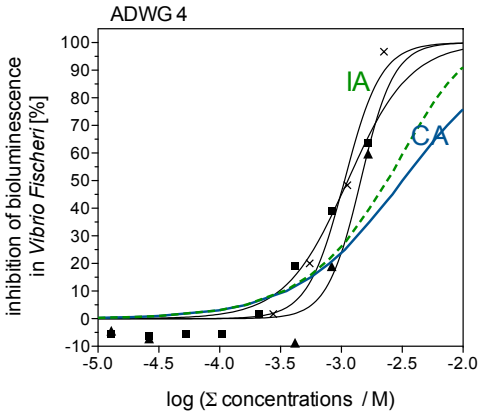


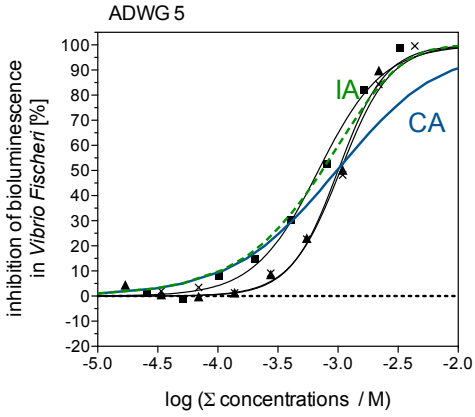
Figure SI-5 The guideline values are not correlated to the EC_{50} values in the bioluminescence inhibition assay with Vibrio fischeri.

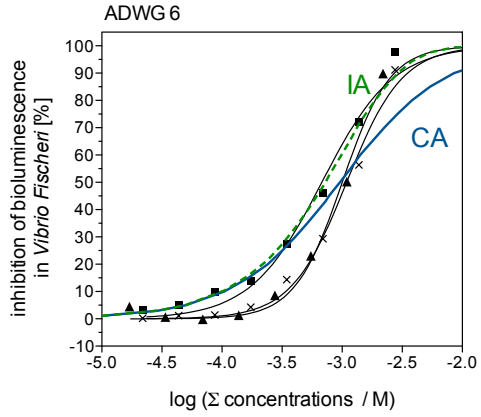

ADWG 1
IA
CA
inhibition of bioluminescence in *Vibrio Fischeri* [%]
log (Σ concentrations / M)


ADWG 2
IA
CA
inhibition of bioluminescence in *Vibrio Fischeri* [%]
log (Σ concentrations / M)


ADWG 3
IA
CA
inhibition of bioluminescence in *Vibrio Fischeri* [%]
log (Σ concentrations / M)


ADWG 4
IA
CA
inhibition of bioluminescence in *Vibrio Fischeri* [%]
log (Σ concentrations / M)


ADWG 5
IA
CA
inhibition of bioluminescence in *Vibrio Fischeri* [%]
log (Σ concentrations / M)


ADWG 6
IA
CA
inhibition of bioluminescence in *Vibrio Fischeri* [%]
log (Σ concentrations / M)

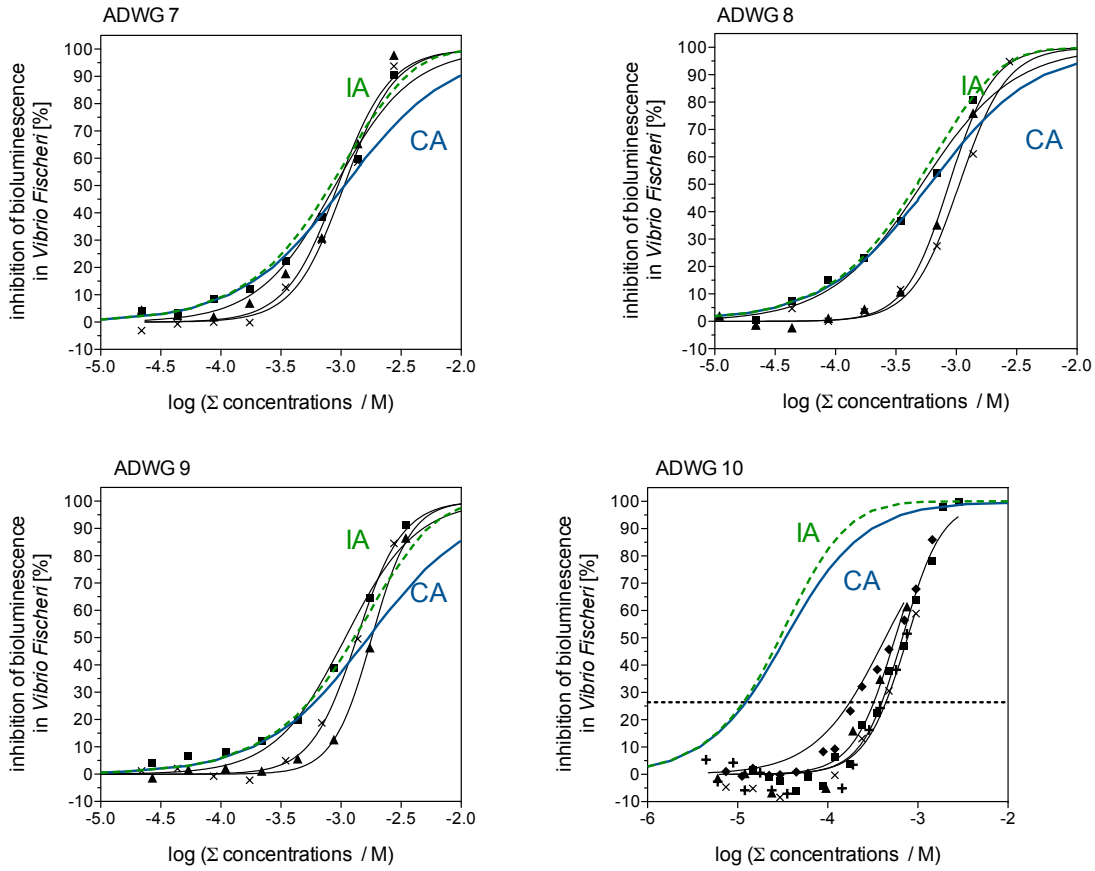


Figure SI-6 Concentration-effect curves of ADWG mixtures.

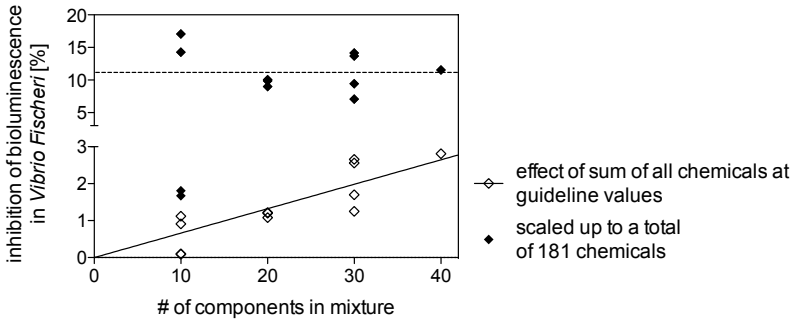


Figure SI-7 Validation of the proposed guideline value with the experimental ADWG mixtures (10 to 40 compounds).

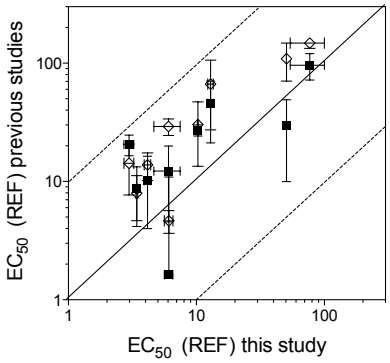
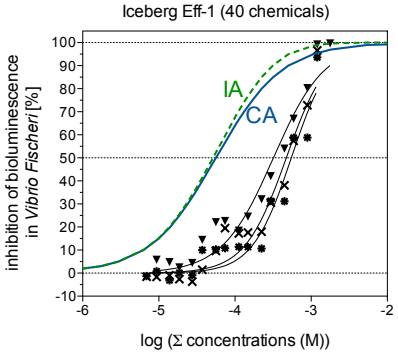
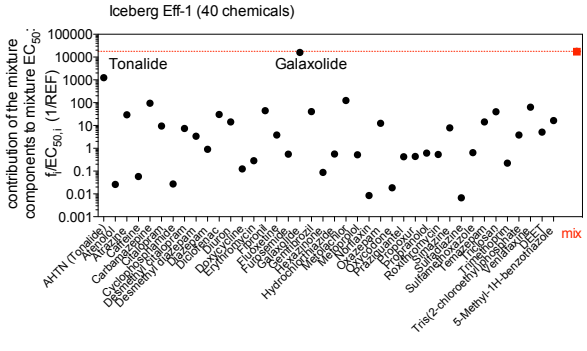
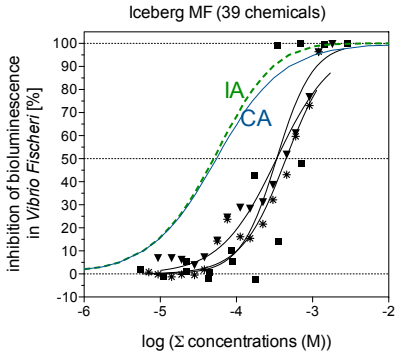
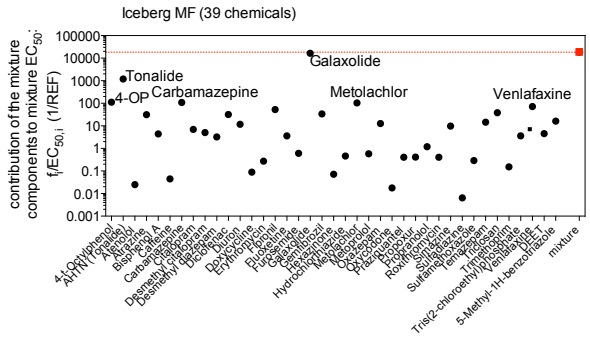


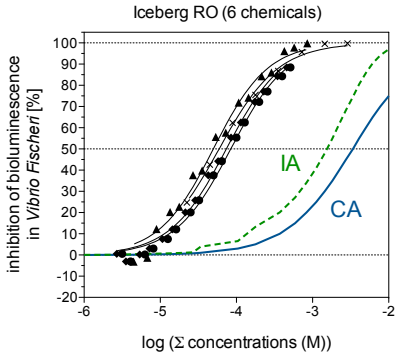
Figure SI-8 Comparison of the median effect concentrations EC_{50} in the present study with previous work at the same sampling sites (samplings in 2010 to 2012), black squares: data from (Macova et al. 2011), empty black diamonds: data from (Escher et al. 2012).

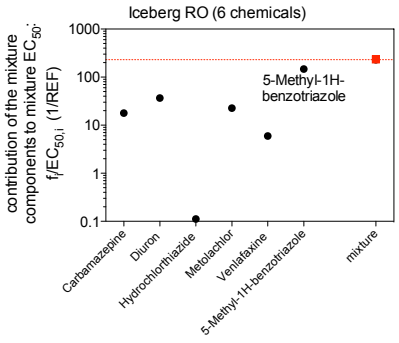


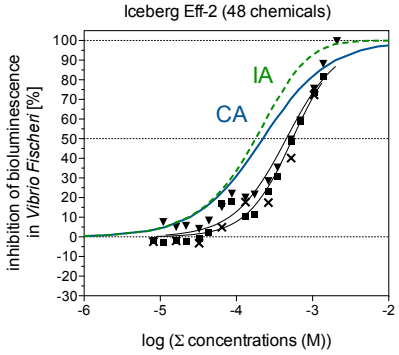


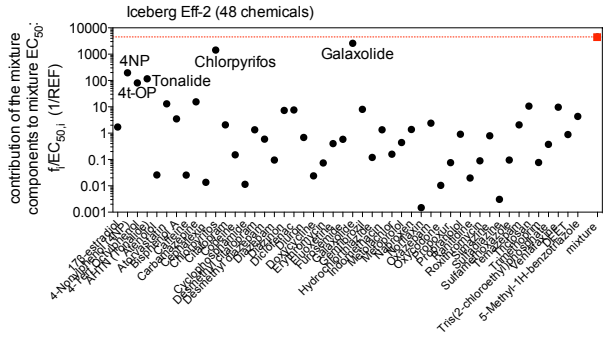

Iceberg MF (39 chemicals)
inhibition of bioluminescence in Vibrio Fischeri [%]
log (Σ concentrations (M))
IA
CA

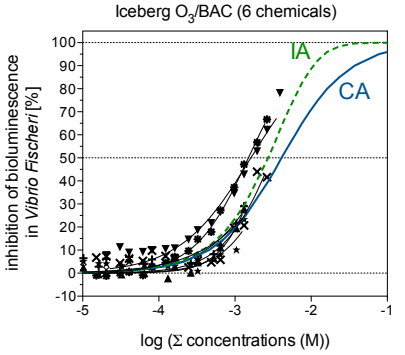

Iceberg MF (39 chemicals)
contribution of the mixture components to mixture EC50: fi/EC50,i (1/REF)
Tonalide
4-OP
Carbamazepine
Galaxolide
Metolachlor
Venlafaxine
mixture

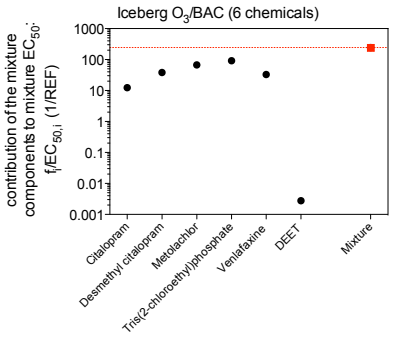

Iceberg RO (6 chemicals)
inhibition of bioluminescence in Vibrio Fischeri [%]
log (Σ concentrations (M))
IA
CA


Iceberg RO (6 chemicals)
contribution of the mixture components to mixture EC50: fi/EC50,i (1/REF)
5-Methyl-1H-benzotriazole
Carbamazepine
Diuron
Hydrochlorthiazide
Metolachlor
Venlafaxine
mixture


Iceberg Eff-2 (48 chemicals)
inhibition of bioluminescence in Vibrio Fischeri [%]
log (Σ concentrations (M))
IA
CA


Iceberg Eff-2 (48 chemicals)
contribution of the mixture components to mixture EC50: fi/EC50,i (1/REF)
4NP
4t-OP
Tonalide
Chlorpyrifos
Galaxolide
mixture


Iceberg O3/BAC (6 chemicals)
inhibition of bioluminescence in Vibrio Fischeri [%]
log (Σ concentrations (M))
IA
CA


Iceberg O3/BAC (6 chemicals)
contribution of the mixture components to mixture EC50: fi/EC50,i (1/REF)
Citalopram
Desmethyl citalopram
Metolachlor
Tris(2-chloroethyl)phosphate
Venlafaxine
DEET
Mixture

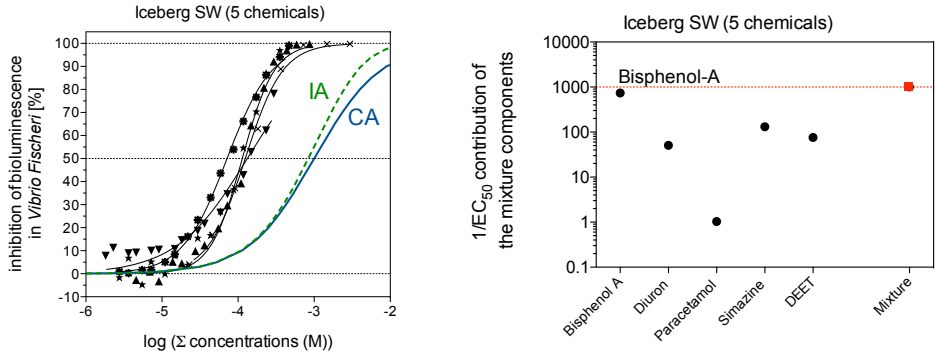


Figure SI-9 Concentration-effect curves of the iceberg mixture of chemicals in the environmental samples (left) and contribution of the individual components to the EC_{50} of the mixture (total effect of mixture indicated in red).

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Most Oxidative Stress Response In Water Samples Comes From Unknown Chemicals: The Need For Effect-Based Water Quality Trigger Values

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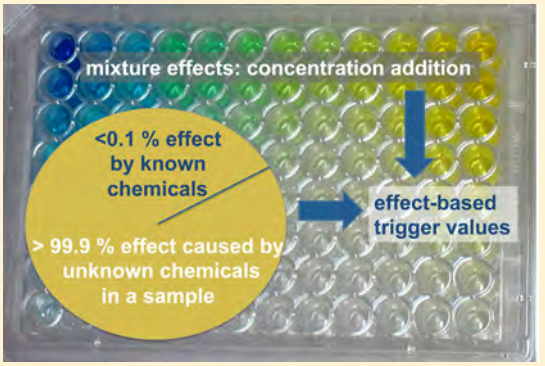
Supporting Information

ABSTRACT: The induction of adaptive stress response pathways is an early and sensitive indicator of the presence of chemical and non-chemical stressors in cells. An important stress response is the Nrf-2 mediated oxidative stress response pathway where electrophilic chemicals or chemicals that cause the formation of reactive oxygen species initiate the production of antioxidants and metabolic detoxification enzymes. The AREc32 cell line is sensitive to chemicals inducing oxidative stress and has been previously applied for water quality monitoring of organic micropollutants and disinfection byproducts. Here we propose an algorithm for the derivation of effect-based water quality trigger values for this end point that is based on the combined effects of mixtures of regulated chemicals. Mixture experiments agreed with predictions by the mixture toxicity concept of concentration addition. The responses in the AREc32 and the concentrations of 269 individual chemicals were quantified in nine environmental samples, ranging from treated effluent, recycled water, stormwater to drinking water. The effects of the detected chemicals could explain less than 0.1% of the observed induction of the oxidative stress response in the sample, affirming the need to use effect-based trigger values that account for all chemicals present.



INTRODUCTION

Bioanalytical tools are increasingly applied for water quality assessment in a research context because they take account of all chemicals present in a sample that are triggering the given effect end point.[1] Thus bioanalytical tools account for known and unknown micropollutants. Unknowns include not only those chemicals that are not regulated or for which no chemical analytical method is available, but also those that are present at very low concentrations, below the analytical detection limits, and which can contribute to the mixture effects in a complex water sample, as well as transformation products that are formed during water treatment or in the environment.[2]

However, bioanalytical tools have not been used for regulatory purposes in the water quality field because there are no effect-based water quality guidelines or trigger values available yet. For receptor-mediated toxicity, for example, for dioxin-like effects, the toxic equivalents (TEQ) concept is well accepted,[3] and the TEQ concept is occasionally applied in water quality legislation, for example, in British Columbia.[4] For each dioxin and dioxin-like chemical, a toxic equivalency factor is defined.[3] By summing up the product of the toxic equivalency factor times the concentration of each chemical in a mixture, one can predict the toxic potential of the mixture.

Analogously, the relative effect potencies can be derived directly from an in vitro bioassay. Bioanalytical equivalent concentrations (BEQ) can be calculated from the relative effect potencies and the measured concentrations (BEQ_{chem}) or directly from the bioassay results (BEQ_{bio}).[5] BEQs are an effective communication tool as they express the effect in an unknown sample by relating it to the effect elicited by a known reference chemical. The BEQ concept is typically applied to chemicals that act via receptor mediated mechanisms but conceptually it can also be adapted to nonspecific toxicity[6] or reactive modes of toxic action.

For many biological end points such as inhibition of photosynthesis or estrogenic effects, the BEQ_{chem} of the known chemicals can explain a large fraction of the BEQ_{bio},[1] but for nonspecific effects such as cytotoxicity often only a very small fraction of effects can be explained by typically quantified chemicals.[7,8]

"Water quality guidelines" or "water quality criteria" provide recommendations on safe levels of chemicals in water. They are typically developed by national and international agencies and provide guidance but are not legally enforceable.[1] They must be

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Table 1. Chemicals for the Designed Mixture Experiments, Their Water Quality Guideline Values in the Australian Drinking Water Guidelines (ADWG)[23] and the Australian Guidelines for Water Recycling (AGWR),[24] Their Physicochemical Properties, Their Effect Concentrations EC_{10} for Cytotoxicity and $EC_{IR1.5}$ for Induction of Oxidative Stress

compound	CAS Number	ADWG guideline value (μg/L)	AGWR guideline value (μg/L)	logK_{ow}[b]	logD_{lipw} (pH 7) (L/kg)[c]	cytotoxicity EC_{10} (μM)[d]	induction of oxidative stress $EC_{IR1.5}$ (μM)	slope (1/μM)
Pharmaceuticals								
atenolol	29122−68−7		25	0.16	−0.73	4060	724 ± 39	6.90 ± 0.37 × 10^{-4}
atorvastatin	134523−00−5		5	6.36	5.56	411	31.1 ± 2.5	1.61 ± 0.13 × 10^{-2}
cephalexin	15686−71−2		35	0.65	0.32	>30000	295 ± 16	1.70 ± 0.09 × 10^{-3}
citalopram	59729−33−8		4	3.74	2.89	123	35.8 ± 2.0	1.40 ± 0.08 × 10^{-2}
fluoxetine	2−84−9		10	4.05	3.21	≈30	4.8 ± 0.6	1.05 ± 0.12 × 10^{-1}
metoprolol	37350−58−6		25	1.88	1.02	420	366 ± 23	1.37 ± 0.09 × 10^{-3}
naproxen	22204−53−1		220	3.18	2.33	>10000	3695 ± 350	1.35 ± 0.13 × 10^{-4}
paracetamol	103−90−2		175	0.46	0.54	11600	3628 ± 196	1.38 ± 0.07 × 10^{-4}
propranolol	525−66−6		40	3.48	2.66	>100	25.9 ± 2.4	1.93 ± 0.18 × 10^{-2}
ranitidine	66357−35−5		26	0.27	−0.61	3180	2043 ± 161	2.45 ± 0.19 × 10^{-4}
gemfibrozil	25812−30−0		600	4.77	3.95	no effect up to 5000 μM		
ibuprofen	15687−27−1		400	3.97	3.13	no effect up to 5000 μM		
norflaxin	70458−96−7		400	−1.03	−1.00	no effect up to 5000 μM		
salicylic acid	69−72−7		29	2.26	1.38	no effect up to 5000 μM		
warfarin	81−81−2		15	2.70	1.97	no effect up to 5000 μM		
Pesticides		30	3					
azinophos-methyl	86−50−0	0.7		2.75	2.88	47	53.7 ± 2.2	9.32 ± 0.39 × 10^{-3}
fipronil	120068−37−3	100	100	4.00	4.15	775	21.2 ± 3.2	2.36 ± 0.36 × 10^{-2}
propiconazole	60207−90−1	5	1	3.72	3.86	106	40.5 ± 1.3	1.24 ± 0.04 × 10^{-2}
dichlorvos	62−73−7	7	50	1.43	1.53	84	7.7 ± 0.3	6.48 ± 0.23 × 10^{-2}
propargite	2312−35−8	9		5	5.17	44	51.2 ± 1.9	9.76 ± 0.37 × 10^{-3}
amitraz	33089−61−1	20	40	5.5	5.68	24	101.9 ± 9.7	4.91 ± 0.47 × 10^{-3}
atrazine	1912−24−9	40	2	2.61	2.73	76	105.2 ± 12.0	4.75 ± 0.54 × 10^{-3}
MCPA[a]	94−74−6	4	1	3.25	2.39	262	no effect up to cytotoxic concentrations	
aldicarb	116−06−3	100	100	1.13	1.22	348	no effect up to cytotoxic concentrations	
dicamba	1918−00−9	400	300	2.21	1.32	1500	no effect up to cytotoxic concentrations	
hexazinone	51235−04−2	20	30	1.85	1.00	1410	no effect up to cytotoxic concentrations	
methomyl	16752−77−5	7	50	0.6	0.68	1480	no effect up to cytotoxic concentrations	
dimethoate	60−51−5	90	50	0.78	0.87	660	no effect up to cytotoxic concentrations	
pirimiphos-methyl	23505−41−1		100	4.2	4.35	303	no effect up to cytotoxic concentrations	
dichlorprop	120−36−5	70	50	3.43	2.57	1520	no effect up to cytotoxic concentrations	
fluometuron	2164−17−2	7	10	2.42	2.54	n.d.	no effect up to cytotoxic concentrations	
fenitrothion	122−14−5	0.3	0.3	3.3	3.44	242	no effect up to cytotoxic concentrations	
dieldrin	60−57−1	4	3	5.40	5.58	196	no effect up to cytotoxic concentrations	
ethion	563−12−2	600	100	5.07	5.24	9250	no effect up to cytotoxic concentrations	
piperonyl butoxide[e]	51−03−6		25	4.75	4.92	1410	no effect up to cytotoxic concentrations	

[a]2-Methyl-4-chlorophenoxyacetic acid. [b]Octanol−water partition coefficient (logK_{ow}) from EPIsuite.[27] [c]Liposome-water distribution ratios at pH 7 (logD_{lipw}(pH 7)) are better descriptors of baseline toxicity for ionizable compounds[28] and were calculated from logK_{ow} via the liposome-water partition coefficient (logK_{lipw}) and the speciation calculated with SPARC.[29] More details on the derivation are given in a previous publication:[30] the only modification was that a new QSAR for the prediction of logK_{lipw}[31] was employed. [d]The slope of the log−logistic concentration-effect curve was set to 1 because in many cases not even 50% reduction of cell viability was reached at the highest concentration tested, therefore a robust fit of the slope was not possible. [e]Piperonyl-butoxide is an inhibitor of the cytochrome P450 monooxygenase and thus not strictly a pesticide but it is often used in formulation of pesticides to enhance their effect.

adopted by competent authorities on the national, state or regional level to become legally binding "water quality standards".[1]

There are EU regulations that allow for bioanalytical methods in screening of feed and food for dioxin-like chemical.[9] It is conceivable to adopt the BEQ/TEQ concept also for the development of bioanalytical trigger values for water quality assessment. In an implicit way the BEQ/TEQ concept has already been applied for assessment of the combined risk of estrogenic chemicals in surface waters,[10] but there is no formal adoption of the BEQ/TEQ concept or any other effect-based method in water quality regulations to date. So far all water quality guidelines are solely based on individual chemicals or groups of very closely related chemicals.

Effect-based methods would allow the inclusion of mixtures in guidelines. Of course there is no single effect-based guideline value sufficient but all relevant modes of toxic action need to be included. We have recently proposed an approach to develop

effect-based trigger EC_{50} values (EBT-EC_{50}) for cytotoxicity assessed with the Microtox assay.[8] This was achieved by applying mixture toxicity models to existing water quality guideline values of individual chemicals. The present study provides the scientific basis to expand this approach to adaptive stress response pathways.

Adaptive cellular stress response pathways play a key role in maintaining cell homeostasis and/or for repairing damage by transcriptional activation of cytoprotective genes.[11] Activation and detection of adaptive stress response pathways is typically more sensitive than cytotoxicity and other measures of cellular damage and thus provide early warning signals of cellular exposure to chemicals.[1] Martin et al.[12] evaluated how more than 300 pesticides activated 25 nuclear receptors and 48 transcription factor response elements and found that one particular pathway, the Nrf2 pathway was activated by the largest number of test chemicals.

In mammals, the NF-E2-related factor 2 (Nrf2) regulates the cellular defense mechanism against oxidative stress through activation of detoxification and antioxidant genes.[13−15] 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 reactive oxygen species and environmental carcinogens.

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.[16] These cells are derived from the human breast cancer cell line MCF-7, 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. We have recently adopted the AREc32 assay for water quality assessment[17] and have applied it for the evaluation of the formation of disinfection byproducts.[18]

Prior to developing effect-based trigger values for the end point of oxidative stress response, it must be assessed how chemicals act jointly in mixtures in the AREc32. While a multitude of studies have demonstrated the validity of the mixture toxicity concepts of concentration addition (CA) for chemicals with the same mode of action and independent action (IA) for chemicals with different modes of toxic action,[19] to our knowledge, there have been no studies undertaken assessing mixture effects on bioassays indicative of adaptive stress response pathways.[20] There are various types of stressors that can induce the Nrf2-ARE pathway, among them reactive oxygen species and electrophilic chemicals, but the commonality in the toxicity pathways is that they all disrupt the association of the repressor protein Keap to the transcription factor Nrf2, which is key to the activation of the antioxidant response element ARE.[21] As there is no direct receptor binding of a chemical required to activate this pathway and there are a diverse set of sensor mechanisms,[22] our working hypothesis is that all stressors act together to activate Nrf2 according to the reference mixture concept of CA. IA is not applicable because different modes of action that are not mediated by Nrf2 would not show any effect in AREc32 apart from cytotoxicity. To test this hypothesis we selected 15 pharmaceuticals and 20 pesticides, evaluated them for their ability to induce the oxidative stress response as individual chemicals followed by mixture experiments with 18 different equipotent and equimolar mixtures compositions as well as mixtures of up to 20 chemicals in ratios of their guideline values and compared the results of the mixture experiments to predictions for CA.

In a second step, we evaluated the relevance of the oxidative stress response for chemicals occurring in environmental samples. Nine water samples collected across the entire water cycle from sewage to surface water to drinking water in South East Queensland, Australia, were evaluated with the AREc32 and 269 organic micropollutants were quantified by chemical analysis, mainly those that are regulated in the Australian Drinking Water Guidelines of 2011 (ADWG)[23] or the Australian guidelines for water recycling: managing health and environmental risks (phase 2) - augmentation of drinking water supplies" (AGWR).[24] The concentrations of those chemicals detected in the water samples were mixed in the detected concentration ratios and also characterized with the AREc32 to estimate the contribution that known chemicals typically have for the overall toxicity of an environmental sample (iceberg experiments).

Finally, based on the results obtained in the experimental part we propose an algorithm to derive effect-based trigger values and illustrate the concept on the example of recycled and drinking water in Queensland.

EXPERIMENTAL SECTION

Chemicals. Chemicals were selected from a total of 381 organic chemicals listed in the ADWG (181 chemicals)[23] or AGWR (349 chemicals).[24] More information on chemicals and how they were selected is given in Table 1 and the Supporting Information (SI), Table SI-1.

AREc32 Bioassay. The AREc32 cell line is a stably transfected human breast cancer cell line MCF7 with an ARE reporter plasmid coupled to a reporter gene encoding for luciferase and was provided by Prof. Roland Wolf.[16] The experimental details are given in SI Section SI-1. The amount of luciferase produced is directly proportional to the ARE activated and thus also the chemical stressor present. In parallel to induction, cytotoxicity was measured with the MTS assay and EC_{10} values for cytotoxicity derived with a log−logistic concentration-effect curve as described previously.[17] Only concentrations that were below the EC_{10} were used for the induction experiments.

The induction ratio IR of the luciferase is defined as the ratio of the relative light units (RLU) of the chemical, mixture or extract divided by the average RLU of the controls (eq 1).

$$\mathrm{IR} = \frac{\mathrm{RLU(sample)}}{\frac{\sum_{i=1}^{n}\mathrm{RLU(control)}}{n}} \quad (1)$$

As there was often only a small window of concentration between induction and cytotoxicity, only the linear part of the concentration-effect (IR) relationship up to an IR of 5 was evaluated with a linear regression through IR 1 (eq 2). The assessment end point is the concentration that induces an IR of 1.5 ($EC_{IR1.5}$).[17]

$$\mathrm{IR} = 1 + \mathrm{slope}\cdot\mathrm{concentration} \quad (2)$$

Each experiment (individual compounds, mixtures and water samples) was run in two to four replicates on independent plates at different days and all experimental data were evaluated with a common concentration-IR regression. The resulting slope and its standard error of the regression fit was converted into $EC_{IR1.5}$ and the standard error of $EC_{IR1.5}$ was calculated by error propagation as described in SI Section SI-1.

Mixture Experiments. All mixture experiments were conducted at a fixed concentration ratio and full concentration-effect curves were measured (using the sum of concentrations as dose-metric). Chemicals were mixed in equipotent concentration ratios, ratios of the ADWG and AGWR guideline values (Table 1, if they differed, the ADWG value was used) and ratios of the concentrations detected in the environmental samples.

