Benchmarking Organic Micropollutants in Wastewater, Recycled Water and Drinking Water with In Vitro Bioassays

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Supporting Information

Received: September 2, 2013

Revised: December 9, 2013

Accepted: December 10, 2013

Published: December 10, 2013

ABSTRACT: Thousands of organic micropollutants and their transformation products occur in water. Although often present at low concentrations, individual compounds contribute to mixture effects. Cell-based bioassays that target health-relevant biological endpoints may therefore complement chemical analysis for water quality assessment. The objective of this study was to evaluate cell-based bioassays for their suitability to benchmark water quality and to assess efficacy of water treatment processes. The selected bioassays cover relevant steps in the toxicity pathways including induction of xenobiotic metabolism, specific and reactive modes of toxic action, activation of adaptive stress response pathways and system responses. Twenty laboratories applied 103 unique *in vitro* bioassays to a common set of 10 water samples collected in Australia, including wastewater treatment plant effluent, two types of recycled water (reverse osmosis and ozonation/activated carbon filtration), stormwater, surface water, and drinking water. Sixty-five bioassays (63%) showed positive results in at least one sample, typically in wastewater treatment plant effluent, and only five (5%) were positive in the control (ultrapure water). Each water type had a characteristic bioanalytical profile with particular groups of toxicity pathways either consistently responsive or not responsive across test systems. The most responsive health-relevant endpoints were related to xenobiotic metabolism (pregnane X and aryl hydrocarbon receptors), hormone-mediated modes of action (mainly related to the estrogen, glucocorticoid, and antiandrogen activities), reactive modes of action (genotoxicity) and adaptive stress response pathway (oxidative stress response). This study has demonstrated that selected cell-based bioassays are suitable to benchmark water quality and it is recommended to use a purpose-tailored panel of bioassays for routine monitoring.

INTRODUCTION

The Tox21, a joint program of the National Institutes of Health, U.S. Environmental Protection Agency (EPA), and U.S. Food and Drug Administration¹ and the U.S. EPA ToxCast Program² aim to advance molecular toxicology, systems biology, and computational toxicology to overcome shortcomings of traditional *in vivo* toxicity testing of chemicals. Parallel initiatives exist in Europe, for example, the EU project ChemScreen.³ Jointly these programs bring a paradigm shift in toxicity testing as *in vitro* methods help elucidate mechanisms of toxicity, prioritize chemicals for further testing and develop predictive models in order to refine, reduce, or replace future *in vivo* testing. These programs rely heavily on high-throughput screening (HTS) using cell-based and cell-free *in vitro* bioassays of large numbers of chemicals to elucidate their toxicity pathways. While Tox21 focuses on the bioanalytical profiling of individual chemicals, these tools should also be applicable to environmental samples of unknown and complex composition, and this study brings together for the first time selected bioassays from Tox21 with established bioassays for water quality assessment.⁴

Cell-based bioassays have been developed to target all steps of the toxicity pathway (Figure 1).⁵ While the induction of xenobiotic metabolism may not lead to cytotoxicity, it is an indicator of the presence of pollutants. Some bioassays provide measures of mechanisms of toxicity by visualizing the interaction of stressors, for example, chemicals, with specific biological targets, for example, binding to endocrine receptors or reaction with DNA. The exposed cells may respond through induction of adaptive stress response pathways, a feature that can be used for the assessment of environmental pollutants, although it is not an adverse effect per se. Cell viability, growth and/or proliferation are indicators of adverse effects on a cellular level. If the cell represents a specialized tissue, this may give an indication of tissue-specific impairment.

Cellular responses will not always imply higher-level effects but are a prerequisite for whole organism and population responses.⁶ The direct detection of initiating key events in a

Cellular toxicity pathway:

Figure 1. Classification of *in vitro* bioassays according to cellular toxicity pathways (adapted from refs 4, 5, and 117).

bioassay only provides a measure of a potential adverse effect because repair and defense mechanisms may ultimately prevent toxicity. From a precautionary perspective, however, the potential to cause an adverse effect is a crucial assessment endpoint. Cell-based bioassays are not suitable to replace regulatory *in vivo* tests but provide hazard information for screening and prioritization of chemicals.⁷

A large variety of cell-based bioassays have been applied for water quality assessment in the past⁴ but many of the endpoints evaluated under HTS toxicology programs⁸ are yet to be included. Many studies rely on a small set of bioassays and each study uses different types of water samples, sample preparation methods, bioassays, and data evaluation methods. Only a few cell-based bioassays have standardized protocols, such as the OECD or ISO guidelines. The goals of this study were to evaluate as many bioassays as practically achievable using one set of water samples with one sample preparation method and to recommend a screening test battery for water quality testing.

We selected cell-based bioassays using three criteria. First, we selected bioassays that have previously been used for water quality assessment. Second, a comprehensive literature review allowed us to identify cell-based bioassays that responded to environmentally relevant organic micropollutants but had not been used for water quality assessment prior to this study. Third, for final selection of endpoints, we screened 25 nuclear receptors (NR) and 48 transcription factor (TF) response elements in HepG2 human liver carcinoma cell lines⁹ to ensure inclusion of endpoints relevant for the particular samples tested.

We evaluated 10 samples, including nine ambient water samples ranging from effluent, recycled water to drinking water, plus one procedural blank. The samples were extracted and concentrated with an optimized solid phase extraction (SPE) method and sent to a total of 20 worldwide laboratories applying 103 bioassays for bioanalytical testing. All experimental data were evaluated using a common method specifically developed for this study to harmonize the different approaches to data evaluation. The results were used not only to validate and compare the different bioassays for application with water samples but also to benchmark water quality. The data may also serve to compare the efficacy of different water treatment processes for removal of organic micropollutants.

It was not the goal of this study to directly compare bioassay protocols and performance of bioassays but rather to obtain an overview of the biological endpoints responsive to typical water contaminants. One goal was to cover the major health-relevant toxicity pathways introduced in Figure 1 and to evaluate which pathways were relevant for water quality testing. The study was further expected to identify the most robust and responsive bioassays, thus, not only mammalian but also bacterial assays were included. The outcome includes recommendations on the makeup of a screening test battery and on indicator bioassays that appear to be particularly relevant for further investigation in water quality monitoring programs.

