Global Water
Research Coalition

Effect Based Monitoring in Water Safety Planning

PROJECT REPORT

Effect Based Monitoring in Water Safety Planning

WP3.2: Medium-to-high throughput bioanalytical tools and decision-making tool for selection of bioassays

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Disclaimer

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GWRC in brief

In 2002, twelve leading research organisations established an international water research alliance: the Global Water Research Coalition (GWRC). GWRC is a non-profit organisation that serves as a focal point for the global collaboration for research planning and execution on water and wastewater related issues.

The Coalition focuses on water supply and wastewater issues and renewable water resources: the urban water cycle. The function of the GWRC is to leverage funding and expertise among the participating research organisations, coordinate research strategies, secure additional funding not available to single country research foundations, and actively manage a centralised approach to global issues. GWRC offers its members the opportunity to leverage resources through cooperative planning and implementation of research.

The GWRC Members are: Canadian Water Association (Canada), KWR – Water B.V. (Netherlands), PUB – Public Utilities Board (Singapore), Stowa- Foundation for Applied Water Research (Netherlands), SUEZ - CIRSEE (France), TZW - Water Technology Center (Germany), UK Water Industry Research (UK), Veolia Research and Innovation (VERI) (France), Water Research Australia (Australia), Water Research Commission (South Africa), The Water Research Foundation (USA), and the Water Services Association of Australia.

The US Environmental Protection Agency has been a formal partner of the GWRC since 2003. The Global Water Research Coalition is affiliated with the International Water Association (IWA).

GWRC members represents the interests and needs of 500 million consumers and have access to research programs with a cumulative annual budget of more than €150 million. The research portfolio of the GWRC members spans the entire urban water cycle and covers all aspects of resource management.

Executive summary

Effect-based monitoring using bioanalytical tools (i.e., in vitro bioassays and well plate-based in vivo assays) are recommended to complement chemical analysis for water quality monitoring. However, there are many different bioassays available, which raises questions about which bioassays and how many should be applied for water quality assessment. Therefore, this report aimed to identify bioassays commonly applied to water extracts and develop a decision-making tool to provide guidance on bioassay selection.

An extensive literature search using Web of Science and Scopus was conducted in January 2020 to identify applicable bioassays. The suitability of each collected paper was screened based on outlined criteria, with 124 suitable studies identified. Surface water (65% of studies) and wastewater (52% of studies) were the most commonly studied water types, with the majority of studies applying solid-phase extraction (SPE) (89%) to extract water samples prior to bioanalysis.

Based on the literature search, commonly applied assays indicative of xenobiotic metabolism, receptor-mediated effects, reactive toxicity, adaptive stress responses and apical effects were compared and their ability to detect effects in different water extracts was evaluated. Assays indicative of activation of the aryl hydrocarbon receptor (AhR), activation of the pregnane X receptor (PXR), activation of the estrogen receptor (ER), activation of the androgen receptor (AR), phytotoxicity, oxidative stress response and bacterial toxicity were able to detect effects in wastewater, surface water and drinking water after sufficient enrichment. In contrast, mammalian reporter gene assays indicative of activation of the thyroid receptor (TR) and activation of the mineralocorticoid receptor (MR) did not induce a response in any of the tested water extracts.

While a large number of bioassays are available, a practical test battery of at least three or four bioassays representative of effects commonly detected in water extracts and aligned with relevant steps of adverse outcome pathways are recommended. In the case of wastewater and water reuse for non-potable use, we recommend assays indicative of activation of AhR, activation of ER and oxidative stress response as they are responsive to a range of water types, represent different stages of the cellular toxicity pathway and are widely used. In the context of drinking water treatment or water reuse for potable use, an assay indicative of either genotoxicity or mutagenicity is recommended in addition to activation of AhR, activation of ER and oxidative stress response. For research applications, more bioassays may be included in a test battery and screening might possible with fewer bioassays.

As multiple assays indicative of the same endpoint are available, a decision-making tool was developed that groups assays into three test batteries based on assay sensitivity, with test battery selection depending on the sampling campaign context and purpose. To assist with the selection of a suitable bioassay for each endpoint, tables were provided that summarised key features of commonly used assays, including availability of an effect-based trigger value (EBT), whether the assay is commonly used for water quality monitoring and assay sensitivity.

Abbrevations: ACC: activity cut-off concentration; AChE: acetylcholinesterase; AH: amiodarone hydrochloride; AhR: aryl hydrocarbon receptor; AO: advanced oxidation; AR: androgen receptor; BAC: biological activated carbon; BEQ: bioanalytical equivalent concentration; CAR: constitutive androstane receptor; DBP: disinfection by-products; DOC: dissolved organic carbon; DWTP: drinking water treatment plants; EAR: exposure-activity ratio; EBT: effect-based trigger value; EC: effect concentration; EQ: equivalent concentration; ER: estrogen receptor; FET: fish embryo toxicity; GR: glucocorticoid receptor; H2O2: hydrogen peroxide; IR: induction ratio; LLE: liquid-liquid extraction; LOD: limit of detection; LOEC: lowest observed effect concentration; LOQ: limit of quantification; MR: mineralocorticoid receptor; ND: not detected; O3: ozonation; PPAR: peroxisome proliferator-activated receptor; PR: progesterone receptor; PSII: photosystem II; PXR: pregnane X receptor; RAR: retinoic acid receptor; RXR: retinoid X receptor; REF: relative enrichment factor; RO: reverse osmosis; SPE: solid-phase extraction; SR: suppression ratio; T3: triiodothyronine; TCDD: 2,3,7,8-tetrachlorodibenzodioxin; TNFα: tumour necrosis factor alpha; TR: thyroid receptor; TU: toxic units; UV: ultraviolet; WWTP: wastewater treatment plant ; YAES: yeast anti-estrogen screen; YAS: yeast androgen screen; YDS: yeast dioxin screen; YES: yeast estrogen screen

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1 Introduction

The aquatic environment can contain a diverse range of micropollutants including pesticides, pharmaceuticals and industrial compounds, while water treatment processes, such as disinfection, can form disinfection by-products (DBPs) or other micropollutant transformation products (e.g., Glassmeyer *et al.*, 2017; Leusch *et al.*, 2018b). As a result, wastewater effluent, surface water and even drinking water can contain a complex mixture of micropollutants, often at low concentrations, and targeted chemical analysis cannot detect all chemicals present. Suspect screening and non-target analysis can identify a larger number of compounds, but cannot provide any information about the potential toxic effects of the micropollutant mixture (Escher *et al.*, 2020). Effect-based monitoring using bioanalytical tools (i.e., in vitro bioassays and well plate-based in vivo assays) can be applied in parallel to chemical analysis to detect the effect of all known and unknown chemicals that are active in a particular bioassay.

Bioassays can account for mixture effects and are risk-scaled as more potent chemicals have a greater effect in the assay. Consequently, effect-based methods have been recommended to complement chemical analysis in water quality monitoring (Brack *et al.*, 2019). Bioassays based on different stages of cellular toxicity pathways including induction of xenobiotic metabolism, receptor-mediated effects, adaptive stress responses and apical effects have been widely applied to evaluate the effect of different water extracts (e.g., Escher *et al.*, 2014; Rosenmai *et al.*, 2018; Alygizakis *et al.*, 2019). However, there are many different assays available, including multiple assays indicative of the same endpoint.

This raises questions about **which bioassays and how many should be applied for water quality assessment**. Consequently, the aim of the current report is to identify bioassays commonly applied to water extracts and develop a decision-making tool to provide guidance on assay selection. As the field progresses and new bioassays are developed, they can be integrated in this modular approach and decision-making tool. Many of the bioassays included have also been applied to support health risk assessment of chemicals (Wetmore, 2015; Bell *et al.*, 2018) and this information is useful to estimate how the in vitro endpoints are connected to health consequences. However, it must be stressed that the use of in vitro bioassays to water samples does not allow any prediction of health risk.

To identify applicable bioassays, a literature search was conducted on 14th January 2020 using both Web of Science and Scopus. We searched for *water AND "in vitro bioassay*" OR "bioanalytical tool*" OR "effect-based method*" OR "cell-based bioassay" OR "effect-based monitor*"* as the "topic" in Web of Science and "title, abstract, keyword" in Scopus. This identified 623 papers. Further, the terms *"in vitro assay" and "wastewater" OR "sewage" OR "drinking water" OR "recycled water" or "surface water"* were also searched in Web of Science and Scopus. This brought the total to 760 papers. An additional 24 papers missed in the Web of Science and Scopus searches were also added, bringing the total to 784 papers (Figure 1 A).

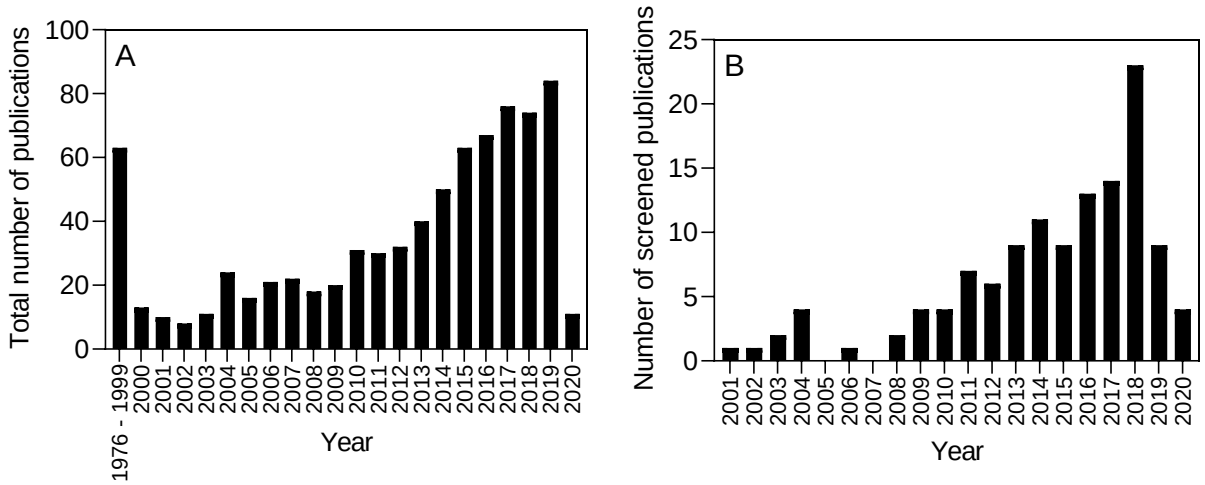

Figure 1: A) Total number of publications by year identified in the Scopus and Web of Science search and B) the number of screened publications by year

The suitability of each paper was screened based on:

- Use of high-throughput *in vitro* bioassays (e.g., 96-well or 384-well plate) or well plate-based *in vivo* assays. High-throughput assays are essential for routine water quality monitoring.
- Application to drinking water, surface water, wastewater, recycled water[1] or groundwater. These water types were selected to cover the potential inputs and outputs of drinking water treatment plants (DWTPs), wastewater treatment plants (WWTPs) and advanced water treatment plants for water reuse.
- Water sample extracted by solid-phase extraction (SPE), passive sampling or liquid-liquid extraction (LLE), rather using whole or unextracted water. Unextracted water may contain metals, salt and other inorganics, in addition to micropollutants, meaning that the response in an unextracted water sample cannot be attributed to micropollutants alone.
- Data presented as an effect concentration (EC) or equivalent concentration (EQ). This information is essential to compare between studies that applied the same assay, so any studies that only reported positive/negative results, as was often the case for mutagenicity and genotoxicity assays, were excluded. Note that more recently mutagenicity and genotoxicity tests such as the Ames and umuC assays have also gone beyond positive/negative results and provide EC values.

The screening process reduced the number of suitable studies to 124. The reviewed studies came from 23 countries, with studies from all continents except for South America. Around half of the studies were from Europe. The studies were published in 27 different journals, with the majority published in Water Research (20%) and Science of the Total Environment (15%). Over half of the studies are very recent, having been published since 2016 (Figure 1 B).

Surface water (65% of studies) followed by wastewater (52% of studies) were the most common water types, with only 23% of studies testing drinking water using bioanalytical tools. Further, the majority of studies applied SPE (89%) to extract the water samples prior to bioanalysis. Consequently, only studies that applied SPE or LLE were included in the summary tables in the Appendix, though studies that have applied passive sampling are discussed throughout the review. Of the applied bioanalytical tools, assays indicative of activation of the estrogen receptor (ER) were the most commonly applied (77% of studies), followed

[1] For the purpose of this review, water recycling for direct or indirect drinking water augmentation is considered. This includes processes such as membrane filtration (e.g., reverse osmosis), advanced oxidation (ozonation, UV, hydrogen peroxide)

by activation of the androgen receptor (AR) (39% of studies) and activation of the aryl hydrocarbon receptor (AhR) (27% of studies).

The following report will review the suitability of current assays indicative of different stages of cellular toxicity pathways and apical effects to detect effects in different water extracts. Many of the reviewed assays are based on mammalian cell lines, though some assays, such as assays indicative of reactive toxicity, are bacterial. Many of the assays indicative of apical effects used well plate-based *in vivo* assays. While some assay results are expressed in different dose-metrics in the literature, we standardised the units to allow comparison between different assays of similar endpoints.

The results for assays indicative of xenobiotic metabolism and receptor-mediated effects were translated in this report to **bioanalytical equivalent concentrations (BEQ**) in units of ng/L or µg/L, which relates the effect concentrations of the water sample (EC(sample)) to the effect of the assay reference compound (EC(ref)) with BEQ = EC(ref)/EC(sample) (Escher *et al.*, 2018b). The larger the BEQ, the greater the effect. The effects are reported as BEQ to allow the comparison of water quality testing with different bioassays. As the sensitivity of the assays varies, their BEQ will normalise some of that variability but it must be noted that sensitivity differences remain due to differences in relative effect potencies of the many chemicals in a water sample.

For assays indicative of adaptive stress responses and apical endpoints, the effect was expressed as an **effect concentration (EC)** in units of **Relative Enrichment Factor (REF).** REF takes into consideration sample enrichment and subsequently dilution in the bioassay, with a REF of 1 referring to the native sample and a REF 10 indicating a water sample needs to be enriched 10 times for an effect to be observed. The lower the EC value, the greater the effect. This is because less enrichment is required to detect an effect in the assay. The effect for assays indicative of adaptive stress responses and reactive toxicity was expressed as the concentration causing an induction ratio of 1.5 ($EC_{IR1.5}$), while the concentration causing 50% effect (EC_{50}) was typically reported for assays indicative of apical effects. EC_{50}, EC_{20}, and EC_{10} can be converted into each other provided that the sigmoidal dose-response model is known and if it is not, we have assumed a log-logistic slope of 1 to bring diverse datasets to a common EC. It would also be possible to derive BEQs for those endpoints and there are some publications that have done so, but there is less consensus as to what constitutes a reference compound and therefore converting to BEQ with different reference compounds would make the studies even less comparable. Therefore, the ECs were compared directly, but one must keep in mind that there are differences in sensitivity of the different reporter gene assays that were compared for each endpoint.

2 Xenobiotic metabolism

The presence of chemicals can induce biotransformation processes in cells to metabolize, detoxify or in some case bioactivate chemicals (Omiecinski *et al.*, 2011). Some important xenobiotic metabolism receptors include the aryl hydrocarbon receptor (AhR), peroxisome-proliferator activated receptor (PPAR), pregnane X receptor (PXR) and constitutive androstane receptor (CAR). Assays indicative of xenobiotic metabolism may not result in cell death, but instead can act as sensitive indicators of chemicals, with many receptors considered capable of binding a wide range of chemicals. To date, only one study has applied assays indicative of CAR to environmental extracts (Escher *et al.*, 2014), with effects detected in wastewater, surface water and drinking water extracts using a yeast-based CAR assay. Therefore, this section will focus on assays indicative of the three xenobiotic metabolism receptors commonly applied to water samples, AhR, PPAR and PXR.