The experimental concentration-IR curves were compared with predictions for mixture effects according to the model of concentration addition (CA). For a multicomponent mixture of "i" components present in the fraction p_i ($\sum p_i = 1$), the $EC_{IR1.5}$ of the mixture, $EC_{IR1.5,CA}$ according to the CA model is:

$$EC_{IR1.5,CA} = \frac{1}{\sum_{i=1}^{n} \frac{p_i}{EC_{IR1.5,i}}} \quad (3)$$

The mixture model of independent action (IA) is not appropriate for the AREc32 induction of oxidative stress because only compounds that induce oxidative stress will give a signal in the test at all and other compounds will just have an IR of 1. More details are discussed in the SI, Section SI-2.

A measure of the deviation between the observed and predicted mixture effect is the index on prediction quality IPQ.[25] The IPQ is zero if there is perfect agreement and is positive if the prediction for CA has a higher $EC_{IR1.5}$ than the experiment and negative if it is the other way around.

$$\text{If } EC_{IR1.5,CA} > EC_{IR1.5,experimental} \text{ then IPQ} = \frac{EC_{IR1.5,CA}}{EC_{IR1.5,experimental}} - 1 \quad (4)$$

$$\text{If } EC_{IR1.5,CA} < EC_{IR1.5,experimental} \text{ then IPQ} = 1 - \frac{EC_{IR1.5,experimental}}{EC_{IR1.5,CA}} \quad (5)$$

Environmental Samples. Nine grab water samples were collected in December 2011 and January 2012 from various sites in South East Queensland plus one laboratory blank. The sample "Eff-1" is a secondary treated effluent that serves as influent to an Advanced Water Treatment Plant, where samples were taken after microfiltration (MF), reverse osmosis (RO) and advanced oxidation (AO). The second plant investigated was an enhanced water treatment plant, where secondary treated effluent (Eff-2) was treated with ozone and biologically activated carbon (O_3/BAC). The sample "River" and "DW" correspond to the influent and outlet of a metropolitan drinking water treatment plant, and sample "SW" is a stormwater sample. The laboratory blank was ultrapure water.

The samples were previously assessed with the Microtox assay and 269 chemicals (pharmaceuticals, pesticides, endocrine disruptors and some consumer products) were quantified using the standard GC-MS/MS and LC-MS/MS methods by the commercial NATA accredited analytical laboratory Queensland Health Forensic and Scientific Services (QHFSS).[8] Target chemicals were pharmaceuticals, pesticides, endocrine disruptors, and some consumer products. Information on the chemicals analyzed for, the analytical methods and their detection limits are given in SI Table SI-2.

The water samples were adjusted to pH 3 and enriched with solid phase extraction (SPE) using OASIS HLB as described previously.[8] The dose-metric of sample extracts is the dimensionless relative enrichment factor REF (eq 6),[6] and for all samples an $EC_{IR1.5}$ with units of REF was derived using the linear concentration-effect relationship given in eq 2.

$$REF = \frac{\text{water volume equivalent transfered to bioassay}}{\text{total volume of bioassay}} \quad (6)$$

The $EC_{IR1.5}$ of the samples can be converted into bioanalytical equivalent concentrations (more specifically tBHQ equivalent concentration, tBQH-EQ) by dividing the $EC_{IR1.5}$ of the reference compound tBHQ (1.1 μM, see SI, Section SI-1) by the $EC_{IR1.5}$ of the sample (eq 7).

$$tBHQ - EQ = \frac{EC_{IR1.5}(\text{reference compound tBHQ})}{EC_{IR1.5}(\text{sample})} \quad (7)$$

In addition, "iceberg" mixtures were prepared of the detected chemicals in the concentration ratios detected. Full concentration-effect curves were run with the iceberg mixtures and the tBHQ-EQ were extrapolated to the detected concentration. A comparison between the tBHQ-EQ of the water sample and the associated iceberg chemical mixture will allow an estimation of the fraction of bioactive unknown chemical pollutants in a water sample.

RESULTS AND DISCUSSION

Induction of Oxidative Stress Response by Pharmaceuticals and Pesticides. Twenty pesticides and 15 pharmaceuticals that are included in the combined ADWG + AGWR list of regulated chemicals and/or have been found in environmental water samples were evaluated for their capacity to induce the oxidative stress response (Table 1). While for the reference compound tBHQ, there is a wide window of concentration between induction of oxidative stress response and cytotoxicity,[17] this window was often very narrow for the investigated chemicals. Therefore, cytotoxicity could mask inductive effects at higher concentrations and even chemicals that have the potential to induce oxidative stress will not do so in a visible way if cytotoxicity caused by another mode of toxic action occurs at lower concentrations. Figure 1 shows a representative example of a concentration-effect curve for atorvastatin. All other single chemicals are depicted in the SI, Table SI-3. If at all inducing, the IR of the single chemicals hardly reached more than an IR 5 before the cytotoxicity overwhelmed the stress response.

The EC_{10} values for cytotoxicity were derived from the log−logistic concentration-effect curves of cell viability assessed with the MTS assay (Table 1) and are discussed in more detail in SI Section SI-3. The $EC_{IR1.5}$ for induction was subsequently derived from the linear portion of the concentration-effect curves up to concentrations of the EC_{10} for cytotoxicity (Figure 1B).

Ten of the 15 pharmaceuticals were identified as inducers of the oxidative stress response, whereas only 5 out of the 20 pesticides were shown to be active (Table 1). Two pesticides, amitraz and atrazine, showed an upward trend in their concentration-IR curves but did not exceed the threshold of IR 1.5. The selection of the pesticides was guided by a previous publication on the induction of the oxidative stress response mediated via Nrf2 in a multifactorial assay using HepG2 cells.[12] All pesticides that were active in AREc32 were also active in HepG2. Those that were inactive in HepG2 were also inactive in AREc32. However, some of the pesticides active in HepG2

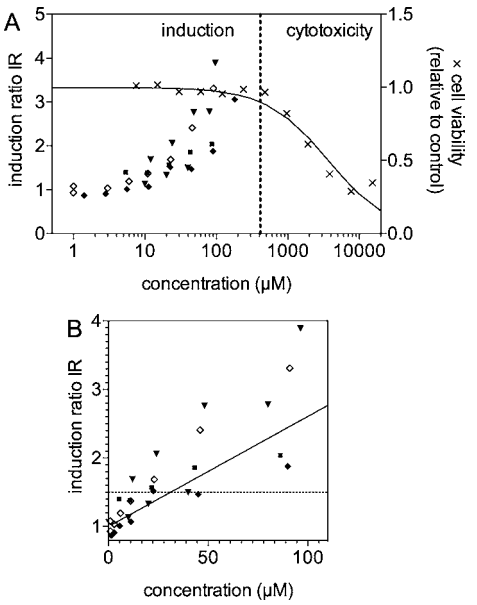

Figure 1. Typical concentration effect curve on the example of atorvastatin; A. cytotoxicity (symbol x displayed on right *y*-axis, drawn line is the best-fit curve) and induction (remaining symbols, where the different symbols refer to independent experiments, displayed on the left *y*-axis) on a logarithmic concentration scale. The vertical dashed line shows the EC_{10} for cytotoxicity of 410 μM, above which any induction experiment would be invalid because cytotoxicity started to take over. B. Linear portion of the induction curve on a nonlogarithmic concentration scale (same symbols as in A).

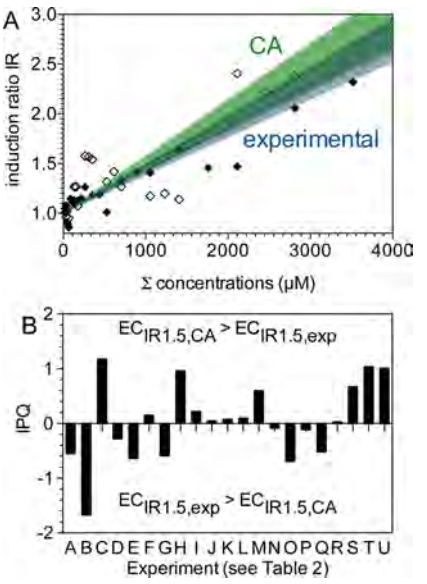

Figure 2. A. Example of one mixture experiment with 10 pharmaceuticals in an equipotent concentration ratio (Experiment different symbols refer to two independent experiments). The green line is the prediction for CA, and the dotted blue line the experimental best fit. The green and blue shaded areas indicated the 95% confidence intervals of the prediction (CA, green) and best fit (experimental, blue). B. Index on prediction quality IPQ for all equipotent and equimolar mixtures (data from Table 2).

were inactive in AREc32 and overall the activity of the active compounds was lower in AREc32 than in HepG2 (SI, Figure SI-4). HepG2 is metabolically active while AREc32 expresses only low levels of metabolic enzymes,[26] which can explain the difference. If some chemicals such as benzo[a]pyrene were treated with a commercially rat liver S9 metabolic enzyme mixture, their induction increased substantially (unpublished results). However, the S9 metabolic enzyme mixture detoxifies many reactive chemicals and therefore we did not add S9 to the samples in the present study.

Is Concentration Addition an Appropriate Model for the Mixture Effects in the Oxidative Stress Response Pathway? To evaluate if the model of concentration addition is applicable to the mixture effects observed with the AREc32, we tested 10 different 5-component and 5, 10-component mixtures of exclusively pharmaceuticals, three equipotent mixtures of 5 pesticides and mixtures of 5 or 10 pharmaceuticals with 5 pesticides. An example of a concentration effect relationship is shown in Figure 2A and all mixture results are compiled in Table 2 (and SI, Table SI-4 for the exact composition of the mixture and all concentration-effect curves). There was an overlap of the 95% confidence intervals of experimental and CA prediction in the example of Figure 2A and in 12 out of the 21 equipotent or equimolar mixtures.

For those mixtures where there was a discrepancy between CA prediction and experimental data, the deviation was fairly small and it was not systematic as the IPQ demonstrates (Figure 2B). The IPQ was close to zero for 9 out of these 21 mixtures (mixtures A to U, IPQ mean ± standard error 0.02 ± 0.05), indicating a good agreement between experiment and model. Of the remaining 12 mixtures, 6 overpredicted the toxicity and 6 underpredicted the toxicity.

An IPQ of −1/+1 means a 2 times over/underprediction of toxicity and an IPQ of 2 means a factor of 3 between experiment and CA model. The IPQ was between 0.5 and 1.2 in 11 mixtures and only in one case larger than 1.5, indicating satisfactory agreement between experiments and predictions. Belden et al.[32] analyzed a large set of literature data on 303 mixture toxicity experiments with pesticides and found that in 88% of the cases, there was $-1 < IPQ < 1$, which is similar to the present, albeit smaller, data set.

We can conclude that the mixture concept of concentration addition is valid for chemicals that are active in AREc32, independent of the chemical class.

Do Inactive Compounds Modulate the Activity of Inducers of the Oxidative Stress Response? After it has been established that compounds that are inducing the Nrf2 pathway act concentration additive, the question remains how inactive compounds influence the activity of active compounds. To our understanding the mixture concept of independent action (IA) is not suitable for the AREc32 because chemicals that do not activate the oxidative stress response will not show any induction. In fact, if the five nonpotent pharmaceuticals were added to a 5- and 10-component mixture of potent activators, no difference in the concentration-effect curves was observed (SI, Figure SI-5). Even if the IA prediction were modeled (SI, Section SI-2), it would not predict much different mixture effects than CA.

How Do Chemicals Mixed at Ratios of Their ADWG/AGWR Guideline Values Interact? When the 10 active pharmaceuticals were mixed together at their ADWG/AGWR guideline values, the experimental mixture effects were slightly lower than the corresponding effect predicted with CA but the IPQ was not larger than in the case of the equipotent mixtures (Table 2), thus we can conclude that CA reasonably well describes the combined effects of these mixtures.

Table 2. Mixture Experiments (Each Line of the Table Refers to a Different Mixture with the No. of Components and the Composition Given in Detail in the SI, Table SI-4), the Reported $EC_{IR1.5}$ Values Are Mean ± Standard Deviation

	no. of	mixture components	mixture ratio	$EC_{IR1.5,exp}$	$EC_{IR1.5,CA}$	IPQ
A	5	pharmaceuticals	equipotent	141 ± 10	91 ± 18	−0.55
B	5	pharmaceuticals	equipotent	2584 ± 302	969 ± 957	−1.67
C	5	pharmaceuticals	equipotent, most potent	35 ± 3	77 ± 5	1.18
D	5	pharmaceuticals	equipotent, least potent	2552 ± 221	2000 ± 148	−0.28
E	5	pharmaceuticals	equipotent	2117 ± 159	1293 ± 98	−0.64
F	5	pharmaceuticals	equipotent	794 ± 102	914 ± 44	0.15
G	5	pharmaceuticals	equipotent	842 ± 142	531 ± 40	−0.57
H	5	pharmaceuticals	equipotent	663 ± 54	1301 ± 99	0.96
I	5	pesticides	equipotent	28 ± 1	34 ± 3	0.22
J	5	pesticides	equipotent	33 ± 1	34 ± 3	0.05
K	5	pesticides	equipotent	32 ± 1	34 ± 3	0.08
L	2 × 5	pharmaceuticals	5 equipotent x 2 equimolar	611 ± 29	670 ± 74	0.10
M	2 × 5	pharmaceuticals	5 equipotent x 2 equimolar	497 ± 22	794 ± 102	0.60
N	2 × 5	pharmaceuticals	5 equipotent x 2 equimolar	903 ± 147	834 ± 73	−0.08
O[a]	10	pharmaceuticals	equipotent	1764 ± 92	1043 ± 83	−0.69
P[a]	10	pharmaceuticals	equipotent	1159 ± 67	1043 ± 83	−0.11
Q[a]	10	pharmaceuticals	equipotent	1010 ± 91	1043 ± 83	−0.52
R[a]	10	pharmaceuticals	equipotent	1189 ± 191	1043 ± 83	0.03
S	10	5 pharm. and 5 pest.	5 equipotent x 2 equimolar	469 ± 14	785 ± 72	0.67
T	15	10 pharm. and 5 pest.	5/10 equipotent x 2 equipotent	695 ± 27	1417 ± 111	1.01
U	15	10 pharm. and 5 pest.	5/10 equipotent x 2 equipotent	470 ± 14	942 ± 53	1.04
V[b]	10	pharmaceuticals	ADWG	248 ± 13	138 ± 11	−0.79
W[b]	10	pharmaceuticals	ADWG	364 ± 58	138 ± 12	−1.64
X[b]	10	pharmaceuticals	ADWG	242 ± 19	138 ± 12	−0.76
Y	10	pharmaceuticals	ADWG	383 ± 25	149 ± 12	−1.58
Z[c]	20	pesticides	ADWG	149 ± 6	331 ± 18	1.22
Z1[c]	20	pesticides	ADWG	111 ± 3	331 ± 18	1.98
Z2[c]	20	pesticides	ADWG	98 ± 3	331 ± 18	2.37

[a,b,c]Mixtures with the same subscript have the same mixture ratio but were mixed up independently from stocks of individual compounds.

In contrast, the mixture of 5 potent and 15 nonpotent pesticides at concentration ratios of their ADWG guideline values gave a consistently higher experimental effect than predicted by CA of the 5 potent compounds (SI Table SI-4 and Table 2). The IPQs for these mixtures were larger, up to 2.4 (Table 2). It is conceivable that the components that were considered nonpotent had in fact a low intrinsic potency that was masked by cytotoxicity but could contribute to the mixture effect. Amitraz and atrazine are examples of such compounds and they were components of this mixture with 0.6% and 1.7%, respectively (SI Table SI-4). While their $EC_{IR1.5}$ had to be extrapolated because they did not exceed the threshold of IR 1.5 and cytotoxicity started below the extrapolated $EC_{IR1.5}$, they clearly showed an upward trend in IR with increasing concentration. Thus, the higher toxicity than predicted by CA is likely to be due to apparently nonpotent but intrinsically active compounds adding in a concentration additive manner to the overall effect.

Comparison of Environmental Samples and the Mixture Effects of the Known Components. The nine water samples were initially assessed for cytotoxicity and then concentrations were chosen for the induction experiments that were below the EC_{10} for cytotoxicity. The EC_{10} values (Table 3) are subject to high uncertainty due to the variable nature of cell viability and low effects (see SI, Table SI-5 for all concentration-effect curves) and in five samples (RO, AO, O3/BAC, SW, Blank) no cytotoxicity was observed up to a REF of 250. The AREc32 cells were 1 to 2 orders of magnitude less sensitive than the bacteria *Vibrio fischeri* (SI, Figure SI-6),[8] presumably due to lower bioavailability in the presence of cell growth medium that contains proteins. Therefore the mammalian cell line would not be sensitive enough for cytotoxicity assessment of environmental samples but its low cytotoxicity is beneficial for the induction experiments.

All samples apart from the laboratory blank showed an induction of Nrf2 (Table 3 and SI, Table SI-4). The $EC_{IR1.5}$ values for induction were approximately 1 order of magnitude lower than the cytotoxicity EC_{10} values (SI Figure SI-7A), indicating higher sensitivity of the induction end point than of cytotoxicity. The fairly uniform ratio of 10 (median 10.8, 10th percentile 6.5, 90th percentile 17) between EC_{10} and $EC_{IR1.5}$ is surprising at first sight as the single chemicals were much more variable (SI, Figure SI-7B, median 2.9, 10th percentile 0.6, 90th percentile 56). It must be noted that single chemicals' effects could only be included in this analysis when they were dominated by oxidative stress, while in the mixtures of many components in environmental samples we can expect that in addition to chemicals that are potent activators of Nrf2 there are others that are not strong inducers and others, where cytotoxicity dominates.

The $EC_{IR1.5}$ were close to 2 in the secondary treated effluents, which means that these samples needed to be enriched by a factor of 2 to exceed the threshold of effect IR 1.5, and the $EC_{IR1.5}$ increased in each treatment train, indicating that the chemicals causing oxidative stress were removed by advanced treatment. The $EC_{IR1.5}$ decreased by more than a factor of 3 during drinking water treatment, which can be attributed to the formation of disinfection byproducts by

Table 3. Cytotoxicity and Induction of Oxidative Stress in Environmental Samples and Iceberg Mixtures

sample	Eff-1	MF	RO	AO	Eff-2	O_3/BAC	river	DW	SW	blank
sampling site	secondary treated effluent (influent to MF)	after microfiltration	after reverse osmosis	after advanced oxidation	secondary effluent (influent to O_3/BAC)	after ozonation and biologically activated carbon filtration	drinking water plant influent (river)	drinking water plant outlet	storm water	laboratory blank
cytotoxicity EC_{10} (REF) of sample	15	29	>250	>250	29	>250	238	54.2	>250	>250
$EC_{IR1.5}$ (REF) of sample	2.0 ± 0.7	4.5 ± 0.3	27.6 ± 7.5	94 ± 21	1.7 ± 0.4	21.6 ± 16.7	17.0 ± 3.1	5.0 ± 1.4	6.1	>200
tBHQ-EQ (μmol/L) of sample	0.59 ± 0.32	0.25 ± 0.10	0.04 ± 0.03	0.012 ± 0.005	0.67 ± 0.31	0.05 ± 0.05	0.07 ± 0.03	0.23 ± 0.11	0.19 ± 0.09	<0.01
# of chemicals detected	40	39	6	0	48	6	0	0	5	0
concentration of detected chemicals (μM)[a]	$4.1 \pm 0.2 \times 10^{-2}$	$4.3 \pm 0.2 \times 10^{-2}$	$2.7 \pm 0.1 \times 10^{-3}$	<LOD	$9.5 \pm 0.5 \times 10^{-2}$	$1.7 \pm 0.1 \times 10^{-3}$	<LOD	<LOD	$1.8 \pm 0.9 \cdot 10^{-3}$	<LOD
$EC_{IR1.5}$ (μM) of iceberg mix	$2.6 \pm 0.8 \times 10^{3}$	$2.6 \pm 0.8 \times 10^{3}$	$1.4 \pm 0.1 \times 10^{2}$		$1.5 \pm 0.3 \times 10^{3}$	$8.6 \pm 0.8 \times 10^{2}$			$2.8 \pm 1.3 \times 10^{2}$	
$EC_{IR1.5}$(REF) of iceberg mix[b]	$6.3 \pm 0.2 \times 10^{4}$	$6.1 \pm 0.2 \times 10^{4}$	$5.4 \pm 0.2 \times 10^{4}$		$1.6 \pm 0.4 \times 10^{4}$	$5.0 \pm 0.5 \times 10^{5}$			$1.5 \pm 0.7 \times 10^{5}$	
tBHQ-EQ (μmol/L) of iceberg mix[b]	$1.8 \pm 0.9 \times 10^{-5}$	$1.9 \pm 0.9 \times 10^{-5}$	$2.1 \pm 1.0 \times 10^{-5}$		$7.1 \pm 3.2 \times 10^{-5}$	$2.3 \pm 2.3 \times 10^{-6}$			$7.6 \pm 4.7 \times 10^{-6}$	
% contribution of detected chemicals to overall effect	0.003%	0.007%	0.051%		0.011%	0.004%			0.004%	

[a]Sum of detected concentrations, for error propagation assumed error of mix 5%. [b]Error calculated with error propagation.

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chlorination and chloramination.[18] Stormwater (SW) had a lower effect than the secondary treated effluents and was within the range of the MF samples. Overall the effects observed were in the same range as levels found in similar samples in the AREc32 validation study.[17]

269 chemicals were quantified in the nine samples, the detailed results are give in the SI, Table SI-6. In 6 samples 5−48 chemicals were detected and the associated sum of the molar concentrations is given in Table 3. The detected chemicals were mixed in the concentration ratios detected and full concentration-effect curves were determined of these iceberg mixtures with AREc32. A comparison of the effect caused by these iceberg mixtures and the entire water sample will tell us, which fraction of the effect can be explained by the known chemicals, that is, which fraction of the iceberg is visible.

The resulting $EC_{IR1.5}$ of the iceberg mixtures in units of total concentrations (μM) for induction were converted to units of REF by division by the total concentration of the detected chemicals in each sample (Table 3). The $EC_{IR1.5}$ were then converted into tBHQ-EQ and the fraction of bioanalytical equivalent concentration explained by the detected chemicals was calculated by dividing the tBHQ-EQ of the iceberg mixtures by the tBHQ-EQ of the sample. The detected chemicals could only explain 0.003% to 0.051% of observed bioanalytical equivalent concentration (Figure 3). This seems

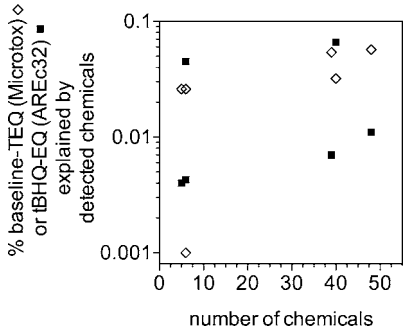

Figure 3. Fraction of bioanalytical equivalents (tBHQ-EQ or baseline-TEQ) explained by chemical analysis, empty diamonds refer to baseline-TEQ quantified with the Microtox assay,[8] filled squares refer to tBHQ-EQ (from Table 3).

low at first glance but is very similar to what was previously found in these samples with the Microtox assay, where the bioanalytical equivalents were expressed in baseline toxicity equivalent concentrations (baseline-TEQ).[8]

It is also possible that endotoxins that are produced by cyanobacteria and could be contained in water samples and be coextracted by SPE could contribute to the oxidative stress response. Their inflammatory activity is confirmed[33] and more recently general links between inflammation and oxidative stress have been established.[34] Endotoxins are known to be present in sewage and may only be removed by advanced water treatment.[35,36]

Chemicals that have specific modes of action by, for example, binding to the estrogen receptor or blocking the photosynthesis by binding to photosystem II are usually a small and well-defined group of chemicals with some structural similarity. In many cases, especially in more polluted samples like raw sewage or wastewater treatment plant effluent, a substantial fraction (over 50%) of the observed effect in an environmental sample can be explained by known and detected chemicals (for a summary of literature data see ref 8). However, even as these chemicals start to fall below detection limits during advanced treatment, they have not fully disappeared as it could been shown that the fraction of BEQ explained by chemical analysis decreases with decreasing pollutant levels even for specific modes of action.[37] In contrast, all chemicals in a mixture will contribute to nonspecific effects such as cytotoxicity, thus it will never be possible to quantify all contributing chemicals individually. The induction of oxidative stress response can be categorized as a response to a reactive mode of toxic action but it seems relatively nonspecific as many chemicals that can produce reactive oxygen species directly or indirectly (e.g., via inhibition of photosynthesis) as well as soft electrophiles can induce the Nrf2 activation, which in turn triggers the synthesis of antioxidants and metabolic enzymes. Thus, it is conceivable that a substantial fraction of chemicals can induce this pathway, which is a priori beneficial as it stimulates defense mechanisms. If, however, the oxidative stress becomes too pressing, the defense mechanisms cannot compensate any more. This will result in apoptosis and necrosis. Thus, the onset of the oxidative stress response is not a toxic effect as such but can be seen as an early warning indicator of potential adverse effects. As discussed above, 50% of the 300 tested pesticides induced the Nrf2 oxidative stress response pathway in a metabolically active HepG2 liver cell line[12] and even caffeine is a known inducer of the Nrf2 pathway.[38] Together with our findings that a substantial fraction of tested pharmaceuticals were active, it appears reasonable to assume that a substantial fraction of all chemicals present can be active in AREc32. Consequently, only a very small fraction of oxidative stress response can be explained by known organic micropollutants, especially given that transformation products often gain reactive properties during chemical transformation.[2]

Derivation of Effect-Based Trigger Values for the AREc32 Assay. Effect-based trigger values cannot be derived from the adverse effect in vivo because there is as yet no quantitative relationship between the induction of oxidative stress response (neither in vitro nor in vivo) and adverse effects. But we can anchor the effect-based trigger value to existing guideline values that were derived from concentrations that are not causing any adverse in vivo effects, even if in most cases the causative adverse process would not be oxidative stress.

As it would be practically difficult to mix the 181 chemicals of the ADWG or 346 chemicals of the AGWR, as an alternative, we propose to extrapolate a trigger value from the 10- and 20-component experimental mixtures in ratios of guideline values (Table 2). Assuming that the chemicals in our experiments are representative for all chemicals in the given guideline document, we can use the sum of the concentrations of the guideline values to extrapolate what effect these total concentrations would have in the AREc32 assay using the experimental $EC_{IR1.5}$ values and those predicted with CA from Table 2.

The effect-based trigger values EBT-$EC_{IR1.5}$ can then be calculated with eq 8 in analogy to the previously proposed EBT-EC_{50} for cytotoxicity assessed with the Microtox assay.[8] Here, the $EC_{IR1.5mixture}$ refers to the experimental mixture $EC_{IR1.5}$ values of the ADWG/AGWR mixtures (Table 2) and the sum of the concentrations of the guideline values was normalized to the number of chemicals contained in the associated guideline, *n*.

$$\text{EBT} - \text{EC}_{\text{IR1.5}} = \frac{\text{EC}_{\text{IR1.5,mixture}}}{\text{EF}}\left(\frac{1}{n}\sum_{i=1}^{n}\text{guideline value}_i\right) \quad (8)$$

The extrapolation factor EF (eq 9) accounts for the number of active chemicals, that is, Nrf2-inducing chemicals, to be included in the assessment, m, and the fraction of guideline value f that is acceptable in this m-component mixture.[8]

$$\text{EF} = f \cdot m \quad (9)$$

As discussed previously,[8] the EF needs to be set to a number that is acceptable to the regulatory community as part of a management decision but we can give some scientific guidance for the choice. For example, if we include m = 1000 active chemicals at f = 0.05, that is, 5% of their guideline concentration, the EF will be 50. The $\text{EC}_{\text{IR1.5,mixture}}$ was derived from mixtures of active inducers of the Nrf2 pathway. In a real sample we can expect no more than 50% of the chemicals to be active inducers of the Nrf2 pathway thus including all chemicals in the derivation of the EBT-$\text{EC}_{\text{IR1.5}}$ is a conservative and precautionary approach.

The sum of the concentrations in the ADWG is 147 μM, n is 181, and if all $\text{EC}_{\text{IR1.5}}$ values from Table 2 were implemented in eq 8, the calculated EBT-$\text{EC}_{\text{IR1.5}}$ with an EF 50 would range from 2.4 to 9.4 REF, with an average of 5.6 REF. The sum of the concentrations in the AGWR is 275 μM, n is 384 and the calculated EBT-$\text{EC}_{\text{IR1.5}}$ would range from 2.8 to 9.6 REF with an average of 5.7 REF. Given the uncertainty and variability of the predictions, we propose to choose a REF of 6 as tentative EBT-$\text{EC}_{\text{IR1.5}}$ for both drinking and recycled water. Thus, if the experimental $\text{EC}_{\text{IR1.5}}$ in a recycled water sample or in a drinking water sample were below 6 REF, then concern is indicated and more refined testing including chemical analyses should be performed.

The proposed EBT-$\text{EC}_{\text{IR1.5}}$ of 6 REF was compared with the measured $\text{EC}_{\text{IR1.5}}$ in the water samples tested in the present study. The samples AO and O_3/BAC correspond to recycled water and their $\text{EC}_{\text{IR1.5}}$ was 94 REF and 22 REF, respectively, thus both samples were above the EBT-$\text{EC}_{\text{IR1.5}}$, thus there is no concern. For drinking water, we have previously demonstrated that the formed disinfection byproducts also cause induction of oxidative stress, which decrease the $\text{EC}_{\text{IR1.5}}$ in disinfected samples. The derived trigger value EBT-$\text{EC}_{\text{IR1.5}}$ is only valid for organic micropollutants. Therefore, the influent of the drinking water treatment plant (sample River), which showed an $\text{EC}_{\text{IR1.5}}$ of 17, was used to assess the compliance of drinking water with the EBT-$\text{EC}_{\text{IR1.5}}$, and compliance was achieved.

The proposed algorithm was developed for Australian Guidelines (ADWG[23] and AGWR[24]) but can be adapted to any set of guideline values provided that evidence is available in a representative subset of chemicals that were tested in the AREc32 assay to provide an input for the extrapolation model. The present paper provides the scientific basis and a conceptual approach to derive effect-based trigger values. The next steps to make the proposed concept attractive for risk assessors would include gaining experience upon the applicability to different case studies and evaluating its robustness for practical use. It must be stressed though that an effect-based trigger value can never stand-alone but a battery of biological end points should be combined and the EBT does not replace chemical analysis but is a tool for initial screening that prioritizes samples for further more comprehensive assessment.

■ ASSOCIATED CONTENT

Supporting Information

Additional information on the chemicals and their physicochemical properties, information on the bioassay and the reference compound tBHQ, alternative mixture toxicity model of independent action, composition, concentration-effect curves and experimental and modeled $\text{EC}_{\text{IR1.5}}$ for all mixture experiments, of the samples and the iceberg mixtures as well as chemical analytical results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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Supporting Information

Most Oxidative Stress Response In Water Samples Comes From Unknown Chemicals: The Need For Effect-Based Water Quality Trigger Values

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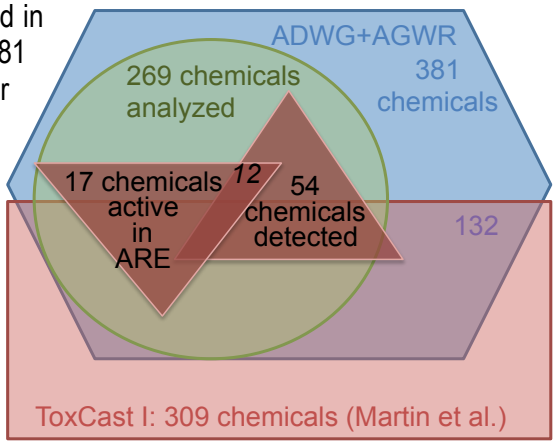
Table of Content

Table SI-1 Chemicals used in the present study, CAS numbers and manufacturer information

Chemicals were selected from a total of 381 chemicals listed in the Australian Drinking Water Guidelines of 2011 (ADWG, 181 organic chemicals)[2] or the "Australian guidelines for water recycling: managing health and environmental risks (phase 2) - augmentation of drinking water supplies" (AGWR, 349 organic chemicals).[3] We call the combined list ADWG+AGWR and if the guideline values differed between the ADWG and the AGWR, the ADWG value was chosen in the combined list. In addition, some pesticides were included in the list of tested compounds that were previously characterized by Martin et al.[1]



Up to 54 chemicals that have been detected in the environmental water samples (out of 269 target analytes) and were on the ADWG+AGWR list were used to prepare the iceberg mixtures, Out of the 35 chemicals used for the characterization of the activity in AREc32 (all of which were on the ADWG+AGWR list), 17 were active and were used for the designed mixture experiments. 12 of the active compounds overlapped with the detected chemicals. All chemicals that were used in the experiments are listed below.