MATERIALS AND METHODS

Samples. Ten grab samples of water were collected in December 2011 and January 2012 (Supporting Information (SI), Section S1 and Table S1). Sample Eff1 is a secondary treated sewage effluent (activated sludge treatment) that serves as influent to a Water Reclamation Plant (WRP) that produces high quality recycled water for indirect potable reuse. Three samples were taken at different stages of treatment: after microfiltration (MF), reverse osmosis (RO) and advanced oxidation (AO) using $\text{H}_2\text{O}_2/\text{UV}$. Bioanalytical assessments had been previously undertaken at this WRP.^{10,11}

The second investigated WRP treats secondary sewage effluent (Eff2) by ozonation followed by biologically activated carbon filtration (O_3/BAC) to produce recycled water for irrigation and industrial usage. The fate of micropollutants in this plant has been previously characterized in more detail.^{12–14}

River water (RW) and drinking water (DW) samples were collected at the inlet and outlet of a metropolitan drinking water treatment plant applying chlorination and chloramination, which was also previously assessed with bioanalytical tools.^{10,15}

The stormwater sample (SW) was collected from a stormwater drain in Brisbane, Australia, that receives runoff from a residential catchment.¹⁶ The laboratory blank consisted of ultrapure water (Milli-Q water) run through the same SPE process as the samples. Fourteen liters were collected for each of Eff1, MF, Eff2, and SW, while 28 L were collected for the remaining samples.

Sample Preparation and Distribution to the Participating Laboratories. The SPE was performed according to Macova et al.¹⁰ using the sorbent materials Oasis HLB (Waters) followed by Supelclean coconut charcoal (Sigma-Aldrich), a combination that was confirmed previously to extract a broad range of organic micropollutants.¹⁷ Details and information on sample preparation and the logistics of sample distribution are summarized in the SI, Section S1. All distributed samples were labeled with codes for blind sample processing.

Sample Characterization. 293 organic micropollutants were previously characterized in these samples.¹⁸ Dissolved organic carbon concentrations are reported in the SI, Table S1.

Bioassays. The majority of selected bioassays was based on mammalian, bacterial or yeast cells. A zebrafish embryo test was included because fish embryos are considered as nonprotected life stages and as ethically accepted alternative to the testing of (adult) animals.¹⁹ Only one bioassay employed a naked enzyme (acetylcholinesterase inhibition assay).

All applied bioassays and their associated experimental methods are listed in Table 1 and categorized according to the toxicity pathways outlined in Figure 1. For bioassays where the protocol was modified or applied for the first time, Section S2 in the SI and Table S2 give additional information on the experimental procedures.

Concentration-Effect Assessment. A critical aspect when working with diverse biological endpoints is a consistent data evaluation process. It was the goal of the present study to harmonize the data evaluation as much as possible, which is challenging given the different types of endpoints measured but a prerequisite for quantitative comparison between different bioassays.

The concentrations of samples were expressed in units of relative enrichment factor REF. The REF is the product of the enrichment factor of the SPE process and the dilution of the extract in the bioassay (for derivation of equations, see SI, Section S3). A REF > 1 means that the sample is enriched in the bioassay (e.g., a REF of 10 means the sample was concentrated 10-fold in the bioassay), a REF < 1 means it was diluted in the bioassay, and a REF of 1 is equivalent to the organic micropollutants in the ambient (undiluted and unconcentrated) sample stripped from inorganics, metals and most colloidal organic matter by SPE.

For each bioassay, the measured responses were plotted against the sample concentration expressed as REF (Figure 2). For assays with a known maximum response, responses were converted to percent of maximum effect. For all endpoints that relate to cell viability and cell population growth, the controls can be expressed as 0% effect, while 100% relates to “no growth” or “all cells dead”. For reporter gene assays that measure the binding to a receptor or the transactivation of a receptor, 0% refers to the basal activity of the receptor, while 100% is defined using an appropriate reference compound that can saturate the receptor without causing cytotoxicity.

The ideal case would be a full concentration-effect curve covering 0% to 100% of effect (Figure 2A), which typically has a sigmoidal shape that can be described with a log-logistic equation. Any log-logistic concentration-effect curve will be linear with respect to (nonlogarithmic) concentrations at low effect level (up to 20–30% effect). As the water samples investigated in the present study often showed only very low effect levels, the linear form of the concentration-effect curves was used for derivation of the effect concentration causing 10% of maximum effect (EC_{10}) of the samples

Table 1. Bioassays Used and Their Toxicity Pathway Classifications^a

#	laboratory	bioassay	literature reference for method development	experimental approach (literature reference/more information in SI)
Xenobiotic Metabolism				
1	ATG	PXR-cisFACTORIAL	46	9
2	ATG	PXR-transFACTORIAL	46	9
3	IRCM	HG5LN PXR	47	48
4	ATG	CAR-transFACTORIAL	46	9
5	CAPIM	CAR-yeast	49	see SI, Section S2-A.
6	ATG	PPAR α -transFACTORIAL	46	9
7	ATG	PPAR γ -transFACTORIAL	46	9
8	IRCM	HELN-PPAR γ	50	28
9	BDS	CALUX-PPAR α	51	52
10	BDS, CSIRO	CALUX-PPAR γ 2	53	52
11	HK	MCF7-PPAR	54	54
12	GU	PPAR γ -GeneBLAzer	27	55
13	GU	Anti-PPAR γ -GeneBLAzer	27	see SI, Section S2-B.
14	CAPIM	AhR-yeast	56	57 and SI, Section S2-B.
15	UQ, RECETOX	CAFLUX	58	RECETOX: 59 UQ: 60
16	RECETOX	H4IIEluc	61	59
17	HK	MCF7DRE	54	54
18	ATG	AhR-cisFACTORIAL	46	9
19	UFZ	DART cyp1a induction	62	63
Specific Modes of Action				
20	UQ, SWISS	algae photosynthesis inhibition	31	64
21	UQ	acetylcholinesterase inhibition	65	30
Specific MOA: ER				
22	GU, CSIRO, BDS, IWW	ER-CALUX	66	33
23	UQ	E-SCREEN	67	10
24	SWISS, CSIRO, UA	YES	68	69
25	CAPIM	hER yeast	49	70
26	CAPIM	medER yeast	49	70
27	IRCM	HELN-ER α	71	72
28	IRCM	HELN-ER β	71	73
29	ATG	ERE-cisFACTORIAL	46	9
30	RECETOX	hER α -HeLa-9903	74	74
31	HK	MCF7-ERE	54	54
32	ATG	ER α -transFACTORIAL	46	9
33	NJU	Steroidogenesis (estrogens)	75	76
34	UFZ	DART cyp19a1b	77	63
35	UF, USF, UCR, SCCWRP	ER α -GeneBLAzer	27	55 see SI, Section S2-C.
36	CSIRO, GU	Anti ER-CALUX	66	17
Specific MOA: AR				
37	GU, BDS, CSIRO	AR-CALUX	66,78	33
38	IRCM	HELN-AR	79	80
39	HK	MCF7-ARE	54	54
40	UA, CSIRO	YAS	81	82
41	UF, USF, UCR, SCCWRP	AR-GeneBLAzer	27	55 see SI, Section S2-C.
42	ATG	AR-transFACTORIAL	46	9
43	RECETOX	MDA-kb2	83	84
44	RECETOX	Anti-MDA-kb2	83	84
45	CSIRO, GU	Anti-AR-CALUX	66,78	17
Specific MOA: GR				
46	GU, BDS, CSIRO	GR-CALUX	66	33
47	UA	GR Switchgear		see SI, Section S2-D.
48	ATG	GR-transFACTORIAL	46	9
49	RECETOX	GR-MDA-kb2	83	84
50	GU, UF, USF, SCCWRP	GR-GeneBLAzer	27	55
51	GU	Anti-GR-GeneBLAzer	27	
52	GU	Anti-GR-CALUX	66	33
Specific MOA: PR				
53	UF, USF, UCR, SCCWRP	PR-GeneBLAzer	27	55 see SI, Section S2-B.
54	GU, BDS, CSIRO	PR-CALUX	66	33