2.1 Activation of aryl hydrocarbon receptor (AhR)

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that is necessary for virtually all of the toxicity of halogenated aromatic hydrocarbons such as polychlorinated and brominated dibenzo-p-dioxins and biphenyls as well as polycyclic aromatic hydrocarbons (PAHs). It activates target genes encoding for the metabolic enzymes CYP1A1, CYP1B1 and NADPH-quinone oxidoreductase (NQO1) but there is also cross talk with Nrf2, the master regulator of antioxidant response, and the hypoxia-inducible factor HIF1α. Its activation contributes to carcinogenicity because cytochrome P450 monooxygenase (CYP) can convert many of its ligands to reactive intermediates capable of causing DNA damage. Assays indicative of activation of AhR are traditionally applied to detect the presence of dioxin-like chemicals, but recent studies have shown that many

environmental chemicals can activate AhR (Martin *et al.*, 2010). Some of the more common reporter gene assays that have been applied to evaluate activation of AhR in water extracts are provided in Table 1, along with the reported concentration causing 10% effect (EC_{10}). Generally, AhR CAFLUX (mouse H1.G1.1c3 and rat H4.G1.1c2), AhR CALUX (rat H4L1.1c4) and H4IIE-luc were similarly sensitive to individual chemicals, with EC_{10} values in the low ng/L range for reference compound 2,3,7,8-tetrachlorodibenzodioxin (TCDD). It should be noted that there are other AhR CALUX cell lines used (e.g. H1L6.1c2 (Mehinto *et al.*, 2017) and H4L1.1c2 (Daniels *et al.*, 2018)), but these have not been as widely applied to date. The AhR reporter gene using human HepG2 cells was around two orders of magnitude less sensitive than the other reporter gene assays (Rosenmai *et al.*, 2018) and has only been applied in a limited number of studies.

In addition to the assays in Table 1, the PAH CALUX assay, which uses H4IIE cells, has also been applied to wastewater effluent (Alygizakis *et al.*, 2019) and surface water (De Baat *et al.*, 2019) extracts. The assay reference compound is benzo(a)pyrene (EC_{50} 3.0×10^{-9} M (Pieterse *et al.*, 2013)), with all results expressed as benzo(a)pyrene EQ. Further, studies have also applied yeast-based activation of AhR assays, such as the yeast dioxin screen (YDS). The reference compound for the YDS is β-naphthoflavone, with an EC_{50} of 3.0×10^{-8} M (Stalter *et al.*, 2011). An assay indicative of activation of AhR, AhR_LUC, was also included in the US EPA ToxCast database. This is based on the human HepG2 cell line (He *et al.*, 2011). TCDD was not measured in ToxCast, but the EC_{10} values of common chemicals run in both AhR_LUC and AhR CALUX were generally within an order of magnitude (Neale *et al.*, 2020a).

Table 1: Common cell-based reporter gene assays applied to evaluate aryl hydrocarbon receptor (AhR) activity in water extracts

Assay	Cell line	Detection method	TCDD EC_{10} (M)	TCDD EC_{10} (ng/L)	EC Reference
AhR CAFLUX	H1.G1.1c3	Fluorescence	6.50×10^{-13}	0.21	(Jia *et al.*, 2015)
AhR CAFLUX	H4.G1.1c2	Fluorescence	6.87×10^{-13}	0.22	(Neale *et al.*, 2015; Konig *et al.*, 2017)
AhR CALUX	H4L1.1c4	Luminescence	5.92×10^{-13}	0.19	(Nivala *et al.*, 2018)
AhR reporter gene assay	HepG2	Luminescence	$6.22\times10^{-11*}$	20	(Rosenmai *et al.*, 2018)
H4IIE-luc	H4IIE	Luminescence	1.60×10^{-13}	0.05	(Lee *et al.*, 2015)

** Presented EC_{10} value converted from EC_{50} value assuming a slope of the log-logistic concentration response curve of 1.*

A summary of reported AhR activity in wastewater, surface water, recycled water and drinking water is provided in Table A1 in the Appendix. A number of studies have evaluated activation of AhR in passive sampler extracts (e.g. Jarošová *et al.*, 2012; Hamers *et al.*, 2018), but only studies that have applied SPE were included in Table A1. Based on the more sensitive reporter gene assays, the TCDD-EQ ranged from 0.1 – 3.3 ng/L in wastewater influent, 0.007 – 1.2 ng/L in treated wastewater, 0.004 - 0.36 ng/L in recycled water and 0.002 – 0.19 ng/L in surface water. The reported WWTP removal efficacy ranged from 13 - 90% (Jalova *et al.*, 2013; Nivala *et al.*, 2018). TCDD EQ in drinking water ranged from <0.004 to 0.17 ng/L. Based on EC_{10} values, effects were detected after 0.7 - 0.8 times enrichment in wastewater influent, between 0.8 to 31 times enrichment in wastewater effluent and 2 to 35 times enrichment in surface water.

2.2 Activation of peroxisome proliferator-activated receptor γ (PPARγ)

The peroxisome proliferator-activated receptor (PPAR) is also a transcription factor that belongs to the superfamily of nuclear receptors and is involved in the regulation of glucose and lipid metabolism and not so much in xenobiotic metabolism (Scarsi *et al.*, 2007). As the name indicates, the main function of PPAR is the delivery of peroxisomes, which are important for fatty acid oxidation and thus relevant for lipid metabolism. There are three isoforms of PPAR – PPARα, PPARß (also called δ) and PPARγ, which are encoded by different genes, show different tissue expression and perform slightly different functions. PPARα is expressed predominantly in metabolically active tissues like liver and kidney cells where its ligands include fatty acids, hypolipidemic drugs and xenobiotics (Seimandi *et al.*, 2005). PPARγ is the key receptor in maintaining glucose and lipid homeostasis and its activation increases the insulin resistance of the cell (Scarsi *et al.*, 2007). To date, most studies have applied assays indicative of binding to PPARγ to environmental water extracts, with only a few studies applying assays

indicative of PPARα (Escher *et al.*, 2014; Alygizakis *et al.*, 2019). Therefore, this section will focus on PPARγ, with two assays frequently applied, PPARγ CALUX and PPARγ GeneBLAzer (Table 2). The EC_{10} value for reference compound antidiabetic pharmaceutical rosiglitazone was over an order of magnitude lower for PPARγ GeneBLAzer. PPARγ activity has been detected in wastewater influent, wastewater effluent and surface water, with activity in recycled water and drinking water below the limit of detection (Table A2). Activity in wastewater influent ranged from 500 - 936 ng/L rosiglitazone EQ, with wastewater effluent ranging from 83 - 640 ng/L rosiglitazone EQ (Table A2). The studies that have evaluated the removal of PPARγ during wastewater treatment found between 69 to >94% removal (Bain *et al.*, 2014; Nivala *et al.*, 2018). The rosiglitazone EQ in surface water varied from 0.6 to 172 ng/L, with sites downstream of WWTPs having the highest effect. Based on EC_{10} values, effects were detected after 0.2 to 0.3 times enrichment in wastewater influent, between 1.5 to >30 times enrichment in wastewater effluent and 1 to 90 times enrichment in surface water

Table 2: Common cell-based reporter gene assays applied to evaluate peroxisome proliferator-activated receptor (PPARγ) activity in water extracts.

Assay	Cell line	Detection method	Rosiglitazone EC_{10} (M)	Rosiglitazone EC_{10} (ng/L)	EC Reference
PPARγ CALUX	U2OS	Luminescence	1.00×10^{-8}	3,600	(Gijsbers *et al.*, 2011)
PPARγ GeneBLAzer	HEK 293	Fluorescence	3.30×10^{-10}	118	(Jia *et al.*, 2015)

2.3 Activation of pregnane X receptor (PXR)

The pregnane X-receptor (PXR) is a promiscuous nuclear receptor with a large ligand binding pocket that can help protect the cell by triggering detoxification pathways (Grimaldi *et al.*, 2015). PXR controls the transcription of a large array of genes encoding for Phase I metabolic enzymes, especially the CYP3A family, which plays an important role in drug metabolism. Two reporter gene assays have been applied to evaluate PXR activity in water extracts, HG5LN hPXR and PXR CALUX. The reference compound for HG5LN hPXR is pharmaceutical SR12813 (EC_{10} 1.58×10^{-8} M (Neale *et al.*, 2015)), while the reference compound for PXR CALUX is nicardipine. EC_{10}/PC_{10} values for industrial compound di(2-ethylhexyl)-phthalate (DEHP) for both assays were presented in Escher *et al.* (2018a), with a slightly lower value for HG5LN hPXR (Table 3). PXR activity was detected in all water samples tested, except for recycled water after reverse osmosis and advanced oxidation (RO/AO) (Table A3). The reported PXR activity in wastewater was 3.8 - 4.7 µg/L SR12813 EQ or 20-240 µg/L nicardipine EQ, while the activity in surface water ranged from <0.02 to 2.3 µg/L SR12813 EQ (Table A3). Based on the EC_{10} values in the HG5LN hPXR assay, effects in wastewater were detected after around 2 times enrichment in wastewater effluent and between 3 to 30 times enrichment in surface water and 2.5 times enrichment in drinking water.

Table 3: Common cell-based reporter gene assays applied to evaluate activation of the pregnane X receptor (PXR) in water extracts.

Assay	Cell line	Detection method	di(2-ethylhexyl)-phthalate (DEHP) EC_{10}/PC_{10} (M)	di(2-ethylhexyl)-phthalate (DEHP) EC_{10}/PC_{10} (µg/L)	EC Reference
HG5LN hPXR	HG5LN (HeLa)	Luminescence	2.77×10^{-7}	108	(Escher *et al.*, 2018a)
PXR CALUX	U2OS	Luminescence	3.97×10^{-7}	155	(Escher *et al.*, 2018a)

3 Hormone receptor-mediated effects

Hormonal pathways are essential for processes related to growth, sexual development, metabolism and homeostasis. Endocrine disrupting chemicals, including synthetic hormones, industrial chemicals and pesticides, can interfere with hormonal

systems by interacting with hormone receptors (le Maire *et al.*, 2010). This includes activating and inhibiting hormone receptors. To date, most of the research has focused on ER, followed by AR. However, other relevant nuclear receptors include the glucocorticoid receptor (GR), progesterone receptor (PR), thyroid receptor (TR), mineralocorticoid receptor (MR), retinoic acid receptor (RAR) and retinoid X receptor (RXR). Further information about the sensitivity of assays indicative of ER, AR, PR, GR and TR can be found in the review by Leusch *et al.* (2017). In addition to the more commonly applied hormone receptors, this section will also focus on assays indicative of MR, RAR and RXR. Due to the large number of studies that have applied assays indicative of hormone receptor-mediated effects we decided to focus on the more commonly applied assays.

3.1 Estrogen receptor

3.1.1 Agonist

Nuclear receptors ERα and ERβ are important for the growth and homeostasis of the uterus and mammary glands, as well as bones and cardiovascular system (le Maire *et al.*, 2010). The majority of assays applied to environmental water extracts focus on ERα (e.g. ERα CALUX, ERα GeneBLAzer), though the T47D-KBluc assay uses the T47D cell line, which expresses both ERα and ERβ (Wilson *et al.*, 2004).

Estrogenic activity was by far the most commonly studied endpoint in water extracts, with 77% of the studies reviewed measuring estrogenic activity. As many different assays have been applied in the literature, we focused on assays that have been applied to water samples in four or more studies. The exception was the embryonic zebrafish assay EASZY, which has only been included in two studies, but was included to represent a whole organism assay. A summary of the included activation of ER assays is provided in Table 4, with similar responsiveness for the mammalian reporter gene assays. The reference compound 17β-estradiol EC_{10} value varied between 0.13 ng/L for T47D-KBluc to 2.1 ng/L for HeLa-9903. The yeast estrogen screen (YES) and EASZY showed activity only at higher concentrations. Further information about the level of response of many of these assays can be found in Leusch *et al.* (2017)

Table 4: Common assays applied to evaluate estrogenic activity in water extracts.

Assay	Cell line/test system	Detection method	17β-estradiol EC_{10} (M)	17β-estradiol EC_{10} (ng/L)	EC Reference
Yeast reporter gene					
YES	Yeast	Absorbance	$3.75\times10^{-11*}$	10.2*	(Escher *et al.*, 2008b)
Mammalian reporter gene					
ERα CALUX	U2OS	Luminescence	7.13×10^{-13}	0.19	(Jia *et al.*, 2015)
ERα GeneBLAzer	HEK 293	Fluorescence	9.87×10^{-12}	2.7	(Nivala *et al.*, 2018)
HeLa-9903	HeLa	Luminescence	$7.78\times10^{-12*}$	2.1*	(Valcarcel *et al.*, 2018)
MELN	MCF-7	Luminescence	2.42×10^{-12}	0.66	(Neale *et al.*, 2015)
MVLN	MCF-7	Luminescence	$3.16\times10^{-12*}$	0.86*	(Shue *et al.*, 2009)
T47D-KBluc	T47D	Luminescence	$4.63\times10^{-13*}$	0.13*	(Liu *et al.*, 2018)
Cell proliferation					
E-Screen	MCF7	Absorbance (cell viability measured using CellTiter (MTS))	$8.18\times10^{-13*}$	0.22*	(Macova *et al.*, 2010)
Whole organism					
EASZY	Embryonic zebrafish	Fluorescence	EC_{50} 6.20×10^{-10}	EC_{50} 168	(Brion *et al.*, 2019)

**Presented EC_{10} value converted from EC_{50} value assuming a slope of the log-logistic concentration response curve of 1.*

Reported estrogenic activity in units of ng/L 17β-estradiol EQ (EEQ) in wastewater influent, wastewater effluent, recycled water, surface water and drinking water is provided in Table A4. Focusing on the mammalian reporter gene assays, the estrogenic activity in wastewater influent ranged from <0.02 to 122 ng/L EEQ, while the activity was mostly between 0.1 to 10 ng/L EEQ in treated effluent. Estrogenic activity is typically well removed during wastewater treatment, with 80 to >99% removal efficacy reported in the literature for a variety of WWTPs (Jugan *et al.*, 2009; Jalova *et al.*, 2013; Bain *et al.*, 2014; Hamilton *et al.*, 2016; Houtman *et al.*, 2018; Nivala *et al.*, 2018). Estrogenic activity was mostly below the limit of detection in recycled water. Estrogenicity varied greatly in surface water, with values from 0.005 ng/L EEQ up to 190 ng/L EEQ, with factors such as proximity to wastewater effluent discharges impacting the observed effects. Finally, low estrogenic activity (<0.01 ng/L EEQ) was often detected in treated drinking water, with one study from China finding 5.2 ng/L EEQ in treated drinking water (Shi *et al.*, 2018). A number of studies have measured estrogenic activity in both source water and treated drinking water, with 39 to 99% removal efficacy observed (Escher *et al.*, 2014; Huang *et al.*, 2016; Lv *et al.*, 2016; Xiao *et al.*, 2016; Conley *et al.*, 2017b; Xiao *et al.*, 2017; Shi *et al.*, 2018; Neale *et al.*, 2020b). Based on EC_{10} values, estrogenic activity was detected after 0.1 to 6.4 times enrichment in wastewater effluent, 0.5 to 145 times enrichment in surface water and 20 to 110 times enrichment in drinking water.

3.1.2 Antagonist

In contrast to estrogenic activity, anti-estrogenic activity is much less studied in environmental water extracts, with only 14% of the reviewed studied measuring this endpoint. Three assays commonly applied to evaluate anti-estrogenic activity include the yeast anti-estrogen screen (YAES) and the mammalian reporter gene ERα CALUX and ERα GeneBLAzer. Based on the reference compound tamoxifen, the ERα CALUX was the most responsive assay, with a concentration causing a suppression ratio of 0.2 ($EC_{SPR0.2}$) value of 0.56 µg/L. Cytotoxicity masked anti-estrogenic activity in wastewater influent, while many treated wastewater effluent samples were below detection (Table A5). Using the YAES assay, two studies found between 13 to 97 µg/L tamoxifen EQ in treated effluent (Conroy *et al.*, 2007; Fang *et al.*, 2012). Anti-estrogenic activity was either low or below detection in surface water, while no anti-estrogenic activity was detected in drinking water.