Chemical	CAS	Provider	Catalogue number	Grade	Chemical Category
17β-estradiol	50-28-2	Sigma	E8875-1g	≥98%	Pharmaceutical
2,4-Dichlorophenoxyacetic acid (2,4,D)	94-74-6	Sigma-Aldrich	45555-250MG	Pestanal	Herbicide
4-Nonylphenol (4NP)	94-75-7	Sigma-Aldrich	31518-250MG	Pestanal	Herbicide
4-Tert Octylphenol	104-40-5	Sigma-Aldrich	46018-1G	Pestanal	Consumer/industrial chemical
Acetylsalicylic acid (Aspirin)	50-78-2	Aldrich	239631-1G	>99%	Pharmaceutical
Atenolol	29122-68-7	Sigma	A7655-1G	>98%	Pharmaceutical
Atorvastatin	134523-00-5	Dr. Ehrenstorfer GmbH	C10318000	Ref Std	Pharmaceutical
Atrazine (total) including metabolites	1912-24-9	Sigma-Aldrich	45330-250MG-R	Pestanal	Herbicide
Bisphenol A	80-05-7	Sigma-Aldrich	239658-50g	>99%	Consumer/industrial chemical
Caffeine	58-08-2	Sigma-Aldrich	C1778-1VL	Sigma Ref Std	Pharmaceutical
Carbamazepine	298-46-4	Sigma-Aldrich	49939-1G		
Cephalexin	15686-71-2	Fluka	33989-100MG-R	Vetranal	Antibiotics
Chlorpyrifos	2921-88-2	Fluka	45395-250MG	Pestanal	Organophosphate Insecticide
Citalopram	59729-32-7	USP	1134233	Ref Std	Pharmaceutical
Codeine	76-57-3	Cerilliant	C-006	Certified Reference Material	Pharmaceutical
Cyclophosphamide	6055-19-2	Sigma	C7397-1G	Ref Std	Pharmaceutical
DEET (N,N-diethyltoluamide (NN-diethyl-3-methylbenzamide))	134-62-3	Fluka	36542-250mg	Pestanal	Consumer/industrial chemical
Desmethyl citalopram	144025-14-9	Cerilliant	D-047	Certified Reference Material	Pharmaceutical Metabolite
Desmethyl diazepam	1088-11-5	Cerilliant	N-905	Certified Reference Material	Pharmaceutical Metabolite

Chemical	CAS	Provider	Catalogue number	Grade	Chemical Category
Diazepam (Valium)	439-14-5	Sigma	D0899-100mg	Ref Std	Pharmaceutical
Diazinon	333-41-5	Fluka	45428-250mg	Pestanal	Organophosphate Insecticide
Dicamba	1918-00-9	Sigma-Aldrich	45430-250mg	Pestanal	Organochlorine Herbicide
Diclofenac	15307-86-5	Dr. Ehrenstorfer GmbH	C 12537000	Ref Std	Pharmaceutical
Diuron	330-54-1	Sigma-Aldrich	45463-250mg	Pestanal	
Doxycycline	24390-14-5	Sigma-Aldrich	33429-100MG-R	Vetranal	Antibiotics
Erythromycin	114-07-8	Fluka	16221-500mg	Pharmaceutical secondary standard	Antibiotics
Fipronil	120068-37-3	Fluka	46451-100mg	Pestanal	Insecticide
Fluoxetine hydrochloride (Prozac)	56296-78-7	Fluka	34012-10mg-R	Vetranal	Pharmaceutical
Furosemide	54-31-9	Fluka	09205-1g	Pharmaceutical Secondary Standard	Pharmaceutical
Galaxolide (1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[g]-2-benzopyran)	1222-05-5	Dr. Ehrenstorfer GmbH	C 1421300	Ref Std	Consumer/industrial chemical
Gemfibrozil	25812-30-0	Sigma-Aldrich	G9518-5G	USEPA	Pharmaceutical
Hexazinone	51235-04-2	Sigma-Aldrich	36129-100MG	Pestanal	Herbicide
Hydrochlorthiazide	58-93-5	Sigma-Aldrich	08213-1G	Pharmaceutical secondary standard	Pharmaceutical
Ibuprofen	15687-27-1	Sigma-Aldrich	32424-100MG	Vetranal	Pharmaceutical
Indomethacin	53-86-1	Sigma-Aldrich	I8280-5G	USP Testing Spec	Pharmaceutical
Lincomycin	7179-49-9	Sigma-Aldrich	31727-250MG	Vetranal	Antibiotics
MCPA (2-Methyl-4-chlorophenoxyacetic acid)	94-74-6	Sigma-Aldrich	45555-250MG	Pestanal	Herbicide
Metolachlor	51218-45-2	Novachem	P-158NB-250	96.4%	Herbicide
Metoprolol	56392-17-7	Sigma-Aldrich	77376-1G	Pharmaceutical Secondary Standard	Pharmaceutical
Naproxen	22204-53-1	Sigma-Aldrich	36405-500MG	Pharm Sec Std	Pharmaceutical
Norfloxacin	70458-96-7	Sigma-Aldrich	33899-100MG-R	Vetranal	Antibiotics
Oxazepam	604-75-1	Cerilliant	O-902	USDEA	Pharmaceutical
Oxycodone	76-42-6	Cerilliant	O-002		Pharmaceutical
Oxytetracycline	6153-64-6	Sigma-Aldrich	16221-500MG	Pharmaceutical secondary standard	Antibiotics
Paracetamol (acetaminophen)	103-90-2	Sigma-Aldrich	A3035-1VL	Analysis Standard	Pharmaceutical
Picloram	1918-02-1	Sigma-Aldrich	36774-250MG-R	Pestanal	Herbicide
Praziquantel	55268-74-1	Sigma-Aldrich	46648-250MG	Vetranal	Pharmaceutical
Propoxur	114-26-1	Sigma-Aldrich	45644-250MG	Pestanal	Carbamate Insecticide
Propranolol	318-98-9	Sigma-Aldrich	P0884-1G	>99%	Pharmaceutical
Ranitidine	66357-59-3	Sigma-Aldrich	44404-500MG	Pharm Sec Std	Pharmaceutical
Roxithromycin	80214-83-1	Sigma-Aldrich	R4393-1G	>90%	Antibiotics
Simazine	122-34-9	Sigma-Aldrich	32059-250MG	Pestanal	Pharmaceutical

Chemical	CAS	Provider	Catalogue number	Grade	Chemical Category
Sulfadiazine	68-35-9	Sigma-Aldrich	35033-100MG		
Sulfamethoxazole	723-46-6	Sigma-Aldrich	31737-250MG	Vetranal	Antibiotics
Sulfasalazine	599-79-1	Sigma-Aldrich	S0883-10G	>98%	Pharmaceutical
Temazepam	846-50-4	Sigma-Aldrich	T8275-100MG	USDEA	Pharmaceutical
Tolutriazole (5-Methyl-1H-benzotriazole)	136-85-6	Sigma-Aldrich	196304-10g	98%	Consumer product
Tonalide (AHTN, 6-Acetyl-1, 1, 2, 4, 4, 7-hexamethyltetraline)	21145-77-7	Sigma-Aldrich	CDS009866-50mg	CPR	Consumer product
Tributylphosphate	126-73-8	Sigma-Aldrich	240494-5ML	>99%	Consumer/industrial chemical
Triclopyr	55335-06-3	Sigma-Aldrich	32016-250MG	Pestanal	Herbicide
Triclosan	3380-34-5	Sigma-Aldrich	72779-5G-F	>97%	Consumer/industrial chemical
Trimethoprim	738-70-5	Sigma-Aldrich	46984-250MG	Vetranal	Antibiotics
Tris(2-chloroethyl)phosphate (TCEP)	115-96-8	Sigma-Aldrich	119660-25G	97%	Consumer/industrial chemical
Venlafaxine	99300-78-4	Sigma-Aldrich	V7264-10MG	>98%	Pharmaceutical

Section SI-1: Additional information on the bioassay

The cells were grown as described previously.[4] The AREc32 cells, seeded at a density of 12,000 cells per 100 µL in a 96-well microtiter plate were dosed with varying concentrations of the chosen pharmaceuticals and their mixtures. All stocks and extracts were made up in methanol, aliquoted into glass vials with inserts, the methanol was blown down, and the residue redissolved in growth medium and then transferred to make serial dilutions in a mixing block or microtiter plate. After 24h of incubation at 37ºC and 5% CO_2 the cell viability was assessed using the MTS (tetrazolium) assay (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, Promega). An EC_{10}, the effect concentration causing 10% reduction of cell viability, was derived from a log-logistic dose response curve.[4] Only concentrations that were below the EC_{10} for cytotoxicity were evaluated for induction of the oxidative stress response.

The induction of the oxidative stress response is proportional to the quantity of luciferase formed. In all experiments with pesticides we used the Luciferase Assay System (Promega E1500) for quantification of luciferase, while for all other samples the solutions were prepared in our laboratory with the following chemicals purchased from. Sigma Aldrich, USA.

After 24h incubation at 37˚C and 5% CO_2, the luciferase response was quantified by first lysing the cells with 30 µL of cell lysis reagent (25mM Tris buffer adjusted to pH 7.8, 1% Triton-X 100, 2 mM EDTA, 2 mM DTT, 10% glycerol, MilliQ water). The lysed cells were then treated with 100 µL of luciferase reagent (20 mM Tricine at pH 7.8, 2.67 mM $MgSO_4.7H_20$, 0.1 mM EDTA, 33.3 mM DTT, 261 µM Coenzyme A, 530 µM ATP, 470 µM luciferin (Promega Cooperation, USA) and MilliQ water; the solution's pH was adjusted to 7.8 just before addition to cells). The microtiter plate was immediately read in a FLUOstar Optima plate reader (BMG Labtech) and the luminescence was quantified.

The $EC_{IR1.5}$ was derived from the concentration-IR regression (equation 1 in main manuscript) with equation S-1 and the standard error of $EC_{IR1.5}$ was derived after error propagation with equation S-2.

$$EC_{IR1.5} = \frac{0.5}{slope} \qquad \text{(S-1)}$$

$$\sigma_{EC_{IR1.5}} = \frac{0.5\sigma_{slope}}{slope^2} \qquad \text{(S-2)}$$

For quality control/quality analysis (QC/QA) purposes, on each microtiter plate a concentration effect curve of *t*-butylhydroquinone (tBHQ, CAS Registry No. 1948-33-0, 97%) was measured and monitored over time. Figure SI-1 gives an account of all 29 experiments with tBHQ performed during this study. Each experiment used an average of three to five plates. There were five experiments (experiments 1,3,11,12,14) at the beginning of the study where the tBHQ had degraded resulting in $EC_{IR1.5}$ > 2 µM. These $EC_{IR1.5}$ data were omitted in the statistics but the experimental data of the samples on the plates was used anyway for the dose-response assessment because the deviation of the $EC_{IR1.5}$ was an artifact of tBHQ degradation and the data of the samples were consistent with replicates. The median $EC_{IR1.5}$ of the 25 experiments with tBHQ was 1.1 µM and the 10th percentile was 0.7 µM and 90th percentile was 1.7 µM, which served as validity criteria. This $EC_{IR1.5}$ was consistent with the initial validation study of the bioassay (1.32 µM).[4]

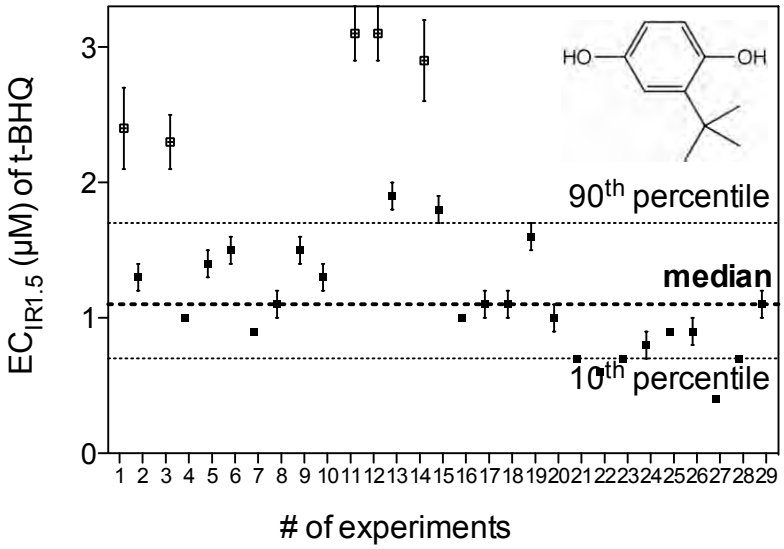


Figure SI-1 Long-term record of the QC/QA reference compound tBHQ.

Section SI-2: Alternative mixture toxicity model of independent action

The mixture model of independent action (IA) is not appropriate for the AREc32 induction of oxidative stress because only compounds with the target mode of action that induce oxidative stress will give a signal in the test at all and other compounds will just have an IR of 1, the same as the controls. Nevertheless we coded the model of IA for comparison. As the control and inactive compounds have an IR of 1, we subtracted the value of 1 from the IR before applying the IA model (eq. SI-1) and then added the 1 at the end results. The model of IA is defined by equation SI-3,

$$\text{effect}_{\text{mixture}} = 1 - \prod_{i=1}^{n} 1 - \text{effect}_i \qquad \text{(SI-3)}$$

where effect_i is the fractional biological effect of component "i" at the concentration in the mixture and Π stands for the product (multiplication).

When comparing the predictions for CA and IA, there is not much difference (Figure SI-2) and it is difficult to differentiate between the leveling off due to cytotoxicity and a true independent action effect, but since IA is toxicologically not relevant it is not further discussed in the paper.

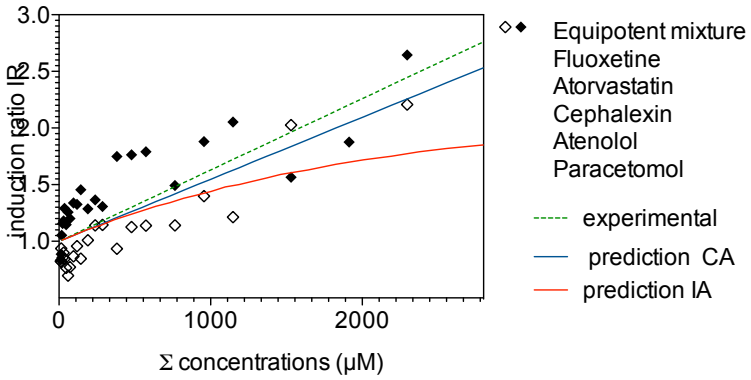


Figure SI-2 Comparison of the predictions for CA (blue line) and IA (red curve) for one of the 5-component mixtures (the corresponding filled and empty symbols correspond to 2 independent replicates done on two plates at the same day, the green dotted line is the best fit linear regression through all data points).

Section SI-3 Discussion on cytotoxicity.

In Table 1, there are 15 pharmaceuticals and 20 pesticides, totaling 35 compounds. 9 Pharmaceuticals were not cytotoxic up to the highest concentration tested and one pesticide did not give any valid data, leaving 6 pharmaceuticals and 19 pesticide data for an analysis of cytotoxicity. Cytotoxicity increased slightly (slope = 0.03, p = 0.02, thus significantly different from zero) with hydrophobicity, expressed in terms of the liposome-water distribution ratio D_{lipw}(pH7) (Figure SI-3A). However, there was no strong correlation with D_{lipw}(pH7) (r^2 = 0.22) unlike in the case of bacterial cytotoxicity such as the bioluminescence inhibition of *Vibrio fischeri* (Microtox assay) (slope = 0.72, r^2 = 0.97).[5] Also the EC_{10} for AREc32 were higher than the EC_{50} in the bacterial cytotoxicity assay. This difference in sensitivity is presumably due to different sensitivity of the two cell types as well as reduced bioavailability of chemicals in the mammalian cell system. For mammalian cells to grow, the medium must be supplemented with fetal calf serum, which contains high amounts of proteins and small amounts of lipids to both of which the chemicals may bind and hence not be available for cellular uptake.

10 of the 15 pharmaceuticals were active inducers of the oxidative stress response and 5 of the 20 pesticides were active, with another two showing an increasing concentration-IR trend but no exceedance of the threshold of IR 1.5. Thus only 17 data points were available for a comparison of induction and cytotoxicity. Thus only 17 data points were available for a comparison of induction and cytotoxicity. The induction endpoint was typically more sensitive than cytotoxicity (Figure SI-3B) but there were exceptions like ranitidine, where cytotoxicity and induction occurred at similar concentrations. In these cases these $EC_{IR1.5}$ must be treated with some caution as the $EC_{IR1.5}$ will not be as precise as others where there is a wider window between induction and cytotoxicity.

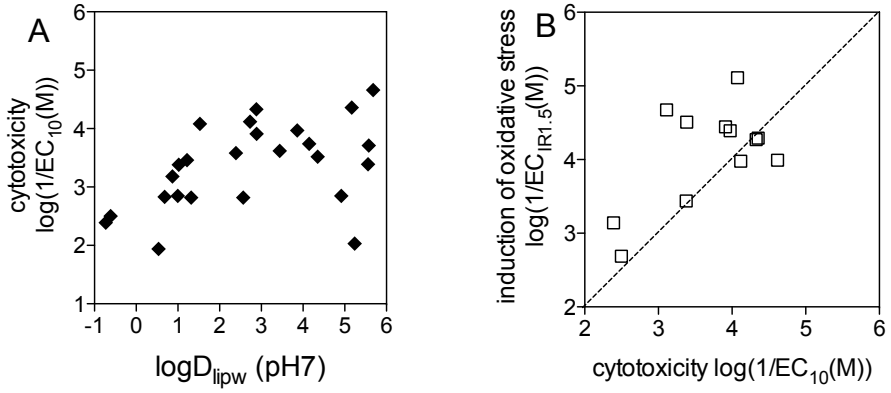


Figure SI-3. A. Correlation between cytotoxicity EC_{10} and the hydrophobicity of the test chemicals (Table 1) expressed by the liposome-water distribution ratio $logD_{lipw}$(pH7). B. Relationship between cytotoxicity EC_{10} and $EC_{IR1.5}$ for induction of the oxidative stress response. The dotted line is the 1:1 line for equal concentrations in both endpoints.

Table SI-2 Analyzed chemicals (in alphabetical order of analyte name) with methods and limit of reporting (LOR). For more details see Tang et al.[5]

Analytical method	Analyte	Units	LOR
Endocrine Disrupting Compounds by GC-MS	17-α-Ethynylestradiol	ng/L	5
Endocrine Disrupting Compounds by GC-MS	17-β-Estradiol	ng/L	5
GC-MS Screen	1H-Benzotriazole	µg/L	0.2
GC-MS Screen	1H-Benzotriazole, 1-methyl	µg/L	0.2
GC-MS Screen	1H-Benzotriazole, 5-methyl	µg/L	0.2
Pesticides by GC-MS	2,4-D	µg/L	0.1
Pesticides by GC-MS	2,4-DB	µg/L	0.1
Pesticides by GC-MS	2,4-DP (Dichlorprop)	µg/L	0.1
Pesticides by GC-MS	2,4,5-T	µg/L	0.1
GC-MS Screen	2,6-Di-t-butyl-p-cresol (BHT)	µg/L	0.5
GC-MS Screen	2,6-Di-t-butylphenol	µg/L	0.2
Pesticides by GC-MS	3-Hydroxycarbofuran	µg/L	0.1
Herbicides and Other Compounds by LC-MS	3,4-Dichloroaniline	µg/L	0.01
GC-MS Screen	4-Chloro-3,5-dimethylphenol	µg/L	0.1
Endocrine Disrupting Compounds by GC-MS	4-*t*-Octylphenol	ng/L	10
Pharmaceuticals by LC-MS	Acesulfame	µg/L	0.01
Pharmaceuticals by LC-MS	Acetylsalicylic acid	µg/L	0.01
Pesticides by GC-MS	Aldicarb	µg/L	0.1
Pesticides by GC-MS	Aldicarb sulfone (Aldoxycarb)	µg/L	0.1
Pesticides by GC-MS	Aldicarb sulfoxide	µg/L	0.1
Pesticides by GC-MS	Aldrin (HHDN)	µg/L	0.1
Pesticides by GC-MS	Allethrin	µg/L	0.1
Herbicides and Other Compounds by LC-MS	Ametryn	µg/L	0.01
Herbicides and Other Compounds by LC-MS	Amitraz	µg/L	0.1
Endocrine Disrupting Compounds by GC-MS	Androsterone	ng/L	5
Pharmaceuticals by LC-MS	Atenolol	µg/L	0.01
Pharmaceuticals by LC-MS	Atorvastatin	µg/L	0.01
Herbicides and Other Compounds by LC-MS	Atrazine	µg/L	0.01
Pesticides by GC-MS	Azinphos-ethyl	µg/L	0.1
Pesticides by GC-MS	Azinphos-methyl	µg/L	0.1
Pesticides by GC-MS	Benalaxyl	µg/L	0.1
Pesticides by GC-MS	Bendiocarb	µg/L	0.1
Pesticides by GC-MS	Bifenthrin	µg/L	0.1
Pesticides by GC-MS	Bioresmethrin	µg/L	0.1
GC-MS Screen	Bisphenol A	µg/L	0.1
Pesticides by GC-MS	Bitertanol	µg/L	0.1
Herbicides and Other Compounds by LC-MS	Bromacil	µg/L	0.01

Analytical method	Analyte	Units	LOR
Pesticides by GC-MS	Bromophos-ethyl	µg/L	0.1
Pesticides by GC-MS	Cadusafos	µg/L	0.1
Pharmaceuticals by LC-MS	Caffeine	µg/L	0.02
Pesticides by GC-MS	Captan	µg/L	0.2
Pharmaceuticals by LC-MS	Carbamazepine	µg/L	0.01
Pesticides by GC-MS	Carbaryl	µg/L	0.01
Pesticides by GC-MS	Carbofuran	µg/L	0.1
Pesticides by GC-MS	Carbophenothion	µg/L	0.1
Pharmaceuticals by LC-MS	Cephalexin	µg/L	0.01
Pharmaceuticals by LC-MS	Chloramphenicol	µg/L	0.1
Pesticides by GC-MS	Chlordene	µg/L	0.1
Pesticides by GC-MS	Chlordene Epoxide	µg/L	0.1
Pesticides by GC-MS	Chlordene-1-hydroxy	µg/L	0.1
Pesticides by GC-MS	Chlordene-1-hydroxy-2,3-epoxide	µg/L	0.1
Pesticides by GC-MS	Chlorfenvinphos	µg/L	0.1
Pesticides by GC-MS	Chlorpyrifos	µg/L	0.1
Pesticides by GC-MS	Chlorpyrifos oxon	µg/L	0.1
Pesticides by GC-MS	Chlorpyrifos-methyl	µg/L	0.1
Pharmaceuticals by LC-MS	Chlortetracycline	µg/L	0.2
Pharmaceuticals by LC-MS	Ciprofloxacin	µg/L	0.15
Pesticides by GC-MS	*cis* -Nonachlor	µg/L	0.1
Pesticides by GC-MS	*cis*-Chlordane	µg/L	0.1
Pharmaceuticals by LC-MS	Citalopram	µg/L	0.01
Pesticides by GC-MS	Clopyralid	µg/L	0.1
Pharmaceuticals by LC-MS	Codeine	µg/L	0.1
Pesticides by GC-MS	Coumaphos	µg/L	0.1
Pharmaceuticals by LC-MS	Cyclophosphamide	µg/L	0.01
Pesticides by GC-MS	Cyfluthrin	µg/L	0.1
Pesticides by GC-MS	Cyhalothrin	µg/L	0.2
Pesticides by GC-MS	Cypermethrin	µg/L	0.1
Herbicides and Other Compounds by LC-MS	Dalapon (2,2-DPA)	µg/L	0.05
Pharmaceuticals by LC-MS	Dapsone	µg/L	0.01
Pharmaceuticals by LC-MS	DEET	µg/L	0.01
Pesticides by GC-MS	DEET	µg/L	0.0
Pesticides by GC-MS	Deltamethrin	µg/L	0.1
Pesticides by GC-MS	Demeton-S-methyl	µg/L	0.1
Herbicides and Other Compounds by LC-MS	Desethyl Atrazine	µg/L	0.01
Herbicides and Other Compounds by LC-MS	Desisopropyl Atrazine	µg/L	0.01
Pharmaceuticals by LC-MS	Desmethyl Citalopram	µg/L	0.01

Analytical method	Analyte	Units	LOR
Pharmaceuticals by LC-MS	Desmethyl Diazepam	µg/L	0.01
Pharmaceuticals by LC-MS	Diazepam	µg/L	0.01
Herbicides and Other Compounds by LC-MS	Diazinon	µg/L	0.02
Pesticides by GC-MS	Dicamba	µg/L	0.1
Pesticides by GC-MS	Dichlofluanid	µg/L	0.1
Pesticides by GC-MS	Dichlorvos	µg/L	0.1
Pharmaceuticals by LC-MS	Diclofenac	µg/L	0.01
Pesticides by GC-MS	Diclofop-methyl	µg/L	0.1
Pesticides by GC-MS	Dicloran	µg/L	0.1
Pesticides by GC-MS	Dicofol	µg/L	3.0
Pesticides by GC-MS	Dieldrin (HEOD)	µg/L	0.05
Pesticides by GC-MS	Dimethoate	µg/L	0.1
Pesticides by GC-MS	Dimethomorph	µg/L	0.2
Pesticides by GC-MS	Dioxathion	µg/L	0.1
Pesticides by GC-MS	Disulfoton	µg/L	0.1
Herbicides and Other Compounds by LC-MS	Diuron	µg/L	0.01
Pharmaceuticals by LC-MS	Doxylamine	µg/L	0.01
Pesticides by GC-MS	Endosulfan Ether	µg/L	0.1
Pesticides by GC-MS	Endosulfan Lactone	µg/L	0.1
Pesticides by GC-MS	Endosulfan Sulfate	µg/L	0.05
Pesticides by GC-MS	Endrin	µg/L	0.1
Pesticides by GC-MS	Endrin aldehyde	µg/L	0.1
Pharmaceuticals by LC-MS	Enrofloxacin	µg/L	0.02
Pharmaceuticals by LC-MS	Erythromycin	µg/L	0.01
Pharmaceuticals by LC-MS	Erythromycin anhydrate	µg/L	0.01
Endocrine Disrupting Compounds by GC-MS	Estriol	ng/L	5
Endocrine Disrupting Compounds by GC-MS	Estrone	ng/L	5
Pesticides by GC-MS	Ethion	µg/L	0.1
Pesticides by GC-MS	Ethoprophos	µg/L	0.1
Endocrine Disrupting Compounds by GC-MS	Etiocholanolone	ng/L	5
Pesticides by GC-MS	Etrimphos	µg/L	0.1
Pesticides by GC-MS	Famphur	µg/L	0.1
Pesticides by GC-MS	Fenamiphos	µg/L	0.1
Pesticides by GC-MS	Fenchlorphos	µg/L	0.1
Pesticides by GC-MS	Fenitrothion	µg/L	0.1
Pesticides by GC-MS	Fenoprop (2,4,5-TP)	µg/L	0.1
Pesticides by GC-MS	Fenthion (methyl)	µg/L	0.1
Pesticides by GC-MS	Fenthion-ethyl	µg/L	0.1
Pesticides by GC-MS	Fenvalerate	µg/L	0.1

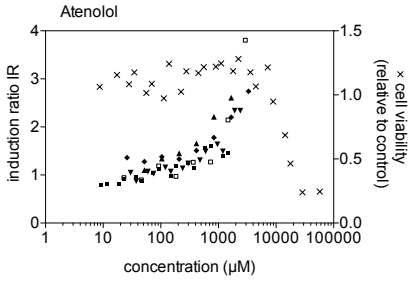
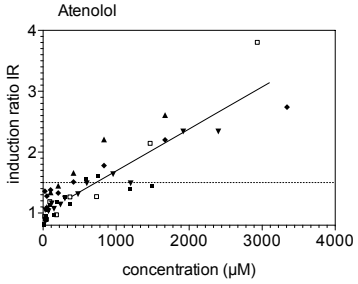
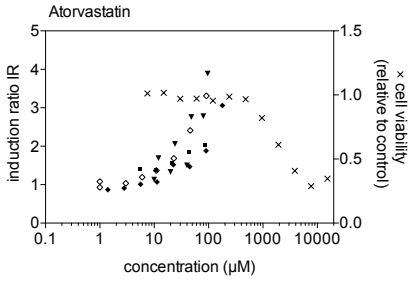
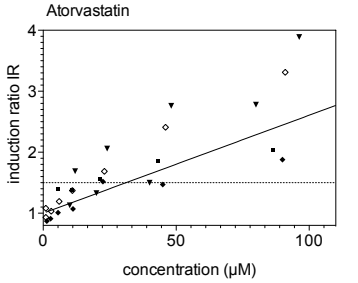
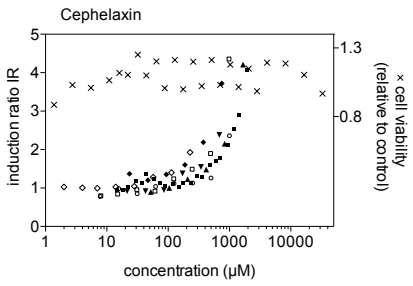
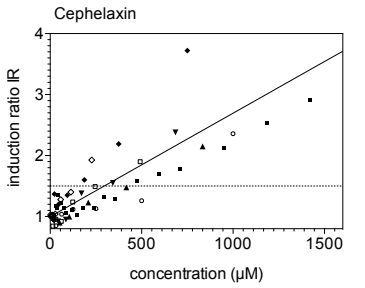
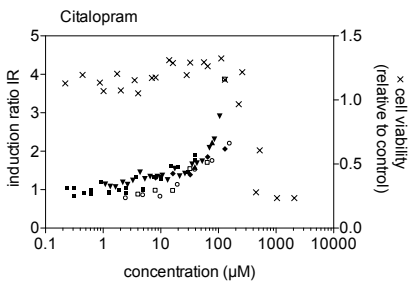
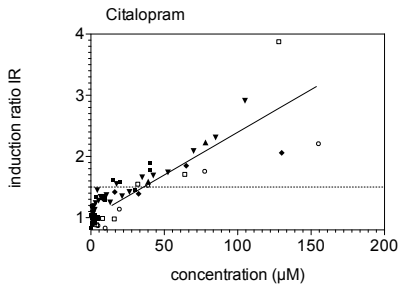
Analytical method	Analyte	Units	LOR
Pesticides by GC-MS	Fipronil	µg/L	0.1
Pesticides by GC-MS	Fluazifop-butyl	µg/L	0.1
Herbicides and Other Compounds by LC-MS	Fluometuron	µg/L	0.01
Pharmaceuticals by LC-MS	Fluoxetine	µg/L	0.01
Pesticides by GC-MS	Fluroxypyr	µg/L	0.1
Pesticides by GC-MS	Fluvalinate	µg/L	0.1
Pharmaceuticals by LC-MS	Fluvastatin	µg/L	0.01
Pharmaceuticals by LC-MS	Frusemide	µg/L	0.01
Pesticides by GC-MS	Furalaxyl	µg/L	0.1
Pharmaceuticals by LC-MS	Gabapentin	µg/L	0.05
FRAGRANCES by GC-MS	Galaxolide	µg/L	0.1
Pharmaceuticals by LC-MS	Gemfibrozol	µg/L	0.01
Herbicides and Other Compounds by LC-MS	Haloxyfop (acid)	µg/L	0.01
Herbicides and Other Compounds by LC-MS	Haloxyfop-2-etotyl	µg/L	0.01
Herbicides and Other Compounds by LC-MS	Haloxyfop-methyl	µg/L	0.01
Pesticides by GC-MS	HCB	µg/L	0.1
Pesticides by GC-MS	Heptachlor	µg/L	0.03
Pesticides by GC-MS	Heptachlor Epoxide	µg/L	0.03
Herbicides and Other Compounds by LC-MS	Hexazinone	µg/L	0.01
Pharmaceuticals by LC-MS	Hydrochlorthiazide	µg/L	0.01
Pharmaceuticals by LC-MS	Ibuprofen	µg/L	0.07
Pharmaceuticals by LC-MS	Ifosfamide	µg/L	0.01
Herbicides and Other Compounds by LC-MS	Imidacloprid	µg/L	0.01
Pharmaceuticals by LC-MS	Indomethacin	µg/L	0.01
Pharmaceuticals by LC-MS	Iopromide	µg/L	0.2
Pesticides by GC-MS	Isofenphos	µg/L	0.1
Pesticides by GC-MS	Lambda-cyhalothrin	µg/L	0.1
Pharmaceuticals by LC-MS	Lincomycin	µg/L	0.01
Pesticides by GC-MS	Lindane (γ-HCH)	µg/L	0.1
Pesticides by GC-MS	Malathion (Maldison)	µg/L	0.1
Pesticides by GC-MS	MCPA	µg/L	0.1
Pesticides by GC-MS	MCPB	µg/L	0.1
Pesticides by GC-MS	Mecoprop	µg/L	0.1
Pesticides by GC-MS	Metalaxyl	µg/L	0.1
Pesticides by GC-MS	Methidathion	µg/L	0.1
Pesticides by GC-MS	Methiocarb	µg/L	0.1
Pesticides by GC-MS	Methomyl	µg/L	0.1
Pesticides by GC-MS	Methomyl oxime	µg/L	0.5
Pesticides by GC-MS	Methoprene	µg/L	0.1