Table 1. continued

#	laboratory	bioassay	literature reference for method development	experimental approach (literature reference/more information in SI)
Specific MOA: PR				
55	GU	Anti-PR-CALUX	66	
56	NJU	steroidogenesis (progesterone)	75	76
57	NJU	steroidogenesis (17 α OH-progesterone)	75	76
Specific MOA: TR				
58	BDS, GU	TR-CALUX	66	17
59	UQ	T-SCREEN	85	
60	ATG	THR α 1-transFACTORIAL	46	9
61	IRCM	HELN-TR		86
Specific MOA: Reproductive and Developmental Effects				
62	HK	MCF7-RARE	54	54
63	UQ	P19/A15	87	see SI, Section S2-E.
64	ATG	ROR β -transFACTORIAL	46	9
65	CAPIM	hRAR-Yeast Assay	49	88
Reactive MOA				
66	UQ, RCEES	umuC TA1535/pSK1002	89	UQ: 90 RCEES: 89
67	UQ	umuC TA1535/pSK1002 +S9	89	90
68	RCEES	umuC NM5004	91	89
69	RECETOX	SOS chromotest	92	93
70	UA, IWW	Ames TA98	94	IWW: 95
71	UA, IWW	Ames TA98+ S9	94	IWW: 95,96
72	UA	Ames TAmix	94	
73	UA	Ames TAmix +S9	94	
74	UQ, IWW	Ames TA100	94	UQ: 97 IWW: 95
75	AWQC	micronucleus assay	98	99
76	CSIRO	ROS formation RTG2	100	101
77	UQ	protein damage <i>E.coli</i>	102	38
Adaptive Stress Response				
78	ATG	HSE-cisFACTORIAL	46	9
79	UFZ	hspb11 induction DART	63	63
80	ATG	HIF-1 α -cisFACTORIAL	46	9
81	UA	Hypoxia-Switchgear		see SI, Section S2-F.
82	ATG	NF-kB-cisFACTORIAL	46	9
83	UQ	NF-kB-Geneblazer	27	55 see SI, Section S2-C.
84	BDS	NF-kB-CALUX	103	33
85	GU	Jurkat E6.1 IkB	none	see SI, Section S2-G.
86	UQ	AREc32	104	39
87	UA	Nrf2-keap	105	see SI, Section S2-H.
88	ATG	Nrf2/ARE-cisFACTORIAL	46	9
89	BDS	Nrf2-CALUX	106	52
90	ATG	p53-cisFACTORIAL	46	9
91	BDS	p53-CALUX	106	52
92	BDS	p53-CALUX +S9	106	52
93	UF	p53-GeneBLazer	27	55 see SI, Section S2-C.
Cytotoxicity and Indicators of System Response				
94	UQ	AREc32 cell viability	39	39
95	GU	Caco 2 NRU	107	17 see SI, Section S2-I.
96	CSIRO	RTG2MTT	108	
97	UFZ	DART 48h lethality	109	110
98	UFZ	DART 120h sublethal	111	110
99	GU	SK-N-SH cytotoxicity	112	see SI, Section S2-J.
100	GU	THP1 cytokine	113	17 see SI, Section S2-K.
101	UQ	algae growth inhibition	114	64
102	UQ, SWISS	<i>Vibrio fischeri</i> (Microtox)	115	SWISS: 69 UQ: 18
103	RCEES	<i>Photobacterium phosphoreum</i>	116	

^a Assay numbers (#) are equivalent to the numbers in Figure 3. Literature references are provided for the method development and for how the assay was performed. Modifications of the assay methods are summarized in the Supporting Information, SI, Table S2.

(Figure 2C). A detailed derivation of EC₁₀ is provided in the SI, Section S3-A.

For assays where the maximum response was unknown or unachievable, the responses were normalized to growth medium

Figure 2. Overview of the concentration-effect models applied to derive benchmark effect concentrations (EC).

controls (including the same percentage of solvent as in the corresponding samples) and assessed via the induction ratio (IR; that is, fold induction relative to control). The maximum response was unknown if no appropriate reference compound existed, or if cytotoxicity quenched the reading of the reporter activity. The problem also arose if the endpoint is inducible or, by nature, there is no clear upper limit, for example, for DNA damage or adaptive stress response. In practice, linear regression through the control effect level ($IR = 1$) was used for derivation of the effect concentration that causes an IR of 1.5, or $EC_{IR1.5}$ (Figure 2D). A detailed derivation of $EC_{IR1.5}$ is provided in the SI, Section S3-B together with a discussion of the comparability of EC_{10} versus $EC_{IR1.5}$ (SI Table S3).

A bioassay can be run in antagonistic mode if the receptors are occupied with a constant concentration of a known and potent agonist. Varying concentrations of sample are added and if the signal of the control is suppressed the sample can be considered to exhibit an antagonistic effect. The effect concentration causing a suppression ratio SR of 0.2, $EC_{SR0.2}$, was used to describe all antagonistic effects and correspondingly an $EC_{CD0.2}$ was defined for endpoints that are based on chaperone dissociation (e.g., I κ B dissociation from NF κ B). A detailed derivation is provided in the SI, Section S3-C and Figure S1.

Data Presentation. A heatmap presenting all measured EC values was generated using the R Software package gplots (www.r-project.org/). Hierarchical clustering was performed using the “complete linkage” method to find similar clusters of water samples.

RESULTS

Repeatability of Bioassays. A number of bioassays were performed simultaneously in multiple laboratories. As discussed in detail in the SI, Section S4 and Table S4, the results were consistent between laboratories and therefore the results of the same bioassays were averaged.

Initial Screening of Nuclear Receptors and Transcription Factors. The FACTORIAL bioassays⁹ were used for an initial profiling of the water samples after enrichment by SPE to a REF of 4. As discussed in the SI, Section S5 and Figure S2, an IR of 1.5 is regarded as the threshold for positive effects. In the SI, Figure S3, the activity profiles of all samples are depicted.