Table 5: Common assays applied to evaluate anti-estrogenic activity in water extracts.

Assay	Cell line/test system	Detection method	Tamoxifen $EC_{SPR0.2}$ (M)	Tamoxifen $EC_{SPR0.2}$ (µg/L)	EC Reference
Yeast reporter gene					
YAES	Yeast	Absorbance	6.00×10^{-7}	223	(Conroy *et al.*, 2007)
Mammalian reporter gene					
ERα CALUX	U2OS	Luminescence	1.50×10^{-9}	0.56	(Jia *et al.*, 2015)
ERα GeneBLAzer	HEK 293	Fluorescence	5.86×10^{-6}	2177	(Neale *et al.*, 2020b)

3.2 Androgen receptor

3.2.1 Agonist

The AR is expressed in a range of tissues and has implications for the development and maintenance of a number of systems, including the reproductive, immune, musculoskeletal and cardiovascular systems (Davey and Grossmann, 2016). Androgenic activity is the second most studied endpoint, with 39% of studies applying assays indicative of activation of AR. While a number of assays have been applied to evaluate androgenic activity, the assays most commonly applied to water extracts include the yeast androgen screen (YAS) and the mammalian reporter gene assays AR CALUX, AR GeneBLAzer and MDA-kB2. Based on the reference compound dihydrotestosterone (DHT), the mammalian reporter gene assays were more sensitive than YAS. Further information about assay sensitivity can be found in Leusch *et al.* (2017). Androgenic activity in wastewater, surface water and drinking water extracts is summarised in Table A6. Between 30 to 350 ng/L DHT EQ were detected in wastewater influent, with low or no androgenic activity typically present in wastewater effluent. The reported WWTP removal efficacy ranges from 95 to >99.9% (Jalova *et al.*, 2013; Bain *et al.*, 2014; Houtman *et al.*, 2018), explaining the low activity in treated effluent. Based on the mammalian reporter gene assays, only low androgenic activity was detected in surface water, with 0.25 to 12 ng/L

DHT EQ reported (Table A6). Based on the DHT EC_{10} values in Table 6, this means samples would need to be enriched between 0.8 to 164 times in the assay to detect an effect in surface water. No androgenic activity was detected in recycled water, with only one study detecting androgenic activity in drinking water at 0.13 ng/L DHT (Brand *et al.*, 2013). Based on the DHT EC_{10} for AR CALUX in Table 6, this equates to an EC_{10} of 223 REF, meaning the sample would need to be enriched over 200 times.

Table 6: Common assays applied to evaluate androgenic activity in water extracts.

Assay	Cell line/test system	Detection method	DHT EC_{10} (M)	DHT EC_{10} (ng/L)	EC Reference
Yeast reporter gene					
YAS	Yeast	Absorbance	2.86×10^{-10}	83	(Sohoni and Sumpter, 1998)
Mammalian reporter gene					
AR CALUX	U2OS	Luminescence	1.00×10^{-10}	29	(Jia *et al.*, 2015)
AR GeneBLAzer	HEK 293	Fluorescence	1.40×10^{-10}	41	(Leusch *et al.*, 2017)
MDA-kB2	MDA-MB-453	Luminescence	3.12×10^{-11}	9.1	(Neale *et al.*, 2017b)

3.2.2 Antagonist

Anti-androgenic activity was assessed in 27 of the reviewed studies (22% of studies). Based reference compound flutamide $EC_{SPR0.2}$ values in Table 7, both yeast and mammalian reporter gene assays were similarly sensitive. Reported anti-androgenic activity in environmental extracts is provided in Table A7. Anti-androgenic activity was highest in wastewater effluent, with between 0.5 to 360 µg/L flutamide EQ reported for the mammalian reporter gene assays (Table A7). Based on the flutamide $EC_{SPR0.2}$ values in Table 7, this indicates between 0.16 to 600 times enrichment in the assay would be required to detect an effect. Anti-androgenic activity in surface water ranged from 0.3 to 257 µg/L flutamide EQ, while no anti-androgenic activity was detected in drinking water or recycled water.

Table 7: Common assays applied to evaluate anti-androgenic activity in water extracts.

Assay	Cell line/test system	Detection method	Flutamide $EC_{SPR0.2}$ (M)	Flutamide $EC_{SPR0.2}$ (µg/L)	EC Reference
Yeast reporter gene					
YAAS	Yeast	Absorbance	$7.50\times10^{-7*}$	207*	(Stalter *et al.*, 2011)
Mammalian reporter gene					
AR CALUX	U2OS	Luminescence	1.10×10^{-6}	304	(Jia *et al.*, 2015)
AR GeneBLAzer	HEK 293	Fluorescence	$5.50\times10^{-7*}$	152*	(Leusch *et al.*, 2017)
MDA-kB2	MDA-MB-453	Luminescence	2.07×10^{-7}	57	(Neale *et al.*, 2017a)

**Presented $EC_{SPR0.2}$ value converted from EC_{50} value assuming a slope of the log-logistic concentration response curve of 1.*

3.3 Glucocorticoid receptor

3.3.1 Agonist

The GR is a corticosteroid receptor that controls the actions of glucocorticoids, and a wide range of environmental contaminants can interfere with glucocorticoid activity (Zhang *et al.*, 2019). Mammalian reporter gene assays have been applied to evaluate glucocorticoid activity in environmental water extracts (Table 8), with GR CALUX and GR GeneBLAzer most commonly applied. Pharmaceutical dexamethasone serves as the assay reference compound, with the lowest EC_{10} reported for GR GeneBLAzer. The mammalian reporter gene CV-1 GR assay has also been recently applied to detect glucocorticoid activity in different water samples (Conley *et al.*, 2017a; Medlock Kakaley *et al.*, 2020), but no EC value for dexamethasone was available, so this assay was not included in Table 8.

Glucocorticoid activity in wastewater influent ranged from 37 to 121 ng/L dexamethasone EQ, with between 11 to 628 ng/L dexamethasone EQ reported in wastewater effluent (Table A8). The studies that evaluated WWTP treatment efficacy found between -7 to 66% removal (Bain *et al.*, 2014; Roberts *et al.*, 2015; Houtman *et al.*, 2018), indicating much poorer removal for glucocorticoid activity compared to estrogenic activity and androgenic activity. As a result, glucocorticoid activity was frequently detected in surface water, with between 9 to 170 ng/L dexamethasone EQ in an effluent impacted river (Daniels *et al.*, 2018). Based on the GeneBLAzer dexamethasone EC_{10} value in Table 8, this equates to 0.5 to 9 times enrichment in the assay. Glucocorticoid activity was not observed in drinking water, ground water or recycled water (Table A8).

Table 8: Common assays applied to evaluate glucocorticoid activity in water extracts.

Assay	Cell line	Detection method	Dexamethasone EC_{10} (M)	Dexamethasone EC_{10} (ng/L)	EC Reference
GR CALUX	U2OS	Luminescence	8.00×10^{-10}	314	(Jia *et al.*, 2015)
GR GeneBLAzer	HEK 293T	Fluorescence	2.08×10^{-10}	82	(Nivala *et al.*, 2018)
GR Switchgear	HT1080	Luminescence	5.00×10^{-10}	196	(Jia *et al.*, 2015)

3.3.2 Antagonist

Only two assays, GR CALUX and GR GeneBLAzer, were applied in the literature to evaluate anti- glucocorticoid activity, with GR GeneBLAzer much more sensitive than GR CALUX based on the reference compound mifepristone $EC_{SR0.2}$ values (Table 9). Anti-glucocorticoid activity was only detected in surface water in two studies (Konig *et al.*, 2017; Jia *et al.*, 2019), with between 2.5 to 610 µg/L mifepristone EQ reported. Anti-glucocorticoid activity was not detected in wastewater effluent, recycled water or drinking water, with cytotoxicity masking the effect in wastewater influent (Table A9).

Table 9: Common assays applied to evaluate anti-glucocorticoid activity in water extracts

Assay	Cell line	Detection method	Mifepristone $EC_{SPR0.2}$ (M)	Mifepristone $EC_{SPR0.2}$ (ng/L)	EC Reference
GR CALUX	U2OS	Luminescence	2.90×10^{-9}	1246	(Jia *et al.*, 2015)
GR GeneBLAzer	HEK 293T	Fluorescence	1.00×10^{-10}	43	(Jia *et al.*, 2015)

3.4 Progesterone receptor

3.4.1 Agonist

Two assays, PR CALUX and PR GeneBLAzer, have been applied to evaluate progestagenic activity in environmental water extracts, with both assays having similar EC_{10} values for synthetic hormone levonorgestrel (Table 10). Progestagenic activity in water extracts has been reported in different equivalent concentrations, including progesterone EQ, levonorgestrel EQ, promegestone EQ and org2058 EQ. To assist with comparison, the results from the literature were converted to levonorgestrel EQ based on published potency data. Up to 3.2 ng/L levonorgestrel EQ was detected in wastewater influent, while between 0.43 to 7.1 ng/L levonorgestrel EQ was detected in treated effluent (Table A10). A number of studies have found increased progestagenic activity after wastewater treatment (Roberts *et al.*, 2015; Houtman *et al.*, 2018), while Bain *et al.* (2014) found between 12 to >93% removal efficacy in three WWTPs in Australia. Up to 9.6 ng/L levonorgestrel EQ was detected in surface water from the Netherlands, though progestagenic activity was often below the assay detection limit or masked by cytotoxicity in surface water. No progestagenic activity was detected in recycled water or drinking water (Table A10).

Table 10: Common assays applied to evaluate progestagenic activity in water extracts

Assay	Cell line	Detection method	Levonorgestrel EC_{10} (M)	Levonorgestrel EC_{10} (ng/L)	EC Reference
PR CALUX	U2OS	Luminescence	$3.44\times10^{-11*}$	10.8	(Scott *et al.*, 2014)
PR GeneBLAzer	HEK 293T	Fluorescence	$1.22\times10^{-11*}$	3.8	(Leusch *et al.*, 2018b)

**Presented EC_{10} value converted from EC_{50} value assuming a slope of the log-logistic concentration response curve of 1.*

3.4.2 Antagonist

Two assays, PR CALUX and PR GeneBLAzer, have been applied to evaluate anti-progestagenic activity in environmental extracts. Based on the reference compound mifepristone $EC_{SR0.2}$ value, PR CALUX was more sensitive than PR GeneBLAzer. Most water extracts either had no response or were masked by cytotoxicity in PR CALUX or PR GeneBLAzer when run in antagonist mode (Table A11). Only one study reported anti-progestagenic activity in wastewater effluent (Alygizakis *et al.*, 2019), with 9 of 12 samples active, while two studies detected anti-progestagenic activity in Australian surface waters (Scott *et al.*, 2014; Scott *et al.*, 2018).

Table 11: Common assays applied to evaluate anti-progestagenic activity in water extracts

Assay	Cell line	Detection method	Mifepristone $EC_{SPR0.2}$ (M)	Mifepristone $EC_{SPR0.2}$ (ng/L)	EC Reference
PR CALUX	U2OS	Luminescence	2.00×10^{-11}	8.6	(Jia *et al.*, 2015)
PR GeneBLAzer	HEK 293T	Fluorescence	3.00×10^{-10}	129	(Nivala *et al.*, 2018)

3.5 Thyroid receptor

3.5.1 Agonist

A number of assays have been applied to evaluate thyroid activity in environmental water extracts including yeast reporter gene assays, mammalian reporter gene assays, cell proliferation assays and a whole organism assay using embryonic xenopus (XETA) (Table A12). Based on reference compound triiodothyronine (T3), the reporter gene assays were the most sensitive. However, effects in mammalian reporter gene assays have only been observed in wastewater influent, with 25 ng/L T3 EQ reported in French wastewater using the PC-DR-LUC assay (Table A12). All other reporter gene assays did not detect thyroid activity in wastewater effluent, surface water or recycled water. In contrast, between 1100 – 1340 ng/L T3 EQ was detected in wastewater effluent using the XETA assay, with 960 ng/L T3 EQ detected in surface water (Valitalo *et al.*, 2017; Leusch *et al.*, 2018a). This suggests that the XETA assay, which incorporates toxicokinetic processes, may be more suitable to evaluate thyroid activity in water extracts than mammalian reporter gene assays.

Table 12: Common assays applied to evaluate thyroid activity in water extracts

Assay	Cell line/test system	Detection method	Triiodothyronine EC_{10} (M)	Triiodothyronine EC_{10} (ng/L)	EC Reference
Yeast reporter gene					
Yeast two-hybrid	Yeast	Absorbance	2.60×10^{-8}	17,000	(Li *et al.*, 2008)
Mammalian reporter gene					
TRβ CALUX	U2OS	Luminescence	8.60×10^{-12}	5.6	(Jia *et al.*, 2015)
TRβ GeneBLAzer	HEK 293	Fluorescence	6.00×10^{-11}	41	(Leusch *et al.*, 2017)
GH3.TRE-Luc	GH3	Luminescence	$6.67\times10^{-12*}$	4.3*	(Leusch *et al.*, 2018a)
PC-DR-LUC	PC12	Luminescence	$2.00\times10^{-11*}$	13*	(Jugan *et al.*, 2009)
Cell proliferation					
T-Screen	GH3	Fluorescence (cell viability measured using alamarBlue (Resazurin))	2.80×10^{-10}	182	(Jia *et al.*, 2015)
Whole organism					
XETA	Embryonic Xenopus	Fluorescence	EC_{50} 4.50×10^{-9}	EC_{50} 3,000	(Leusch *et al.*, 2018a)

**Presented EC_{10} value converted from EC_{50} value assuming a slope of the log-logistic concentration response curve of 1.*

3.5.2 Antagonist

Three assays have been applied to evaluate anti-thyroid activity in environmental water extracts, the yeast reporter gene yeast two-hybrid assay and the mammalian reporter gene assays GH3.TRE-Luc and TRβ GeneBLAzer. The anti-thyroid activity assay reference compound is the pharmaceutical amiodarone hydrochloride, with the EC_{50} values lower for the mammalian reporter gene assays (Table 13). Anti-thyroid activity was detected in wastewater influent (60 – 422 µg/L AH EQ), wastewater effluent (13-35 µg/L AH EQ) and surface water (3.3 – 16 µg/L AH EQ) using the yeast two-hybrid assay (Table A13). In contrast, only wastewater effluent had a response in TRβ GeneBLAzer, with none of the samples having a response in GH3.TRE-Luc in antagonist mode.

Table 13: Common assays applied to evaluate anti-thyroid activity in water extracts

Assay	Cell line/test system	Detection method	Amiodarone hydrochloride EC_{50} (M)	Amiodarone hydrochloride EC_{50} (µg/L)	EC Reference
Yeast reporter gene					
Yeast two-hybrid	Yeast	Absorbance	3.10×10^{-5}	21,000	(Li *et al.*, 2008)
Mammalian reporter gene					
TRβ GeneBLAzer	HEK 293	Fluorescence	7.30×10^{-6}	5,000	(Leusch *et al.*, 2018a)
GH3.TRE-Luc	GH3	Luminescence	8.40×10^{-6}	5,700	(Leusch *et al.*, 2018a)

3.6 Mineralocorticoid receptor

Similar to GR, MR is a corticosteroid receptor that controls the action of mineralocorticoids (Zhang *et al.*, 2019). Currently there is only one assay used to assess mineralocorticoid activity in water extracts, HG5LN-hMR, which can be run in both agonist and antagonist mode. The reference compound in agonist mode is the hormone aldosterone (EC_{50} 9.80×10^{-10} M (Leusch *et al.*, 2018b)), while pharmaceutical spironolactone (EC_{50} 2.89×10^{-9} M (Bellet *et al.*, 2012)) is the antagonist reference compound. To date, mineralocorticoid activity has not been detected in wastewater, surface water or drinking water (Bellet *et al.*, 2012; Creusot

et al., 2014; Leusch *et al.*, 2018b). However, anti-mineralocorticoid activity has been detected in wastewater influent, wastewater effluent and surface water (Table A14), with between up to 0.9 to 2.3 µg/L spironolactone EQ reported. Anti-mineralocorticoid activity was below the limit of detection in drinking water.