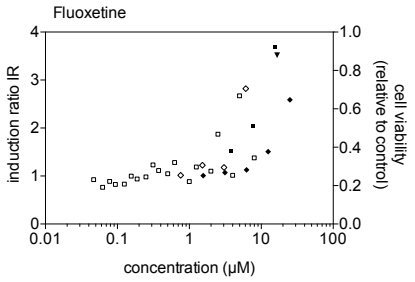
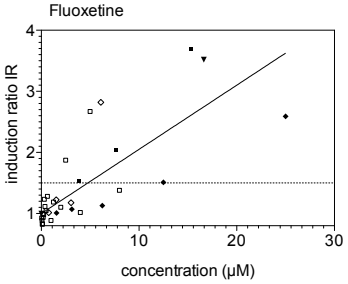
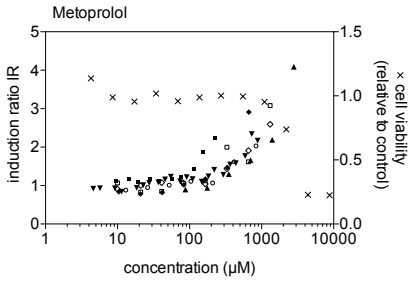
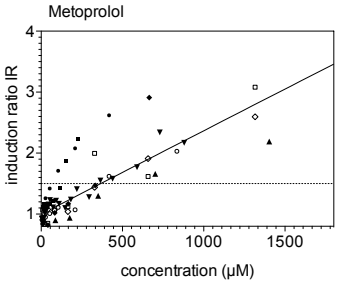
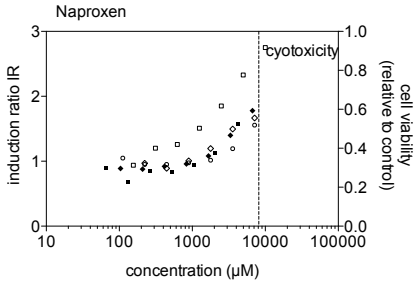
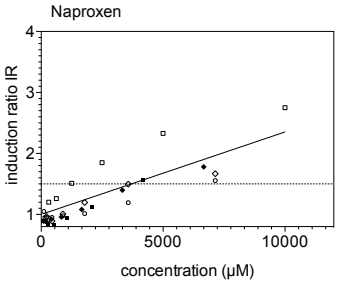
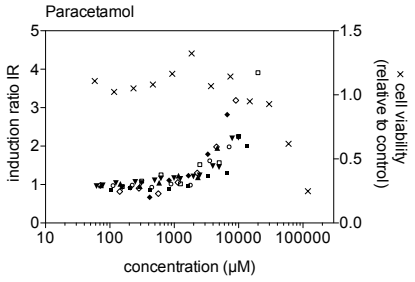
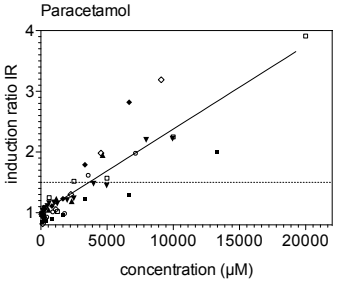
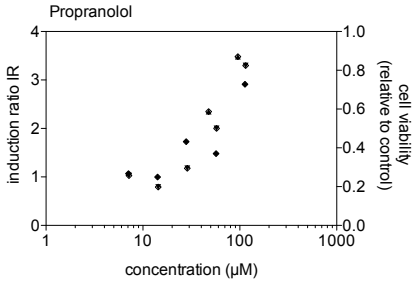
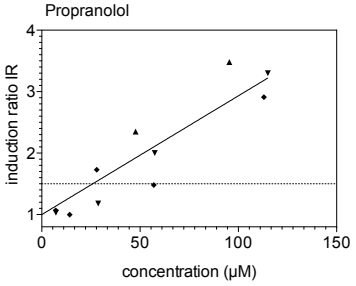
Analytical method	Analyte	Units	LOR
Pesticides by GC-MS	Methoxychlor	µg/L	0.1
Herbicides and Other Compounds by LC-MS	Metolachlor	µg/L	0.01
Pharmaceuticals by LC-MS	Metoprolol	µg/L	0.01
Pesticides by GC-MS	Metribuzin	µg/L	0.1
Pesticides by GC-MS	Mevinphos	µg/L	0.1
GC-MS Screen	Moclobemide	µg/L	0.5
Pesticides by GC-MS	Molinate	µg/L	0.1
Pesticides by GC-MS	Monocrotophos	µg/L	0.5
FRAGRANCES by GC-MS	Musk Ketone	µg/L	0.1
FRAGRANCES by GC-MS	Musk Xylene	µg/L	0.1
GC-MS Screen	N-Butyl benzenesulfonamide	µg/L	0.1
GC-MS Screen	N-Butyltoluenesulfonamide	µg/L	0.1
Pharmaceuticals by LC-MS	Naproxen	µg/L	0.1
Endocrine Disrupting Compounds by GC-MS	Nonylphenol	ng/L	100
Pharmaceuticals by LC-MS	Norfloxacin	µg/L	0.05
Endocrine Disrupting Compounds by GC-MS	Norgestrel	ng/L	10
Pesticides by GC-MS	*o,p*-DDD	µg/L	0.1
Pesticides by GC-MS	*o,p*-DDE	µg/L	0.1
Pesticides by GC-MS	*o,p*-DDT	µg/L	0.1
Pesticides by GC-MS	Omethoate	µg/L	0.5
Pesticides by GC-MS	Oxadiazon	µg/L	0.1
Pesticides by GC-MS	Oxamyl	µg/L	0.5
Pesticides by GC-MS	Oxamyl oxime	µg/L	0.5
Pharmaceuticals by LC-MS	Oxazepam	µg/L	0.01
Pesticides by GC-MS	Oxychlordane	µg/L	0.1
Pharmaceuticals by LC-MS	Oxycodone	µg/L	0.01
Pesticides by GC-MS	Oxydemeton-methyl	µg/L	0.1
Pesticides by GC-MS	Oxyfluorfen	µg/L	0.1
Pharmaceuticals by LC-MS	Oxytetracycline	µg/L	0.4
Pesticides by GC-MS	*p,p*-DDD	µg/L	0.1
Pesticides by GC-MS	*p,p*-DDE	µg/L	0.1
Pesticides by GC-MS	*p,p*-DDT	µg/L	0.1
Pharmaceuticals by LC-MS	Paracetamol	µg/L	0.02
Pesticides by GC-MS	Parathion (ethyl)	µg/L	0.1
Pesticides by GC-MS	Parathion-methyl	µg/L	0.1
Pesticides by GC-MS	Pendimethalin	µg/L	0.1
Pesticides by GC-MS	Permethrin	µg/L	0.1
Pesticides by GC-MS	Phenothrin	µg/L	0.1
Pharmaceutical by LC-MS	Phenytoin	µg/L	0.01

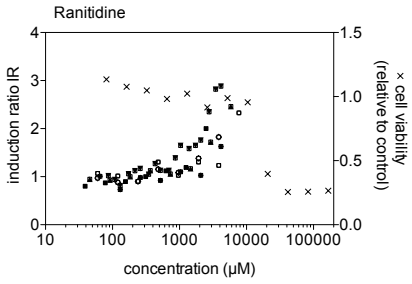
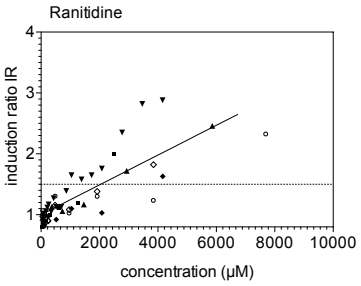
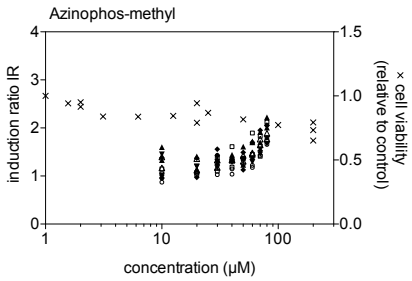
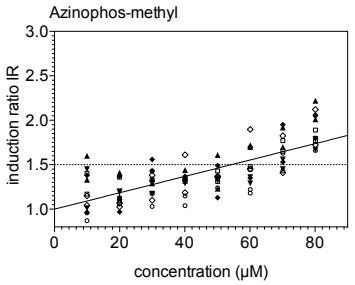
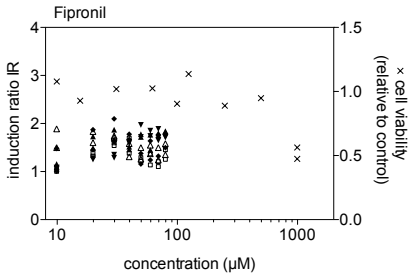
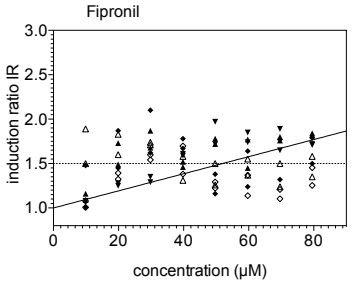
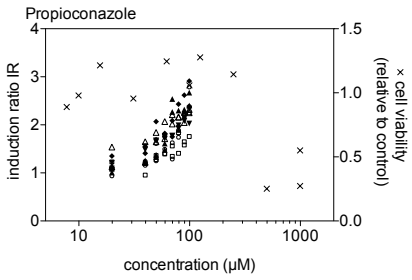
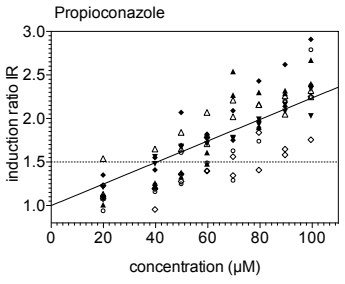
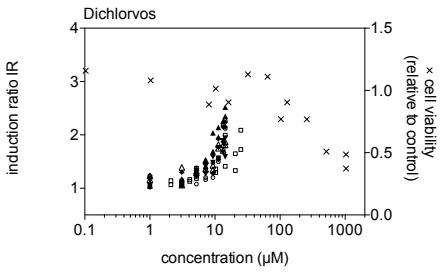
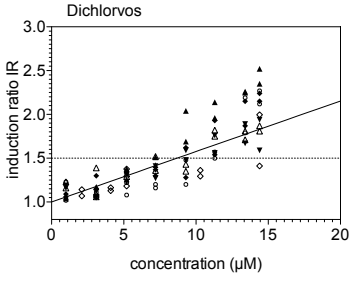
Analytical method	Analyte	Units	LOR
Pesticides by GC-MS	Phorate	µg/L	0.1
Pesticides by GC-MS	Phosmet	µg/L	0.1
Pesticides by GC-MS	Phosphamidon	µg/L	0.1
Pesticides by GC-MS	Picloram	µg/L	0.1
Pesticides by GC-MS	Piperonyl Butoxide	µg/L	0.1
Pesticides by GC-MS	Pirimicarb	µg/L	0.1
Pesticides by GC-MS	Pirimiphos-methyl	µg/L	0.1
Pharmaceuticals by LC-MS	Praziquantel	µg/L	0.01
Pharmaceuticals by LC-MS	Primidone	µg/L	0.01
Pesticides by GC-MS	Procymidone	µg/L	0.1
Pesticides by GC-MS	Profenofos	µg/L	0.1
Pesticides by GC-MS	Promecarb	µg/L	0.1
Herbicides and Other Compounds by LC-MS	Prometryn	µg/L	0.01
Pesticides by GC-MS	Propanil	µg/L	0.1
Pesticides by GC-MS	Propargite	µg/L	0.2
Pesticides by GC-MS	Propazine	µg/L	0.1
Pesticides by GC-MS	Propiconazole	µg/L	0.1
Pesticides by GC-MS	Propoxur	µg/L	0.01
Pesticides by GC-MS	Propoxur	µg/L	0.01
Pharmaceuticals by LC-MS	Propranolol	µg/L	0.01
Pesticides by GC-MS	Prothiophos	µg/L	0.1
Pesticides by GC-MS	Pyrazophos	µg/L	0.1
Pharmaceuticals by LC-MS	Ranitidine	µg/L	0.05
Pesticides by GC-MS	Rotenone	µg/L	0.1
Pharmaceuticals by LC-MS	Roxithromycin	µg/L	0.02
Pharmaceuticals by LC-MS	Salicylic acid	µg/L	0.1
Pharmaceuticals by LC-MS	Sertraline	µg/L	0.01
Herbicides and Other Compounds by LC-MS	Simazine	µg/L	0.01
Pharmaceuticals by LC-MS	Simvastatin	µg/L	0.1
Pharmaceuticals by LC-MS	Sulfasalazine	µg/L	0.01
Pharmaceuticals by LC-MS	Sulfsalazine	µg/L	0.01
Pharmaceuticals by LC-MS	Sulphadiazine	µg/L	0.01
Pharmaceuticals by LC-MS	Sulphamethoxazole	µg/L	0.01
Pharmaceuticals by LC-MS	Sulphathiazole	µg/L	0.01
Pesticides by GC-MS	Sulprofos	µg/L	0.1
Pesticides by GC-MS	Tebuconazole	µg/L	0.1
Herbicides and Other Compounds by LC-MS	Tebuthiuron	µg/L	0.01
Pharmaceuticals by LC-MS	Temazepam	µg/L	0.01
Pesticides by GC-MS	Terbufos	µg/L	0.1

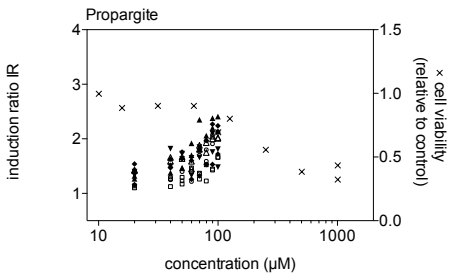
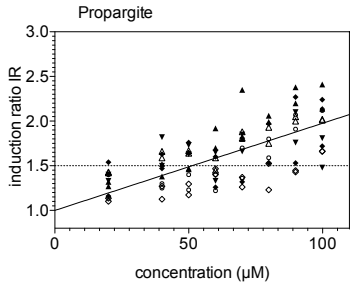
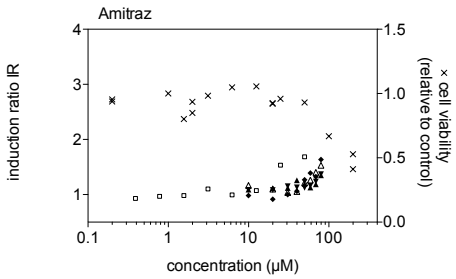
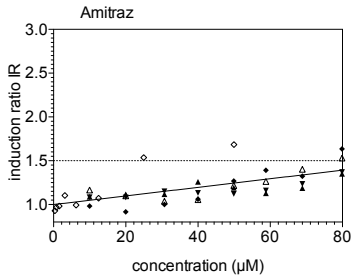
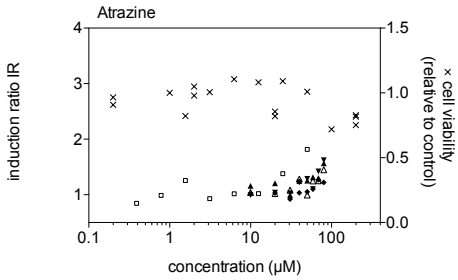
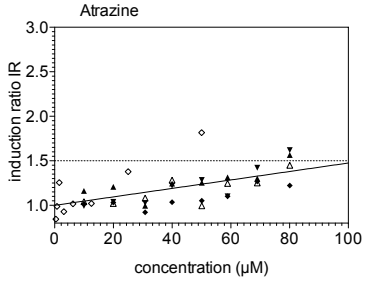
Analytical method	Analyte	Units	LOR
Pesticides by GC-MS	Terbufos	µg/L	0.1
Pesticides by GC-MS	Terbuthylazine	µg/L	0.1
Herbicides and Other Compounds by LC-MS	Terbutryn	µg/L	0.01
Endocrine Disrupting Compounds by GC-MS	Testosterone	ng/L	10
Pesticides by GC-MS	Tetrachlorvinphos	µg/L	0.1
Pharmaceuticals by LC-MS	Tetracycline	µg/L	0.1
Pesticides by GC-MS	Tetradifon	µg/L	0.1
Pesticides by GC-MS	Tetramethrin	µg/L	0.1
Pesticides by GC-MS	Thiabendazole	µg/L	0.2
Pesticides by GC-MS	Thiodicarb	µg/L	0.1
FRAGRANCES by GC-MS	Tonalid	µg/L	0.1
Pharmaceuticals by LC-MS	Tramadol	µg/L	0.01
Pesticides by GC-MS	*trans*-Chlordane	µg/L	0.1
Pesticides by GC-MS	*trans*-Nonachlor	µg/L	0.1
Pesticides by GC-MS	Transfluthrin	µg/L	0.1
GC-MS Screen	Tri-n-butyl phosphate	µg/L	0.1
Pesticides by GC-MS	Triadimefon	µg/L	0.3
Pesticides by GC-MS	Triadimenol	µg/L	0.1
Pesticides by GC-MS	Triallate	µg/L	0.1
Pesticides by GC-MS	Triclopyr	µg/L	0.1
GC-MS Screen	Triclosan	µg/L	0.01
GC-MS Screen	Triclosan methyl ether	µg/L	0.1
GC-MS Screen	Triethyl phosphate	µg/L	0.1
Pesticides by GC-MS	Trifluralin	µg/L	0.1
Pharmaceuticals by LC-MS	Trimethoprim	µg/L	0.01
GC-MS Screen	Tris(chloroethyl) phosphate	µg/L	0.1
GC-MS Screen	Tris(chloropropyl) phosphate isomers	µg/L	0.1
GC-MS Screen	Tris(dichloropropyl) phosphate	µg/L	0.1
Pharmaceuticals by LC-MS	Tylosin	µg/L	0.05
Pharmaceuticals by LC-MS	Venlafaxine	µg/L	0.01
Pesticides by GC-MS	Vinclozolin	µg/L	0.1
Pharmaceuticals by LC-MS	Warfarin	µg/L	0.01
Pesticides by GC-MS	α-Endosulfan	µg/L	0.05
Pesticides by GC-MS	β-Endosulfan	µg/L	0.05
Pesticides by GC-MS	α-HCH (α-BHC)	µg/L	0.1
Pesticides by GC-MS	β-HCH (β-BHC)	µg/L	0.1
Pesticides by GC-MS	δ-HCH (δ-BHC)	µg/L	0.1

Table SI-3 Concentration-effect curves of all investigated chemicals that showed induction of ARE (Table 1 in manuscript). The symbol x stands for cell viability and corresponds to the right y-axis. The different symbols (circles, triangles, squares, diamonds) stand for independent experiments and the regression line corresponds to a common fit of all data points.
All EC_{10} and $EC_{IR1.5}$ values are tabulated in Table 1 in the main manuscript.

compound	Cytotoxicity and induction on a logarithmic concentration scale	Linear concentration-effect curve for induction
Atenolol		
Atorvastatin		
Cephalexin		
Citalopram		

compound	Cytotoxicity and induction on a logarithmic concentration scale	Linear concentration-effect curve for induction
Fluoxetine		
Metoprolol		
Naproxen		
Paracetamol		
Propranolol		

compound	Cytotoxicity and induction on a logarithmic concentration scale	Linear concentration-effect curve for induction
Ranitidine		
Azinophos-methyl		
Fipronil		
Propiconazole		
Dichlorvos		

compound	Cytotoxicity and induction on a logarithmic concentration scale	Linear concentration-effect curve for induction
Propargite		
Amitraz		
Atrazine		

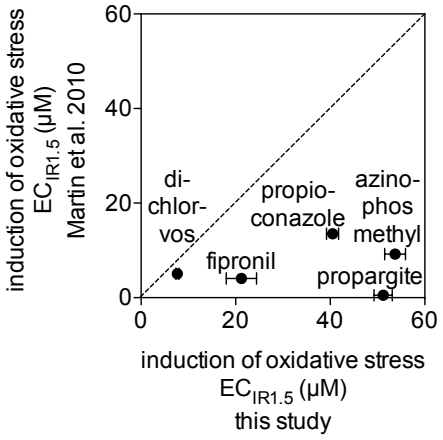
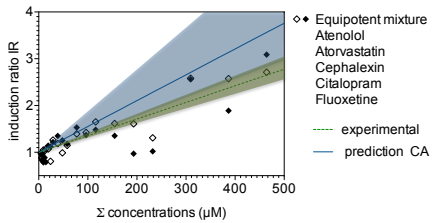
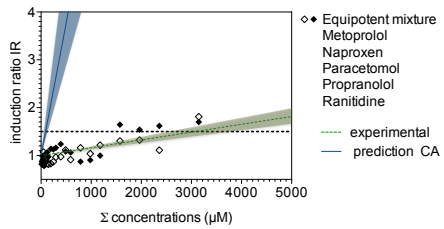
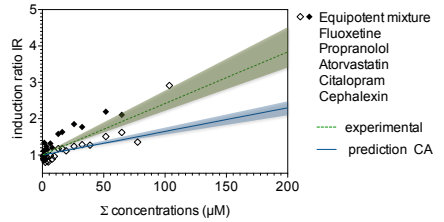
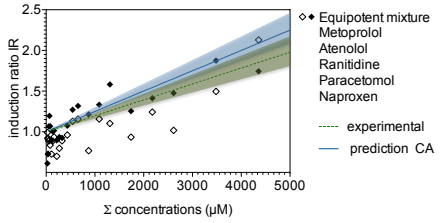
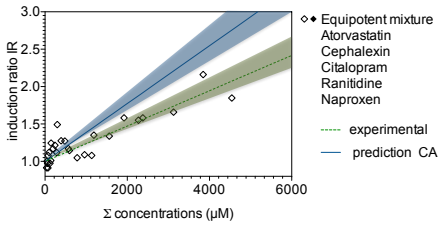
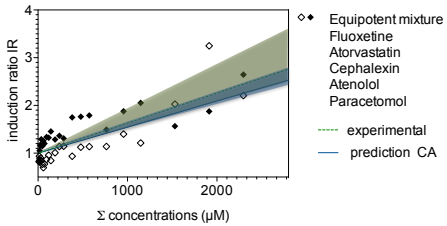
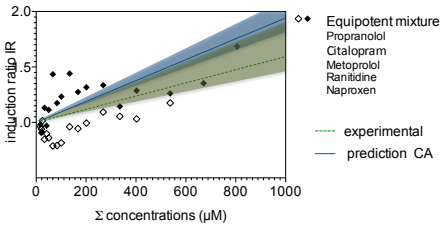
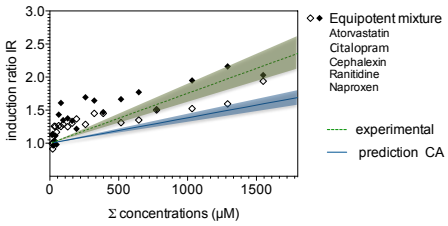
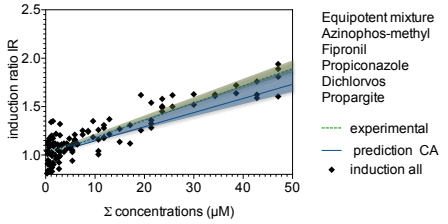
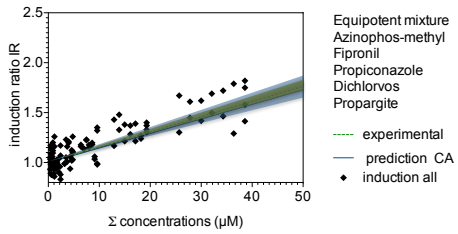
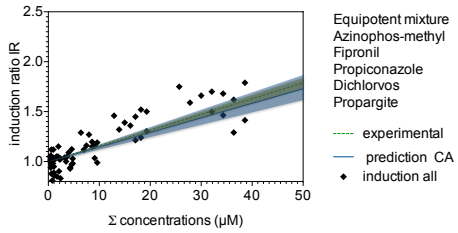
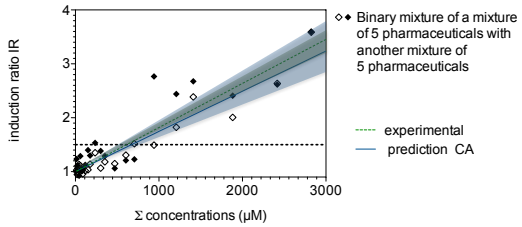
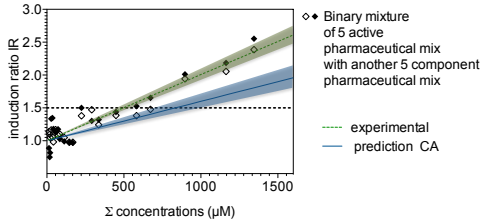


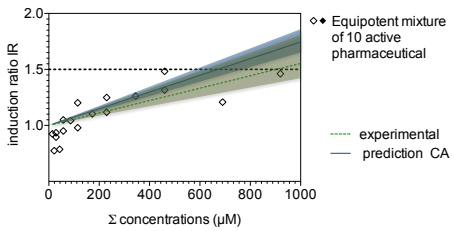
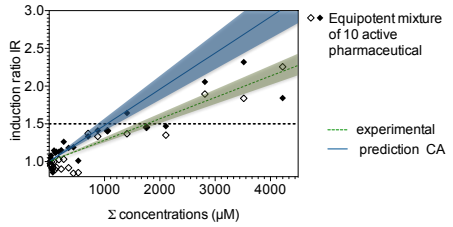
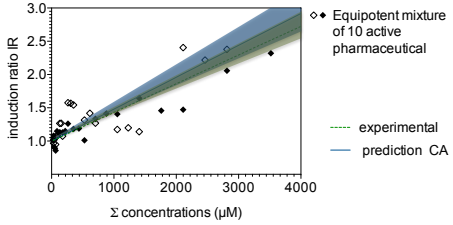
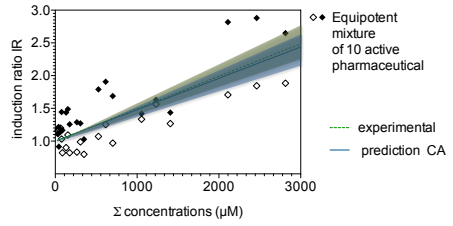
Figure SI-4 Comparison of the $EC_{IR1.5}$ between a previous study[1] using HepG2 cells and the present study with AREc32.

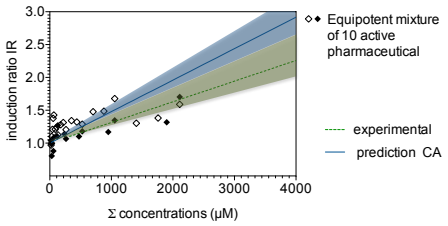
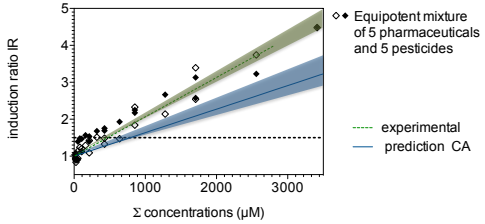
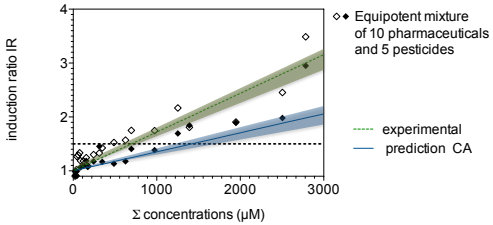
Table SI-4. Composition, concentration-effect curves and experimental and modeled $EC_{IR1.5}$ for all mixture experiments.
The blue shaded areas are the confidence intervals for the CA prediction, the green shaded are the confidence intervals of the experimental concentration effect curve. The different symbols stand for independent experiments and the regression line corresponds to a common fit of all data points. Some of the more complex mixtures were prepared several times and tested individually in duplicates.