The blank did not induce any of the tested endpoints indicating that the sample extraction and enrichment process did not negatively influence the test outcome. The Eff1 and Eff2 samples caused an activation of five of 25 nuclear receptors (NR) and 5 of 48 transcription factors (TF) tested (SI Figure S3A and C). The active NRs were the pregnane X receptor (PXR), the peroxisome proliferator-activated receptor (PPAR γ), the estrogen receptor (ER α) and marginally the glucocorticoid receptor (GR) and the liver X receptor (LXR). The active TFs were related to the aryl hydrocarbon receptor (AhR), PXR, the oxidative stress response (nrf2/ARE), the estrogen response element (ERE), and the RAR-related orphan receptor (ROR).

The screening provides strong support to expand the test battery to include additional endpoints to those routinely employed.⁴ Particularly the PXR and AhR, which are related to xenobiotic metabolism, warrant more attention as these had the highest activity in the prescreening assay. The LXR is relevant as its activation induces the PPAR.²⁰ The PPAR pathway is related to obesity²¹ and has gained much attention in recent years and therefore various PPAR-related endpoints have been included in the test battery if not specifically LXR. The oxidative stress response pathway appeared to be of high relevance and has rarely been investigated with water samples prior to this study.

In response to the findings of this screening, the active three NRs and five TFs and two others associated to relevant pathways (CAR, PPAR α , AR, GR, THR α 1, ROR β , HSE, HIF1 α , NF κ B, p53) were included in the detailed dose–response analysis.

Responsiveness of the Bioassays. Figure 3, Table 2, and Table S5 in the SI give an overview of all results of the 101 different bioassays tested plus the two bacterial cytotoxicity assays.

Figure 3. Summary of results in 101 bioassays (excluding inactive FACTORIAL and the bacterial cytotoxicity assays). Plotted are the effect concentrations (EC_{10} , $EC_{IR1.5}$, or $EC_{SR0.2}$) in units of REF (relative enrichment factors). The colors encode for the magnitude of the EC. Green stands for high effect concentrations (low potency) and transitions to red for low effect concentrations (high potency). Dark green are EC values that were >30 REF (which means that the sample that is enriched 30 times still does not show an effect), green from 10 to 30 REF, light green from 3 to 10 REF. A sample that has its EC at concentrations of the native sample up to three times enriched is denoted in yellow. Samples that have to be diluted for the EC are orange for up to 3 times diluted (REF 1 to 0.3) and red for over 3 times diluted. Numbers on the right refer to bioassay numbers in Table 1.

A positive response is defined here as $EC_{IR1.5}$ or EC_{10} lower than the highest tested REF, thus no extrapolations were performed.

The first two questions that we have to answer to judge the suitability of bioassays for water quality assessment are (A) “do “polluted” samples induce a response?” and (B) “is the response acceptably low in control samples?” (A) Sixty-five bioassays showed a response in at least one of the water samples. For Eff2, which can be considered as a moderately “polluted” sample, the number of positive results was 60. (B) No solvent blank caused any effect and the procedural blank with ultrapure water only produced effect in five bioassays (4.9%).

Procedural Blank. Even small impurities leaching out of the material or present in the solvent would likely contribute to the nonspecific effect of the blank. Here we applied two different SPE sorbent materials (HLB and coconut charcoal), which were eluted separately and required twice the amount of solvent. This consideration rationalizes the low but positive results of the blanks in the two bioluminescence inhibition assays with marine luminescent bacteria, which were higher than previously seen when only one type of solid-phase material were applied.¹⁰

Furthermore, the yeast-based assays AhR-yeast and CAR-yeast showed responses in the blanks but only at much higher REF

Table 2. Summary of Responsive and Non-Responsive Bioassays (Total = Number of Bioassays, In Parentheses Number of Replicates), + = Number of Positive Responses, – = Number of Negative Responses

toxicity pathway	MOA	inducing chemicals/ positive controls	total	+	–	positive response	negative response
xenobiotic metabolism	pregnane X receptor (PXR)	steroids/phenobarbital, various pharma-ceuticals	3	3	0	PXR-cisFACTORTIAL, PXR-transFACTORTIAL, HGSLN PXR	-
	constitutive androstane receptor (CAR)	phthalates, fibrate pharmaceuticals	2 (1)	1	1	CAR-yeast	CAR-transFACTORTIAL
	peroxisome proliferator-activated receptor (PPAR)		7 (1)	2	5	PPAR γ -transFACTORTIAL, HELN-PPAR γ	PPAR α -transFACTORTIAL, CALUX-PPAR α , CALUX-PPAR γ , PPAR γ GeneBLAzer, MCF7-PPAR
	PPAR suppression		1	0	1	-	Anti-PPAR γ GeneBLAzer
specific MOA	aryl hydrocarbon receptor (AhR)	PAHs, PCDDs, coplanar PCBs	6 (1)	6	0	AhR-yeast, CAFLUX, H4IIELuc, MCF7-DRE (transient), AhR-cisFACTORTIAL, DART cyp1a induction	-
	acetylcholinesterase (AChE)	insecticides	1	0	1	-	AChE enzyme inhibition
	photosystem II	herbicides	1 (1)	1	0	IPAM	-
specific receptor-mediated MOA	estrogen receptor (ER)	human hormones and industrial chemicals (xenoestrogens), 17 β -estradiol	14 (9)	14	0	ER-CALUX, E-SCREEN, YES, HELN ER α , HELN ER δ , ERE-cisFACTORTIAL, hER α -HeLa-9903, MCF7-ERE, ER α -transFACTORTIAL, Steroidogenesis, DART cyp19alb (aromatase), ER-GeneBLAzer, hER yeast, medER yeast	-
	ER suppression	4-Hydroxy-tamoxifen	1 (1)	1	0	Anti-ER-CALUX	-
	androgen receptor (AR)	(Dihydro)-testosterone	7 (6)	1	6	MDA-kb2 (but coexpression with GR)	AR-CALUX, HELN-AR, MCF7-ARE, Yeast Androgen Screen (YAS), AR-GeneBLAzer, AR-transFACTORTIAL
	AR suppression	Flutamide	2 (1)	2	0	Anti-AR-CALUX, anti MDA-kb2	PR-CALUX, PR-GeneBLAzer
reactive modes of action	progesterone receptor (PR)	Levonorgestrel	4 (5)	2	2	Steroidogenesis, induction of progesterone and of 17 α OH-progesterone	-
	PR suppression	Mifepristone	1	1	0	Anti-PR-CALUX	-
	glucocorticoid receptor (GR)	Dexamethasone	5 (6)	5	0	GR-CALUX, GR Switchgear, GR-transFACTORTIAL, GR-MDA-kb2 (AR suppressed), GR-GeneBLAzer	-
	GR suppression	Mifepristone	2	2	0	Anti-GR-CALUX, anti-GR-GeneBLAzer	-
	thyroid receptor (TR)	3,3'-5-Triiodo-L-thyronine	4 (1)	0	4	-	TR-CALUX, T-SCREEN, THR α 1-transFACTORTIAL, HELN-TR
	RAR/RXR (Reproductive and developmental effects)	Retinoic acid	4	1	3	hRAR-yeast assay	MCF7-RARE, P19/A15, ROR δ -transFACTORTIAL,
	genotoxicity	4-Nitroquinoline-N-oxide	11 (4)	11	0	umuC $\pm$ S9, SOS chromotest, Ames $\pm$ S9, micronucleus assay	-
	oxidative stress	PAHs, electrophilic chemicals, <i>t</i> -butyl hydroquinone	1	1	0	oxidative stress in RTG2 cells	-
	protein damage	Sea-Nine	1	0	1	-	protein damage <i>E.coli</i> GSH $\pm$
	heat shock response	oxygen depletion (can be caused by metals)	2	0	2	-	HSE-cisFACTORTIAL, hspb11 induction in DART after 120h
adaptive stress response pathway	hypoxia	tunicamycin, caplain	2	0	2	-	HIF-1 α -cisFACTORTIAL, Hypoxia-Switchgear
	endoplasmic reticulum stress	high salt, glycol	0	0	0	-	-
	inflammation	metals, PCBs, smoke, particles	4	1	3	Jurkat E6.1 IkB	NF-kB-CALUX, NF-kB-GeneBLAzer, NF-kB-cisFACTORTIAL
	oxidative stress	reactive oxygen species, <i>t</i> -butyl hydroquinone	4	3	1	AREc32, Nrf2/ARE-cisFACTORTIAL, Nrf2-CALUX	Nrf2-keap