3.7 Retinoic acid receptor and retinoid X receptor

Only a handful of studies have applied assays indicative of the retinoic acid receptor (RAR) and retinoid X receptor (RXR). For example, wastewater effluent, surface water and drinking water had no response in the RXR CALUX and HELN-RARa-RXR assays (Leusch *et al.*, 2018b). In contrast, some surface water extracts from Serbia had a response in the RAR GeneBLAzer and RXR GeneBLAzer assays, with <0.02 - 0.15 ng/L all-trans retinoic acid EQ and 7 ng/L 9-cis-retinoic acid-EQ reported, respectively (Konig *et al.*, 2017). This equates to 41 - 170 times enrichment in the assay for RAR GeneBLAzer and 240 times enrichment in the assay for RAR GeneBLAzer. RAR activity was also detected in surface water using the yeast two-hybrid RAR assay (<0.4 - 8 ng/L all-trans retinoic acid EQ) (Chinathamby *et al.*, 2013) and an *in vitro* reporter gene bioassay using P19/A15 cells (<10 - 29 ng/L all-trans retinoic acid EQ) (Javurek *et al.*, 2015). Escher *et al.* (2014) also applied the P19/A15 assay to wastewater, recycled water, surface water and drinking water extracts, with only one wastewater effluent sample inducing 10% effect after 25 times enrichment in the assay.

4 Other receptor-mediated effects

In addition to hormone receptor mediated effects, other relevant specific modes of action include phytotoxicity and neurotoxicity.

4.1 Phytotoxicity

While not directly relevant for human health, several studies have applied algal assays to assess photosystem inhibition (PSII) in a range of water matrices (e.g., Tang and Escher, 2014; Hamers *et al.*, 2018). Most studies have assessed PSII inhibition using the combined algae assay with PSII inhibition measured after two hours using imaging pulse-amplitude modulated (PAM) fluorometry using green microalgae *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum* and *Pseudokirchneriella subcapitata*) (Escher *et al.*, 2008a). The assay reference compound is the herbicide diuron, with reported EC_{50} values ranging from 1.40 to 4.3 µg/L (Jia *et al.*, 2015; Allan *et al.*, 2017). In contrast, only two studies used Max-I-PAM with *Chlorella vulgaris*, with EC_{50} values of 16.0-16.8 µg/L reported (Macova *et al.*, 2010; Leusch *et al.*, 2014a). Consequently, *Raphidocelis subcapitata* is more sensitive. PSII inhibition is commonly expressed in diuron equivalent concentrations, with 0.04-2.2 µg/L diuron EQ detected in wastewater influent, <0.03-1.3 µg/L diuron EQ detected in wastewater effluent and 0.01-1.3 µg/L diuron EQ detected in surface water (Table A15). Based on the EC_{10} value, this translates to an effect being detected after 0.3 to 99 times enrichment. Low activity was reported in drinking water (0.02-0.05 µg/L diuron EQ) and RO treated recycled water (<0.004-0.05 05 µg/L diuron EQ) (Table A15). The observed effect in water extracts are primarily explained by PSII herbicides, with other chemicals only having a minor contribution to the observed effect (Tang and Escher, 2014; Neale *et al.*, 2017b). Therefore, the PSII inhibition assay could be applied in cases where agricultural activities may potentially impact source water quality.

4.2 Neurotoxicity

The cell-free enzymatic acetylcholinesterase (AChE) inhibition assay (Ellman *et al.*, 1961) is often used to assess neurotoxicity in environmental water extracts (Macova *et al.*, 2011; Tousova *et al.*, 2017). Organophosphate insecticide parathion is commonly used as the assay reference compound and the reported parathion EC_{50} values range from 26.3 to 120 µg/L (Escher *et al.*, 2008b; Macova *et al.*, 2010). AChE inhibition was detected in wastewater influent, wastewater effluent, recycled water, surface water and drinking water extracts (Table A16), but the assay does suffer from false-positive and false-negatives that limits its application. For example, Neale and Escher (2013) found that dissolved organic carbon (DOC) concentrations as low as 2 mg of carbon/L (mgC/L) caused quenching in the assay. DOC can be co-extracted during sample treatment, so the AChE

inhibition assay is not recommended for DOC-rich samples, such as wastewater or surface water. Cell-based neurotoxicity assays would be more applicable, with work currently underway to identify suitable *in vitro* bioassays (Legradi *et al.*, 2018). In addition to receptor-mediated neurotoxicity, it can also act via other toxicological mechanisms, such as blocking of the release of neurotransmitters or inhibiting their re-uptake in vesicles.

4.3 Other assays

In addition to the above studies, new bioassays have been developed specifically to detect modes of action relevant to pharmaceuticals. For example, Bernhard *et al.* (2017) has developed beta-blocker and non-steroidal anti-inflammatory drug (NSAID) assays, with a limit of detection of 2 µg/L metoprolol equivalents and 0.5 µg/L diclofenac equivalents, respectively. The assays were applied to SPE enriched wastewater effluent, with 3.2 to 4.2 µg/L metoprolol equivalents and 3.5 µg/L diclofenac equivalents detected. Further, Zhang *et al.* (2018) applied a TGFα shedding assay to assess the biological activity of pharmaceuticals that bind to G protein-coupled receptors. SPE enriched wastewater from Japan and the UK were applied, with most samples found to be angiotensin (AT1), dopamine (D2), adrenergic (β1), acetylcholine (M1), and histamine (H1) receptor antagonists. These assays have not been widely applied to date.

5 Reactive toxicity

Reactive toxicity occurs when chemicals form covalent bonds with DNA, proteins and membrane lipids. If the target is DNA, genototoxicity and mutagenicity may result. Reactive chemicals occur at low concentrations in water because they are at the same time degradable, but reactive intermediated can also form by metabolic activation. DBPs, which form due to the reaction of disinfectants, such as chlorine and chloramine, with organic matter in water, are reactive chemicals and are responsive in a number of assays indicative of reactive toxicity (Stalter *et al.*, 2016a), including oxidative stress, not addressed here directly but indirectly via the adaptive stress responses (Chapter 6).

Two common bacterial assays used to assess reactive toxicity in environmental extracts include the umuC assay to detect genotoxicity and the Ames assay to detect mutagenicity. Both assays can be run either with or without rat liver S9 fraction, which is used to simulate metabolic activation. There are also other reactive toxicity assays, such as γ-H2AX foci and micronucleus assays, but these assays have not been widely applied to water extracts to date, so were not considered in the review.

5.1 Genotoxicity

The umuC assay, which is also known as umu or SOS/umu, is used to assess DNA damage via the inducible SOS response. SOS genes are repressed under normal conditions, but are released in the presence of DNA damage to help repair any damage (Michel, 2005). A number of *Salmonella typhimurium* strains including TA1535/pSK1002, NM2009, NM3009, NM5004 and the *E. coli* SOS chromotest have been applied to water extracts (Escher *et al.*, 2014; Han *et al.*, 2016). However, most studies have used TA1535/pSK1002 either with or without metabolic activation, so this section will focus on the TA1535/pSK1002 strain. The reference compound without metabolic activation is 4-nitroquinoline N-oxide (4NQO) ($EC_{IR1.5}$ 9.47×10^{-8} M (Macova *et al.*, 2011)), while 2-aminoanthracene (2-AA) ($EC_{IR1.5}$ 2.42×10^{-7} M (Tang *et al.*, 2014)) is often used as the reference compound with metabolic activation.

The umuC assay with has been applied to wastewater, surface water, recycled water and drinking water (Table A17). Wastewater influent and wastewater effluent were the most responsive, with many samples not having a response up to the maximum REF in surface water or highly treated recycled water (e.g., RO or ozone and biological activated carbon (O_3/BAC)). Increased genotoxicity after disinfection in an Australia DWTP was observed by Neale *et al.* (2012). Genotoxicity increased from 0.05 µg/L 4NQO EQ (-S9) and 0.18 µg/L 2-AA EQ (+S9) at the inlet to 0.43 µg/L 4NQO EQ (-S9) and 1.29 µg/L 2-AA EQ (+S9) at the outlet. The effect at the outlet was observed after 23 to 33 times enrichment, with genotoxicity without metabolic activation more sensitive. This suggests that with sufficient enrichment, the umuC assay can be used to assess DBP formation during

drinking water treatment. Tap water also induced a response in the umuC assay without S9 after 29 to 65 times enrichment (Stalter *et al.*, 2016b). Focusing on single DBPs, the addition of S9 did not increase genotoxicity and in some cases reduced the potency (Stalter *et al.*, 2016a), suggesting many DBPs are direct genotoxicants.

5.2 Mutagenicity

The bacterial Ames assay has been widely used to assess mutagenicity in a range of water extracts. However, many studies simply report a positive or negative response (e.g., Berninger *et al.*, 2019; Albergamo *et al.*, 2020), without determining an effect concentration or equivalent concentration. Consequently, this review focused on studies that have reported an effect concentration, with studies reporting the effect concentration inducing a revertant ratio of 1.5 ($EC_{RR1.5}$) included in Table A18. The *Salmonella typhimurium* strains applied in Table A18 include TA98, which responds to frameshift mutations, and TA100 and TAmix, which both respond to base pair substitutions (Kamber *et al.*, 2009). Escher *et al.* (2014) found increasing mutagenicity (e.g., lower $EC_{RR1.5}$ values) in treated drinking water compared to source water for all strains tested, with little difference between strains or with or without S9. Effects were detected after 3 to 13 times enrichment in drinking water, 5 to >30 times enrichment in surface water and 0.6 to >100 times enrichment in wastewater effluent (Table A18). Further, Ames strains TA98, TA100 and YG7108, which is responsive to nitrosamines, were tested with and without metabolic activation in source water and treated drinking water extracts from three French DWTPs (Neale *et al.*, 2020b). However, none of the samples had an effect up to the maximum REF of 200.

6 Adaptive stress responses

Adaptive stress responses pathways are activated to help restore cells back to homeostasis after damage from stressors, including organic chemicals (Simmons *et al.*, 2009). This review will focus on three adaptive stress response pathways commonly applied to environmental water extracts: oxidative stress response (Nrf2), p53 response for genotoxicity and NF-κB response for inflammation. Assays indicative of adaptive stress responses hypoxia and heat shock response did not have a response in drinking water, surface water, wastewater or recycled water extracts (Escher *et al.*, 2014).

6.1 Oxidative stress response

Five mammalian reporter gene assays have been applied to evaluate the oxidative stress response in environmental water extracts (Table 14, Table A19). tert-Butylhydroquinone (tBHQ) is often used as the assay reference compound, with similar $EC_{IR1.5}$ or lowest observed effect concentration (LOEC) at an induction factor of 1.5 for AREc32, ARE GeneBLAzer, Nrf2 CALUX and Nrf2 reporter gene assay. The tBHQ $EC_{IR1.5}$ value for Nrf2-MDA-MB was over an order of magnitude higher (e.g., less toxic) and the assay did not have an effect in drinking water, surface water, wastewater effluent or recycled water (Escher *et al.*, 2014; Jia *et al.*, 2015). Consequently, Nrf2-MDA-MB does not appear to be suitable for environmental water extracts.

Effects in drinking water have been detected after 3 to 102 times enrichment (Table A19), with drinking water treated with membrane filtration often not inducing the oxidative stress response up to the maximum REF (REF 100 to 150) (Albergamo *et al.*, 2020; Neale *et al.*, 2020b). In contrast, an increase in the oxidative stress response has been observed after drinking water disinfection (Neale *et al.*, 2012; Escher *et al.*, 2013; Hebert *et al.*, 2018), indicating that oxidative stress response assays can detect formed DBPs. The $EC_{IR1.5}$ in surface water varied from 7 to 93 REF, while effects were observed in wastewater influent and effluent from $EC_{IR1.5}$ 0.3 to 5 REF and $EC_{IR1.5}$ 2 to 47 REF, respectively (Table A20). The reported oxidative stress response WWTP removal efficacy ranged from 81 to 85% (Nivala *et al.*, 2018). Effects in recycled water ranged from $EC_{IR1.5}$ 4 to 74 REF.

Table 14: Common assays applied to evaluate the oxidative stress response in water extracts

Assay	Cell line	Detection method	tBHQ $EC_{IR1.5}$ (M)	tBHQ $EC_{IR1.5}$ (µg/L)	EC Reference
AREc32	MCF-7	Luminescence	1.32×10^{-6}	219	(Escher *et al.*, 2012)
ARE GeneBLAzer	HepG2	Fluorescence	2.44×10^{-6}	406	(Neale *et al.*, 2015)
Nrf2 CALUX	U2OS	Luminescence	$1.00\times10^{-6*}$	166	(van der Linden *et al.*, 2014)
Nrf2 reporter gene assay	HepG2	Luminescence	2.00×10^{-6}	332	(Lundqvist *et al.*, 2019a)
Nrf2-MDA-MB	MDA-MB-231-745	Luminescence	3.30×10^{-5}	5490	(Jia *et al.*, 2015)

**LOEC at an induction factor of 1.5*

6.2 p53 response

Two assays, p53 GeneBLAzer and p53 CALUX, have been applied to environmental water extracts (Table 15). The two assays use different reference compounds, making it difficult to compare assay sensitivity, with p53 CALUX results reported in both actinomycin D and cyclophosphamide EQ. Most studies report either no effect or cytotoxicity in p53 response assays (Table A20). The p53 response in the Danube River was observed after 65 times enrichment in the p53 GeneBLAzer, which gave an mitomycin EQ of 235 ng/L (Neale *et al.*, 2015). p53 activity was also detected in wastewater influent and effluent in Finland using the p53 CALUX after the addition of +S9 for metabolic activation (Valitalo *et al.*, 2017), with the results expressed as cyclophosphamide equivalents.

Table 15: Common assays applied to evaluate the p53 response in water extracts

Assay	Cell line	Detection method	Reference compound	$EC_{10}^{*}/EC_{IR1.5}^{\dagger}$ (M)	$EC_{10}^{*}/EC_{IR1.5}^{\dagger}$ (µg/L)	EC Reference
p53 CALUX (+/- S9)	U2OS	Luminescence	Actinomycin D	$2.00\times10^{-9*}$	2.5^{*}	(Pieterse *et al.*, 2015)
p53 GeneBLAzer	HCT-116	Fluorescence	Mitomycin C	$4.53\times10^{-8\dagger}$	$15^{\dagger}$	(Neale *et al.*, 2015)

6.3 NF-κB response

Three reporter gene assays have been applied to evaluate the NF-κB response in environmental water extracts (Table 16). The tumor necrosis factor alpha (TNFα) $EC_{IR1.5}$ value for NFκB GeneBLAzer was 4.5 times lower than the NFκB reporter gene assay developed by Lundqvist *et al.* (2019b) (Table 16), while no reference compound data was available for NFκB CALUX. Extracts of drinking water, surface water and wastewater were active in the NFκB GeneBLAzer assay, while only wastewater influent was active in the NFκB reporter gene assay and no samples were active in NFκB CALUX (Table A21). While effects are often observed at low enrichment factors in NFκB GeneBLAzer, the assay may not be suitable to evaluate the effects of micropollutants. Endotoxins are NF-κB activators and a recent study showed that co-extracted endotoxins likely explained most of the effect in surface water extracts in the NFκB GeneBLAzer assay (Neale *et al.*, 2018b).