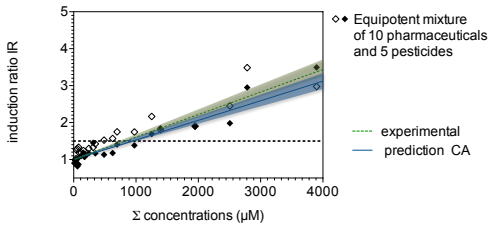
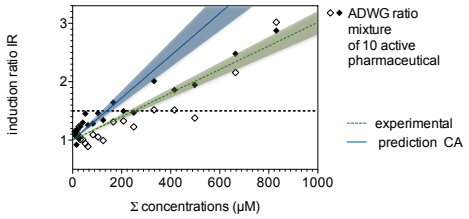
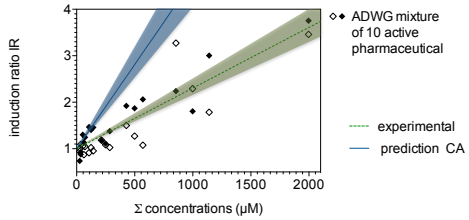
#	i	Composition p_i (%)		type	mixture ratio	$EC_{IR1.5,exp}$	$EC_{IR1.5,CA}$	IPQ
A	5	65.3% Atenolol, 1.5% Atorvastatin, 12% Cephalexin, 18.9% Citalopram, 1.9% Fluoxetine		pharma-ceuticals	equipotent	141±10	91±18	-0.55
B	5	2.5% Metoprolol, 40.2% Naproxen, 34% Paracetamol, 1.7% Propranolol, 21.6% Ranitidine		pharma-ceuticals	equipotent	2584±302	969±957	-1.67
C	5	9% Atorvastatin, 73.7% Cephalexin, 11.6% Citalopram, 1.3% Fluoxetine, 4.0% Propranolol		pharma-ceuticals	equipotent, most potent	35±3	77±5	1.18
D	5	11.6% Atenolol, 2.3% Metoprolol, 36.7% Naproxen, 29.9% Paracetamol, 20% Ranitidine		pharma-ceuticals	equipotent, least potent	2552±221	2000±148	-0.28

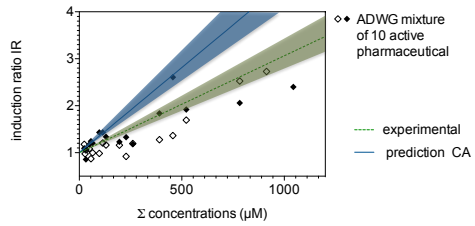
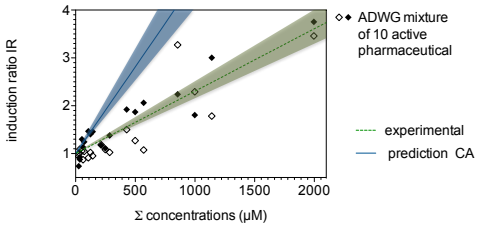
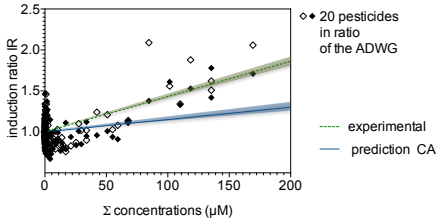
#	i	Composition p_i (%)		type	mixture ratio	$EC_{IR1.5,exp}$	$EC_{IR1.5,CA}$	IPQ
E	5	0.5% Atorvastatin, 3.8% Cephalexin, 1% Citalopram, 62.3% Naproxen, 32.8% Ranitidine		pharma-ceuticals	equipotent	2117±159	1293±98	-0.64
F	5	26.4% Atenolol, 0.6% Atorvastatin, 5.0% Cephalexin, 0.1% Fluoxetine (Prozac), 67.9% Paracetamol		pharma-ceuticals	equipotent	794±102	914±44	0.15
G	5	4.5% Citalopram, 3.7% Metoprolol, 59.6% Naproxen, 0.6% Propranolol, 31.7% Ranitidine		pharma-ceuticals	equipotent	842±142	531±40	-0.57
H	5	0.5% Atorvastatin, 3.7% Cephalexin, 0.6% Citalopram, 62.1% Naproxen, 33.2% Ranitidine		pharma-ceuticals	equipotent	663±54	1301±99	0.96
I	5	22.5% Azinophos-methyl, 3.2% Dichlorvos, 18.4% Fipronil, 30.6% Propargite, 25.3% Propiconazole		pesti-cides	equipotent	28±1	34±3	0.22

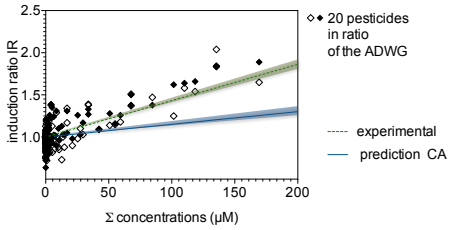
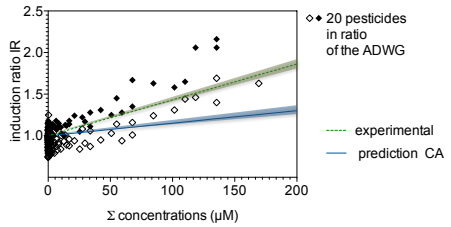
#	i	Composition p_i (%)		type	mixture ratio	$EC_{IR1.5,exp}$	$EC_{IR1.5,CA}$	IPQ
J	5	22.5% Azinophos-methyl, 3.2% Dichlorvos, 18.4% Fipronil, 30.6% Propargite, 25.3% Propiconazole		pesti-cides	equipotent	33±1	34±3	0.05
K	5	22.5% Azinophos-methyl, 3.2% Dichlorvos, 18.4% Fipronil, 30.6% Propargite, 25.3% Propiconazole		pesti-cides	equipotent	32±1	34±3	0.08
L	2x5	(32.6% Atenolol, 0.7% Atorvastatin, 6% Cephalexin, 9.4% Citalopram, 0.9% Fluoxetine) x (1.3% Metoprolol, 20.1% Naproxen, 17% Paracetamol, 0.8% Propranolol, 10.8% Ranitidine)		pharma-ceuticals	5 equipotent x 2 equimolar	611±29	670±74	0.10
M	2x5	(4.73% Atorvastatin, 36.84% Cephalexin, 5.78% Citalopram, 0.64% Fluoxetine, 2.01% Propranolol) x (5.80% Atenolol, 1.15% Metoprolol, 18.36% Naproxen, 14.93% Paracetamol, 9.76% Ranitidine)		pharma-ceuticals	5 equipotent x 2 equimolar	497±22	794±102	0.60

#	i	Composition p_i (%)		type	mixture ratio	$EC_{IR1.5,exp}$	$EC_{IR1.5,CA}$	IPQ
N	2x5	(13.20% Atenolol, 0.30% Atorvastatin, 2.49% Cephalexin, 0.04% Fluoxetine, 33.97% Paracetamol) x (2.23% Citalopram, 1.86% Metoprolol, 29.79% Naproxen, 0.28% Propranolol, 15.83% Ranitidine)		pharma-ceuticals	5 equipotent x 2 equimolar	903±147	834±73	-0.08
O	10	6.95% Atenolol, 0.28% Atorvastatin, 2.79% Cephalexin, 0.33% Citalopram, 0.05% Fluoxetine, 3.56% Metoprolol, 34.93% Naproxen, 31.26% Paracetamol, 0.27% Propranolol, 19.58% Ranitidine		pharma-ceuticals	equipotent	1764±92	1043±83	-0.69
P	10	Same as above		pharma-ceuticals	equipotent	1159±67	1043±83	-0.11
Q	10	Same as above		pharma-ceuticals	equipotent	1010±91	1043±83	-0.52

#	i	Composition p_i (%)		type	mixture ratio	$EC_{IR1.5,exp}$	$EC_{IR1.5,CA}$	IPQ
R	10	Same as above		pharma-ceuticals	equipotent	1189±191	1043±83	0.03
S	10	(0.24% Atorvastatin,1.83% Cephalexin, 0.29% Citalopram, 31.06% Naproxen, 16.58% Ranitidine) x (14.27% amitraz, 14.17% azinophos-methyl, 2.20% dichlorvos, 7.80% fipronil, 11.55% propioconazole)		5 pharm. & 5 pest.	5 equipotent x 2 equimolar	469±14	785±72	0.67
T	15	(3.47% Atenolol, 0.14% Atorvastatin, 1.40% Cephalexin, 0.17% Citalopram, 0.03% Fluoxetine, 1.78% Metoprolol, 17.47% Naproxen, 15.63% Paracetamol, 0.13% Propranolol, 9.79% Ranitidine) x (14.27% amitraz, 14.17% azinophos-methyl, 2.20% dichlorvos, 7.80% fipronil, 11.55% propioconazole)		10 pharm. & 5 pest.	5/10 equipotent x 2 equipotent	695±27	1417±111	1.04

#	i	Composition p_i (%)		type	mixture ratio	$EC_{IR1.5,exp}$	$EC_{IR1.5,CA}$	IPQ
U	15	(4.17% Atenolol, 0.17% Atorvastatin, 1.68% Cephalexin, 0.20% Citalopram, 0.03% Fluoxetine, 2.13% Metoprolol, 20.96% Naproxen, 18.76% Paracetamol, 0.16% Propranolol, 11.75% Ranitidine) x (11.34% amitraz, 6.24% azinophos-methyl, 9.24% dichlorvos, 1.76% fipronil, 11.42% propioconazole)		10 pharm. & 5 pest.	5/10 equipotent x 2 equipotent	470±14	942±53	1.01
V	10	4.90% Atenolol, 0.83% Atorvastatin, 6.21% Cephalexin, 0.63% Citalopram, 1.76% Fluoxetine, 4.01% Metoprolol, 39.10% Naproxen, 31.10% Paracetamol, 6.44% Propranolol, 5.01% Ranitidine		pharma-ceuticals	ADWG	248±13	138±11	-0.79
W	10	Same as above		pharma-ceuticals	ADWG	383±25	138±11	-1.64

#	i	Composition p_i (%)		type	mixture ratio	$EC_{IR1.5,exp}$	$EC_{IR1.5,CA}$	IPQ
X	10	Same as above		pharma-ceuticals	ADWG	242±19	138±12	-0.76
Y	10	Same as above		pharma-ceuticals	ADWG	383±25	149±12	-1.58
Z	20	0.40% Aldicarb, 0.56% Amitraz, 1.70% Atrazine, 1.74% Azinophos-methyl, 8.31% Dicamba, 7.82% Dichlorprop, 0.42% Dichlorvos, 0.01% Dieldrin, 0.56% Dimethoate, 0.19% Ethion, 0.46% Fenitrothion, 0.03% Fipronil, 3.96% Fluomethuron, 25.49% Hexazinone, 0.18% MCPA, 2.27% Methomyl, 32.58% Piperonyl butoxide, 5.42% Pirimiphos methyl, 0.37% Propargite, 7.52% Propiconazole		pesti-cides	ADWG	149±6	331±18	1.22

#	i	Composition p_i (%)		type	mixture ratio	$EC_{IR1.5,exp}$	$EC_{IR1.5,CA}$	IPQ
Z1	20	same as above		pesti-cides	ADWG	111±3	331±18	1.98
Z2	20	same as above		pesti-cides	ADWG	98±3	331±18	2.37

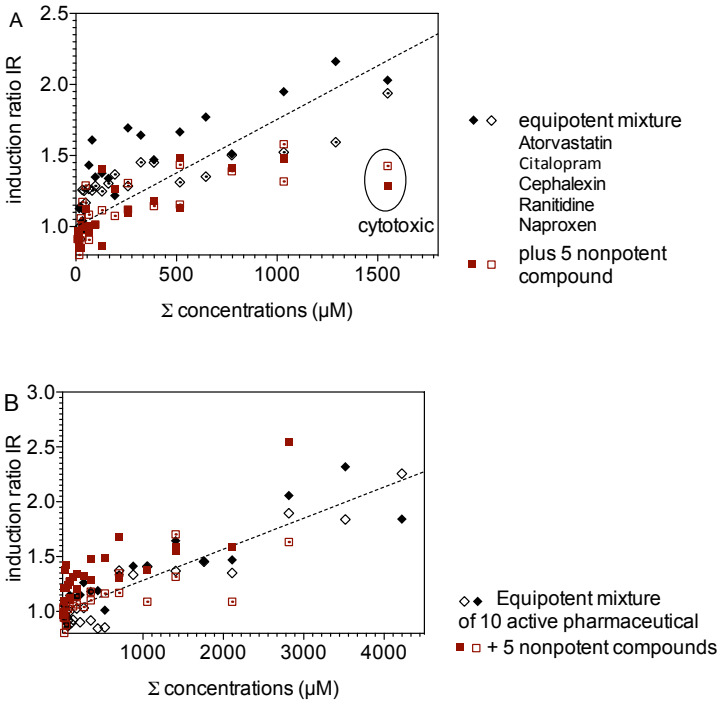


Figure SI-5. Mixture of potent and non-potent pharmaceuticals, A. 5 potent plus 5 nonpotent, B. 10 potent plus 5 nonpotent (the corresponding filled and empty symbols correspond to 2 independent replicates done on two plates at the same day).

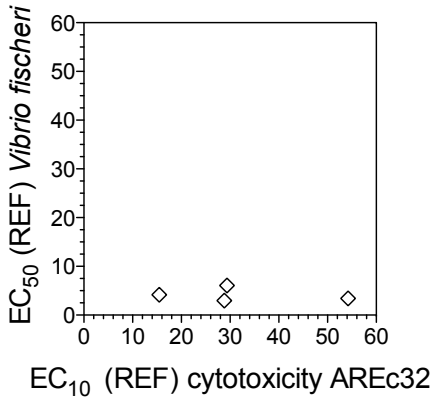


Figure SI-6. Relationship between cytotoxicity EC of water samples, EC_{50} in the marine luminescent bacterium *Vibrio fischeri* after 30 min incubation versus the EC_{10} in AREc32 after 24h of incubation. The EC_{10} in AREc32 are extrapolated and therefore highly uncertain and thus we do not report error bars. The error bars in EC_{50} for *V. fischeri* are smaller than the symbols.

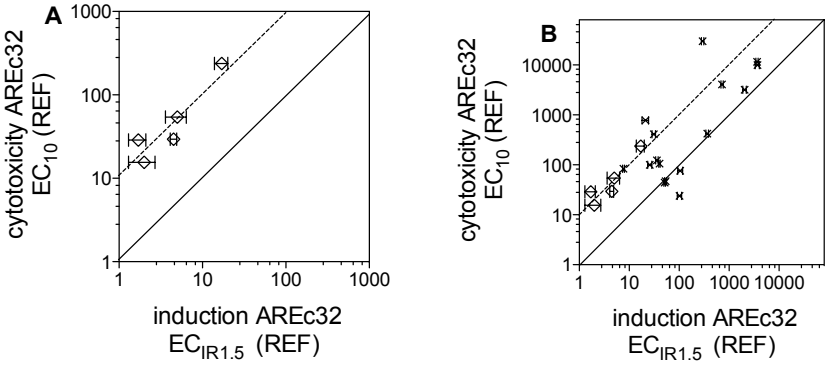
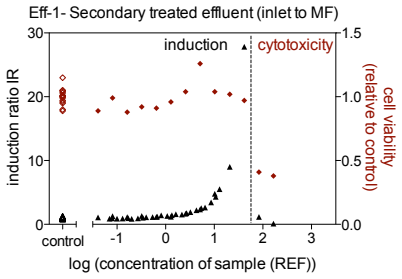
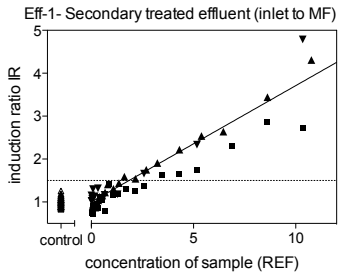
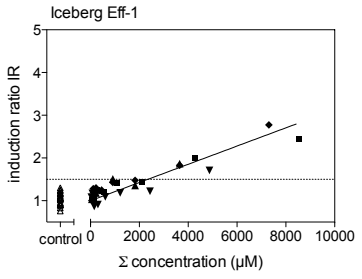
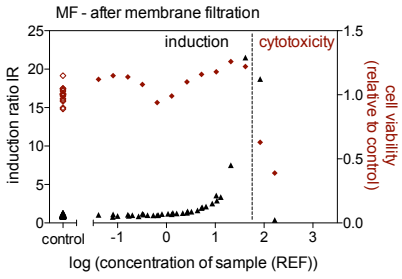
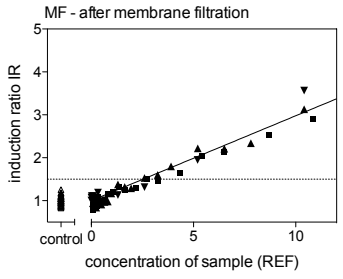
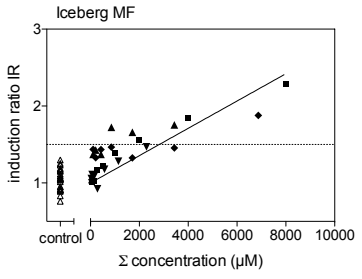
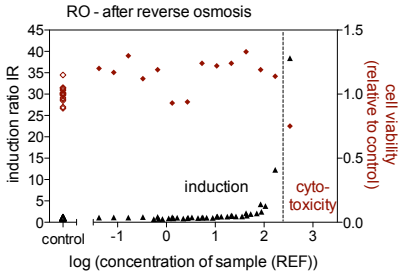
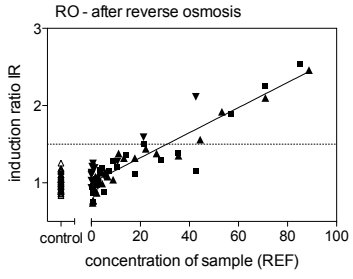
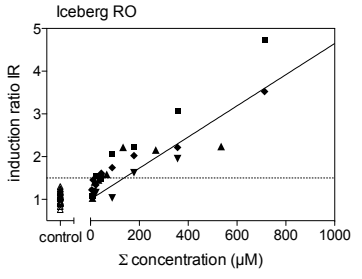
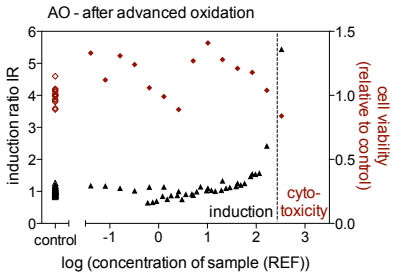
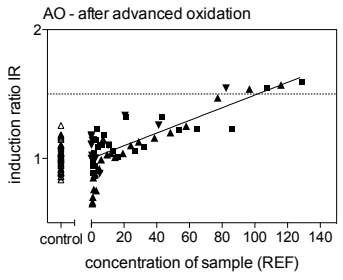
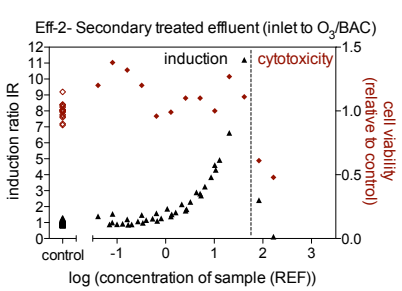
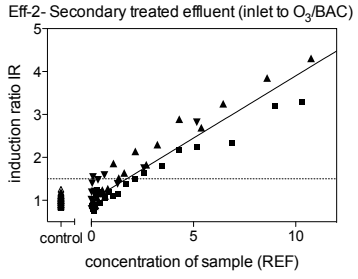
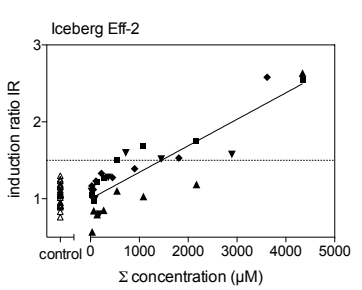


Figure SI-7. Relationship between cytotoxicity EC_{10} and induction $EC_{IR1.5}$ of water samples (empty diamonds, A and B) and single chemicals (symbol x, B) in AREc32. The EC_{10} in AREc32 are extrapolated and therefore highly uncertain and thus we do not report error bars. The error bars of the $EC_{IR1.5}$ are standard errors from the error propagation of the concentration-IR regression. The drawn line is the one-to-one relationship, the broken line is the 1:10 relationship.

Table SI-5 Concentration-effect curves of the water samples and the iceberg mixtures. Different symbols stand for independent experiments and the regression line corresponds to a common fit of all data points.

Samples Cytotoxicity and induction on a logarithmic concentration scale	Samples Linear concentration-effect curve for induction	Iceberg mixtures
		
		
		
		No chemicals > LOR
		

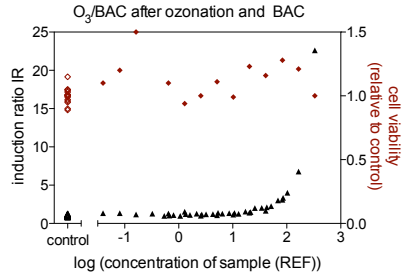
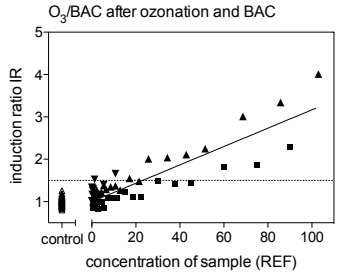
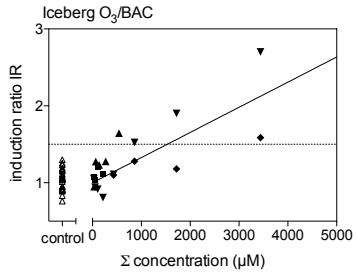
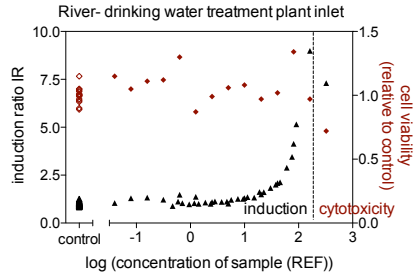
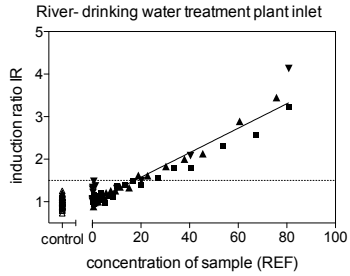
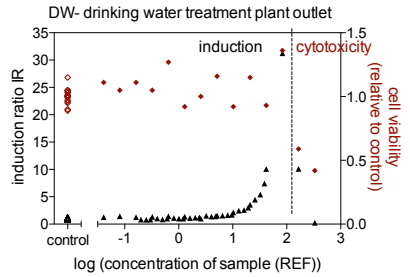
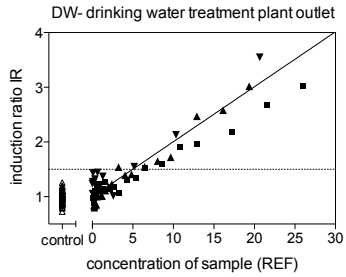
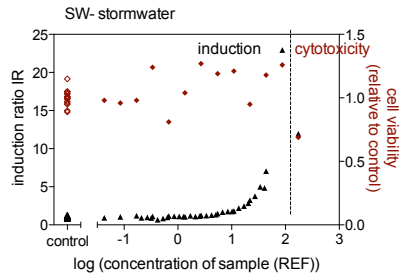
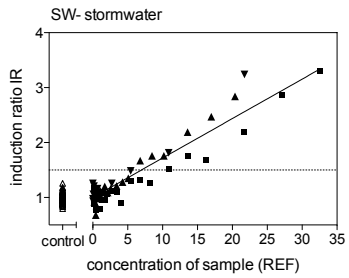
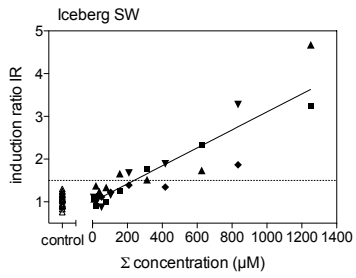
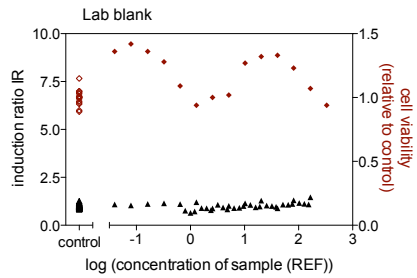
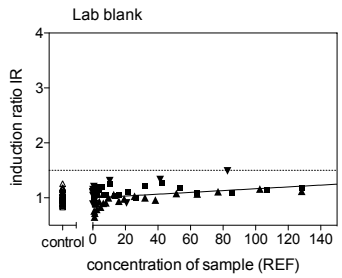
Samples Cytotoxicity and induction on a logarithmic concentration scale	Samples Linear concentration-effect curve for induction	Iceberg mixtures
		
		No chemicals > LOR
		No chemicals > LOR
		
		No chemicals > LOR

Table SI-6 Detected chemicals in the six environmental samples where chemicals were present at concentrations about the limit of reporting (LOR).[5]

Analyte	Units	LOR	Eff-1 (secondary treated effluent, influent to MF)	MF (after micro-filtration)	RO (after reverse osmosis)	Eff-2 (secondary effluent (influent to O_3/BAC)	O_3/BAC (after ozonation and biologically activated carbon filtration)	SW (storm-water)
number of chemicals detected			40	39	6	48	6	5
17-β-Estradiol	µg/L	0.005				0.006		
Nonylphenol	µg/L	0.1				0.13		
4-*t*-Octylphenol	µg/L	0.1		0.017		0.11		
Tonalid	µg/L	0.1	0.1	0.1		0.1		
Atenolol	µg/L	0.01	0.10	0.10		0.94		
Atorvastatin	µg/L	0.01				0.04		
Atrazine	µg/L	0.01	0.35	0.39				
Bisphenol A	µg/L	0.01		0.018		0.13		0.20
Caffeine	µg/L	0.02	0.05	0.04		0.21		
Carbamazepine	µg/L	0.01	1.6	1.9	0.02	2.5		
Cephalexin	µg/L	0.01				0.12		
Chlorpyrifos	µg/L	0.1				5.6		
Citalopram	µg/L	0.01	0.13	0.10		0.27	0.02	
Codeine	µg/L	0.1				0.24		
Cyclophos-phamide	µg/L	0.01	0.01			0.04		
Desmethyl Citalopram	µg/L	0.01	0.14	0.10		0.24	0.01	
Desmethyl Diazepam	µg/L	0.01	0.03	0.03		0.05		
Diazepam	µg/L	0.01	0.01			0.01		
Diazinon	µg/L	0.1				0.16		
Diclofenac	µg/L	0.01	0.11	0.12		0.26		
Diuron	µg/L	0.01	0.16	0.14	0.03	0.07		0.04
Doxylamine	µg/L	0.01	0.24	0.18		0.44		
Erythromycin	µg/L	0.01	0.02	0.02		0.05		
Fipronil	µg/L	0.1	0.1	0.1				
Fluoxetine	µg/L	0.01	0.03	0.03		0.03		
Frusemide	µg/L	0.01	0.13	0.15		1.3		
Galaxolide	µg/L	0.1	1.0	1.1		1.6		
Gemfibrozol	µg/L	0.01	0.08	0.07		0.15		
Hexazinone	µg/L	0.01	0.02	0.02				
Hydrochlor-thiazide	µg/L	0.01	0.76	0.65	0.01	1.5		
Indomethacin	µg/L	0.01				0.08		
Lincomycin	µg/L	0.01						
Metolachlor	µg/L	0.01	0.82	0.73	0.01	0.01	0.01	
Metoprolol	µg/L	0.01	0.12	0.14		0.97		
Naproxen	µg/L	0.1				0.32		
Norfloxacin	µg/L	0.05	0.06			0.10		
Oxazepam	µg/L	0.01	0.60	0.57		1.1		
Oxycodone	µg/L	0.01	0.03	0.03		0.16		
Paracetamol	µg/L	0.02						0.02
Praziquantel	µg/L	0.01	0.01	0.01				
Propoxur	µg/L	0.01	0.03	0.03		0.05		
Propranolol	µg/L	0.01	0.01	0.02		0.14		
Ranitidine	µg/L	0.05				0.70		
Roxithromycin	µg/L	0.02	0.05	0.04		0.08		

Analyte	Units	LOR	Eff-1 (secondary treated effluent, influent to MF)	MF (after micro-filtration)	RO (after reverse osmosis)	Eff-2 (secondary effluent (influent to O_3/BAC)	O_3/BAC (after ozonation and biologically activated carbon filtration)	SW (storm-water)
Simazine	µg/L	0.01	0.18	0.23		0.17		0.02
Sulphadiazine	µg/L	0.01	0.03	0.03		0.13		
Sufamethoxazole	µg/L	0.01	0.15	0.07		0.21		
Temazepam	µg/L	0.01	0.47	0.50		0.65		
Triclosan	µg/L	0.01	0.02	0.02		0.05		
Trimethoprim	µg/L	0.01	0.07	0.05		0.23		
Tris(chloroethyl) phosphate	µg/L	0.1	0.4	0.4		0.4	0.3	
Venlafaxine	µg/L	0.01	1.6	1.9	0.01	2.4	0.10	
DEET	µg/L	0.01	0.11	0.10		0.18	0.03	0.11
1H-Benzo-triazole, 5-methyl	µg/L	0.2	0.53	0.54	0.32	1.3		

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REALISTIC ENVIRONMENTAL MIXTURES OF MICROPOLLUTANTS IN SURFACE, DRINKING, AND RECYCLED WATER: HERBICIDES DOMINATE THE MIXTURE TOXICITY TOWARD ALGAE

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(Submitted 10 October 2013; Returned for Revision 6 December 2013; Accepted 11 March 2014)

Abstract: Mixture toxicity studies with herbicides have focused on a few priority components that are most likely to cause environmental impacts, and experimental mixtures were often designed as equipotent mixtures; however, real-world mixtures are made up of chemicals with different modes of toxic action at arbitrary concentration ratios. The toxicological significance of environmentally realistic mixtures has only been scarcely studied. Few studies have simultaneously compared the mixture effect of water samples with designed reference mixtures comprised of the ratios of analytically detected concentrations in toxicity tests. In the present study, the authors address the effect of herbicides and other chemicals on inhibition of photosynthesis and algal growth rate. The authors tested water samples including secondary treated wastewater effluent, recycled water, drinking water, and storm water in the combined algae assay. The detected chemicals were mixed in the concentration ratios detected, and the biological effects of the water samples were compared with the designed mixtures of individual detected chemicals to quantify the fraction of effect caused by unknown chemicals. The results showed that herbicides dominated the algal toxicity in these environmentally realistic mixtures, and the contribution by the non-herbicides was negligible. A 2-stage model, which used concentration addition within the groups of herbicides and non-herbicides followed by the model of independent action to predict the mixture effect of the two groups, could predict the experimental mixture toxicity effectively, but the concentration addition model for herbicides was robust and sufficient for complex mixtures. Therefore, the authors used the bioanalytical equivalency concept to derive effect-based trigger values for algal toxicity for monitoring water quality in recycled and surface water. All water samples tested would be compliant with the proposed trigger values associated with the appropriate guidelines. *Environ Toxicol Chem* 2014;33:1427–1436. © 2014 SETAC

Keywords: Concentration addition Effect-based monitoring Mixture toxicity Photosynthesis inhibition Trigger values

INTRODUCTION

Pesticides and mixture toxicity

Regulation of surface [1], drinking [2,3], and recycled water [4] around the world focuses predominantly on individual chemicals; however, there are concerns about the potential adverse effects from the interactions of chemicals present simultaneously in mixtures at low concentrations [5,6]. The combined effects from pesticides in aquatic systems have been well studied during the past 20 yr [7–9]. Studies on the toxicity of multi-component mixtures showed that the observed effect of a mixture usually displayed higher toxicity than the single components [10,11]. Two concepts were established that systematically link the toxicity of the individual components of a mixture to its mixture toxicity, termed concentration addition (CA) and independent action (IA) [6]. Concentration addition applies to chemicals with the same mode of action that, in mixtures, behave as if they were dilutions of each other that differ only in their relative potencies [5,12]. Concentration addition forms the conceptual basis of the toxicity equivalency approach used for the hazard and risk assessment of, for example, dioxin or polychlorinated biphenyl mixtures [13]. The general notion for pesticide mixtures with a common target mechanism, such as groups of herbicides or groups of insecticides, is that CA provides a more reliable tool for predicting and assessing the joint toxicity, because the pesticides act on the same target and exhibit the same mode of action [6]. Belden et al. [8] reviewed the predictive power of CA on 207 pesticide mixture experiments that were composed regardless of the mode of action; they concluded that only in less than 5% of the published studies the experimental toxicity exceeded the CA predictions by a factor of 2 or more. For those pesticide mixtures that exhibited a similar mode of action, the CA predictions agreed well with the observed toxicity. For mixtures of dissimilarly acting components, the combined effect was calculated from the effects caused by individual mixture components by the statistical concept of independent random events, or IA [14,15]. Belden et al. [8] found that the IA model was slightly more accurate than the CA model for pesticide mixtures with different modes of action; however, the differences between the CA and IA models were small. Because all of these studies were based mainly on pesticides with known modes of action in controlled experiments, the assessment of the effect of environmentally realistic mixtures remains uncertain.

Lack of mixture toxicity studies of environmentally realistic mixtures

Studies evaluating field exposures traditionally indicated that most toxicity is likely the result of a few components within a mixture that are present at high concentrations relative to their effective concentration [16,17]. However, there are concerns that chemicals present at concentrations below their expected biological effect level or below their analytically quantification limit would increase the overall toxicity of a realistic environmental mixture. Because of the high number of chemicals potentially present in an environmental sample, many studies chose to analyze

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only the priority pesticides that are most likely to cause environmental impacts [18]. In addition, many studies focused on designed "reference mixtures" in which all the components were known to act either by an identical or by completely different molecular mechanisms of action [12,14,15].