Table 2. continued

toxicity pathway	MOA	inducing chemicals/ positive controls	total	+	-	positive response	negative response
	DNA damage	electrophilic chemicals, UV radiation, <i>nulcin-3</i>	4 (1)	0	4		p53-cisFACTORIAL, p53-CALUX-S9, p53-CALUX +S9, p53-GeneBLAzer
cytotoxicity indicative of system response	bacterial cytotoxicity	all	2 (1)	2	0	<i>Vibrio fischeri</i> (Microtox), <i>Photobacterium phosphoreum</i>	-
	algal growth	all (+herbicides)	1 (1)	1	0	algae growth inhibition	-
	cytotoxicity (mammalian cells)	all	4	4	0	ARE32 cell viability, Caco 2 NRU, RTG2MTT, DART 48h lethality	-
	development	all	1	1	0	DART 120h sublethal	-
	neurotoxicity	insecticides	1	0	1		SK-N-SH cytotoxicity
	immunotoxicity	immunosuppressive chemicals	1	0	1		THP1 cytokine

than the samples. A positive blank value was observed in one of the various Ames assays and is most likely due to measurement uncertainty as this value was derived from only one data point.

Bacterial Cytotoxicity Screening Assays. The bacterial cytotoxicity assays with *Vibrio fischeri* and *Photobacterium phosphoreum* were used as quick bioanalytical assessment tools, as these tests are rapid with only 15–30 min of exposure. Bioluminescence tests are nonspecific assays, as all stressors can impair the energy production and thus decrease bioluminescence. They provide a high responsiveness, that is, often the indicate effects for the “diluted” sample at REFs of less than 1. However, the high sensitivity if compared to other cellular assays may result from a higher bioavailability, that is, absence of serum proteins typically used in in vitro assay. This increased bioavailability could result in a detection of trace amounts of coextracted dissolved organic carbon (DOC). In particular the low-molecular fraction of assimilable organic carbon can add to the observed effects as previously demonstrated.²² However, the high sensitivity may also result from specific interactions with bacterial physiology. Therefore, and because the control sample exhibited some effects as well, the luminescence assays have been excluded from the heatmap (see below).

Heatmap. The summary of 101 EC values (excluding the two bacterial cytotoxicity assays) in each of the 10 samples is presented in form of a heatmap (Figure 3, data summarized in SI, Table S5). The similarity of bioanalytical fingerprints between different water samples was characterized by hierarchical clustering.

Evidently, quantitative comparison is difficult because EC were expressed as EC₁₀ or EC_{IR1.5} and these two values are only directly comparable if the maximum IR is around 6 (see discussion in SI, Section S3-B). Therefore hierarchical clustering was only performed on samples and not on bioassays.

The closest similarity existed between the blank and the highly treated AO sample, while the RO sample clustered with this group on the next level of hierarchy (Figure 3). Surface water and ozonated recycled water clustered together. Both of these groups (cluster RW + O₃/BAC and cluster RO + Blank + AO) clustered closely on the next level of similarity. Of the more polluted samples, Eff1 and MF were highly similar. This is not unexpected, as microfiltration, the only treatment step separating the two samples, is ineffective at removing micropollutants. Slightly higher effects were, however, observed in the MF sample likely due to disinfection by chloramination of the membrane to avoid biofouling.¹⁰ On the next level of hierarchical clustering, the two WWTP effluents Eff1 + MF and Eff2 showed high similarity.

The largest separation was observed between the cluster of Eff1 + Eff2 + MF and all other samples (Figure 3), clearly demonstrating that cell-based bioassays can distinguish between wastewater and reclaimed water samples.

The bioanalytical fingerprints can also help distinguish between different water types: WWTP effluents not only showed the highest effects but also distinct responses related to known environmental pollutants, including pesticides, industrial chemicals, pharmaceuticals, and personal care products, for example, the activation of the aryl hydrocarbon receptor by PAHs or methylmercaptoaniline,²³ the activation of the estrogen receptor by natural hormones and xenoestrogens, the activation of the glucocorticoid receptor by dexamethasone and organotin compounds,²⁴ or photosynthesis inhibition by herbicides. The specific effects, caused by chemicals that bind to receptors, were decreased substantially in the WRPs.

Stormwater had a slightly different pattern to WWTP effluents but was also dominated by pesticides, as represented for example by herbicidal activity that was absent in other samples of its cluster.

In other studies on stormwater one could even identify sewer cross-contamination by bioanalytical profiling.¹⁶

In contrast, disinfection by chlorination lead to disinfection byproducts, which showed distinct bioassay response patterns with clearly increased genotoxicity and oxidative stress responses in DW as compared to its source water (RW), while specific-receptor mediated effects were low in RW and almost fully disappeared in DW (Figure 3). This is consistent with previous bioanalytical profiling of the drinking water treatment process.¹⁵

■ DISCUSSION

Multiplexed Assays As a Screening Tool. The FACTORIAL assay was applied here for the first time to water samples and yielded interesting fingerprints of effects that were consistent with the results of the other bioassays. However, more work is required to implement reference chemicals and include a more rigorous dose–response assessment. The effect fingerprints were qualitatively consistent with responses seen in the initial ToxCast I screening of 320 pesticides, where 73% of the pesticides were responsive in PXR, 52% in Nrf2/ARE and 46% in PPAR γ .⁹

Benchmarking Water Quality. A detailed discussion of the responses of each bioassay is provided in the SI, Section S6. Here follows a summary of the responsive and nonresponsive endpoints in relation to the associated step in the toxicity pathway (Table 2). It should be noted that, although responsiveness is related to assay sensitivity, even the most sensitive assay will not respond in the absence of chemicals capable of activating the bioassay endpoint. In other words, the absence of a measurable effect does not necessarily prove an assay insufficiently sensitive. Relative assay sensitivity can be assessed by comparing the effect concentrations and limits of detection of reference chemicals between assays. While such comparison was not possible for all assays tested in the present study, the results obtained can give some indication regarding the suitability of bioassays for monitoring purposes.