Table 16: Common assays applied to evaluate the NF-κB response in water extracts

Assay	Cell line	Detection method	TNFα $EC_{IR1.5}$ (ng/L)	EC Reference
NFκB GeneBLAzer	THP-1	Fluorescence	20	(Neale *et al.*, 2015)
NFκB reporter gene assay	HepG2	Luminescence	90	(Lundqvist *et al.*, 2019b)
NFκB CALUX	-	Luminescence	-	-

7 Apical effects

In addition to assays indicative of different stages of the cellular toxicity pathway, whole organism assays indicative of apical effects are commonly applied to water quality monitoring. Further, some organisms, such as zebrafish, are used as a model species for human health risk assessment (Bambino and Chu, 2017).These assays can provide information about mortality, growth and development and capture effects from multiple toxicity pathways resulting in the same apical effect (Wernersson *et al.*, 2015). While these assays are often used for direct toxicity assessment, we have focused on assays applied to water extracts, including bacterial toxicity, algal growth inhibition and fish embryo toxicity. These assays can be run in well plates. The Daphnia immobilisation assay has also been applied to passive sampler extracts (Hamers *et al.*, 2018; De Baat *et al.*, 2019), but not SPE extracts to our knowledge, so it was not included in the review.

7.1 Bacterial toxicity

Bacterial bioluminescence inhibition assays, such as the Microtox (*Aliivibrio fischeri*) and BLT-Screen (*Photobacterium leiognathi*) assays, have been applied to drinking water, surface water and wastewater extracts (Tang *et al.*, 2013; van de Merwe and Leusch, 2015). While these assays are simple, they are fast (15-30 minutes) and are responsive to organic micropollutants. For example, 50% inhibition of bioluminescence was observed after 0.5 to 17 times enrichment in wastewater influent, 2 to 27 times enrichment in wastewater effluent, 8 to 87 times enrichment in surface water and 3 to 40 times enrichment in drinking water (Table A22). Bacterial toxicity assays can detect the formation of DBPs, with increasing effect (e.g., decreasing EC_{50} values) reported throughout DWTPs (Escher *et al.*, 2012; Neale *et al.*, 2012). While bacterial toxicity assays only provide information about non-specific effects and should be complemented with assays indicative of specific effects, their advantage is that they can also be used in locations that only have access to microbiology laboratory facilities.

In addition to bacterial bioluminescence inhibition assays, luminescent bacterial biosensors indicative of DNA damage, oxidative stress and protein damage have been developed in recent years (Woutersen *et al.*, 2011). There is increasing interest in applying these biosensors as online water quality monitoring tools, though further work is required to improve sensitivity.

7.2 Algal growth inhibition

Algal growth inhibition assays using green microalgae *Raphidocelis subcapitata* have been applied to wastewater effluent, recycled water and surface water extracts, with only one study testing drinking water extracts (Table A23). Most studied applied the combined algae assay, with growth inhibition measured after 24 h based on Escher *et al.* (2008a), while one study used 72 h algal growth inhibition based on the OECD guidelines (OECD, 2011). The combined algae assay was responsive to wastewater effluent extracts, with 10% growth inhibition observed at a REF from 0.7 to 13, while 10% growth inhibition in surface water occurred at a REF from 1.3 to 51 (Table A23). No effects were observed in groundwater or recycled water treated with RO/AO or O_3/BAC up to the maximum tested REF.

7.3 Fish embryo toxicity

The fish embryo toxicity (FET) assay has only been applied for water quality monitoring in a limited number of studies. Escher *et al.* (2014) applied the 48 h FET assay to drinking water, surface water, wastewater effluent and drinking water, but only one wastewater effluent extract had an effect, with 10% mortality observed at a REF of 5. Further, the 48 h FET assay was applied to surface water extracts from the Danube River, with the EC_{50} value ranging from REF 111 to 665 (Neale *et al.*, 2015). The EC_{50} values were around an order of magnitude lower in surface water from four river basins in Europe in the 96 h FET assay (Tousova *et al.*, 2017). Both Tousova *et al.* (2017) and Neale *et al.* (2015) applied large volume SPE, with 50 to 500 L of water concentrated. The 48 h FET assay has also been applied to surface water and wastewater passive sampler extracts, with styrene divinylbenzene Speedisk samplers proving more responsive than silicone rubber samplers (Hamers *et al.*, 2018).

8 Multiplexed high-throughput assays

In addition to assays indicative of a single endpoint, some studies have applied multiplex high-throughput screening assays, such as Attagene cis-Factorial and trans-Factorial assays to drinking water, surface water and wastewater (Escher *et al.*, 2014; Blackwell *et al.*, 2019; Medlock Kakaley *et al.*, 2020). These assays include many targeted endpoints covering different stages of the cellular toxicity pathway. Escher *et al.* (2014) found PXR, ERα, PPARy, AhR and the antioxidant response element were the most responsive in wastewater, surface water and recycled water extracts. Blackwell *et al.* (2019) confirmed that PXR and AhR related endpoints were most commonly activated in surface water, followed by ERα and PPARγ related endpoints (Figure 2). Medlock Kakaley *et al.* (2020) found that PXR and PXR signaling pathway (PXRE) were the only nuclear receptors activated in the intake to a DWTP, with no effect in treated water. These results support the finding from individual reporter gene assays, with assays indicative of activation of PXR, AhR and ERα found to be responsive to a wide range of water types. They also confirm that the iterative strategy over the last years of implementing various reporter gene assays has resulted in a sufficient number of endpoints and has not overlooked important endpoints relevant for water quality. However, compared to targeted reporter gene assays, Blackwell *et al.* (2019) found that the multiplex assays were less sensitive when applied to the same surface water samples. Thus, they might be less suitable for surveillance monitoring, where reporter gene assays will remain the first choice. Since these multiplexed assays cover a wider range of endpoints, they are especially suitable for screening purposes to assure that new endpoints are not overlooked when a new type of water is investigated.

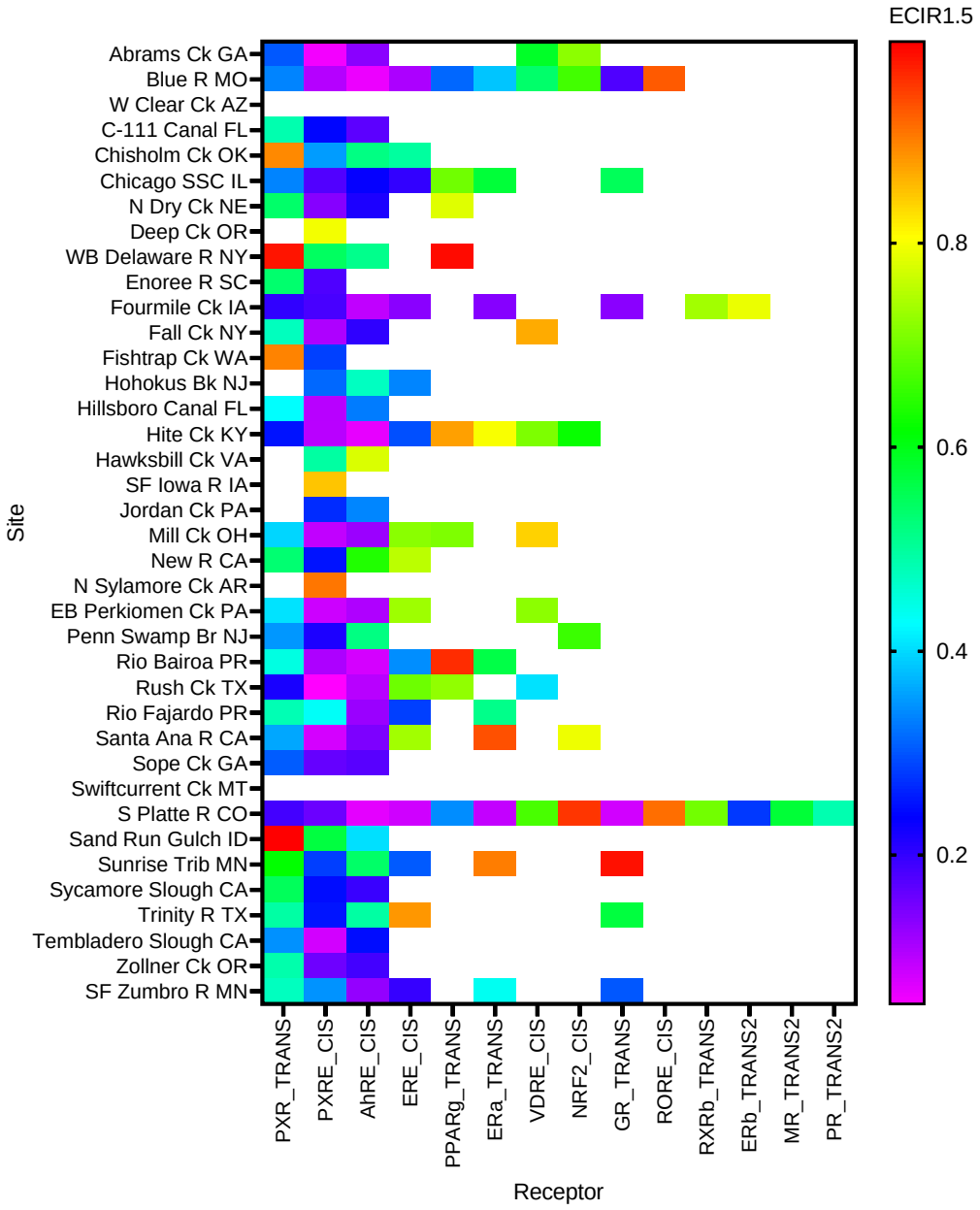

Figure 2: Heat map showing derived $EC_{IR1.5}$ values from Blackwell et al. (2019) for commonly activated Attagene endpoints in surface water extracts.

9 Effects estimated by chemical analysis

While all of the studies reviewed above have applied bioassays to evaluate water quality, an alternative approach is to predict the effect based on chemical analysis data and single chemical effect data. Common examples of this approach include bioanalytical equivalent concentration from chemical analysis (BEQ_{chem}) (e.g., Tang *et al.*, 2013; Neale *et al.*, 2015; Tousova *et al.*, 2017), toxic units (TU) (Ginebreda *et al.*, 2014) and exposure-activity ratio (EAR) analysis (e.g., Blackwell *et al.*, 2019; Bradley *et al.*, 2019; Brunner *et al.*, 2019). The definition of these indicator is outlined in Figure 3, showing also how one can translate between them.

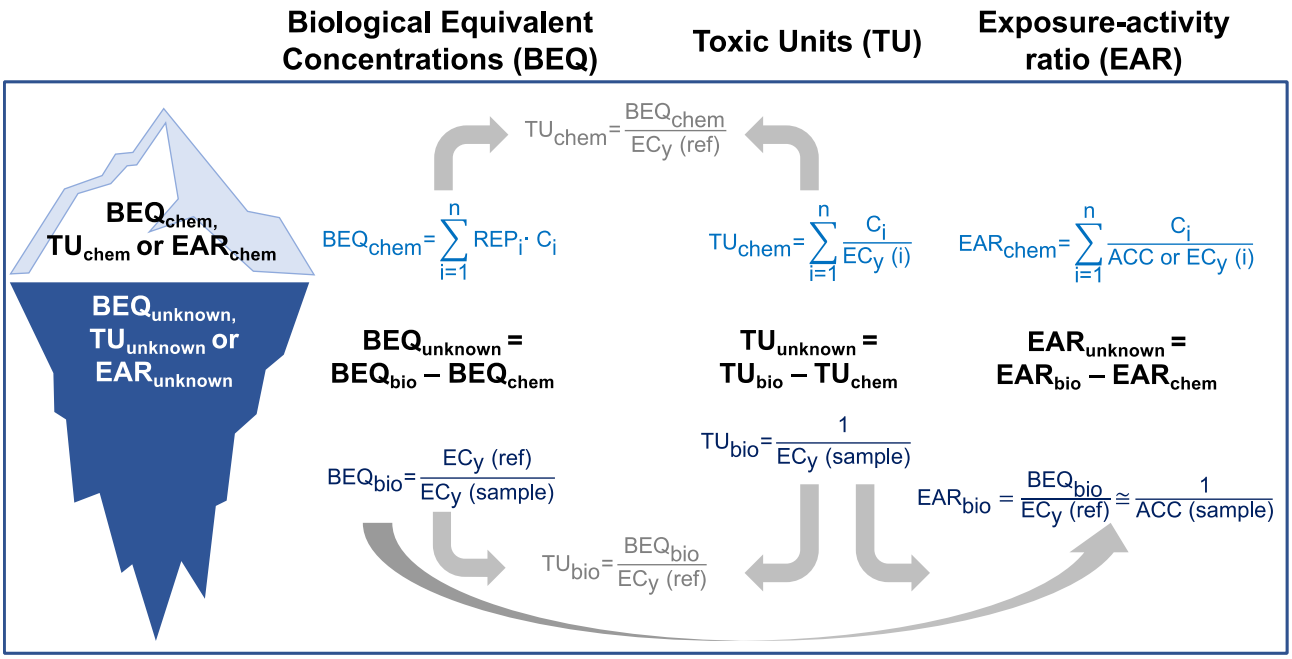

Figure 3: Various typically used effect estimates from chemical analysis and effect potencies of single chemicals and how they related to measured bioassay activity. Figure adapted from Villeneuve et al. (2019) and Neale and Escher (2020). BEQ: bioanalytical equivalent concentrations; TU: Toxic Unit; EAR: exposure-activity ratio; REPi: relative effect potency Ci: concentration of chemical i; ECy: effect concentration triggering effect y; ACC: activity cut-off concentration.

EAR compares the detected environmental concentration (e.g., exposure) to the activity concentration at the cut-off (ACC (Filer *et al.*, 2017)) from the US EPA ToxCast database and sums this up for all detected chemicals with available activity data (Blackwell *et al.*, 2017). Water samples with an EAR of 1 or higher indicate that the chemicals present can induce an effect *in vitro* and this approach can be used to prioritise chemicals contributing to the effect (Corsi *et al.*, 2019; Bradley *et al.*, 2020). The BEQ_{chem} approach is similar and uses the detected chemical concentration and the potency of the detected chemical relative to the assay reference compound (Escher *et al.*, 2013). BEQ_{chem} can be compared to the BEQ from bioanalysis (BEQ_{bio}) using iceberg modelling to determine the fraction of the effect that can be explained by detected chemicals (Neale *et al.*, 2018a). Both approaches require experimental single chemical effect data, which can be sourced from the literature or the US EPA ToxCast database. Both EAR and BEQ_{chem} are able to predict the majority of the effect for assays where few potent chemicals drive the effect, such as receptor-mediated effects (e.g. estrogenic activity, PSII inhibition). For example, natural and synthetic estrogenic hormones (e.g., 17β-estradiol, estrone and 17α-ethinylestradiol) often explain most of the effect in the activation of ER assay (e.g., Murk *et al.*, 2002; Leusch *et al.*, 2010; Konemann *et al.*, 2018), while PSII herbicides, such as diuron, atrazine, simazine and terbuthylazine, often explain most of the effect in the phytotoxicity assay (e.g., Bengtson Nash *et al.*, 2006; Neale *et al.*, 2017b). However, EAR and BEQ_{chem} underestimate the effect of water samples for assays where many chemicals can contribute, such as assays indicative of xenobiotic metabolism, adaptive stress responses or apical effects. Often less than 1% of the observed effect was explained by detected chemicals in these assays (e.g., Escher *et al.*, 2013; Creusot *et al.*, 2014; Neale *et al.*, 2017b), highlighting the importance of applying bioassays for water quality monitoring.

10 Decision-Making Tool

Due to the complex mixture of chemicals commonly present in environmental water extracts, a single bioassay cannot capture all the effects that can be induced by these complex mixtures. While a large number of assays are available, a practical test battery of at least three or four bioassays representative of effects commonly detected in water samples and aligned with relevant steps of adverse outcome pathways are recommended.

While it is possible that other relevant effects may be missed with only three to four bioassays, aligning the selected bioassay with results from multiplex assays (Section 8) can help prevent any common effects being overlooked. Further, assay selection will depend both on the context (e.g., water type, treatment type) and the purpose of the sampling campaign (e.g., to assess product quality or treatment process efficacy).