Real-world mixtures, however, are made up of chemicals with both similar and dissimilar mechanisms of action [19]. The toxicological significance of realistic environmental mixture has only been scarcely studied. Junghans et al. [7] studied the interactions of 25 detected pesticides (22 herbicides and 3 insecticides) on algal reproduction of *Scenedesmus vacuolatus*. Olmstead and LeBlanc [19] chose 9 frequently detected chemicals from a survey of 82 organic contaminants in 139 freshwater streams and mixed them in the observed median effect concentrations (EC50s) and tested the mixtures on *Daphnia magna*. These mixture toxicity studies were based on occurrence modeling or chemical survey; to our knowledge, however, there are no studies on herbicide toxicity which simultaneously compare the mixture effect of realistic environmental samples with designed reference mixtures composed of the ratios of the detected concentrations of all components from the corresponding environmental samples.

Selection of appropriate models for environmentally realistic mixtures: CA versus two-step prediction model

Junghans [20] proposed to use a two-step prediction (TSP) model for mixtures that consist of components exhibiting both similar and dissimilar modes of toxic action. The TSP model sorts chemicals that act similarly into common clusters and applies CA for the chemicals within individual clusters as a first step. In the second step, the predictions of individual clusters are combined using an IA model. This approach was used to evaluate the integral effect of a mixture of 18 triazine herbicides, 9 chloroacetanilide herbicides, 8 sulfonylurea herbicides, and 6 quinolones; it also has been applied to a mixture of 5 pharmaceuticals and 1 phenylurea herbicide in the algae chlorophyll fluorescence test [21] and to a 10-chemical mixture consisting of acetylcholinesterase inhibitors, narcosis inhibitors, and seedling root inhibitors in the *D. magnia* mortality test [22]. These studies showed that the TSP model could better predict toxicity than CA or IA alone and suggested that TSP is a more reliable model for mixtures with various modes of action. The concentration ratios in these studies were mainly equipotent, however, and it remains unclear if the TSP model holds true for environmentally realistic mixtures for algal toxicity assessment or if it is sufficient to invoke CA of the herbicides present.

We tested 10 water samples collected in South East Queensland, Australia, which included secondary treated wastewater effluent, recycled water, river water, drinking water, and storm water. Previously, 293 chemicals were quantified analytically [23], and the detected chemicals were mixed in the concentration ratios detected and also tested in the combined algae test [24]. These designed mixtures were termed "iceberg mixtures" because they constitute the known "tip of the iceberg" of known chemicals; many chemicals in an environmental sample are likely to be unknown and, following the analogy, constitute the submerged, invisible part of the iceberg. The biological effects of the water samples could then be compared with the iceberg mixtures to quantify the fraction of effect caused by known and unknown chemicals.

In addition, we performed a mixture toxicity analysis of the interactions of the detected chemicals in the mixture. We first separated the analytically detected chemicals into 2 groups: herbicides that inhibit photosynthesis by binding to the photosystem II (PSII) and all other chemicals (termed "non-herbicides" hereafter). Then, the CA prediction model was applied to the experimental values of the detected PSII herbicides, and a CA prediction based on effect concentrations estimated with a quantitative structure–activity relationship (QSAR) model was used for non-herbicides, assuming that all non-herbicides act as baseline toxicants. Then we used an IA model to predict the combined effect of PSII herbicides and non-herbicides to evaluate whether the TSP model could explain toxicity of the real environmental mixtures.

Development of effect-based trigger values for herbicidal toxicity

The ultimate goal of the present study was to develop effect-based trigger values for herbicides in water. The lack of bioassay-based trigger values for regulatory authorities has hindered the application of bioanalytical tools for monitoring water quality.

Guideline values for individual chemicals represent the concentrations of chemicals that do not result in any significant health risk during a person's lifetime exposure to drinking water [2,3] or in any ecological risk in surface water [1]. Although humans are not specifically affected by herbicides, guideline values exist for 12 PSII herbicides in the Australian Drinking Water Guidelines [3] and the Australian Guidelines for Water Recycling [4]. Herbicides are particularly toxic to algae, and therefore algae constitute an ideal model system to quantify the effects caused by herbicides even though algal toxicity is not of direct human health relevance. Further, the Australian and New Zealand Guidelines for Fresh and Marine Water Quality [1] list guideline values for 6 PSII herbicides. In aquatic ecosystems, algae are among the most sensitive species for herbicides and thus constitute an ideal test organism for the derivation of effect-based trigger values. Exceedence of an effect-based trigger value would indicate a more detailed analysis, such as chemical analysis or more definitive toxicity assessment, is required.

We have previously proposed algorithms to establish effect-based trigger values as a first-tier screening tool in augmentation to existing chemical analysis [23,25]. Effect-based trigger effect concentrations (EBT-EC) were derived for non-specific toxicity and adaptive stress responses in which a large fraction of chemicals present in the environment contributed to the effect and only a very small fraction of effects could be explained by known chemicals [23,25]. Effect-based trigger bioanalytical equivalent concentrations (EBT-BEQ) are typically used for receptor-mediated effects for which a reference chemical with a clear maximum and minimum potency can be defined [26]. In the present study, we expand the existing approaches to the combined algae test and propose EBT-BEQs for mixtures of herbicides and non-herbicides.

MATERIALS AND METHODS

Chemicals

Diuron ([3-3,4-dichlorophenyl]-3,3-dimethylurea; CAS number 330-54-1; 99.5% purity) was used as positive control for the combined algae test [24]. The 64 chemicals used in the mixture experiments are listed in the Supplemental Data, Table S1. These chemicals were quantified with gas chromatography–mass spectrometry and liquid chromatography–mass spectrometry in a previous study [23].

Water

Nine grab water samples and 1 blank were collected in December 2011 and January 2012 from various sites in South East Queensland, Australia. The details of the sample selection

are described by Tang et al. [23]. Briefly, 4 samples were collected from an Advanced Water Treatment Plant [27]. These included the secondary treated wastewater effluent that serves as the influent to the plant (sample Eff-1), the sample after microfiltration (sample MF), and a sample taken after reverse osmosis (sample RO). The product water was disinfected with UV and hydrogen peroxide (advanced oxidation; sample AO). Two samples were collected from an Enhanced Water Treatment Plant [28], the secondary treated wastewater effluent (sample Eff-2), and after ozonation followed by biological activated carbon filtration (sample O_3/BAC). The product water of this plant is mainly for industrial reuse. Two samples were collected from a drinking water treatment plant [29], river water (sample RW) and drinking water (sample DW), and represented the influent and effluent of the drinking water plant. Storm water (sample SW) was sampled from Fitzgibbon, Brisbane, Australia, after a rainfall event [30]. A laboratory blank (sample LB) consisting of ultrapure MilliQ water was also collected.

The water samples were extracted using 2 types of cartridges set up in sequence: Oasis® HLB cartridge (500 mg; Waters) followed by Supelclean® coconut charcoal cartridge (2 g; Sigma-Aldrich). The details of the extraction and elution procedures were described by Tang et al. [23]. The solid phase extraction (SPE) sample extracts were composed of a mixture of known and unknown chemicals at unknown concentrations. Dose-metric is the relative enrichment factor (REF), which is a measure of how much a sample would have to be enriched ($REF > 1$) or diluted ($REF < 1$) to achieve a given effect (Equation 1).

$$REF = \frac{\text{water volume equivalent in bioassay}}{\text{total volume of medium in bioassay}} \quad (1)$$

Combined algae test with Pseudokirchneriella subcapitata

The green algae *P. subcapitata* (CSIRO culture collection) was maintained in batch cultures at 23 °C and 170 ± 20 μE m^{-2} s^{-1} in Talaquil medium. The combined algae test was conducted according to Escher et al. [24]. The photosynthesis yield (Y) was determined using a Maxi-Imaging-PAM (IPAM; Walz GmbH), while the growth rate (μ) was determined by measuring absorbance at 600 nm using a FluoStar Omega plate reader (BMG Labtech). Readings were taken at 0 h, 2 h, and 24 h. Previous experiments have confirmed exponential growth during this exposure period, and more frequent measurement of the microtitre plates would disturb the algal growth. The inhibition of the photosynthetic yield after 2 h and the inhibition of growth rate over 24 h were calculated using Equations 2 and 3, respectively.

$$\text{inhibition}_{\text{IPAM}} = 1 - \frac{Y_{\text{sample}}}{Y_{\text{control}}} \quad (2)$$

$$\text{inhibition}_{\text{growth rate}} = 1 - \frac{\mu_{\text{sample}}}{\mu_{\text{control}}} \quad (3)$$

Concentration–response assessment of reference compounds and water extracts were performed in 96-well plates with a dilution series of 8 concentrations. Each plate consisted of a positive control and 3 negative controls with the same dilution series. Each experiment consisted of at least 2 replicates and was repeated at least 3 times on separate days.

Relative effect potencies (REP_i) for the individual PSII herbicide describe the potency of the different PSII herbicides relative to the reference compound diuron and were calculated with Equation 4 [31]. Diuron was used as the reference compound because the literature has shown that it is the most potent PSII herbicide in the combined algae test [31,32].

$$REP_i = \frac{EC50(\text{diuron})}{EC50(i)} \quad (4)$$

QSAR

The baseline toxicity QSAR has the form given in Equation 5. Typically, the hydrophobicity descriptor chosen would be the octanol–water partition coefficient (K_{ow}), but it has been demonstrated that the liposome–water partition coefficient (K_{lipw}) is a better descriptor because it allows for the development of a common QSAR for both polar and nonpolar baseline toxicants [33]. In the study we replaced the K_{lipw} by the liposome-water distribution ratio at pH 7 (D_{lipw}[pH7]) because some of the chemicals are acids or bases that are charged at pH 7.

$$\log(1/EC50\,[M]) = \text{slope} \times \log D_{lipw}(pH7) + \text{intercept} \quad (5)$$

The K_{lipw} values of the baseline toxicants that were used to establish the QSAR were measured values [33], and the D_{lipw}(pH7) values of all chemicals evaluated in the present study were calculated and reported in Tang et al. [23].

A measure of the specificity of the effect of a compound *i* is the toxic ratio (TR_i), which is the quotient of the EC50 predicted with the baseline toxicity QSAR ($EC50_{\text{baseline-QSAR}}$ (i)), and the experimental EC50 ($EC50_{\text{experimental}}$ (i); Equation 6). If the TR_i exceeds 10, then the chemical *i* is considered to exhibit a specific mode of toxic action [34].

$$TR_i = \frac{EC50_{\text{baseline QSAR}}\,(i)}{EC50_{\text{experimental}}\,(i)} \quad (6)$$

Mixture toxicity predictions

Predictions of mixture effects according to the CA model can be calculated according to Equation 7 for a mixture of n components i, present in fractions p_i, yielding the EC50 of the CA mixture, $EC50_{CA}$.

$$EC50_{CA} = \frac{1}{\sum_{i=1}^{n} \frac{p_i}{EC50_i}} \quad (7)$$

The $EC50_{CA}$ was computed for the group of herbicides and non-herbicides independently. A concentration–effect curve was constructed for each group (herbicides and non-herbicides) and then combined with the model of independent action (Equation 8).

$$\text{Effect}_{IA} = 1 - [(1 - \text{effect}_{\text{herbicides}}) \times (1 - \text{effect}_{\text{non-herbicides}})] \quad (8)$$

Bioanalytical equivalent concentrations

Mixtures of compounds that act concentration-additive can also be described by the bioanalytical equivalency concept; that is, the equivalent concentration of a reference compound, in case of the combined algae test diuron, can be calculated directly from the bioassay response of a sample (Equation 9) or by summing the REP_i multiplied by the concentration C_i of each known mixture component i (Equation 10). The resulting diuron equivalent concentrations (DEQ_{bio} and DEQ_{chem}) can then be

compared to assess which fraction of effect cannot be explained by detected chemicals.

$$\mathrm{DEQ_{bio}} = \frac{\mathrm{EC50(diuron)}}{\mathrm{EC50(sample)}} \quad (9)$$

$$\mathrm{DEQ_{chem}} = \sum_{i=1}^{n} \mathrm{REP_i} \times \mathrm{C_i} \quad (10)$$

The DEQ values can be derived for both endpoints, IPAM and growth rate. Because outcomes are similar, we derived DEQs only for the endpoint of photosynthesis inhibition (IPAM).

RESULTS AND DISCUSSIONS

QSAR for non-herbicides as baseline toxicants and PSII herbicide toxicity in the combined algae test

The baseline toxicity QSARs derived for the 6 non-herbicides acting as baseline toxicants (Equation 11 for IPAM and Equation 12 for growth rate) were statistically not different from the previously published QSAR for the combined algae test [24] (Supplemental Data, Figure S1, with 95% confidence intervals of slope and intercept overlapping between Escher et al. [24] and the present study).

$$\log(1/\mathrm{EC50_{IPAM}[M]}) = (0.55 \pm 0.16) \times \log D_{\mathrm{lipw}} + (1.39 \pm 0.50); \quad r^2 = 0.75,\ n = 6,\ F = 12 \quad (11)$$

$$\log(1/\mathrm{EC50_{growth\ rate}[M]}) = (0.51 \pm 0.15) \times \log D_{\mathrm{lipw}} + (2.14 \pm 0.46); \quad r^2 = 0.75,\ n = 6,\ F = 12 \quad (12)$$

The toxicity of the PSII herbicides is higher than that of the baseline toxicants because of their specific mode of action (Figure 1). The toxic ratio analysis showed that PSII herbicides displayed log $\mathrm{TR_{IPAM}}$ values of 3 to 5 for photosynthesis inhibition and $\mathrm{TR_{growth\ rate}}$ of 2 to 4 for growth rate inhibition (Supplemental Data, Table S2 and Figure S2). The PSII herbicides demonstrated higher photosynthesis inhibition because they act specifically by binding to the quinone-binding site ($\mathrm{Q_B}$) site on the D1 protein and prevent quinone from binding to this site [35], whereas baseline toxicants acted non-specifically on photosynthesis and algal growth rate (Supplemental Data, Figure S3).

The REP values in relation to the reference compound diuron were determined for the 12 PSII herbicides (Table 1). Atrazine, hexazinone, and simazine had REP values of 0.12, 0.26, and 0.04, respectively; that is, they were less potent than diuron. These REP values are very similar to the literature values considering differences in algal species and exposure times (Supplemental Data, Table S3).

Equipotent mixtures of herbicides

A mixture with a constant concentration ratio of the 12 PSII herbicides was mixed in proportion according to the relative potencies of the herbicides (equipotent mixture) and was compared with the CA prediction. The predicted $\mathrm{EC50_{CA}}$ of photosynthesis inhibition of 2.0×10^{-7} M agreed well with the experimental EC50 of $1.2 \pm 0.2 \times 10^{-7}$ M (Supplemental Data, Figure S4), which meets the expectation that the CA model can accurately predict the mixture effects of compounds with the same mode of action.

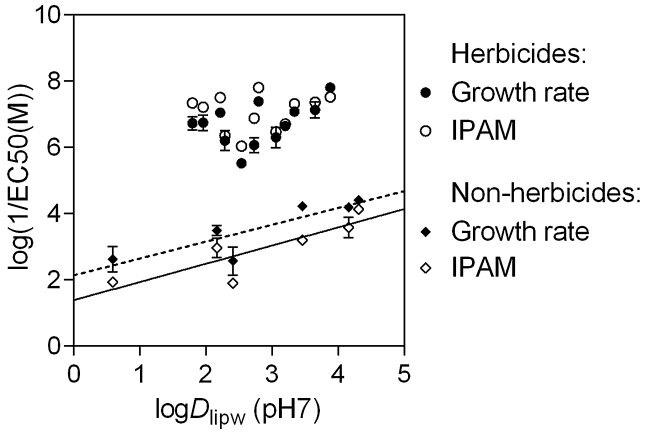

Figure 1. Quantitative structure–activity relationship analysis of the 6 tested non-herbicides acting as baseline toxicants in the combined algae test in comparison with the 12 photosystem II (PSII) herbicides. The endpoints of photosynthesis inhibition after 2 h of exposure ($\mathrm{EC50_{IPAM}}$) are indicated as open circles or open diamonds, and the algal growth rate inhibition during 24 h ($\mathrm{EC50_{growth\ rate}}$) are shown as filled circles or filled diamonds. The solid and dotted lines are the linear regression of the photosynthesis inhibition (IPAM) and growth rate of non-herbicides, respectively. D_{lipw}(pH7) = liposome-water distribution ratio at pH 7.

Effects and chemical analysis of water samples

The secondary treated wastewater effluent samples (Eff-1 and Eff-2) displayed similar diuron equivalent concentrations ($\mathrm{DEQ_{bio,sample}}$; 0.33 μg/L and 0.18 μg/L; Table 2) as in previous studies [36,37]. The $\mathrm{DEQ_{bio,sample}}$ was retained after microfiltration (MF) but was significantly reduced after reverse osmosis (RO). Herbicidal activity was below the limit of detection, expressed as DEQ of 0.01 μg/L in the combined algae test after advanced oxidation (AO) and after ozonation and biological activated carbon filtration ($\mathrm{O_3}$/BAC), indicating both types of treatments are efficient in removing organic micropollutants that possess PSII inhibitor properties. No toxicity was found in the drinking water plant influent (RW) and a very low $\mathrm{DEQ_{bio,sample}}$ was found in the drinking water plant outlet (DW). A very low $\mathrm{DEQ_{bio,sample}}$ (0.02 μg/L) was also observed in the same samples taken at another time by Neale et al. [29], a level which is close to the limit of detection expressed as DEQ of 0.01 μg/L. Storm water (SW) showed a similar $\mathrm{DEQ_{bio,sample}}$ of 0.04 μg/L as observed in a previous study [30], with a median of 0.18 μg/L. The observed effects in storm water were similar to secondary treated wastewater effluent, which could be caused by runoff from nearby agriculture.

Through chemical analysis of 269 individual compounds, we identified 5 to 48 compounds in the water samples (Supplemental Data, Table S4) [23]. Of the 40 detected compounds in Eff-1, 4 were PSII herbicides; these were retained in the MF sample, whereas the number of detected PSII herbicides post-RO reduced to 1. All chemicals were removed after the AO step in the Advanced Water Treatment Plant (Table 2). Similarly, 2 of the 48 detected compounds in Eff-2 were PSII herbicides, and no compounds were detected analytically after ozonation and biological activated carbon filtration in the Enhanced Water Treatment Plant. In storm water, 5 compounds were detected, 2 of which were PSII herbicides. Lastly, no PSII herbicides were detected in the river and drinking water samples.

Table 1. Physiochemical properties of PSII herbicides and EC50 values for the combined algae test with *Pseudokirchneriella subcapitata*

Compound	CAS no.	Molecular weight (g/mol)	log K_{OW}	log D_{lipw} (L/kg)	$EC50_{IPAM}$ (M)	$EC50_{growth\ rate}$ (M)	REP[a]
Atrazine	1912-24-9	215.69	2.61	2.73	$1.31 \pm 0.06 \times 10^{-7}$	$8.43 \pm 4.60 \times 10^{-7}$	0.12
Bromacil	314-40-9	261.12	2.11	2.19	$3.12 \pm 0.00 \times 10^{-8}$	$9.49 \pm 1.80 \times 10^{-8}$	0.50
Diuron	330-54-1	233.10	2.68	2.80	$1.56 \pm 0.01 \times 10^{-8}$	$4.23 \pm 0.16 \times 10^{-8}$	1.00
Fluometuron	2164-17-2	232.21	2.42	2.54	$1.02 \pm 0.30 \times 10^{-6}$	$2.01 \pm 0.86 \times 10^{-6}$	0.02
Hexazinone	51235-04-2	252.32	1.85	1.96	$6.08 \pm 0.03 \times 10^{-8}$	$1.51 \pm 1.00 \times 10^{-7}$	0.26
Metribuzin	21087-64-9	214.29	1.70	1.80	$4.59 \pm 0.01 \times 10^{-8}$	$1.63 \pm 0.91 \times 10^{-7}$	0.34
Prometryn	7287-19-6	241.36	3.51	3.65	$4.35 \pm 0.03 \times 10^{-8}$	$6.50 \pm 4.21 \times 10^{-8}$	0.36
Propanil	709-98-8	218.08	3.07	3.20	$1.99 \pm 0.18 \times 10^{-7}$	$3.79 \pm 2.50 \times 10^{-7}$	0.08
Propazine	139-40-2	229.71	2.93	3.06	$3.58 \pm 1.39 \times 10^{-7}$	$5.91 \pm 5.27 \times 10^{-7}$	0.04
Simazine	122-34-9	201.66	2.18	2.29	$3.60 \pm 2.28 \times 10^{-7}$	$4.23 \pm 5.72 \times 10^{-7}$	0.04
Terbuthylazine	5915-41-3	229.71	3.21	3.34	$4.84 \pm 0.02 \times 10^{-8}$	$8.57 \pm 0.76 \times 10^{-8}$	0.32
Terbutryn	886-50-0	241.36	3.74	3.88	$3.04 \pm 0.02 \times 10^{-8}$	$1.53 \pm 0.26 \times 10^{-8}$	0.51

[a] Diuron served as reference chemical to compute REP.
PSII = photosystem II; EC50 = effect concentration; K_{OW} = octanol–water partition coefficient; D_{lipw} = liposome–water distribution ratio; IPAM = photosynthesis inhibition; REP = relative effect potency.

Mixture modeling for herbicides and non-herbicides in the real environmental mixture

To elucidate whether herbicides present in a realistic environmental mixture dominate the overall toxicity or whether the contribution of non-herbicides plays a significant role in the mixture effects, we evaluated the TSP model for the iceberg mixtures, which simulate the known fraction of chemicals in the water samples. Sample Eff-1 was used as an example in Figure 2; herbicides contributed 8% and non-herbicides contributed 92% of the mixture composition in molar fractions. Although the contributing molar fractions of herbicides were low, the CA model alone was sufficient to explain the toxicity of the mixture (Figure 2A), and the contribution of the non-herbicides was practically negligible (Figure 2B). The TSP could perfectly predict the overall toxicity of realistic environmental mixtures (Figure 2C and Table 3), but it was not necessary because the CA effect of the herbicides could already adequately explain the overall mixture effect.

Similarly, in the other 3 polluted water samples (MF, RO, and Eff-2), the CA effect of herbicides clearly dominated the overall mixture effect despite the herbicide molar fractions ranging only from 1.2% to 4.6% (Supplemental Data, Figure S5). Sample SW had 43% (molar fraction) of herbicides in the mixture, and the predicted EC50 value from the CA model of herbicides was similar to the TSP EC50 value similar to other samples.

The results of the present study demonstrated that CA has a strong conceptual and methodological bearing for PSII inhibitors, even in a complex environmental mixture. This experimentally substantiates the suggestion by Chevre et al. [38] to use CA for risk assessment of PSII inhibitor mixtures.

Table 2. Comparison of diuron equivalent concentrations (DEQ_{bio}) for photosynthesis inhibition in environmental samples and iceberg mixtures, with diuron equivalent concentrations calculated from chemical analysis data and relative effect potencies (DEQ_{chem}; Equation 10)

	Sample									
	Eff-1	MF	RO	AO	Eff-2	O_3/BAC	RW	DW	SW	LB
No. of detected chemicals[a]										
PSII herbicides	4	4	1	0	2	0	0	0	2	0
Non-PSII herbicides	36	35	5	0	46	6	0	0	3	0
$DEQ_{chem,herbicides}$ (μg/L)[b]	0.242	0.226	0.028		0.083				0.041	<0.01
$DEQ_{chem,non\text{-}herbicides}$ (μg/L)[c]	9.35×10^{-4}	1.01×10^{-3}	2.53×10^{-6}		2.48×10^{-3}	2.12×10^{-6}			7.13×10^{-6}	
DEQ_{chem} (μg/L)	0.243	0.227	0.028		0.086				0.041	
Contribution of non-herbicides to DEQ_{chem}	0.38%	0.44%	0.01%		2.89%				0.02%	
$DEQ_{bio,sample}$ (μg/L)	0.333 ± 0.040	0.260 ± 0.010	0.021 ± 0.003	<0.01	0.183 ± 0.033	<0.01	<0.01	0.020 ± 0.010	0.112 ± 0.017	<0.01
$DEQ_{bio,iceberg}$ (μg/L)	0.359 ± 0.0231	0.334 ± 0.010	0.180 ± 0.019		0.162 ± 0.005				0.021 ± 0.002	
DEQ explained by detected herbicides $DEQ_{chem}/DEQ_{bio,sample}$	73%	88%	135%		47%				37%	
DEQ explained by detected herbicides and non-herbicides $DEQ_{bio,iceberg}/DEQ_{bio,sample}$	108%	129%	866%		88%				18%	

[a] Chemical analysis data obtained from Tang et al. [23].
[b] $DEQ_{chem,herbicides}$ of herbicides calculated using measured concentrations and the relative effect potencies (REPs) listed in Table 1.
[c] $DEQ_{chem,non\text{-}herbicides}$ of all other chemicals were calculated using the baseline toxicity quantitative structure–activity relationship (Equation 11) to estimate effect concentration (EC50) values, and REPs were calculated in relation to the experimental EC50 of diuron.
PSII = photosystem II; Eff-1 = secondary treated wastewater effluent (influent to MF); MF = after microfiltration; RO = after reverse osmosis; AO = after advanced oxidation; Eff-2 = secondary treated wastewater effluent (influent to O_3/BAC); O_3/BAC = after ozonation and biologically activated carbon filtration; RW = drinking water plant influent; DW = drinking water plant outlet; SW = storm water; LB = laboratory blank.

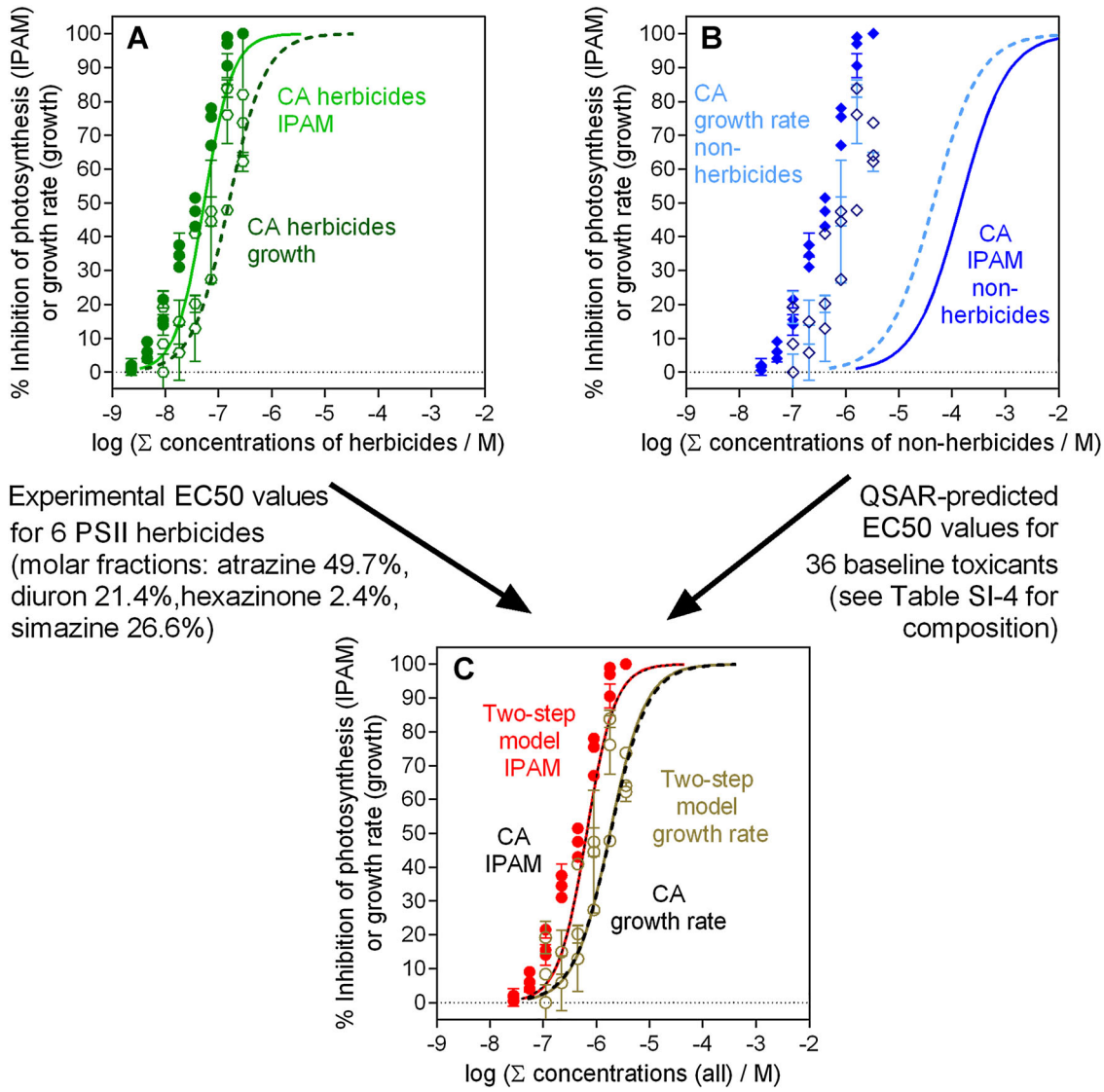

Figure 2. The two-step prediction approach (TSP) illustrated on the example of the secondary treated wastewater effluent sample (Eff-1). First, the photosystem II (PSII) herbicides were separated from the detected chemicals and the rest of chemicals were treated as non-herbicides. Concentration addition (CA) predictions were done separately on (**A**) herbicides using experimental median effect concentration (EC50) data and on (**B**) non-herbicides using EC50 of baseline toxicants predicted with quantitative structure–activity relationship (QSAR) model. Second, the independent action (IA) and CA models were applied to the 2 groups of chemicals for mixture toxicity prediction (**C**). Closed circles and diamonds indicate experimental data for photosynthesis inhibition (IPAM), and the open circles and diamonds are algal growth rate inhibition. Solid lines indicate IPAM and dotted lines indicate algal growth rate inhibition. [Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com]

Table 3. Experimental effect concentration EC50 values (M) of the iceberg mixtures and comparison with the CA and TSP models

		Experimental for herbicide icebergs		CA prediction for herbicides		QSAR for non-herbicide icebergs		CA prediction for non-herbicides		Experimental for the whole iceberg		TSP model	
Sample	Molar fractions of herbicides, non-herbicides	EC50	log EC50	EC50	log EC50	EC50 (M)	log EC50	EC50	log EC50	EC50	log EC50	EC50	log EC50
Eff-1	8.1%, 91.9%	3.62×10^{-8}	–7.49	5.37×10^{-8}	–7.27	3.66×10^{-7}	–6.44	1.45×10^{-4}	–3.84	4.04×10^{-7}	–6.40	6.55×10^{-7}	–6.18
MF	4.6%, 91.4%	3.83×10^{-8}	–7.42	6.38×10^{-8}	–7.20	3.83×10^{-8}	–7.42	1.52×10^{-4}	–3.82	4.45×10^{-7}	–6.35	7.39×10^{-7}	–6.13
RO	4.5%, 95.5%	2.34×10^{-9}	–8.63	1.55×10^{-8}	–7.81	5.00×10^{-8}	–7.30	3.84×10^{-3}	–2.42	5.24×10^{-8}	–7.28	3.48×10^{-7}	–6.46
Eff-2	1.2%, 98.8%	2.56×10^{-8}	–7.60	5.06×10^{-8}	–7.30	2.01×10^{-6}	–5.70	5.62×10^{-4}	–3.25	2.03×10^{-6}	–5.69	4.01×10^{-6}	–5.40
SW	42.5%, 57.5%	1.51×10^{-7}	–6.82	8.52×10^{-8}	–7.07	2.04×10^{-7}	–6.69	1.41×10^{-3}	–2.85	3.53×10^{-7}	–6.45	2.01×10^{-7}	–6.70

TSP = two-step prediction; EC50 = effect concentration; CA = concentration addition; QSAR = quantitative structure–activity relationship; Eff-1 = secondary treated wastewater effluent (influent to MF); MF = after microfiltration; RO = after reverse osmosis; Eff-2 = secondary treated wastewater effluent SW = storm water.