Induction of Xenobiotic Metabolism Pathways. Induction of metabolic pathways is not per se an indicator of toxicity but gives an indication of exposure to bioactive chemicals. Metabolism can detoxify or bioactivate chemicals. Omiecinski et al.²⁵ stressed the relevance and the toxicological implications of a number of xenobiotic metabolism pathways and associated NR, including the PXR, PPAR α , β and γ , AhR, and CAR.

Three and six bioassays were evaluated for the PXR and AhR, respectively, and all showed positive responses in less treated samples and negative responses in recycled water and the blank (Table 2, SI, Section S6-A, Figure S5).

CAR plays a role in both phase I and II metabolism and plays a protective role against toxicity induced by bile acids as well as regulation of physiological functions. The target chemicals for CAR are less clearly defined than for AhR and while a few pesticides (e.g., methoxychlor, carbaryl propazine, 6-deisopropylatrazine) induced the CAR in the CAR-trans-FACTORIAL assay in previous work,⁹ no response was detected in the water samples in the CAR-trans-FACTORIAL assay up to an REF of 4. In contrast, the CAR-yeast showed a response in all samples (EC_{IR1.5} from 0.1 to 9.4) (Table 2, SI, Section S6-A, Figure S5).

For PPAR, only two of seven bioassays (PPAR γ -trans-FACTORIAL and HELN-PPAR γ) gave signals in the four most polluted samples (Table 2, SI, Section S6-A, Figure S5). PPAR is strongly linked to the regulation of glucose and lipid metabolism as well as inflammation, and is less important for xenobiotic metabolism.²⁶ In a high throughput study of 3000 environmentally relevant chemicals, roughly 1% of the tested chemicals were PPAR γ agonists and 8% were PPAR γ

antagonists.²⁷ Organotins²⁸ and polyhalogenated bisphenol A²⁹ were found to induce PPAR α and γ . The higher activity of PPAR γ over PPAR α for water samples is consistent with the finding that 146 of 309 ToxCast phase I chemicals were active in PPAR γ -transFACTORIAL, while the other isoforms were less responsive.⁹

Specific Modes of Toxic Action. Most specific modes of action involve binding to receptors or inhibition of enzymes. In the past, direct enzyme inhibition assays have been popular tools for water quality testing. Recent work on the influence of dissolved organic matter (DOM) on the acetylcholinesterase assay has demonstrated that DOM nonspecifically impacts the assay at relatively low concentrations.³⁰ The implication of these findings is that for most tests with naked enzymes, water samples cannot be concentrated above a REF of 2. In the present study, only the two wastewater samples produced a valid response in this assay. Despite the high relevance of this biological endpoint for many insecticides, it thus proves unsuitable to investigate recycled water samples.

Photosynthesis Inhibition. An important group of environmental contaminants are herbicides that inhibit photosynthesis. While they are specifically designed to target photosynthesis inhibition, herbicides can nevertheless be toxic to humans and are regulated in recycled and drinking water guidelines. The most sensitive species to detect herbicides are algae, for which the inhibition of photosystem II by triazines and phenylurea herbicides can be specifically measured by pulse-amplitude modulated fluorometry.³¹ This endpoint was very responsive in the water samples Eff1, Eff2, MF, SW that were suspected to contain herbicides (SI, Table S5).

Estrogen Receptor. The most relevant receptor-mediated effects are related to endocrine disruption (SI, Section S6-B, Figure S6). Estrogenic effects are by far the most prominent and environmentally relevant endocrine effects for aquatic species but they are overshadowed by other endocrine endpoints when it comes to human health. Fourteen different bioassays indicative of estrogenic effects were evaluated and all were active in four to five samples (Table 2). The absolute responsiveness was highest for ER-CALUX and MCF7-ERE but the effect pattern across the different samples was similar for all bioassays (SI, Table S5). No antiestrogenic activity could be detected in any of the samples, which is typical for samples that contain estrogenic chemicals.¹⁷

Androgen Receptor. Of seven bioassays (bioassay nos. 37–43, Table 1), only the MDA-kb2 produced positive results in the wastewater samples. Both GR and AR are expressed in this cell line and they share the same DNA response element, so it is unclear if the activity in this assay is purely AR-mediated, although incubation with flutamide indicates that the contribution of AR to the overall effect is higher than of GR.

Both bioassays for antiandrogenicity (anti-AR-CALUX and anti-MDA-kb2) were positive in some samples although only at very high REF. The WWTP effluents, which typically have highest antiandrogenic effects, were not responsive in anti-MDA-kb2, presumably due to the interfering agonistic response of effluent, similar to what has been observed with YAS and anti-YAS in an earlier study.³²

Progesterone Receptor. The two transactivation assays for the PR, PR-CALUX, and PR-GeneBLAzer, did not exceed the 10% effect threshold in all samples. However, progestagenic activity has been detected previously in aquatic samples.^{17,33,34} The anti-PR (anti-PR-CALUX) assay was also negative with samples tested to a REF of 2. The increased levels of progesterone and 17 α -hydroxyprogesterone in the H295R bioassay

for steroidogenesis were most likely due to an inhibitory effect on CYP21A.

Glucocorticoid Receptor. ER, AR, and PR are important for the development and functioning of the reproductive system. The GR is more abundant and found in all cell types. Given that the GR has important functions in glucose metabolism and the immune feedback mechanisms, it has been linked to a wide spectrum of diseases, including cardiovascular, inflammatory and immune disease, diabetes, and obesity, and is therefore of high potential relevance. Five bioassays targeting activation of the GR were included in this study, all of which were active in one or more samples (SI, Figure S7). The observed GR activity is in agreement with previous studies on similar water types.^{17,33}

Thyroid Receptor. No assay indicative of modulation of the thyroid hormone system showed response to any of the water samples (SI, Table S5), which comes as no surprise because the most commonly observed thyroid agonists and goitrogens are oxyanions such as the perchlorate and nitrate.³⁵ These inorganic ions do not act via TR binding and are not extracted with SPE.

RAR/RXR. The retinoic acid signaling pathway is crucial for reproduction and development as well as for cell homeostasis and immune function.³⁶ Two receptors are key to this pathway, the retinoic acid receptor (RAR) and the retinoic X receptor (RXR). RXR is a heterodimer partner not only for the RAR but also for other nuclear receptors including PPAR, PXR, CAR, and TR.³⁷

We tested four bioassays that are connected to the retinoic acid-signaling pathway but only the two-hybrid assay, where RAR γ is inserted into yeast with lacZ as reporter gene, showed activity in three samples. The ROR β -transFACTORIAL did not show any response with the water samples tested, although this endpoint tested positive in 30% of the ToxCast I chemicals.⁹ Clearly, the role of RXR for water quality assessment should be further explored in the future.