In the case of wastewater and water reuse for non-potable use, we recommend assays that represent different stages of the cellular toxicity pathway, i.e., xenobiotic metabolism, receptor-mediated effects and adaptive stress responses. A typical test battery would include activation of AhR, activation of ER and oxidative stress response based on the above literature review. These three endpoints are responsive to a range of water types, as demonstrated by both individual and multiplexed assays. They are also commonly applied assays, with 27%, 77% and 21% of reviewed studied applying assays indicative of activation of AhR, activation of ER and oxidative stress response, respectively. Further, proposed effect-based trigger values (EBTs) are available for these endpoints (Brand *et al.*, 2013; Escher *et al.*, 2013; Escher *et al.*, 2015; van der Oost *et al.*, 2017; Escher *et al.*, 2018a). The availability of EBTs will be discussed further in Deliverable 3.4, where the existing EBT will be reviewed. Further, this recommendation aligns with recommendations for testing surface water quality (Brack *et al.*, 2019), and such harmonisation is important given that rivers are receiving effluent input and may at the same time be source water for DWTPs. Further, applying the same test battery allows comparison of effects over time.

Of course, if focus of a study or a monitoring program is on endocrine disruption, further endocrine endpoints can be adde such as GR and anti-AR. Likewise for activation of xenobiotic metabolism, PXR and PPARg are viable alternative to AhR. They

In the context of drinking water treatment or water reuse for potable use, an assay indicative of either genotoxicity or mutagenicity is recommended in addition to activation of AhR, activation of ER and oxidative stress response. It is worth noting that oxidative stress response assays can detect increased effects after drinking water disinfection. However, they cannot replace mutagenicity or genotoxicity testing (with traditional bacterial assays such as the Ames test or umuC assay) but are often also be triggered by genotoxic chemicals, not only those with direct reactive toxicity. Unfortunately, EBT values for these assays are not currently available in the scientific literature.

As discussed above, there are multiple assays available for each endpoint, so a decision-making tool is required to assist users select appropriate assays. Consequently, we suggest a decision-making tool that groups assays into three test batteries (Table 17), with test battery selection depending on the sampling campaign context (water type) and purpose (Table 18). “Sensitivity” stands for assay responsiveness. If bioassays used as bioanalytical tool, the detection limit must be sufficiently low to be able to detect effects in very clean water samples. This can on the one hand be achieved by high enrichment, on the other hand, by using bioassays that respond already to low concentrations of chemcials. For example, if the purpose of a sampling campaign is to assess WWTP product quality alone, a battery of low sensitivity assays, such as yeast reporter gene assays, could be applied as these assays are typically sufficiently sensitive to detect effects in treated effluent (test battery 1). However, a battery of high sensitivity assays, namely mammalian reporter gene assays, are recommended for understanding critical processes in WWTPs or for any purpose in a water reuse context (test battery 2). This is because yeast reporter gene assays are unlikely to be sensitive enough to detect effects after advanced treatment processes. As discussed above, the majority of mammalian reporter gene assays are similarly sensitive so any of the reviewed assays can be applied.

In contrast to wastewater, a battery of high sensitivity assays is required for all sampling purposes in a drinking water context (test battery 3). Test battery 3 adds a genotoxicity or mutagenicity assay, such as Ames or umuC, which will be particularly important if disinfected, e.g., chlorinated, water is being evaluated. It should be noted that the assay detection limit depends on the volume of sample enriched, so larger sample volumes are recommended for cleaner samples, such as drinking water or recycled water. This will be discussed further in Deliverable 3.3.

In all test batteries, the specific effects measures should be accompanied by cytotoxicity assessment. This is because cytotoxicity may cause false negative results (e.g., masking the effect) or false positive results (e.g., “cytotoxicity burst” phenomena (Judson *et al.*, 2016)) If a reporter gene assay is used that cannot be duplexed with a quantitative cytotoxicity assays that reports effect concentrations for cytotoxicity (e.g., inhibitory concentration IC_{10}), then it is imperative to include an assay with an apical endpoint, such as the bacterial bioluminescence inhibition assays discussed in Section 7.1.

Table 17: Recommended endpoints in the different test batteries to apply for water quality monitoring. For battery selection depending on the context and purpose of the sampling campaign, see Table 18 below. Assays indicative of the different endpoints are provided in Tables 19 to 21.

Test battery	Bioassays			
Battery 1	Low sensitivity ER*	Oxidative stress	AhR	
Battery 2	High sensitivity ER*	Oxidative stress	AhR	
Battery 3	High sensitivity ER*	Oxidative stress	AhR	Mutagen/genotoxicity

** High sensitivity ER requires mammalian reporter gene assays; low sensitivity ER includes yeast reporter gene assays.*

Table 18: Battery selection depending on sampling campaign context and purpose. See Table 17 above for description of the different test batteries.

Water context	Purpose		
	Assess product quality	Assess treatment efficacy	Understand treatment processes (eg, CCP)
Wastewater treatment	Battery 1	Battery 1	Battery 2
Water reuse (non-potable)	Battery 2	Battery 2	Battery 2
Drinking water (incl potable reuse)	Battery 3	Battery 3	Battery 3

To assist with the selection of a suitable bioassays for each endpoint Table 19 for estrogenicity, Table 20 for oxidative stress response and Table 21 for AhR activation summarise some key features of commonly used assays, including availability of an EBT, whether the assay is commonly used for water quality monitoring and assay sensitivity. Tables 19 to 21 will need to be updated as new EBTs are developed for assays currently without EBTs. We cannot provide such a table for mutagenicity/genotoxicity assessment because most of the available assays only give yes/no responses and future work will need to focus on suggesting quantitative measures and EBTs for mutagenicity/genotoxicity assays. Either the umuC assay for genotoxicity or the Ames assay for mutagenicity could be applied as both are commonly applied to water samples.

Table 19: Overview of key parameters of common assays applied to evaluate estrogenic activity.

Assay	Cell line/test system	17β-estradiol EC_{10} (ng/L)	Availability of EBT	Commonly applied in case studies	Sensitivity	Experience with water quality testing
Yeast reporter gene						
YES	Yeast	10.2	+	+	Low	Applied in wastewater influent, wastewater effluent, recycled water, surface water, drinking water
Mammalian reporter gene						
ERα CALUX	U2OS	0.19	+	+	High	Applied in wastewater influent, wastewater effluent, recycled water, surface water, ground water, drinking water
ERα GeneBLAzer	HEK 293	2.7	+	+	High	Applied in wastewater influent, wastewater effluent, recycled water, surface water, ground water, riverbank filtrate drinking water
HeLa-9903	HeLa	2.1	+	+	High	Applied in wastewater effluent, recycled water, surface water, drinking water
MELN	MCF-7	0.66	+	+	High	Applied in wastewater influent, wastewater effluent, surface water, drinking water
MVLN	MCF-7	0.86	+	+	High	Applied in wastewater influent, wastewater effluent, surface water
T47D-KBluc	T47D	0.13	-	+	High	Applied in wastewater effluent, surface water, drinking water
Cell proliferation						
E-Screen	MCF7	0.22	+	-*	High	Applied in wastewater influent, wastewater effluent, recycled water, surface water, drinking water
Whole organism						
EASZY	Embryonic zebrafish	EC_{50} 168	+	-	Low	Applied to wastewater effluent, surface water

"+" indicates availability of previously published EBT or commonly applied in case studies; "-" indicates that an EBT is not currently available or the assay is infrequently applied in case studies.

**E-Screen has been widely used in the past, but it is a seven-day test, so is much more time consuming than other assays. Consequently, it is less commonly applied now.*

Table 20: Overview of key parameters of common assays applied to evaluate the oxidative stress response.

Assay	Cell line	tBHQ $EC_{IR1.5}$ (µg/L)	Availability of EBT	Commonly applied in case studies	Sensitivity	Experience with water quality testing
AREc32	MCF-7	219	+	+	High	Applied in wastewater influent, wastewater effluent, recycled water, surface water, ground water, riverbank filtrate, drinking water
ARE GeneBLAzer	HepG2	406	+	+	High	Applied in wastewater effluent and surface water
Nrf2 CALUX	U2OS	166	+	+	High	Applied in wastewater effluent, recycled water, surface water, drinking water
Nrf2 reporter gene assay	HepG2	332	-	-	High	Applied in wastewater influent, wastewater effluent, surface water and drinking water
Nrf2-MDA-MB	MDA-MB-231-745	5490	-	-	Moderate	Applied in wastewater effluent, recycled water, surface water, ground water, drinking water

"+" indicates availability of previously published EBT or commonly applied in case studies; "-" indicates that an EBT is not currently available or the assay is infrequently applied in case studies.

Table 21: Overview of key parameters of common cell-based reporter gene assays applied to evaluate the aryl hydrocarbon receptor (AhR) activity.

Assay	Cell line	TCDD EC_{10} (ng/L)	Benzo(a) pyrene EC_{10} (ng/L)	Availability of EBT	Commonly applied in case studies	Sensitivity	Experience with water quality testing
AhR CAFLUX	H1.G1.1c3, H4.G1.1c2	0.21-0.22		-	+	High	Applied in wastewater influent, wastewater effluent, recycled water, surface water, groundwater, drinking water
AhR CALUX	H4L1.1c4	0.19	211	+	+	High	Applied in wastewater effluent, wastewater effluent, surface water, riverbank filtrate, drinking water
AhR reporter gene assay	HepG2	20		-	-	Low	Applied in wastewater influent, wastewater effluent, surface water, drinking water
H4IIE-luc	H4IIE	0.05		-	+	High	Applied in wastewater influent, wastewater effluent, recycled water, surface water, groundwater, drinking water
PAH CALUX	H4IIE		50	+	-	High	Applied to wastewater effluent*

"+" indicates availability of previously published EBT or commonly applied in case studies; "-" indicates that an EBT is not currently available or the assay was infrequently applied in case studies.

**Also applied to surface water passive sampler extracts.*

Both commercial laboratories (e.g., BioDetection Systems (CALUX test battery), Attagene (cis-Factorial and trans-Factorial assays), Xenometrix (Yeast reporter gene assays, Ames)) and research laboratories offer bioassay testing facilities. Further, the cell lines and reagents required for some assays can be purchased (e.g., Thermo Scientific (GeneBLAzer test battery)), and some assays are available as kits, potentially allowing water utilities to establish and run some of these assays in house. Naming a few companies does not mean that these providers are preferred, they only serve as examples.

Some water utilities may have difficulty accessing laboratories that can run mammalian reporter gene assays but will most likely have access to a microbiology laboratory. In these cases, a simple bacterial toxicity assay, such as Microtox or BLT-Screen, could be applied. Both assays are similar sensitive, have been applied to wastewater, surface water and drinking water (though Microtox is more widely used) and EBTs are available for Microtox (Tang *et al.*, 2013; Escher *et al.*, 2018a). It should be noted that these assays only provide information about non-specific effects and should be complemented with assays indicative of specific effects when possible, but they can be powerful as sum parameter for chemical water quality (e.g., to measure changes over time, or to compare different waters). Since the critical membrane concentrations for baseline toxicity are similar for all cells (Escher et al., 2019), the cytotoxicity in mammalian cell lines occurs often at a similar concentration range as that of bacterial growth inhibition assays.

It should be noted that while test batteries of three or four assays have been common practise and are recommended in most situations, water utilities could also apply more comprehensive test batteries. This could include any assay previously found to have a response in water extracts (refer to the tables in the Appendix) and could also include whole organism assays indicative of apical effects (e.g., algal growth inhibition assay or fish embryo toxicity assay). The selection of additional assays may be related to water quality concerns of the water utility. For example, a phytotoxicity assay could be included if raw drinking water is collected from a catchment impacted by agriculture. An overview of all endpoints reviewed in the current document, excluding

the four endpoints recommended above, and their responsiveness wastewater effluent, surface water and wastewater are summarised in Table 22.

Table 22: Overview of additional reviewed endpoints and their responsiveness in wastewater effluent, surface water and drinking water.

Endpoint	Wastewater effluent	Surface water	Drinking water
PPARγ	+	+	-
PXR	+	+	+
Anti-ER	+	+	-
AR	+	+	+
Anti-AR	+	+	-
GR	+	+	-
Anti-GR	-	+	-
PR	+	+	-
Anti-PR	+	+	-
TR	+*	+*	-
Anti-TR	+	+	-
MR	-	-	-
Anti-MR	+	+	-
RAR/RXR	+	+	-
Phytotoxicity	+	+	+
AChE	+	+	+
p53	+	+	-
NF-κB	+	+	+
Bacterial toxicity	+	+	+
Algal growth	+	+	+
FET	+	+	-

"+" indicates activity detected at least once in wastewater effluent, surface water or drinking water; "-" indicates no effect up to the maximum REF or effect masked by cytotoxicity in wastewater effluent, surface water or drinking water.

**Only in the XETA assay.*

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Appendices

Table A1: Summary of reported AhR activity in units of ng/L TCDD EQ in different environmental water extracts.

NB: Only studies that have applied SPE or liquid-liquid extraction are included.

Matrix	Assay	Activation of AhR (ng/L TCDD EQ)	Reference
Wastewater influent	AhR reporter gene assay	187 - 386	(Lundqvist *et al.*, 2019b)
Wastewater influent	AhR CAFLUX (H1.G1.1c3)	1.1 - 1.8	(Macova *et al.*, 2011)
Wastewater influent	AhR CALUX (H4L1.1c4)	0.25 - 0.27	(Nivala *et al.*, 2018)
Wastewater influent	H4IIE-luc	0.1 - 3.3	(Jalova *et al.*, 2013)
Wastewater influent	H4IIE-luc	<0.11	(Lee *et al.*, 2015)
Wastewater effluent	AhR CAFLUX (H1.G1.1c3)	0.13 - 0.21*	(Escher *et al.*, 2014)
Wastewater effluent	H4IIE-luc	0.04-0.06*	(Escher *et al.*, 2014)
Wastewater effluent	AhR reporter gene assay	97 – 168	(Lundqvist *et al.*, 2019b)
Wastewater effluent	AhR CAFLUX (H1.G1.1c3)	0.007 - 0.03	(Jia *et al.*, 2015)
Wastewater effluent	AhR CAFLUX (H1.G1.1c3)	0.21 - 1.2	(Macova *et al.*, 2011)
Wastewater effluent	AhR CAFLUX (H1.G1.1c3)	0.59 - 0.98	(Reungoat *et al.*, 2010)
Wastewater effluent	AhR CAFLUX (H1.G1.1c3)	0.087	(Macova *et al.*, 2010)
Wastewater effluent	AhR CAFLUX (H4G1.1c2)	0.063 - 0.10	(Neale *et al.*, 2017b)
Wastewater effluent	AhR CALUX (H4L1.1c4)	0.12 - 0.13	(Nivala *et al.*, 2018)
Wastewater effluent	H4IIE-luc	0.1 - 0.7	(Jalova *et al.*, 2013)
Wastewater effluent	H4IIE-luc	<0.1 - 0.44	(Loos *et al.*, 2013)
Wastewater effluent	H4IIE-luc	<0.05 - 0.47	(Maier *et al.*, 2016)
Wastewater effluent	PAH CALUX	52 - 242†	(Alygizakis *et al.*, 2019)
Wastewater effluent	Yeast dioxin screen (YDS)	16 – 158#	(Stalter *et al.*, 2011)
Recycled water (UV, UV/H_2O_2,O_3,O_3/UV)	AhR CAFLUX (H1.G1.1c3)	0.004 - 0.02	(Jia *et al.*, 2015)
Recycled water (RO/AO)	AhR CAFLUX (H1.G1.1c3)	<0.007*	(Escher *et al.*, 2014)
Recycled water (RO/AO)	H4IIE-luc	<0.004*	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	AhR CAFLUX (H1.G1.1c3)	<0.007*	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	H4IIE-luc	<0.004*	(Escher *et al.*, 2014)
Recycled water (O_3)	AhR CAFLUX (H1.G1.1c3)	0.26 - 0.36	(Reungoat *et al.*, 2010)
Recycled water (O_3)	AhR CAFLUX (H1.G1.1c3)	0.1	(Macova *et al.*, 2010)
Recycled water (RO/AO)	AhR CAFLUX (H1.G1.1c3)	0.08	(Macova *et al.*, 2011)
Reclaimed water	H4IIE-luc	<0.02	(Lee *et al.*, 2015)
Surface water	AhR reporter gene assay	53	(Lundqvist *et al.*, 2019a)
Surface water	AhR CAFLUX (H1.G1.1c3)	0.028*	(Escher *et al.*, 2014)
Surface water	AhR CAFLUX (H1.G1.1c3)	0.15 - 0.19	(Macova *et al.*, 2011)
Surface water	AhR CAFLUX (H1.G1.1c3)	0.01 - 0.02	(Konig *et al.*, 2017)
Surface water	AhR CAFLUX (H4.G1.1c2)	0.05 - 0.16	(Konig *et al.*, 2017)
Surface water	AhR CAFLUX (H4G1.1c2)	0.002 - 0.017	(Neale *et al.*, 2015)
Surface water	AhR CAFLUX (H4G1.1c2)	0.026 - 0.077	(Neale *et al.*, 2017b)
Surface water	AhR CALUX (H4L1.1c4)	0.009 - 0.16	(Muller *et al.*, 2018)
Surface water	AhR CALUX (H4L1.1c4)	0.008	(Neale *et al.*, 2018a)
Surface water	H4IIE-luc	0.009*	(Escher *et al.*, 2014)
Surface water	H4IIE-luc	<0.05 - 0.18	(Maier *et al.*, 2016)
Surface water	Yeast dioxin screen (YDS)	<320 – 602#	(Brettschneider *et al.*, 2019)
Riverbank filtrate	AhR CALUX (H4L1.1c4)	0.02 - 0.03	(Albergamo *et al.*, 2020)
Ground water	AhR CAFLUX (H1.G1.1c3)	<0.002	(Jia *et al.*, 2015)
Ground water	H4IIE-luc	<0.005	(Lee *et al.*, 2015)
Drinking water	AhR reporter gene assay	45 - 52	(Lundqvist *et al.*, 2019a)
Drinking water	AhR CAFLUX (H1.G1.1c3)	0.024*	(Escher *et al.*, 2014)
Drinking water	AhR CAFLUX (H1.G1.1c3)	0.17	(Macova *et al.*, 2011)
Drinking water	AhR CALUX (H4L1.1c4)	Effect similar to blanks	(Albergamo *et al.*, 2020)
Drinking water	H4IIE-luc	<0.004*	(Escher *et al.*, 2014)