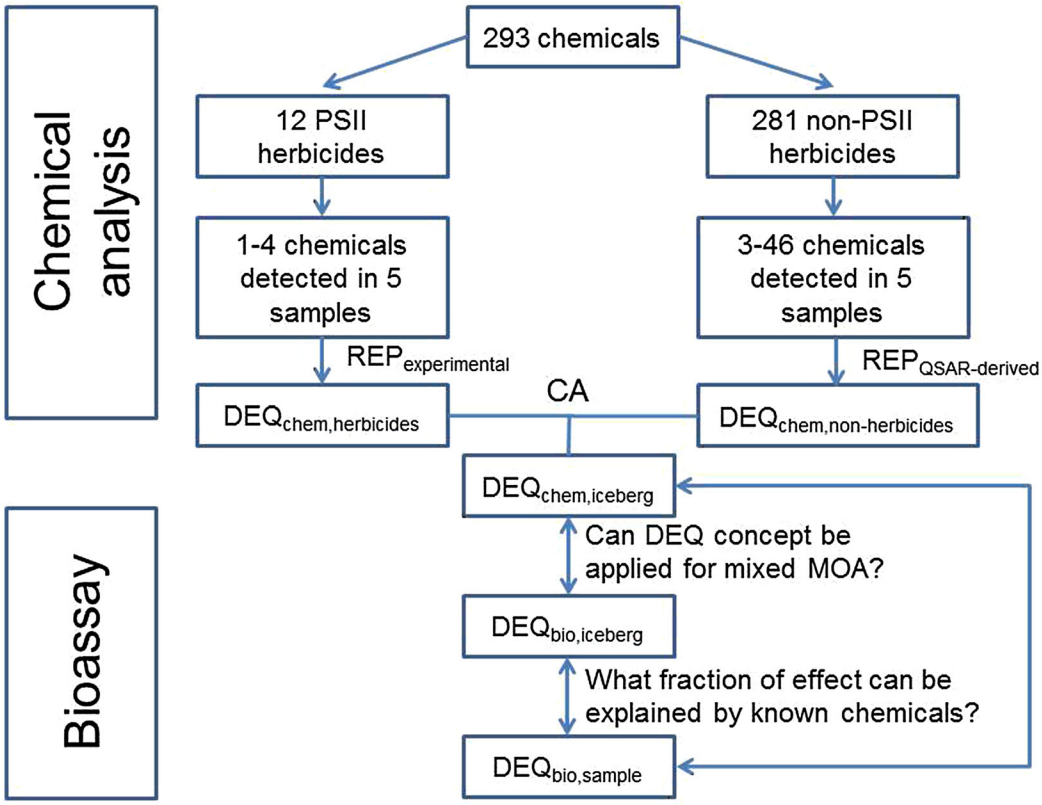

Figure 3. Schematic diagram of the comparison between diuron equivalent concentrations calculated by summing the relative effect potencies multiplied by the concentration of each known mixture component (DEQ_{chem}) for herbicides and non-herbicides ($DEQ_{chem,herbicides}$ and $DEQ_{chem,non\text{-}herbicides}$) and the diuron equivalent concentrations calculated directly from the bioassay for iceberg mixtures and water samples ($DEQ_{bio,iceberg}$ and $DEQ_{bio,sample}$).

How much effect is caused by known chemicals?

After CA was established as a relevant mixture toxicity concept, BEQs were derived for all samples. All steps to compare the DEQ obtained from the bioassay and chemical analysis in environmentally realistic mixtures comprised of PSII herbicides and non-herbicides are described in Figure 3. All of the detected chemicals were mixed in the analytically quantified concentration ratios to create the iceberg mixtures. These designed iceberg mixtures were tested with the combined algae test to compare the biological effects caused by known constituents with the effect of the water samples, which in addition contain unknown chemicals or chemicals below analytical detection limits. There was good agreement between the water sample $DEQ_{bio,sample}$ and the iceberg mixture $DEQ_{bio,iceberg}$ for Eff-1, Eff-2 and MF, explaining 88% to 129% of the observed DEQ (Figure 4A and Table 2). The exceptions were iceberg mixtures that consist of fewer than 6 components—RO and SW samples with 6 and 5 detected chemicals, of which only 1 and 2 were herbicides, respectively (Table 2). Because the number of detected chemicals was

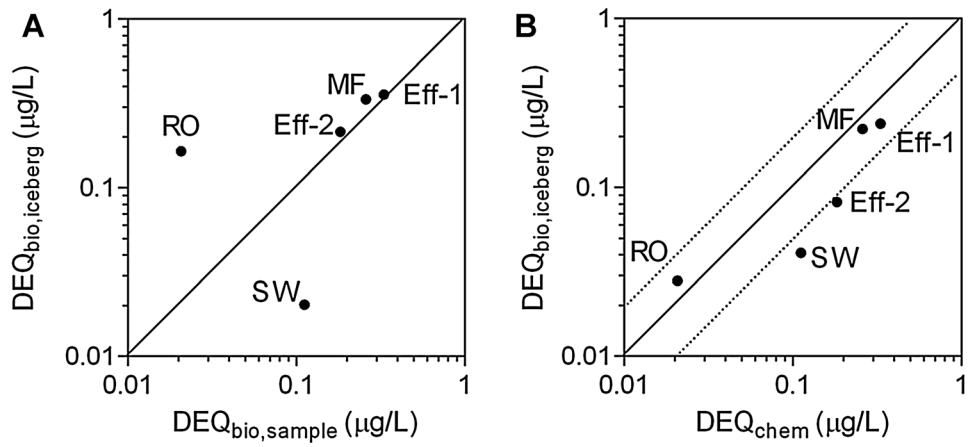

Figure 4. (**A**) Comparison between the diuron equivalent concentrations (DEQ) calculated directly from the bioassay of the iceberg mixtures ($DEQ_{bio,iceberg}$) with the experimental DEQ of the extracted water samples ($DEQ_{bio,sample}$). (**B**) Diuron equivalent concentrations compared between bioassay ($DEQ_{bio,iceberg}$) and chemical data adjusted with the relative potency (DEQ_{chem}) for all water samples. The solid line indicates 1:1 association, and the dashed lines a factor of 2 derivation from ideal agreement. Eff-1 = secondary treated wastewater effluent (influent to MF); MF = after microfiltration; RO = after reverse osmosis; Eff-2 = secondary treated wastewater effluent; SW = storm water.

smaller, a small error in the concentration of the 1 or 2 detected herbicides would cause a major change in effect of the mixture, which can explain the low comparability between $DEQ_{bio,iceberg}$ and $DEQ_{bio,sample}$.

Similarly, when we compared the iceberg mixture $DEQ_{bio,iceberg}$ with DEQ_{chem}, $DEQ_{chem,non\text{-}herbicide}$ values were negligible in comparison with $DEQ_{chem,herbicides}$ values (Table 2 and Figure 4B). Overall, the results of the present study showed that the detected PSII herbicides expressed as $DEQ_{chem,herbicides}$ reflected 37% to 135% of the $DEQ_{bio,sample}$ for Eff-1, Eff-2, MF, RO, and SW samples.

Significant relationships between $DEQ_{chem,herbicides}$ and the experimental DEQs of water samples, $DEQ_{bio,sample}$, were reported in the algal photosynthesis inhibition bioassay; for example, Vermeirssen et al. [31] observed 50% to 85% agreement between DEQ_{chem} from 6 quantified PSII inhibitors and $DEQ_{bio,sample}$ in passive sampler water extracts.

Derivation of EBT-DEQ for herbicides

Because the observed algal toxicity could be fully explained by the CA effect of herbicides in the real water samples, EBT-DEQ can be derived for herbicides because the herbicides dominated the effect and the non-herbicides can be neglected. Guideline values for herbicides are defined for drinking water [3], recycled water [4], and recreational water [1] in Australia. We used these guideline values as an example but the principle can be applied to any other water quality guideline values or standards.

The guideline values for each compound i (GV_i) can be converted to DEQ_i with Equation 13 and are listed in Table 4.

$$DEQ_i = REP_i \times GV_i \quad (13)$$

If the guideline values were derived from a photosynthesis inhibition endpoint, then all resulting DEQ_i were theoretically equal and could be directly applied as EBT-DEQ. This is of course not the case, because the guideline values were derived from human-health endpoints or the entire ecosystem, not just focusing on algal toxicity. Therefore, the DEQ_i varied by several orders of magnitude (Table 4).

Similar to most biological data, the DEQ_i are distributed in a log-normal manner (Figure 5). It is interesting to note that

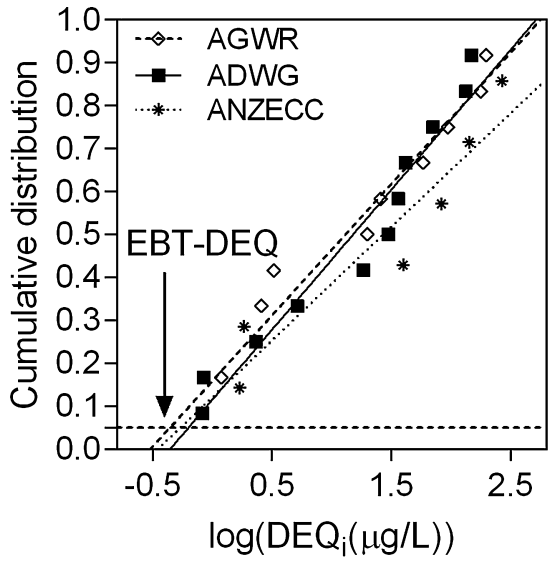

Figure 5. Cumulative frequency distribution of diuron equivalent concentrations for compound i (DEQ_i) and the estimated effect-based trigger DEQ (EBT-DEQ). AGWR = Australian Guidelines for Water Recycling; ADWG = Australian Drinking Water Guidelines; ANZECC = Australian and New Zealand Guidelines for Fresh and Marine Water Quality.

despite the facts that the different guidelines included differing numbers of herbicides and that the guideline values in the Australian Drinking Water Guidelines [3] and Australian Guidelines for Water Recycling [4] are meant to protect human health whereas the Australian and New Zealand Guidelines for Fresh and Marine Water Quality [1] are meant to protect ecosystem health, the cumulative frequency distribution of diuron equivalent concentrations looked remarkably similar (Figure 5). As a precautionary approach we used the 5th percentile of the distribution of log DEQ_i values to derive the EBT-DEQ (Figure 5).

The resulting EBT-DEQs were 0.44 μg/L for drinking water, 0.63 μg/L for recycled water, and 0.54 μg/L for surface water. The proposed EBT-DEQs were compared with the measured $DEQ_{bio,sample}$ in the water samples. All samples would be compliant when compared with their respective EBT-DEQ. The EBT-DEQs were also compared with experimental $DEQ_{bio,}$

Table 4. Guideline values (GV; μg/L) and the calculated EBT-DEQs (μg/L) for the photosynthesis inhibition endpoint in the combined algae assay

		GV_i			DEQ_i		
	REP[a]	ADWG[b]	AGWR[c]	ANZECC[d]	ADWG[b]	AGWR[c]	ANZECC[d]
Atrazine	0.12	20	40	n.a.	2.6	5.2	n.a.
Bromacil	0.50	400	300	600	178.9	134.1	268.3
Diuron	1.00	20	30	40	20.0	30.0	40.0
Fluometuron	0.02	70	50	100	1.2	0.9	1.7
Hexazinone	0.26	400	300	600	94.8	71.1	142.2
Metribuzin	0.34	70	50	5	25.9	18.5	1.8
Prometryn	0.36	n.a.	105	n.a.	n.a.	36.4	n.a.
Propanil	0.08	700	500	1000	58.6	41.9	83.7
Propazine	0.04	50	50	n.a.	2.3	2.3	n.a.
Simazine	0.04	20	20	n.a.	0.8	0.8	n.a.
Terbuthylazine	0.32	10	n.a.	n.a.	3.3	n.a.	n.a.
Terbutryn	0.51	400	300	n.a.	198.5	148.9	n.a.

[a]Relative potency values (REP) from Table 1.
[b]From Australian Drinking Water Guidelines (ADWG) [3].
[c]From Australian Guidelines for Water Recycling (AGWR) [4].
[d]From Australian and New Zealand Guidelines for Fresh and Marine Water Quality—Recreational Waters (ANZECC) [1].
EBT-DEQ = estimated effect-based trigger diuron equivalent concentrations; n.a. = no guideline value available.

$_{sample}$ values from the literature (Supplemental Data, Table S5) [24,29,30,37,39–43] and all water samples were compliant except storm water, which has a wide range of DEQs among sites because of various rainfall events and land use characteristics [30].

CONCLUSION

The present study demonstrated that herbicides dominate the algal toxicity in environmentally realistic mixtures, although only a small number of chemicals were detected analytically. The contribution by the non-herbicides was negligible in both the endpoint of photosynthesis inhibition and that of inhibition of growth rate. A CA model including only the herbicides was sufficient to explain the effects of the entire iceberg mixture, and it was not necessary to invoke the two-step mixture prediction model that combines CA with IA of the groups of herbicides and non-herbicides. Therefore, the BEQ concept is suitable to derive effect-based trigger values for algal toxicity for monitoring water quality. Initial analysis of experimental results obtained in the present study and experimental data from literature showed that all water samples tested would be compliant with the proposed trigger values.

SUPPLEMENTAL DATA

Tables S1–S6.
Figures S1–S5. (517 KB DOCX).

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Which chemicals drive biological effects in wastewater and recycled water?

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ABSTRACT

Removal of organic micropollutants from wastewater during secondary treatment followed by reverse osmosis and UV disinfection was evaluated by a combination of four *in-vitro* cell-based bioassays and chemical analysis of 299 organic compounds. Concentrations detected in recycled water were below the Australian Guidelines for Water Recycling. Thus the detected chemicals were considered not to pose any health risk. The detected pesticides in the wastewater treatment plant effluent and partially advanced treated water explained all observed effects on photosynthesis inhibition. In contrast, mixture toxicity experiments with designed mixtures containing all detected chemicals at their measured concentrations demonstrated that the known chemicals explained less than 3% of the observed cytotoxicity and less than 1% of the oxidative stress response. Pesticides followed by pharmaceuticals and personal care products dominated the observed mixture effects. The detected chemicals were not related to the observed genotoxicity. The large proportion of unknown toxicity calls for effect monitoring complementary to chemical monitoring.

1. Introduction

Indirect potable reuse (IPR) of wastewater has become a necessity in many water-scarce regions of the world (National Research Council, 1998; Rodriguez et al., 2009; Sedlak, 2014). IPR schemes typically rely on advanced treatment of secondary wastewater effluents from wastewater treatment plants (WWTP). Such advanced treatment usually consists of a combination of membrane filtration (e.g., ultrafiltration and reverse osmosis) and oxidation processes (e.g., advanced oxidation, UV disinfection) to remove pathogens and chemicals, including inorganics and heavy metals, nutrients and organic micropollutants (Binnie and Kimber, 2009). Recycled water is then introduced into aquifers or waters and can potentially be used as part of the drinking water supply. As reviewed recently (Rodriguez et al., 2009; van der Bruggen, 2010), a large number of IPR schemes have been implemented in the US, as well as some in the UK, Namibia, and Singapore. To date, no adverse health impacts have been reported related to recycled water (Khan and Roser, 2007).

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In Australia, there are two major IPR projects. On the East Coast, the Western Corridor Recycled Water Project is Australia's largest water recycling scheme and the third-largest advanced water treatment project in the world. It was commissioned in 2008 but ultimately the scheme has not become operational as of 2014 because the 2003–2008 drought in Southeast Queensland ended with a period of heavy rainfalls and floods from mid-2010 onwards. On the West Coast, a pilot IPR scheme has been successfully implemented from 2010 to 2012 that treats secondary effluent from the Beenyup WWTP with ultrafiltration (UF) followed by reverse osmosis (RO) and final UV disinfection. The recycled water is injected into the Leederville aquifer, which is a drinking water source for the city of Perth (Water Corporation, 2013). This scheme has been approved to go to full scale, with stage one expected to be completed in 2016 (http://www.watercorporation.com.au). This managed aquifer recharge scheme is the focus of this paper.

Most IPR schemes extensively investigated potential environmental and human health impact of the replenishment of drinking water reservoirs with recycled water before implementation. Typically a large number of organic micropollutants known to occur in sewage or formed from natural precursors during treatment processes (e.g., disinfection by-products) are monitored through chemical analyses. These include pharmaceuticals and personal care products, pesticides, household and industrial chemicals. While micro- or ultra-filtration mainly remove bacteria, pathogens and high molecular-weight natural organic matter, most organic micropollutants are removed during RO treatment (Gupta and Ali, 2013). However, low molecular weight and non-ionic (neutral) organic molecules (e.g. NDMA, dioxane, halogenated solvents) were less effectively rejected by RO membranes. As a result, these compounds are frequently detected in recycled water at low concentrations (Snyder et al., 2007; Drewes et al., 2008).

Taking a precautionary approach, frequently detected organic micropollutants in recycled water are tightly regulated in many countries. In the US, recycled water has to comply with drinking water guidelines. The Australian Guidelines for Water Recycling (AGWR) lists 348 organic chemicals with health-based guideline values (GVs) (NRMMC & EPHC & NHMRC, 2008). The GVs generally match the Australian Drinking Water Guidelines (ADWG) (NHMRC, 2011) but almost twice as many chemicals are regulated in recycled water. Fifteen regulated organic micropollutants were occasionally detected in recycled water of the Western Corridor Recycled Water Project (Hawker et al., 2011) but concentrations never exceeded GVs. In addition the potential impact on the receiving drinking water reservoir was modeled, and concentrations of organic chemicals were expected to decrease further due to dilution and natural attenuation, mainly by biodegradation and sorption to sediments (Hawker et al., 2011).

In an initial investigation (2005–2008) of the IPR scheme in Perth, 396 parameters were monitored over three years (Van Buynder et al., 2009). While 23 organic chemicals and 6 metals/inorganics were detected in more than 25% of all RO waters investigated, all concentrations of chemicals in RO water were below GVs. The organics detected in RO permeate were mainly disinfection by-products (e.g., NDMA), small volatile organics (e.g., benzene, dioxane) and complexing agents (e.g., EDTA, NTA). Detected concentrations were below GVs and were not considered to pose any appreciable health risk, with one exception, the disinfection by-product NDMA (Linge et al., 2012). However, there remain unknowns because the detected chemicals could only explain a small fraction (~2–5%) of the dissolved organic carbon in the RO permeate (Linge et al., 2012). While up to 95% of dissolved organic carbon in RO permeate could not be accounted for, chemicals below detection limit may have contributed to the residual DOC, along with low molecular-weight natural organic matter originally present in drinking water and wastewater, unknown anthropogenic micropollutants, chemicals used during RO treatment or leached from RO membranes and soluble microbial by-products (Linge et al., 2012).

To bridge this knowledge gap, target and non-target screenings were conducted recently in water post RO and post UV using an Orbitrap MS spectrometer (Busetti et al., 2013). Both target and non-target screenings showed that (a) "suspect" or "unknown" chemicals did not make up the majority of the DOC in RO treated water, and (b) a large number anthropogenic chemicals targeted (i.e., pesticides, biocides, industrial chemicals, pharmaceuticals) were not detected in recycled water, further reducing the risk associated with human consumption of recycled water.

Furthermore, during Perth's Groundwater Replenishment Trial, which ended in 2012, 292 Recycled Water Quality Parameters were monitored over three years. The results of this extensive monitoring program confirmed 100% compliance of all water samples analyzed with the required water quality guidelines (Water Corporation, 2013).

In the present study, chemical analysis was complemented with bioanalytical tools. Cell-based bioassays are widely used for water quality assessment and monitoring (Escher and Leusch, 2012) and have previously been applied to evaluate water quality from samples taken in the investigated IPR scheme (Leusch et al., 2014a, 2014b).

Cell-based bioassays can provide a comprehensive profile of the biological activity of mixtures of organic chemicals and can also give information on the types of effect by choosing cells and assessment endpoints that are associated with defined modes of action (Escher and Leusch, 2012). So far, investigated modes of action have predominantly focused on estrogenic and other endocrine effects as well as genotoxicity (Escher and Leusch, 2012). We previously applied 100 distinctly different bioassays to recycled water and demonstrated that a small number of indicator bioassays can be applied for monitoring of the treatment efficacy as well as for benchmarking the water quality of recycled water against other types of water (Escher et al., 2014a). According to these recommendations six bioassays were initially trialled and four bioassays were selected in this study Non-specific toxicity (cytotoxicity) was evaluated with the bioluminescence inhibition test with *Vibrio fischeri* (Microtox) (Tang et al., 2013). Photosynthesis inhibition using the combined algae test (Escher et al., 2008) was a representative specific mode of action. We also determined estrogenicity with the E-CALUX (Rogers and Denison, 2000) and the activation of the aryl hydrocarbon receptor with the AhR-CAFLUX (Nagy et al., 2002) assay but these two bioassays did not show any responses and were therefore not suitable for the mixture modeling.

Reactive toxicity was assessed with the umuC assay for genotoxicity (Macova et al., 2011) and the AREc32 for oxidative stress response (Escher et al., 2012). In addition, we quantified 299 organic micropollutants during the same sampling campaign.

The aim of the study was to assess which of the detected chemicals drive the biological effect and which fraction of effect remains unexplained by detected chemicals. Therefore we mixed all chemicals that were (a) present at concentrations above the limit of detection (LOD) and (b) included in the AGWR. These chemicals were mixed in the concentration ratios that were detected by analytical chemistry in the various samples. These mixtures were termed "iceberg mixtures" as they constituted the visible "tip of the iceberg" and allowed us to estimate the contribution of unknown chemicals and chemicals below detection limits to the overall mixture effect. We have previously performed such experiments with wastewater and recycled water and were able to show that known chemicals can explain the majority of specific receptor-mediated effects (Tang and Escher, 2013) but for more general endpoints such as cytotoxicity (Tang et al., 2013) and oxidative stress response (Escher et al., 2013) less than 1% of effect could be explained by known chemicals.

In addition to the four biological endpoints evaluated here, estrogenicity is a highly relevant biological endpoint in wastewater and associated water types. However, previous work has demonstrated that no estrogenic activity could be detected in recycled water (Leusch et al., 2014a), and that the estrogenicity in typical source water can be fully explained by known chemicals (Rutishauser et al., 2004). Therefore, and because no estrogenic responses were detected in the investigated waters, this endpoint was omitted in the present study.

The present study does not only apply this iceberg concept to a different and more diverse set of samples in a recycling plant than in our previous studies but goes a step further in that the iceberg mixtures were subdivided into six chemical groups (pharmaceuticals (including personal care products), endocrine disruptors compounds (EDCs), antibiotics, X-ray contrast media (XRCs), pesticides (including transformation products) and others). By comparing the effects of the individual groups and the effects of the combined iceberg mixtures, it could be determined, which chemical group dominates or significantly contributes to the biological effects at any stage of the treatment process.

2. Materials and methods

2.1. Chemicals

The 65 chemicals used in the mixture experiments are listed in the Electronic Supplementary Material (ESM), Table S1. All chemicals were of analytical grade and purchased from Sigma–Aldrich or Novachem, Australia.

2.2. Sampling site

Grab samples were collected from a Wastewater Treatment Plant (WWTP) and an Advanced Water Recycling Plant (AWRP) located in Perth, Western Australia in July 2012 (Fig. 1). The WWTP treats predominately urban residential sewage (Water Corporation, 2013). Briefly, the raw wastewater is treated with grit removal and goes through sedimentation tanks (WWTP influent). This water then undergoes aeration, activated sludge treatment and clarification as a secondary treatment. The majority of the resulting secondary treated effluent (WWTP effluent) is discharged into the ocean and a small portion (~7 ML/day) is fed into the AWRP. The treatment train of the AWRP consists of chloramination for disinfection during treatment process, ultrafiltration, reverse osmosis and ultraviolet light (UV) disinfection. Samples were collected in the following points: after the sedimentation tanks in the WWTP (WWTP influent); after secondary treatment (WWTP effluent); after ultrafiltration (post UF); before reverse osmosis in the holding tank (mixing tank); after reverse osmosis (post RO); after UV disinfection (post UV). The reverse osmosis reject (RO reject) was also collected. The recycled water was injected into the groundwater system at a maximum of 4.5 ML/day. Routine water quality data were assessed by the plant operators at the time of sampling and is given in the ESM, Table S2. In addition, a laboratory blank (LB) and a blank (FB) were made up of ultrapure water.

2.3. Sampling and sample preparation

The water samples were collected in amber glass bottles and preserved with 0.1% sodium thiosulphate and concentrated hydrochloric acid to pH 2.5. The samples were split into two portions, for chemical analyses and bioassays. For bioassays, all samples were filtered with 0.45 μm microfiber glass Duo-Fine filter cartridges (PALL Life Sciences, NY, USA) before solid-phase extraction (SPE) in 20 mL custom-made cartridges from Supelco (Sigma–Aldrich, Sydney, Australia). The extraction material was comprised of 2 g SupelClean coconut

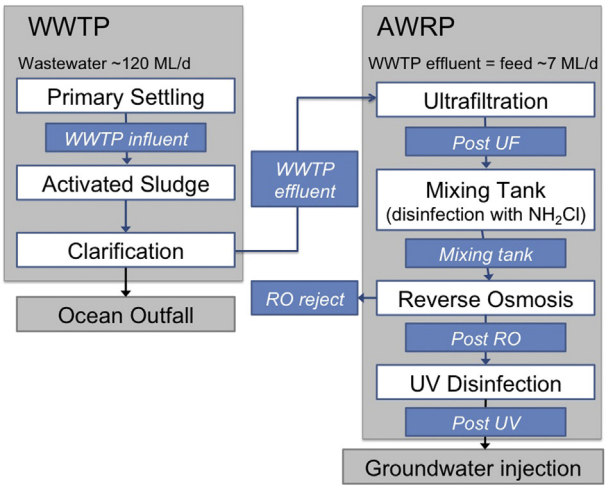

Fig. 1 – Overview of the treatment processes at the Wastewater Treatment Plant (WWTP) and Advanced Water Recycling Plant (AWRP). The blue boxes denote the points where the samples were collected (text in *italics*). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1 – Bioassays used in this study, reference chemicals for the derivation of BEQ and their effect concentrations EC.

Mode of action	Bioassay	Literature reference (assay principle)/(method applied)	Reference compound	Effect concentration EC of reference compound	Bioanalytical equivalent concentration BEQ	Limit of detection expressed as BEQ[a]
Non-specific: cytotoxicity	Bioluminescence inhibition test with *Vibrio fischeri* (Microtox)	(ISO11348-3 1998)/(Tang et al., 2013)	Virtual baseline toxicant (a model chemical with $\log K_{ow} = 3$ and a molecular weight of 300 g mol^{-1})	$EC_{50} = 66 \pm 6.7$ mg/L	Baseline toxicity equivalent concentration (Baseline-TEQ)	0.13 mg/L
Specific: photosynthesis inhibition	Combined algae test with *Pseudokirchneriella subcapitata*	(Muller et al., 2008)/(Escher et al., 2008)	Diuron	$EC_{50} = 1.81 \pm 0.45$ µg/L	Diuron equivalent concentration (DEQ)	0.004 µg/L
Reactive: genotoxicity	umuC assay −S9	(ISO13828 1999)/(Macova et al., 2011)	4-Nitroquinoline-N-oxide (4NQO)	$EC_{IR1.5} = 9.1 \pm 3.8$ µg/L	4NQO equivalent concentration (4NQOEQ)	0.10 µg/L
Reactive: genotoxicity after metabolic activation	umuC assay + S9	(ISO13828 1999)/(Macova et al., 2011)	2-Aminoanthracene (2AA)	$EC_{IR1.5} = 46.7 \pm 27.6$ µg/L	2AA equivalent concentration (2AAEQ)	0.05 µg/L
Reactive: oxidative stress	AREc32 assay	(Wang et al., 2006)/(Escher et al., 2012)	t-butyl-hydroquinone (tBHQ)	$EC_{IR1.5} = 0.15 \pm 0.03$ mg/L	tBHQ equivalent concentration (tBHQEQ)	8.64 µg/L

[a] Limit of detection calculation from the equivalent concentration caused by an effect of 3 times the standard deviation of the controls.

charcoal and 1 g SupelSelect HLB with frits in between. The cartridges were conditioned with 20 mL acetone:hexane mixture (1:1, v:v), followed by 20 mL methanol and 20 mL ultrapure water at pH 3 at a flow rate of 5 mL/min. Samples were then loaded onto the custom-made cartridges using three 8-channel offline peristaltic pumps (Gilson, Middleton, USA) at a flow rate of 3 mL/min. The cartridges were dried under vacuum and wrapped in parafilm and aluminum foil and stored at −20 °C before shipping to the Entox laboratory for elution. The cartridges were eluted with 20 mL methanol and 20 mL acetone:hexane mixture (1:1, v:v) under gravity. The extracts were evaporated under gentle nitrogen flow and solvent-exchanged into 1 mL of methanol.

The SPE extracts were comprised of a mixture of known and unknown chemicals at unknown concentrations. The dose-metric is the relative enrichment factor (REF), which is a measure of how much a sample is enriched (REF > 1) or diluted (REF < 1) in the bioassay as compared to the original sample (equation (1)).

$$\mathrm{REF} = \frac{\text{water volume equivalent in bioassay}}{\text{total volume of medium in bioassay}} \quad (1)$$

2.4. Chemical analysis

Water samples were analyzed using GC/MS–MS and LC/MS–MS at Queensland Health Forensic and Scientific Services and at Curtin Water Quality Research Centre (CWQRC). A total of 299 chemicals were analyzed between the two laboratories. More details on the sample preparation for chemical analysis and analytical methods are given in the ESM Data, Section S1.

2.5. Designed iceberg mixtures

Detected chemicals were mixed in the ratios of concentrations found (ESM, Table S3). The detected chemicals were clustered in six groups: endocrine disrupting compounds (EDCs), antibiotics, X-ray contrast media (XRCs), pesticides (including transformation products), pharmaceuticals (excluding antibiotics but including personal care products such as triclosan and consumer products such as caffeine) and "others" (ESM, Table S3). In addition, the individual chemical group mixtures were mixed according to the contributing fraction into one mixture comprising all detected chemicals termed as "iceberg mixture".