Reactive Toxicity. Testing for reactive toxicity focused on genotoxicity and mutagenicity (Table 2, SI Figure S8). Only one bioassay, the micronucleus assay, detects DNA damage directly; the Ames test relies on reverse mutations and the umuC assay on detection of DNA repair. Three samples were active in the micronucleus assay, Eff2, RW and DW (SI, Figure S8). The SOS chromotest and umuC assays gave consistent results and were responsive at lower REFs but the Ames assay gave more variable responses and even false-positive responses (presumably due to the high inherent degree of endogenous gene mutation in bacteria).

Tests for genotoxicity can be run in the presence and absence of a rat liver metabolic enzyme mix (S9 fraction) to differentiate between chemicals that require metabolic activation and those that are detoxified by metabolism. In the umuC and the Ames assay, there was no discernible difference between response with and without S9.

The *E. coli* assay for protein damage relies on growth inhibition differences between a strain that is glutathione-deficient (GSH-) and the corresponding parent strain (GSH+).^{38,39} These assays were found to be unsuitable for samples with high organic matter content.³⁸ In the present study no effects could be detected although there appeared to be a qualitative difference in growth inhibition between the GSH- and the GSH+ strains.

Only one assay attempted to quantify reactive oxygen species formation in RTG2 cells and results were positive and

consistent with the activation of oxidative stress response pathway discussed below.

Induction of Adaptive Stress Response Pathways. Both the heat shock response and the hypoxia induction were negative in all assays tested (SI, Table S5). No bioassay for endoplasmic reticulum stress could be identified and therefore this potentially relevant endpoint had to be omitted.

Response to inflammation was tested by enzyme-linked immunosorbent assay (ELISA) in the human T-lymphoblast cell line Jurkat E6.1 by quantifying I κ B, which is a chaperone for NF- κ B that keeps NF- κ B inactive and prevents it from entering the nucleus. Five samples tested positive in this assay (SI, Table S5). In contrast, the NF- κ B-CALUX, NF- κ B-GeneBLazer and the NF- κ B-cisFACTORIAL did not respond to any of the samples. These latter assays are relatively new, have not yet been applied for water quality assessment and possibly require further validation work to improve their detection limits.

Three of four bioassays indicative of the oxidative stress response were active in six to eight samples, highlighting the potential importance of this stress response pathway. The AREc32, Nrf2/ARE-cisFACTORIAL and the Nrf2-CALUX were all able to detect effects at low sample enrichment. The data also showed a wide dynamic range between different samples, which makes them ideal water quality indicators, although their relevance to health effects is less evident than for other bioassays.

The p53 protein plays an important role as a tumor suppression factor but all evaluated assays did not show any effect, both, in presence and absence of S9.

General Cytotoxicity and Models for System Response.

The overarching effect overlying each of the cellular toxicity pathways is cytotoxicity (Figure 1). As cytotoxicity normally manifests at higher concentrations than induction of response pathways, this endpoint is best implemented as a quality control measure for all induction assays to verify that cell vitality is not adversely affected. We did not complete full dose-response curves for cytotoxicity in all mammalian reporter gene assays apart from AREc32 for which cytotoxicity was similar to the targeted cytotoxicity assays in a human colon cancer cell line Caco 2 NRU. The fish cell line RTG2 was of relatively low responsiveness. Acute toxicity in the zebrafish embryo (DART 48h lethality) was only observed in two samples Eff2 and SW but at high enrichment factors (REF 5–6).

Cytotoxicity assays may also give information about system toxicity if appropriate cell lines are used, although a recent report by the ACuteTox project suggests little difference in response using different cell lines in vitro.⁴⁰ Here we considered the sublethal endpoint in the zebrafish embryo toxicity test after 120 h of incubation as an indicator of developmental and potentially long-term apical effects. This effect was clearly more responsive than the 48 h acute lethality endpoint in the zebrafish embryo.

The SK-N-SH neuroblastoma cell line⁴¹ is sensitive to chemicals that block the sodium channels and similar cell lines have been used previously in an assay to evaluate paralytic shellfish poisons caused by neurotoxic freshwater cyanobacteria.⁴² We used a simpler version of this assay in this study, evaluating cytotoxicity as a coarse measure of cellular neurotoxicity. This endpoint was not active for our water samples tested to a REF of 2.

Expression of various cytokines in the human acute monocytic leukemia cell line THP-1 gives an indication of potential immunotoxicity.¹⁷ Although a previous study reported

detectable inhibition of IL1 β secretion in chlorinated waters,¹⁷ we did not observe detectable effects in our study up to a REF of 2.

Benchmarking Treatment Efficacy. The bioassay results can be used to assess and monitor treatment processes (see SI, Section S7 for more detail). Best suited for this purpose are bioassays that show a clear decrease in response with increasing treatment and will not fall below the detection limit after treatment. We refer to these assays as “indicator bioassays” from hereon. Reverse osmosis (RO) is known as highly efficient in removing trace constituents, and only 13 indicator bioassays remained above detection limit after RO (but below LOD after AO, SI, Figure S10). After ozonation and BAC treatment, (another) 13 indicator bioassays remained above detection limit (SI, Figures S11 and S12). Between the two water reclamation plants, 18 suitable indicator bioassays were identified, including those indicative of AhR, PXR, CAR, ER, algal toxicity, genotoxicity and oxidative stress (SI, Figures S11 and S12).

In contrast, chlorination and chloramination increased the response in 15 of the 101 bioassays in drinking water samples (SI, Figure S13). The increased effect was most pronounced in the induction of xenobiotic metabolism and the reactive modes of action and oxidative stress response, which is consistent with the formation of chlorinated disinfection byproducts that cause genotoxicity and oxidative stress.^{15,43} This comparison demonstrates that there is no single battery of bioassays that can be applied universally, but rather that a panel of assays should be tailored to fit the needs of each application.