**TCDD EQ calculated using EC_{10}values from Table 1; †PAH CALUX reference compound is benzo(a)pyrene; #Yeast dioxin screen reference compound is β-naphthoflavone.*

Table A2: Summary of reported PPARγ activity in units of ng/L Rosiglitazone EQ in different environmental water extracts.

NB: Only studies that have applied SPE or LLE are included.

Matrix	Assay	PPARγ activity (ng/L Rosiglitazone EQ)	Reference
Wastewater influent	PPARγ CALUX	500 - 800	(Bain *et al.*, 2014)
Wastewater influent	PPARγ GeneBLAzer	719 - 936	(Nivala *et al.*, 2018)
Wastewater effluent	PPARγ CALUX	<119*	(Escher *et al.*, 2014)
Wastewater effluent	PPARγ GeneBLAzer	<59*	(Escher *et al.*, 2014)
Wastewater effluent	PPARγ CALUX	<350 - 640	(Alygizakis *et al.*, 2019)
Wastewater effluent	PPARγ CALUX	<32 - 270	(Bain *et al.*, 2014)
Wastewater effluent	PPARγ GeneBLAzer	<100	(Jia *et al.*, 2015)
Wastewater effluent	PPARγ GeneBLAzer	83 - 134	(Nivala *et al.*, 2018)
Recycled water (RO/AO)	PPARγ CALUX	<119*	(Escher *et al.*, 2014)
Recycled water (RO/AO)	PPARγ GeneBLAzer	<59*	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	PPARγ CALUX	<119*	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	PPARγ GeneBLAzer	<59*	(Escher *et al.*, 2014)
Recycled water (UV, UV/H_2O_2,O_3,O_3/UV)	PPARγ GeneBLAzer	<100	(Jia *et al.*, 2015)
Surface water	PPARγ CALUX	<119*	(Escher *et al.*, 2014)
Surface water	PPARγ GeneBLAzer	<59*	(Escher *et al.*, 2014)
Surface water	PPARγ GeneBLAzer	0.59 - 8.9	(Konig *et al.*, 2017)
Surface water	PPARγ GeneBLAzer	2.0 - 172	(Muller *et al.*, 2018)
Surface water	PPARγ GeneBLAzer	11	(Neale *et al.*, 2018a)
Riverbank filtrate	PPARγ GeneBLAzer	<1.2*	(Albergamo *et al.*, 2020)
Ground water	PPARγ GeneBLAzer	<100	(Jia *et al.*, 2015)
Drinking water	PPARγ GeneBLAzer	<1.2*	(Albergamo *et al.*, 2020)
Drinking water	PPARγ CALUX	<119*	(Escher *et al.*, 2014)
Drinking water	PPARγ GeneBLAzer	<59*	(Escher *et al.*, 2014)

**EC_{10} converted to Rosiglitazone EQ using EC_{10} values provided in Table 2.*

Table A3: Summary of reported PXR activity in units of µg/L SR12813 EQ (HG5LN hPXR) or µg/L nicardipine EQ (PXR CALUX) in different environmental water extracts.

NB: Only studies that have applied SPE or LLE are included.

Matrix	Assay	PXR Activity	Reference
Wastewater effluent	HG5LN hPXR	3.8 - 4.7 µg/L SR12813 EQ*	(Escher *et al.*, 2014)
Wastewater effluent	PXR CALUX	20-240 µg/L nicardipine EQ	(Alygizakis *et al.*, 2019)
Surface water	HG5LN hPXR	2.3 µg/L SR12813 EQ*	(Escher *et al.*, 2014)
Surface water	HG5LN hPXR	<0.02 - 2.3 µg/L SR12813 EQ	(Neale *et al.*, 2015)
Surface water	HG5LN hPXR	0.22 µg/L SR12813 EQ	(Neale *et al.*, 2018a)
Recycled water (RO/AO)	HG5LN hPXR	<0.66 µg/L SR12813 EQ*	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	HG5LN hPXR	0.98 µg/L SR12813 EQ*	(Escher *et al.*, 2014)
Drinking water	HG5LN hPXR	3.2 µg/L SR12813 EQ*	(Escher *et al.*, 2014)

**EC_{10} converted to SR12813 EQ using EC_{10} values provided in Table 3.*

Table A4: Summary of reported estrogenic activity in units of ng/L EEQ in different environmental water extracts.

NB: Only studies that have applied SPE or LLE are included.

Matrix	Assay	Estrogenic activity (ng/L EEQ)	Reference
Wastewater influent	ERα CALUX	1.1 - 120	(Murk *et al.*, 2002)
Wastewater influent	ERα CALUX	28 - 122	(Bain *et al.*, 2014)
Wastewater influent	ERα CALUX	37 - 93	(Houtman *et al.*, 2018)
Wastewater influent	ERα CALUX	19 - 27	(Roberts *et al.*, 2015)
Wastewater influent	ERα CALUX	0.5 - 42	(Valitalo *et al.*, 2017)
Wastewater influent	ERα GeneBLAzer	11 - 24	(Nivala *et al.*, 2018)
Wastewater influent	E-Screen	225	(Hamilton *et al.*, 2016)
Wastewater influent	E-Screen	<0.02 - 100	(Leusch *et al.*, 2014a)
Wastewater influent	E-Screen	3.2 - 19	(Macova *et al.*, 2011)
Wastewater influent	MELN	15 - 94	(Jugan *et al.*, 2009)
Wastewater influent	MELN	46 - 63	(Cargouet *et al.*, 2004)
Wastewater influent	MVLN	5.4 - 124	(Jalova *et al.*, 2013)
Wastewater influent	MVLN	32	(Kusk *et al.*, 2011)
Wastewater influent	YES	Up to 86	(Murk *et al.*, 2002)
Wastewater influent	YES	22 - 55	(Zhang *et al.*, 2011)
Wastewater influent	YES	26 - 29	(Kusk *et al.*, 2011)
Wastewater influent	YES	13 - 23	(Stalter *et al.*, 2011)
Wastewater effluent	EASZY	<6.3-673	(Brion *et al.*, 2019)
Wastewater effluent	ERα CALUX	0.3 - 2.7*	(Escher *et al.*, 2014)
Wastewater effluent	ERα GeneBLAzer	0.64 - 3.4*	(Escher *et al.*, 2014)
Wastewater effluent	E-Screen	0.28 - 0.39*	(Escher *et al.*, 2014)
Wastewater effluent	hERα-HeLa-9903	1.7 - 7.6*	(Escher *et al.*, 2014)
Wastewater effluent	YES	2.3 - 18*	(Escher *et al.*, 2014)
Wastewater effluent	EASZY	22	(Neale *et al.*, 2017b)
Wastewater effluent	ERα CALUX	<0.2 - 0.4	(Gehrmann *et al.*, 2018)
Wastewater effluent	ERα CALUX	0.03 - 22.9	(Konemann *et al.*, 2018)
Wastewater effluent	ERα CALUX	0.03 - 16	(Murk *et al.*, 2002)
Wastewater effluent	ERα CALUX	<0.006 - 7.4	(Alygizakis *et al.*, 2019)
Wastewater effluent	ERα CALUX	0.39 - 5.5	(Bain *et al.*, 2014)
Wastewater effluent	ERα CALUX	<1	(Houtman *et al.*, 2018)
Wastewater effluent	ERα CALUX	<0.2	(Jia *et al.*, 2015)
Wastewater effluent	ERα CALUX	0.1 - 0.2	(Roberts *et al.*, 2015)
Wastewater effluent	ERα CALUX	up to 4.7	(Leusch *et al.*, 2014b)
Wastewater effluent	ERα CALUX	0.61 - 3.1	(Valitalo *et al.*, 2017)
Wastewater effluent	ERα CALUX	0.39 - 1.0	(Van der Linden *et al.*, 2008)
Wastewater effluent	ERα GeneBLAzer	0.03 - 12	(Konemann *et al.*, 2018)
Wastewater effluent	ERα GeneBLAzer	1.5 - 6.5	(Mehinto *et al.*, 2015)
Wastewater effluent	ERα GeneBLAzer	0.42 - 0.71	(Nivala *et al.*, 2018)
Wastewater effluent	ERα GeneBLAzer	2.3 - 17	(Mehinto *et al.*, 2016)
Wastewater effluent	E-Screen	0.2 - 7.8	(Korner *et al.*, 2001)
Wastewater effluent	E-Screen	<0.2	(Hamilton *et al.*, 2016)
Wastewater effluent	E-Screen	<0.02 - >10	(Leusch *et al.*, 2014a)
Wastewater effluent	E-Screen	<0.02-0.34	(Macova *et al.*, 2011)
Wastewater effluent	E-Screen	6.0	(Macova *et al.*, 2010)
Wastewater effluent	E-Screen	0.2 - 34	(Bicchi *et al.*, 2009)
Wastewater effluent	E-Screen	3.1	(Henneberg *et al.*, 2014)
Wastewater effluent	E-Screen	5.7 - 7.6	(Reungoat *et al.*, 2010)
Wastewater effluent	hERα-HeLa-9903	0.03 - 24	(Konemann *et al.*, 2018)
Wastewater effluent	hERα-HeLa-9903	<0.5 - 0.88	(Henneberg *et al.*, 2014)
Wastewater effluent	MELN	0.04 - 20	(Konemann *et al.*, 2018)
Wastewater effluent	MELN	0.8 - 5.7	(Jugan *et al.*, 2009)

Matrix	Assay	Estrogenic activity (ng/L EEQ)	Reference
Wastewater effluent	MELN	2.8	(Miege *et al.*, 2009)
Wastewater effluent	MELN	2.0 - 4.2	(Neale *et al.*, 2017b)
Wastewater effluent	MELN	2 - 24	(Cargouet *et al.*, 2004)
Wastewater effluent	MVLN	0.1 - 5.1	(Jalova *et al.*, 2013)
Wastewater effluent	MVLN	<0.5 - 18	(Jarošová *et al.*, 2014)
Wastewater effluent	MVLN	24	(Furuichi *et al.*, 2004)
Wastewater effluent	MVLN	1.4 - 2.5	(Kusk *et al.*, 2011)
Wastewater effluent	T47D-KBluc	15	(Medlock Kakaley *et al.*, 2020)
Wastewater effluent	YES	<2.8 - 3.2	(Gehrmann *et al.*, 2018)
Wastewater effluent	YES	0.1 - 16	(Murk *et al.*, 2002)
Wastewater effluent	YES	1.3 - 3.3	(Fang *et al.*, 2012)
Wastewater effluent	YES	<0.1 - 6.3	(French *et al.*, 2015)
Wastewater effluent	YES	2.5 - 30	(Zhang *et al.*, 2011)
Wastewater effluent	YES	<LOQ - 53	(Aerni *et al.*, 2004)
Wastewater effluent	YES	2.7 - 3.0	(Kusk *et al.*, 2011)
Wastewater effluent	YES	12 - 20	(Pawlowski *et al.*, 2003)
Wastewater effluent	YES	<1.0 - 9.0	(Huggett *et al.*, 2003)
Wastewater effluent	YES	0.81 - 91	(Escher *et al.*, 2008b)
Wastewater effluent	YES	0.1 - 0.8	(Stalter *et al.*, 2011)
Wastewater effluent	YES	2.2 - 6.2	(Zeng *et al.*, 2016)
Wastewater effluent	ERα GeneBLAzer	<0.6 - 0.78	(Leusch *et al.*, 2018b)
Recycled water (UV, UV/H_2O_2,O_3,O_3/UV)	ERα CALUX	<0.2	(Jia *et al.*, 2015)
Recycled water (RO/UV)	ERα GeneBLAzer	<1.7 - 2.6	(Mehinto *et al.*, 2015)
Recycled water (RO/AO)	ERα CALUX	<0.008*	(Escher *et al.*, 2014)
Recycled water (RO/AO)	ERα GeneBLAzer	<0.14*	(Escher *et al.*, 2014)
Recycled water (RO/AO)	E-Screen	<0.01	(Macova *et al.*, 2011)
Recycled water (RO/AO)	E-Screen	<0.007*	(Escher *et al.*, 2014)
Recycled water (RO/AO)	hERα-HeLa-9903	<0.08*	(Escher *et al.*, 2014)
Recycled water (RO/AO)	YES	<0.34*	(Escher *et al.*, 2014)
Recycled water (RO)	ERα CALUX	0.08 - 0.17	(Leusch *et al.*, 2014b)
Recycled water (RO)	E-Screen	<0.02	(Leusch *et al.*, 2014a)
Recycled water (O_3/BAC)	ERα CALUX	<0.01*	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	ERα GeneBLAzer	<0.14*	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	E-Screen	<0.007*	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	hERα-HeLa-9903	<0.08*	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	YES	<0.34*	(Escher *et al.*, 2014)
Recycled water (O_3)	E-Screen	<0.06	(Macova *et al.*, 2010)
Recycled water (O_3)	E-Screen	<0.06	(Reungoat *et al.*, 2010)
Reclaimed water	ERα GeneBLAzer	0.17	(Lee *et al.*, 2015)
Surface water	ERα GeneBLAzer	0.17 - 4.0	(Neale *et al.*, 2020b)
Surface water	EASZY	<2.0	(Neale *et al.*, 2017b)
Surface water	EASZY	<6.3 - 30	(Brion *et al.*, 2019)
Surface water	ERα CALUX	8.0 - 129	(Shi *et al.*, 2018)
Surface water	ERα CALUX	0.06 - 1.2	(Konemann *et al.*, 2018)
Surface water	ERα CALUX	0.07 - 0.47	(Murk *et al.*, 2002)
Surface water	ERα CALUX	<0.015 - 0.12	(Jia *et al.*, 2019)
Surface water	ERα CALUX	0.10	(Roberts *et al.*, 2015)
Surface water	ERα CALUX	<0.1 - 6.5	(Scott *et al.*, 2014)
Surface water	ERα CALUX	0.18 - 0.50	(Van der Linden *et al.*, 2008)
Surface water	ERα CALUX	0.02*	(Escher *et al.*, 2014)
Surface water	ERα GeneBLAzer	<0.1 - 0.31	(Leusch *et al.*, 2018b)
Surface water	ERα GeneBLAzer	0.02 - 0.96	(Konemann *et al.*, 2018)
Surface water	ERα GeneBLAzer	0.005 - 0.8	(Daniels *et al.*, 2018)
Surface water	ERα GeneBLAzer	<0.5 - 6.3	(Mehinto *et al.*, 2017)