2.6. Bioanalytical assessment

All bioassays were previously applied and characteristics of the bioassays and literature references for the methods are given in Table 1. For each sample, the bioanalytical equivalent concentration BEQ was calculated from the effect concentration EC of the reference compound divided by the EC of the water sample.

$$\mathrm{BEQ_{water}} = \frac{\mathrm{EC(reference\ compound)}}{\mathrm{EC(water\ sample)}} \quad (2)$$

In case of the water samples, the EC is in units of REF and the BEQ is termed $\mathrm{BEQ_{water}}$. Analogously the BEQ of designed

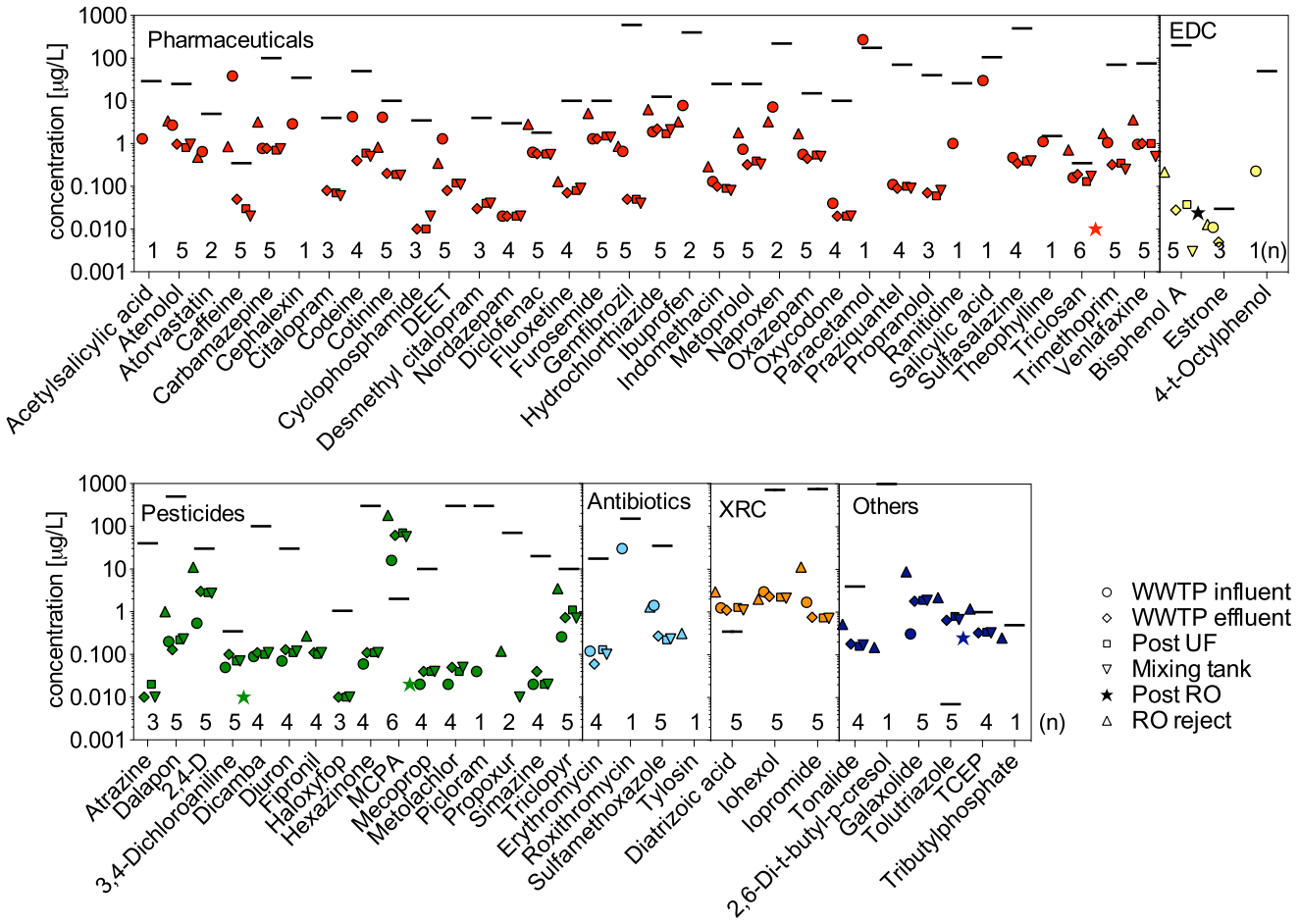

Fig. 2 – Concentration of 65 chemicals detected in at least one water sample and used for the iceberg mixture experiments (Table S3) (from 299 analyzed chemicals and a total of 95 detected chemicals); (*n*) refers to the number of samples with concentrations above the limit of detection. The detected chemicals were clustered in six groups: pharmaceuticals, endocrine disrupting compounds (EDCs), pesticides, antibiotics, x-ray contrast media (XRC), and others. (n) refers to the number of samples that were above the detection limit. The different symbols denote the different water samples (circle: WWTP influent, diamond: WWTP effluent, square: post UV, down-facing triangle: mixing tank, up-facing triangle: RO reject, star: post RO). The black bars denote the guideline values (GV) of the AGWR (NRMMC & EPHC & NHMRC, 2008). The only chemical that was included and does not have an AGWR GV is fipronil (but included in ADWG).

iceberg mixtures $BEQ_{iceberg}$ and the individual chemical groups $BEQ_{group\ i}$ can be derived with equation (3) by using the EC values experimentally obtained from the designed chemical mixtures (in units of mol/L) and converted to the EC in units of REF, EC(iceberg, REF), using the known chemical concentrations C in the mixture equivalent to the measured concentrations in the sample (sum of concentrations in units of mol/L).

$$BEQ_{iceberg} = \frac{EC(\text{reference compound})}{\frac{EC(\text{iceberg, M})}{C(\text{iceberg})}} = \frac{EC(\text{reference compound})}{EC(\text{iceberg, REF})} \quad (3)$$

The reference compounds and the associated BEQ for each bioassay are defined in Table 1. The limits of detection were derived by translating the effect of three times the standard deviation of the controls into the corresponding BEQ values (Table 1).

3. Results and discussion

3.1. Chemical analysis

A total of 299 chemicals were analyzed in the water samples, of which 172 were included in the AGWR (ESM, Figure S1). In the paper, we focus the discussion on the regulated chemicals (ESM, Table S3), while results on additional non-regulated chemicals are compiled in the ESM, Table S4. The highest number of chemicals were detected in WWTP influent, WWTP effluent, post UF and mixing tank (50, 50, 49, 50, respectively, ESM, Figure S1). The concentrations of chemicals in the WWTP influent were typically higher than in WWTP effluent, although due to the higher LOD in the WWTP influent sample, some chemicals were not detected in the WWTP influent but found in the WWTP effluent. UF did not reduce concentrations of chemicals. Instead, RO was found to be a very effective

Table 2 – Summary of all bioassay results expressed as bioanalytical equivalent concentrations.

Sampling site/treatment	WWTP influent	WWTP effluent	Post UF	Mixing tank	Post RO	Post UV	RO reject	Lab blank	Trip blank
V. fischeri bioluminescence inhibition assay									
Baseline-TEQ$_{water}$ (mg L^{-1})	25.9 ± 0.72	9.15 ± 0.07	5.12 ± 0.78	5.83 ± 0.65	0.43 ± 0.09	0.74 ± 0.10	29.9 ± 1.0	0.40 ± 0.04	0.29 ± 0.01
Baseline-TEQ$_{iceberg}$ (mg L^{-1})	0.04 ± 0.02	0.11 ± 0.03	0.16 ± 0.03	0.13 ± 0.02	0.003 ± 0.004	n.t.	0.37 ± 0.07	n.t.	n.t.
Baseline-TEQ$_{EDC}$ (μg L^{-1})	1.81 ± 1.67	0.30 ± 0.07	0.44 ± 0.10	0.04 ± 0.01	0.30 ± 0.09	n.t.	2.22 ± 0.49	n.t.	n.t.
Baseline-TEQ$_{XRC}$ (μg L^{-1})	<0.07	<0.05	<0.05	<0.05	n.t.	n.t.	<0.2	n.t.	n.t.
Baseline-TEQ$_{antibiotics}$ (μg L^{-1})	0.15 ± 0.04	0.09 ± 0.03	0.10 ± 0.05	0.06 ± 0.04	n.t.	n.t.	0.16 ± 0.18	n.t.	n.t.
Baseline-TEQ$_{pesticides}$ (μg L^{-1})	25.4 ± 10.7	96.0 ± 34.1	132 ± 31.0	69.8 ± 35.8	0.14 ± 0.03	n.t.	252 ± 109	n.t.	n.t.
Baseline-TEQ$_{pharmaceuticals}$ (μg L^{-1})	25.6 ± 35.7	9.1 ± 2.7	7.3 ± 2.7	11.7 ± 2.7	0.94 ± 0.67	n.t.	39.0 ± 13.5	n.t.	n.t.
Baseline-TEQ$_{others}$ (μg L^{-1})	0.05 ± 0.01	2.8 ± 0.6	4.0 ± 1.0	2.8 ± 0.9	1.1 ± 0.2	n.t.	15.8 ± 5.6	n.t.	n.t.
IPAM photosynthesis inhibition assay									
DEQ$_{water}$ (μg L^{-1})	0.073 ± 0.023	0.033 ± 0.012	0.025 ± 0.006	0.017 ± 0.004	<0.004	<0.004	0.11 ± 0.02	<0.004	<0.004
DEQ$_{iceberg}$ (μg L^{-1})	0.10 ± 0.04	0.11 ± 0.03	0.10 ± 0.03	0.10 ± 0.03	$2.5 \pm 1.2 \times 10^{-5}$	n.t.	$<5.5 \times 10^{-4}$	n.t.	
DEQ$_{EDC}$ (ng L^{-1})	2.9×10^{-2}	$8.8 \pm 3.3 \times 10^{-4}$	$1.2 \times 10^{-3} \pm 3.0 \times 10^{-4}$	$1.0 \pm 0.3 \times 10^{-4}$	$7.7 \pm 5.0 \times 10^{-4}$	n.t.	n.t.	n.t.	n.t.
DEQ$_{XRC}$ (ng L^{-1})	$<3.4 \times 10^{-3}$	$<2.4 \times 10^{-3}$	$<2.5 \times 10^{-3}$	$<2.3 \times 10^{-3}$	n.t.	n.t.	$<9.4 \times 10^{-3}$	n.t.	n.t.
DEQ$_{antibiotics}$ (ng L^{-1})	0.35 ± 0.15	$<2.6 \times 10^{-3}$	$<2.4 \times 10^{-3}$	$<2.4 \times 10^{-3}$	n.t.	n.t.	$<2.4 \times 10^{-3}$	n.t.	n.t.
DEQ$_{pesticides}$ (ng L^{-1})	30 ± 16	93 ± 24	60 ± 30	61 ± 35	$<1.2 \times 10^{-4}$	n.t.	<0.42	n.t.	n.t.
DEQ$_{pharmaceuticals}$ (ng L^{-1})	0.55 ± 0.36	0.25 ± 0.14	0.19 ± 0.15	0.37 ± 0.12	$<7.8 \times 10^{-5}$	n.t.	1.34 ± 0.72	n.t.	n.t.
DEQ$_{others}$ (ng L^{-1})	$<5.2 \times 10^{-4}$	$<6.0 \times 10^{-3}$	$<6.6 \times 10^{-3}$	$<6.2 \times 10^{-3}$	$<8.2 \times 10^{-4}$	n.t.	$<2.6 \times 10^{-2}$	n.t.	n.t.
umuC genotoxicity assay without metabolic activation									
4NQOEQ$_{water}$ (μg L^{-1})	0.56 ± 0.17	0.24 ± 0.10	0.09 ± 0.02	0.13 ± 0.07	<0.10	<0.10	0.62 ± 0.18	<0.10	<0.10
4NQOEQ$_{iceberg}$ (μg L^{-1})	$<4.2 \times 10^{-3}$	$<6.4 \times 10^{-3}$	$<7.2 \times 10^{-4}$	$<6.1 \times 10^{-4}$	$<3.7 \times 10^{-6}$	n.t.	$<2.1 \times 10^{-3}$	n.t.	n.t.
4NQOEQ$_{EDC}$ (μg L^{-1})	$<3.1 \times 10^{-6}$	$<3.9 \times 10^{-7}$	$<4.6 \times 10^{-7}$	$<4.0 \times 10^{-8}$	$<2.9 \times 10^{-7}$	n.t.	$<2.7 \times 10^{-6}$	n.t.	n.t.
4NQOEQ$_{XRC}$ (μg L^{-1})	$<3.8 \times 10^{-5}$	$<1.4 \times 10^{-5}$	$<1.4 \times 10^{-5}$	$<1.3 \times 10^{-5}$	n.t.	n.t.	$<5.3 \times 10^{-5}$	n.t.	n.t.
4NQOEQ$_{antibiotics}$ (μg L^{-1})	$<9.3 \times 10^{-6}$	$<1.9 \times 10^{-6}$	$<1.7 \times 10^{-6}$	$<1.7 \times 10^{-6}$	n.t.	n.t.	$<8.9 \times 10^{-6}$	n.t.	n.t.
4NQOEQ$_{pesticides}$ (μg L^{-1})	$<4.3 \times 10^{-4}$	$<1.6 \times 10^{-1}$	$<1.9 \times 10^{-1}$	$<1.5 \times 10^{-1}$	$<8.1 \times 10^{-1}$	n.t.	$<4.9 \times 10^{-1}$	n.t.	n.t.
4NQOEQ$_{pharmaceuticals}$ (μg L^{-1})	$<3.9 \times 10^{-3}$	$<5.6 \times 10^{-5}$	$<5.4 \times 10^{-5}$	$<5.4 \times 10^{-5}$	$<5.6 \times 10^{-8}$	n.t.	$<2.5 \times 10^{-4}$	n.t.	n.t.
4NQOEQ$_{others}$ (μg L^{-1})	$<2.0 \times 10^{-6}$	$<2.2 \times 10^{-5}$	$<2.5 \times 10^{-5}$	$<2.3 \times 10^{-5}$	$<3.1 \times 10^{-6}$		$<9.6 \times 10^{-5}$	n.t.	n.t.
AREc32 oxidative stress response assay									
tBHQEQ$_{water}$ (μg L^{-1})	32.4 ± 0.4	19.5 ± 7.0	<8.64	<8.64	<8.64	<8.64	73.3 ± 18.8	<8.64	<8.64
tBHQEQ$_{iceberg}$ (ng L^{-1})	219 ± 47	5.6 ± 1.5	5.6 ± 3.3	6.3 ± 1.4	0.15 ± 0.07	n.t.	25.7 ± 5.02	n.t.	n.t.
tBHQEQ$_{EDC}$ (ng L^{-1})	0.01 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.0007 ± 0.0004	0.005 ± 0.001	n.t.	0.05 ± 0.06	n.t.	n.t.
tBHQEQ$_{XRC}$ (ng L^{-1})	<0.08	<0.06	<29	<27	n.t.	n.t.	<110	n.t.	n.t.
tBHQEQ$_{antibiotics}$ (ng L^{-1})	<0.05	<0.01	<0.01	<0.01	n.t.	n.t.	<0.06	n.t.	n.t.
tBHQEQ$_{pesticides}$ (ng L^{-1})	1.75 ± 0.64	2.79 ± 0.75	4.07 ± 1.65	2.85 ± 0.56	0.003 ± 0.001	n.t.	10.0 ± 3.0	n.t.	n.t.
tBHQEQ$_{pharmaceuticals}$ (ng L^{-1})	<51	1.11 ± 0.76	2.75 ± 0.56	1.44 ± 0.47	0.002 ± 0.001	n.t.	14.6 ± 6.3	n.t.	n.t.
tBHQEQ$_{others}$ (ng L^{-1})	<0.01	0.43 ± 0.18	0.48 ± 0.22	0.46 ± 64.7	0.09 ± 0.03	n.t.	1.84 ± 0.71	n.t.	n.t.

n.t. = not tested.

removal process and only five chemicals were detected in the post RO sample, however, no chemicals were detected post-UV disinfection. In the post RO sample, low levels of the anticorrosive chemical tolytriazole, the plasticizer bisphenol A, the pharmaceutical triclosan and the pesticides MCPA and the pesticide degradation product 3,4-dichloroaniline were detected. Tolytriazole (Busetti et al., 2013; Loi et al., 2013) and bisphenol A (Water Corporation, 2013) were detected in previous monitoring programs but triclosan and the pesticides MCPA and 3,4-dichloraniline were detected in post RO water for the first time in this AWRP.

The chemicals' concentrations in post RO and post UV samples were below the Australian GVs for recycled water (NRMMC & EPHC & NHMRC, 2008). For comparison, the GVs are indicated in Fig. 2 by black bars. If at all, the concentrations exceeded the GVs for recycled water only in WWTP influent or RO reject. An exception was the pesticide MCPA, which exceeded the GV prior to the RO treatment but was two orders of magnitude below the GV in RO water, and was below detection in the post UV sample. Diatrizoic acid was also above GV up to the mixing tank but was below detection after RO.

The majority of detected chemicals fell into the group of pharmaceuticals with 34 out of 44 analyzed pharmaceuticals being detected in at least one sample (Fig. 2). Five pharmaceuticals (citalopram, desmethylcitalopram, cyclophosphamide, fluoxetine and propranolol) were not detected in WWTP influent due to increased LODs in the complex sewage matrix but were present in the WWTP effluent. In general, concentrations were significantly reduced during secondary treatment (Fig. 2) and nine pharmaceuticals (acetylsalicylic acid, acetaminophen, atorvastatin, cephalexin, ibuprofen, naproxen, ranitidine, salicylic acid and theophylline) were below detection limit after secondary treatment. Concentrations of carbamazepine, diclofenac, fluoxetine, gemfibrozil and indomethacin were very similar to previous studies (Busetti et al., 2009). Concentrations of pharmaceuticals remained fairly constant in the first steps of the AWRP because UF cannot efficiently remove organic micropollutants. RO reduced all chemicals to below detection except triclosan, which was detected for the first time at its LOD of 0.01 μg/L. In a previous study, clofibric acid, diazepam and naproxen had been occasionally detected but in less than 25% of the samples (Linge et al., 2012).

Of the EDCs, mainly xenoestrogens were quantified in this study as the previous monitoring had shown that the estrogens ethinyl estradiol, 17β-estradiol and estrone were always below detection (Van Buynder et al., 2009). In the present study, estrone levels of 5 ng/L in the WWTP effluent fell below detection limit thereafter. The surfactant 4-t-octylphenol was only detected in the WWTP influent. The plasticizer bisphenol A was also detected in the blanks. The concentrations of bisphenol A listed in the ESM, Table S3 represent the measured values minus the blank value and are therefore of high uncertainty but positive detections are consistent with previous work (Van Buynder et al., 2009).

Antibiotics were grouped separately from the pharmaceuticals because they are relevant for the formation of resistant bacterial strains. Secondary treatment greatly reduced the concentration of antibiotics with only erythromycin and sulfamethoxazole detected in the WWTP effluent. Concentrations remained stable during the first steps of the AWRP but RO efficiently rejected all antibiotics, which is again consistent with previous work (Busetti and Heitz, 2011; Linge et al., 2012; Busetti et al., 2013).

XRCs are good indicator compounds as they are frequently detected in fairly constant concentrations up to UF but are well removed by RO (Busetti et al., 2010), which was confirmed in the present study (Fig. 2).

Pesticides were generally well removed during treatment with only MCPA and 3,4-dichloraniline detected at very low levels. MCPA was detected at a concentration 50 times higher in the WWTP effluent than in previous work, therefore it is not astonishing that it was detected post RO in the present study, and not previously (Rodriguez et al., 2012; Busetti et al., 2013). Concentrations in WWTP effluent were similar to previous work for atrazine, 2,4-dichlorophenoxyacetic acid and simazine (Rodriguez et al., 2012).

The group of compounds called "others" was comprised of benzothiazoles, fragrance chemicals and flame retardants. 5-Methyl-1H-benzotriazole (tolytriazole) was the only chemical in this group detected at ng/L levels post RO, which is consistent with previous findings (Busetti et al., 2013; Loi et al., 2013). The fragrance chemicals were analyzed for the first time at this plant and while their concentrations were constant during the WWTP and the initial AWRP steps, RO removed them below detection (Fig. 2). Previously, galaxolidon a biological transformation by-products of the musk fragrance galaxolide, was detected in post RO and post UV samples at average concentrations of 31 and 19 ng/L, respectively.

From comparison of the chemical analysis with previous works as discussed above one can conclude that the grab samples taken for the present study are fairly representative and are suitable for bioanalytical assessment and mixture effect studies.

3.2. *Bioanalytical assessment*

The highest effect levels in all bioassays were observed in the WWTP influent and RO reject samples, the effects decreased along treatment train (Table 2 and ESM, Table S5). Apart from Microtox, effects were below detection limits post RO and post UV disinfection.

For the non-specific toxicity, the baseline-TEQ decreased from 26 mg/L in WWTP influent to 9 mg/L after secondary treatment (WWTP effluent). The levels remained low at 5–6 mg/L after ultrafiltration (post UF) and in the mixing tank between UF and RO. The baseline-TEQ was further reduced to less than 1 mg/L post RO and post UV to levels as low as the blanks (Table 2). These levels were similar to what was observed previously in this plant (Leusch et al., 2014a) (ESM, Figure S2A) and in another Australian AWRP (Macova et al., 2011; Escher et al., 2014a; Tang and Escher, 2013), which uses the same treatment processes (ESM, Figure S3A).

A consistent trend was observed in the PSII inhibition endpoint, the highest diuron equivalent concentration (DEQ) was observed in RO reject (0.09 μg/L) and the DEQ decreased along the treatment train from 0.07 μg/L in WWTP influent to 0.03 μg/L in WWTP effluent and 0.02 μg/L post UF and mixing tank (Table 2). The DEQs in post RO and post UV were below the detection limit of 0.004 μg/L. The EC were very similar to previous work (Leusch et al., 2014a) (ESM, Figure S2B),

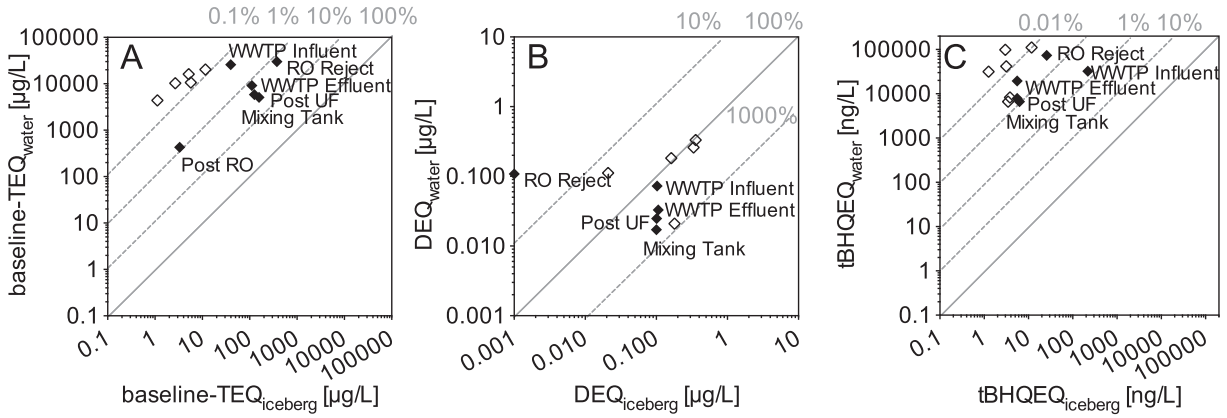

Fig. 3 – Contribution of detected chemicals for (A) non-specific toxicity as baseline-TEQ (Microtox), (B) DEQ (IPAM) and (C) oxidative stress response as tBHQEQ (AREc32). Filled diamonds represent experimental data from the present study, open diamonds represent reported data from other recycled water plants and surface water (Tang et al., 2013; Escher et al., 2014b).

although in the previous study EC_{20} not EC_{50} were measured and the DEQ levels were much lower than in another AWRP (Tang and Escher, 2013) but the removal efficiency by reverse osmosis was again similar (ESM, Figure S3B).

The *umuC* genotoxicity assay only gave responses when metabolism was not activated with metabolic enzymes. The only sample that was active after metabolic activation by rat liver S9 was the WWTP influent with a 2AAEQ of 2 µg/L. The results for 2AAEQs were therefore omitted from Table 2 as they were mainly non-detects. Without metabolic activation, the highest response in the *umuC* assay was found in WWTP influent and RO reject with a 4NQOEQ of 0.6 µg/L (Table 2). The 4NQOEQ levels decreased along the treatment train and were below the detection limit of 0.1 µg/L in post RO and post UV samples. A comparison of the $EC_{IR1.5}$ with previous work on the same AWRP (Leusch et al., 2014a) showed again consistent results (ESM, Figure S2C), although the secondary effluent still showed an effect after metabolic activation in the previous work while it was below detection limit in the present study.

For the oxidative stress response, the highest tBHQ equivalent concentration (tBHQEQ) was observed in the RO reject sample at 73 µg/L (Table 2). The tBHQEQ levels decreased along the treatment train from WWTP influent (32 µg/L) to post RO and post UV samples (<9 µg/L). Again a comparison with the other AWRP (Escher et al., 2013) revealed a consistent pattern of reduction, although the levels in the WWTP effluent were five times lower in the present study and the levels in the post-UV sample were slightly higher but in the same range as the blanks (ESM, Figure S3C).

3.3. *Contribution of known chemicals to the observed biological effects*

The effect concentrations of the designed iceberg mixtures are given in the ESM, Table S5, and the associated BEQ are listed in Table 2. The iceberg mixtures explained less than 3% of the observed cytotoxicity (Fig. 3A and Table 3). A smaller fraction of effect could be explained for WWTP influent as compared to the samples along the AWRP treatment train (Fig. 3A) and the fraction explained was not related to the number of chemicals detected (ESM, Figure S1). The fraction explained in WWTP effluent was similar to previous work (Tang et al., 2013), but larger fractions than in previous work were explained in the other samples (Fig. 3A).

In contrast, the photosynthesis inhibition was higher in the iceberg mixtures than in the samples (Fig. 3B and Table 3), which indicates that PSII-herbicides dominate the mixture effects toward algae, which had previously been confirmed for similar types of samples (Tang and Escher, 2013). The lower effects in the samples as compared to the iceberg mixtures can be rationalized by the fact that the chemical analysis was corrected for SPE recovery while for the bioassays the composition of the samples is unknown and one cannot correct for SPE recovery. While SPE recovery of pesticides is typically close to 100% (Escher et al., 2014b), any recovery lower than 100% will cause the effect of the icebergs appear to be higher than of the extracted samples.

The detected chemicals explained only 0.04%–0.7% of the observed oxidative stress response (Fig. 3C and Table 3), which

Table 3 – Fraction of BEQ explained by detected chemicals ($BEQ_{iceberg}/BEQ_{water}$).

Sampling site/treatment	WWTP influent	WWTP effluent	Post UF	Mixing tank	Post RO	RO reject
V. fischeri bioluminescence inhibition assay $Baseline\text{-}TEQ_{iceberg}/Baseline\text{-}TEQ_{water}$	0.2%	1.2%	3.1%	2.2%	0.8%	1.3%
IPAM photosynthesis inhibition assay $DEQ_{iceberg}/DEQ_{water}$	141%	323%	405%	581%	–	0.1%
AREc32 oxidative stress response assay $tBHQEQ_{iceberg}/tBHQEQ_{water}$	0.68%	0.03%	0.07%	0.09%	–	0.04%

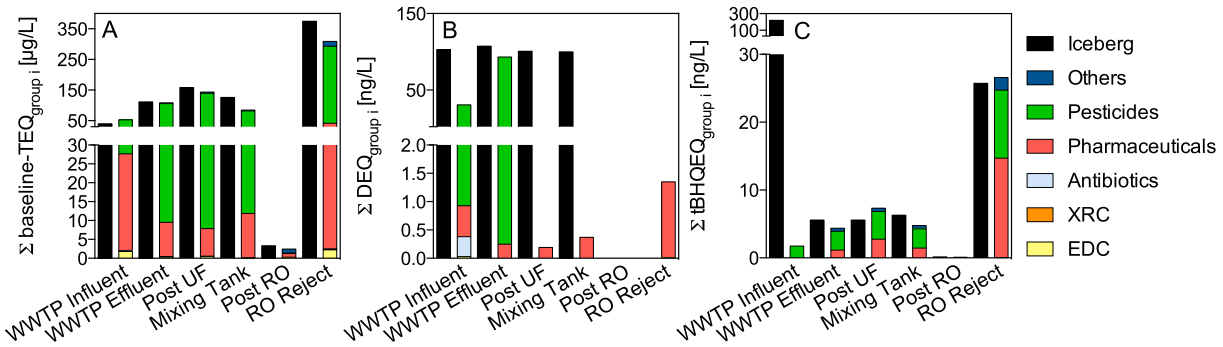

Fig. 4 – Cumulative bioanalytical equivalent concentrations of the iceberg mixtures in comparison with the cumulative BEQs of the six chemical groups: (A) non-specific toxicity (Microtox), (B) photosynthesis inhibition (IPAM), (C) oxidative stress response (AREc32).

was in the same order of magnitude as previous work (Escher et al., 2013). Interestingly the WWTP influent was an outlier with an unusual high fraction explained (0.7%), while for the cytotoxicity assay there was a remarkably low fraction explained (0.2%). This observation is presumably an artifact as the WWTP influent also had a high organic matter content and the detection limits of individual chemicals were higher, so that in some cases chemicals were below the LOD even though they were present in the WWTP effluent (ESM, Figure S1 and Table S3).

Overall, the fraction of BEQ explained by known chemicals was generally higher in this study than in the previous study (empty diamonds in Fig. 3). This can be explained by the fact that a higher number of chemicals were quantified in the present study than in the previous studies (Escher et al., 2013; Tang et al., 2013) and is likely not related to a different composition of the water samples.

3.4. *Contribution of individual chemical groups to the overall iceberg mixtures*

All individual chemical groups were tested in all bioassays. Positive responses were found only in the assays for cytotoxicity, photosynthesis inhibition and oxidative stress response and there was no response in the genotoxicity assay (Table 2).

Fig. 4 shows the cumulative BEQs of the six chemical groups in comparison with the experimental BEQ of the entire iceberg mixture. With one exception, the individual group BEQs summed up to the experimental BEQ of the entire iceberg mixture, which confirms the suitability of the experimental design and concentration addition of individual groups.

For the cytotoxicity endpoint, pesticides and pharmaceuticals had an equal share to the BEQ in the WWTP influent sample, while pesticides dominated in all other samples (Table 2, Fig. 4A). This is consistent with the general notion that many pesticides are more recalcitrant towards secondary treatment than many pharmaceuticals. Of the other four chemical groups only the EDCs had a minor contribution of 3% in the WWTP influent and 12% in the RO reject (Table 2). Post RO the BEQ levels were very low with pharmaceuticals and others dominating the BEQ.

As expected, the group of pesticides dominated the overall DEQ quantified in the photosynthesis inhibition assay (Table 2, Fig. 4B). Antibiotics contributed only 1% to the DEQ in the WWTP influent but were below detection limit thereafter. Pharmaceuticals contributed between 0.3% and 1.8% to the DEQ. Post RO, no photosynthesis inhibition was detected. In the RO reject the pharmaceuticals had a nominal contribution, which must be an artifact of the mixture calculations, which are extrapolations, as the iceberg mixture itself was not active.

For the oxidative stress response, there was generally a good agreement between the BEQ of the iceberg mixtures and the sum of the BEQ of the individual groups (Table 2 and Fig. 4C), with the exception of the WWTP influent sample where the pharmaceuticals were below detection limit, which is probably an extrapolation artifact and not real. In the remaining samples, the pesticides caused approximately 60% of tBHQEQ, the pharmaceuticals 30% and the others 10%, and these proportions did not vary much during treatment despite the overall tBHQEQ varying by more than ten-fold, indicating that there was no preferential removal for any group.

4. Conclusions

A previous study had compared, qualitatively, chemical analysis with *in-vitro* and *in-vivo* bioassays and found that treatment of wastewater in the investigated plant reduced chemicals as well as effects below the detection limit (Leusch et al., 2014a). The present study confirmed previous findings of Leusch et al. (2014a) and went a step further: for the first time chemical monitoring was linked with effect-based assessment in a quantitative manner and related to the individual groups of chemicals.

Mixture toxicity modeling applying the mixture model of concentration addition, which is valid for chemicals acting according to the same mode of action, confirmed previous findings that chemicals typically present in wastewater act concentration-additive in the applied bioassays (Escher et al., 2013; Tang et al., 2013; Tang and Escher, 2013). After this was confirmed, it was possible to quantify (a) which fraction of effect could be explained by the detected chemicals and (b) which groups of chemicals influenced or even dominated the mixture effects.

Although a total of 299 chemicals were screened and a higher fraction of biological effect could be explained than in previous studies (Escher et al., 2013; Tang et al., 2013), the detected chemicals explained less than 3% of cytotoxicity and less than 1% of oxidative stress response. As in earlier work (Tang and Escher, 2013), all responsible chemicals for photosynthesis inhibition were included in the analytical target list. This finding can be rationalized by the fact that pesticides explained the majority of this effect, which was not unexpected because the pesticide group contained several highly potent photosynthesis inhibitors such as diuron, hexazinone and simazine (ESM, Table S3). What was even more interesting is the novel finding that pesticides were also responsible for around two thirds of the effects of the iceberg mixtures in the cytotoxicity and oxidative stress response assays. Thus it appears that in addition to a focus on endocrine disruptors (Leusch et al., 2014a), pesticide monitoring is of high relevance despite the source water being of domestic origin and Australia having separate sewerage systems. This observation has implications for risk assessment and management. Given that even the most thorough chemical analysis could account for only a small fraction of the non-specific toxicity and adaptive stress response, we propose to always complement chemical monitoring with cell-based bioassays, which constitute efficient and high-throughput monitoring tools.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2014.04.043.

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