A Routine Test Battery of Indicator Bioassays. Because a single bioassay is not capable of assessing water quality comprehensively, a set of relevant biological endpoints that are sensitive to micropollutants typically encountered in water samples can be used collectively as indicators of water quality. A battery of bioassays should include integrative endpoints such as cytotoxicity as well as endpoints specific to relevant steps in the cellular toxicity pathway. As a minimum, indicator bioassays should cover examples found responsive to and representative of “induction of xenobiotic metabolism”, “endocrine disruption” and “adaptive stress responses”. Relevant endpoints are proposed in the following:

1. Induction of xenobiotic metabolism. Our results confirmed that activation of the aryl hydrocarbon receptor, already one of the most widely applied endpoints in water quality assessment, is a relevant indicator of the presence of chemicals and should be included in any routine test battery. The pregnane X receptor showed high responsiveness to water samples and responds to a wide range of chemicals and should be further explored for routine application.
2. Endocrine disruption. Specific receptor-mediated modes of action including estrogenic and androgenic effects showed the most promise for routine water quality screening applications. Recent work using GR-CALUX applied to various environmental chemicals and water samples³³ also support our findings that GR activity is present and could be detected in secondary treated effluent with the current battery of GR bioassays. In addition, given the co-occurrence of progestins and synthetic estrogens in hormone replacement therapy, PR activity remains of interest, despite the negative findings

here. Lastly, it is vital to test for antagonistic as well as agonistic effects.

3. Reactive modes of action. Genotoxicity as measured by well-established bioassays such as umuC or SOS chromotest served the purpose well. Bioassays derived from mammalian cells would be more relevant for human health and thus preferable to bacterial assays. As the p53 assays did not show the hoped-for responsiveness it is recommended to further evaluate alternative bioassays.
4. Adaptive stress response pathways. Oxidative stress response appears to be a highly sensitive and yet selective indicator of environmental pollution that responds to a wide range of chemicals as well as to transformation products and disinfection byproducts.⁴⁴ This is consistent with previous chemical testing in Nrf2/ARE-cisFACTOIAL, where almost 50% of the ToxCast chemicals were active.⁹ Thus this mode of action is recommended to be included in any routine test battery, especially if transformation reactions are expected.
5. Cytotoxicity and systemic response. The bacterial cytotoxicity assays (*V. fischeri* and *P. phosphoreum*) are very fast and sensitive screening assays but their high sensitivity and effects caused by controls indicated that the responses may not be of human health relevance. In contrast, cytotoxicity assays with mammalian cells are comparatively less sensitive and clearly the bioassays for toxicity pathways are more relevant. With limitations, specific cell lines may be used as indicators of organ/systemic response. Nevertheless, further work has to be invested in the selection of appropriate tests systems and protocols for cell-based bioassays for organ/systemic responses as these are much less developed than nonspecific cytotoxicity assays and bioassays targeting cellular toxicity pathways. The enzymatic AChE inhibition assay to test for one aspect of neurotoxicity failed completely but there is the potential to implement neurotoxicity endpoints in the zebrafish embryo toxicity test.⁴⁵ A whole organism in vitro assay, such as the zebrafish embryo assay may help to link specific responses from the cellular assays to systemic responses by the observed phenotypes.

We also recommend that more attention be paid to the basal activities of cell lines in use. As metabolism is the most crucial modifier of toxicity, detoxifying many chemicals but activating others, the metabolic capacity of bioassay cell lines needs to be considered when selecting or designing a bioassay. Many available cell lines have low metabolic activity and for these it is advisable to run each experiment in parallel in the presence of an exogenous metabolic mixture, for example, liver S9 fraction.

In summary, an ideal battery of bioassays for water quality assessment and testing should contain sensitive bioassays that cover a wide range of cellular toxicity pathways (Figure 1). For induction of xenobiotic metabolism pathways, we recommend AhR and PXR. For specific modes of action, the receptor-mediated hormonal effects related to the estrogenic, glucocorticoid and antiandrogenic pathways appear to be most relevant as most are responsive to water samples. The oxidative stress response clearly stands out as a highly responsive defense mechanism. Cell viability (“cytotoxicity”) assays should be further developed with a focus on those representative of systemic responses.

■ ASSOCIATED CONTENT

■ Supporting Information

Additional information on the water samples, the bioassay methods, the data evaluation, initial screening, bioassay results (including a large table with all detailed results), and a text on monitoring the treatment efficacy at WRPs is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare the following competing financial interest(s): Attagene and BDS are companies that market the bioassays applied by them in the present study.

■ ACKNOWLEDGMENTS

We thank Unitywater and Seqwater for access to their treatment plants. We acknowledge J.P. Giesy of University of Saskatchewan, Canada, for sharing the H4IIE-luc cells with RECETOX and M. Denison from University of California, Davis, U.S. for sharing the AhR-CAFLUX used at UQ and RECETOX. AREc32 cells were kindly provided by C.R. Wolf from University of Dundee, UK, to U.Q. We thank Michael Bartkow for helpful discussions, Peta Neale, Julien Reungoat, and Jatinder Sidhu for sampling assistance and Daniela Baumberger, Mriga Dutt, Eva Glenn, Ling Jin, and Shane McCarty for experimental assistance. This work was supported, mainly, by the WaterReuse Research Foundation (WRF 10-07), and, in part, by the California Water Resources Control Board (Agreement No. 10-096-250) and the European Union, project Demeau, grant agreement number 308339.

■ ABBREVIATIONS

AO	advanced oxidation
AR	androgen receptor
ASR	adaptive stress response
ATG	Attagene
AWQC	Australian Water Quality Centre
BDS	BioDetection Systems
BEQ	bioanalytical equivalent concentration
CAPIM	Centre for Aquatic Pollution Identification and Management
CAR	constitutive androstane receptor
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CT	cytotoxicity
CYP	cytochrome P450 monooxygenase
DART	embryo toxicity test with the zebrafish <i>Danio rerio</i>
DW	drinking water
EC	effect concentration
EEQ	estradiol equivalent concentration
Eff	effluent

ER	estrogen receptor
GU	Griffith University
GR	glucocorticoid receptor
HK	Hong Kong Baptist University
HTS	high-throughput screening
IR	induction ratio
IRCM	Cancer Research Institute of Montpellier
ISO	International Organization for Standardization
IWW	Institute for Water Research in North-Rhine Westfalia, Germany
MF	microfiltration
MOA	mode of action
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NJU	Nanjing University
NIH	National Institutes of Health
NRU	neutral red uptake
OECD	Organisation for Economic Co-operation and Development
PPAR	peroxisome proliferator-activated receptor
PR	progesterone receptor
PXR	pregnane-X-receptor
RAR	retinoic acid receptor
RCEES	Research Center for Eco-Environmental Sciences
RECETOX	Research Centre for Toxic Compounds in the Environment
REF	relative enrichment factor
RFU	relative fluorescence units
RLU	relative light units
RO	reverse osmosis
ROS	reactive oxygen species
RT-PCR	real-time polymerase chain reaction
RW	river water
SCCWRP	Southern California Coastal Water Research Project
SPE	solid phase extraction
SW	stormwater
SWISS	Swiss Centre for Applied Ecotoxicology
TEQ	toxic equivalent concentration
UA	University of Arizona
UCR	University of California Riverside
UF	University of Florida
UFZ	Helmholtz Centre for Environmental Research
UQ	The University of Queensland
USF	University of South Florida
WRP	water reclamation plant
WWTP	wastewater treatment plant
XM	xenobiotic metabolism

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