Matrix	Assay	Estrogenic activity (ng/L EEQ)	Reference
Surface water	ERα GeneBLAzer	ND - 2.2	(Muller *et al.*, 2018)
Surface water	ERα GeneBLAzer	<0.02 - 1.2	(Scott *et al.*, 2018)
Surface water	ERα GeneBLAzer	1.1	(Hashmi *et al.*, 2018)
Surface water	ERα GeneBLAzer	0.005-0.26	(Konig *et al.*, 2017)
Surface water	ERα GeneBLAzer	<0.23	(Neale *et al.*, 2018a)
Surface water	ERα GeneBLAzer	<0.5 - 4.0	(Mehinto *et al.*, 2016)
Surface water	ERα GeneBLAzer	<0.14*	(Escher *et al.*, 2014)
Surface water	E-Screen	<0.02 - 0.1	(Leusch *et al.*, 2014a)
Surface water	E-Screen	<0.01	(Macova *et al.*, 2011)
Surface water	E-Screen	0.005 - 1.9	(Oh *et al.*, 2006)
Surface water	E-Screen	0.03 - 2.4	(Konig *et al.*, 2017)
Surface water	E-Screen	0.1 - 9.0	(Bicchi *et al.*, 2009)
Surface water	E-Screen	0.04 - 0.8	(Henneberg *et al.*, 2014)
Surface water	E-Screen	6.6 - 85	(Liu *et al.*, 2018)
Surface water	E-Screen	0.01*	(Escher *et al.*, 2014)
Surface water	hERα-HeLa-9903	0.02 - 1.0	(Konemann *et al.*, 2018)
Surface water	hERα-HeLa-9903	<0.02 - 2.0	(Prochazkova *et al.*, 2018)
Surface water	hERα-HeLa-9903	0.15*	(Escher *et al.*, 2014)
Surface water	MELN	0.06 - 4.0	(Konemann *et al.*, 2018)
Surface water	MELN	<0.3 - 1.8	(Jugan *et al.*, 2009)
Surface water	MELN	<0.03 - 1.4	(Miege *et al.*, 2009)
Surface water	MELN	0.15 - 0.95	(Neale *et al.*, 2017b)
Surface water	MELN	0.005 - 1.2	(Neale *et al.*, 2015)
Surface water	MELN	0.02	(Neale *et al.*, 2018a)
Surface water	MELN	<0.009 - 1.9	(Tousova *et al.*, 2017)
Surface water	MELN	12 - 23	(Mnif *et al.*, 2012)
Surface water	MELN	0.3 - 4.5	(Cargouet *et al.*, 2004)
Surface water	MVLN	1.2 - 10	(Furuichi *et al.*, 2004)
Surface water	MVLN	<1.4 - 32	(Shue *et al.*, 2009)
Surface water	T47D-KBluc	<0.032 - 116	(Conley *et al.*, 2017a)
Surface water	T47D-KBluc	0.1 - 0.25	(Medlock Kakaley *et al.*, 2020)
Surface water	T47D-KBluc	12 - 190	(Liu *et al.*, 2018)
Surface water	YES	<LOD - 1.1	(Murk *et al.*, 2002)
Surface water	YES	<0.07 - 0.21	(Brettschneider *et al.*, 2019)
Surface water	YES	<0.2 - 131	(Chen *et al.*, 2016b)
Surface water	YES	<0.1 - 0.99	(French *et al.*, 2015)
Surface water	YES	<0.2 - 82	(Huang *et al.*, 2016)
Surface water	YES	0.23 - 324	(Zhao *et al.*, 2011)
Surface water	YES	<LOQ - 1.9	(Aerni *et al.*, 2004)
Surface water	YES	1.1 - 1.3	(Pawlowski *et al.*, 2003)
Surface water	YES	0.32-3.5	(Escher *et al.*, 2008b)
Surface water	YES	0.2 -18	(Vermeirssen *et al.*, 2005)
Surface water	YES	0.46*	(Escher *et al.*, 2014)
Surface water	YES	<LOD - 5.2	(Xiao *et al.*, 2016)
Surface water	YES	0.20 - 3.0	(Lv *et al.*, 2016)
Surface water	YES	<LOD - 6.2	(Xiao *et al.*, 2017)
Surface water	ERα CALUX	0.01 - 1.1	(Brand *et al.*, 2013)
Surface water	T47D-KBluc	<0.03 - 0.47	(Conley *et al.*, 2017b)
Riverbank filtrate	ERα GeneBLAzer	<0.03*	(Albergamo *et al.*, 2020)
Ground water	ERα CALUX	<0.2	(Jia *et al.*, 2015)
Ground water	ERα GeneBLAzer	<0.15 - 0.45	(Lee *et al.*, 2015)
Drinking water	ERα CALUX	<0.008*	(Escher *et al.*, 2014)
Drinking water	ERα GeneBLAzer	<0.03*	(Albergamo *et al.*, 2020)
Drinking water	ERα GeneBLAzer	<0.14*	(Escher *et al.*, 2014)
Drinking water	E-Screen	<0.01	(Macova *et al.*, 2011)
Drinking water	E-Screen	<0.007*	(Escher *et al.*, 2014)

Matrix	Assay	Estrogenic activity (ng/L EEQ)	Reference
Drinking water	hERα-HeLa-9903	0.35*	(Escher *et al.*, 2014)
Drinking water	ERα CALUX	<LOD - 5.3	(Shi *et al.*, 2018)
Drinking water	ERα CALUX	<0.01 - 0.03	(Brand *et al.*, 2013)
Drinking water	ERα CALUX	<LOD	(Van der Linden *et al.*, 2008)
Drinking water	ERα GeneBLAzer	<0.03	(Leusch *et al.*, 2018b)
Drinking water	ERα GeneBLAzer	<0.03 - 0.04	(Neale *et al.*, 2020b)
Drinking water	hERα-HeLa-9903	<LOD	(Valcarcel *et al.*, 2018)
Drinking water	MELN	<0.3	(Jugan *et al.*, 2009)
Drinking water	T47D-KBluc	<LOD - 0.114	(Van Zijl *et al.*, 2017)
Drinking water	T47D-KBluc	<0.025 - 0.08	(Conley *et al.*, 2017b)
Drinking water	T47D-KBluc	<0.04 - 0.06	(Medlock Kakaley *et al.*, 2020)
Drinking water	YES	<0.34*	(Escher *et al.*, 2014)
Drinking water	YES	<LOD - 0.5	(Xiao *et al.*, 2016)
Drinking water	YES	<LOD	(Van Zijl *et al.*, 2017)
Drinking water	YES	0.02 - 0.09	(Lv *et al.*, 2016)
Drinking water	YES	<LOD - 1.41	(Xiao *et al.*, 2017)

**EC_{10} converted to EEQ using EC_{10} values provided in Table 4.*

Table A5: Summary of reported anti-estrogenic activity in units of µg/L tamoxifen EQ in different environmental water extracts.

NB: Only studies that have applied SPE or LLE are included.

Matrix	Assay	Anti-estrogenic activity (µg/L Tamoxifen EQ)	Reference
Wastewater influent	Anti-ERα GeneBLAzer	Cytotoxic	(Nivala *et al.*, 2018)
Wastewater effluent	Anti ERα CALUX	<0.07*	(Escher *et al.*, 2014)
Wastewater effluent	Anti ERα CALUX	<1.5 - 110	Gehrmann et al. 2018
Wastewater effluent	Anti ERα CALUX	<0.5	(Jia *et al.*, 2015)
Wastewater effluent	Anti-ERα GeneBLAzer	Cytotoxic	(Nivala *et al.*, 2018)
Wastewater effluent	YAES	13 - 97	(Conroy *et al.*, 2007)
Wastewater effluent	YAES	19 - 24	(Fang *et al.*, 2012)
Wastewater effluent	Anti-ERα GeneBLAzer	<2177*	(Leusch *et al.*, 2018b)
Recycled water (UV, UV/H_2O_2,O_3,O_3/UV)	Anti ERα CALUX	<0.5	(Jia *et al.*, 2015)
Recycled water (RO/AO)	Anti ERα CALUX	<0.04*	(Escher *et al.*, 2014)
Recycled water (RO)	Anti ERα CALUX	2.3 - 4.4	(Leusch *et al.*, 2014b)
Recycled water (O_3/BAC)	Anti ERα CALUX	<0.04*	(Escher *et al.*, 2014)
Surface water	Anti-ERα GeneBLAzer	Cytotoxic	(Neale *et al.*, 2020b)
Surface water	Anti ERα CALUX	<0.07 - 1.1	(Jia *et al.*, 2019)
Surface water	Anti ERα CALUX	<5	(Scott *et al.*, 2014)
Surface water	Anti ERα CALUX	<5 - 6	(Daniels *et al.*, 2018)
Surface water	Anti ERα CALUX	<0.04*	(Escher *et al.*, 2014)
Surface water	Anti-ERα GeneBLAzer	<435*	(Leusch *et al.*, 2018b)
Surface water	Anti-ERα GeneBLAzer	<1 - 2.7	(Scott *et al.*, 2018)
Surface water	YAES	<50	(Zhao *et al.*, 2011)
Ground water	Anti ERα CALUX	<0.5	(Jia *et al.*, 2015)
Drinking water	Anti ERα CALUX	<0.04*	(Escher *et al.*, 2014)
Drinking water	Anti-ERα GeneBLAzer	<109*	(Leusch *et al.*, 2018b)
Drinking water	Anti-ERα GeneBLAzer	<22*	(Neale *et al.*, 2020b)

**$EC_{SR0.2}$ values converted to Tamoxifen EQ using $EC_{SR0.2}$ values provided in Table 5.*

Table A6: Summary of reported androgenic activity in units of ng/L dihydrotestorone (DHT) EQ in different environmental water extracts.

NB: Only studies that have applied SPE or LLE are included.

Matrix	Assay	Androgenic activity (ng/L DHT EQ)	Reference
Wastewater influent	AR CALUX	44 - 59	(Sauer *et al.*, 2018)
Wastewater influent	AR CALUX	<0.62 - 67	(Valitalo *et al.*, 2017)
Wastewater influent	AR CALUX	30 - 350	(Bain *et al.*, 2014)
Wastewater influent	AR CALUX	38 - 242	(Houtman *et al.*, 2018)
Wastewater influent	AR CALUX	<25 - 100	(Leusch *et al.*, 2014a)
Wastewater influent	AR CALUX	145 - 232	(Roberts *et al.*, 2015)
Wastewater influent	AR GeneBLAzer	50 - 83*	(Nivala *et al.*, 2018)
Wastewater effluent	AR CALUX	<0.97*	(Escher *et al.*, 2014)
Wastewater effluent	AR GeneBLAzer	<4.1*	(Escher *et al.*, 2014)
Wastewater effluent	MDA-kb2	3.9 - 7.6*	(Escher *et al.*, 2014)
Wastewater effluent	YAS	<2.8*	(Escher *et al.*, 2014)
Wastewater effluent	AR CALUX	0.75 - 0.83	(Van der Linden *et al.*, 2008)
Wastewater effluent	AR CALUX	<LOD - 0.79	(Sauer *et al.*, 2018)
Wastewater effluent	AR CALUX	<0.62	(Valitalo *et al.*, 2017)
Wastewater effluent	AR CALUX	<2 - 2	(Leusch *et al.*, 2014b)
Wastewater effluent	AR CALUX	1	(Houtman *et al.*, 2018)
Wastewater effluent	AR CALUX	<30	(Jia *et al.*, 2015)
Wastewater effluent	AR CALUX	<25	(Leusch *et al.*, 2014a)
Wastewater effluent	AR CALUX	<1.1	(Roberts *et al.*, 2015)
Wastewater effluent	AR CALUX	<1.6 - 2.7	(Gehrmann *et al.*, 2018)
Wastewater effluent	AR GeneBLAzer	Cytotoxic	(Nivala *et al.*, 2018)
Wastewater effluent	AR GeneBLAzer	<2	(Leusch *et al.*, 2018b)
Wastewater effluent	MDA-kb2	<0.77 - 2.1	(Medlock Kakaley *et al.*, 2020)
Wastewater effluent	MDA-kb2	2.6 - 12	(Neale *et al.*, 2017b)
Wastewater effluent	YAS	<5.4 - 138	(French *et al.*, 2015)
Recycled water (UV, UV/H_2O_2,O_3,O_3/UV)	AR CALUX	<30	(Jia *et al.*, 2015)
Recycled water (RO/AO)	AR CALUX	<0.97*	(Escher *et al.*, 2014)
Recycled water (RO/AO)	AR GeneBLAzer	<2.0*	(Escher *et al.*, 2014)
Recycled water (RO/AO)	MDA-kb2	<0.34*	(Escher *et al.*, 2014)
Recycled water (RO/AO)	YAS	<2.8*	(Escher *et al.*, 2014)
Recycled water (RO)	AR CALUX	<25	(Leusch *et al.*, 2014a)
Recycled water (O_3/BAC)	AR CALUX	<0.97*	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	AR GeneBLAzer	<2.0*	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	MDA-kb2	<0.34*	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	YAS	<2.8*	(Escher *et al.*, 2014)
Surface water	AR CALUX	<0.08 - 0.25	(Brand *et al.*, 2013)
Surface water	AR CALUX	<LOD - 12	(Van der Linden *et al.*, 2008)
Surface water	AR CALUX	<LOD - 0. 45	(Sauer *et al.*, 2018)
Surface water	AR CALUX	<0.97*	(Escher *et al.*, 2014)
Surface water	AR CALUX	<25	(Leusch *et al.*, 2014a)
Surface water	AR CALUX	<1.1	(Roberts *et al.*, 2015)
Surface water	AR CALUX	<7	(Scott *et al.*, 2014)
Surface water	AR GeneBLAzer	<0.41*	(Neale *et al.*, 2020b)
Surface water	AR GeneBLAzer	0.29 - 3.2*	(Konig *et al.*, 2017)
Surface water	AR GeneBLAzer	<0.45*	(Neale *et al.*, 2018a)
Surface water	AR GeneBLAzer	2.3*	(Hashmi *et al.*, 2018)
Surface water	AR GeneBLAzer	<0.41 - 4.9*	(Muller *et al.*, 2018)
Surface water	AR GeneBLAzer	< 9	(Scott *et al.*, 2018)
Surface water	AR GeneBLAzer	<1	(Leusch *et al.*, 2018b)

Matrix	Assay	Androgenic activity (ng/L DHT EQ)	Reference
Surface water	AR GeneBLAzer	<2.0*	(Escher *et al.*, 2014)
Surface water	MDA-kb2	<0.82 - 4.8	(Conley *et al.*, 2017a)
Surface water	MDA-kb2	<0.77	(Medlock Kakaley *et al.*, 2020)
Surface water	MDA-kb2	<0.02 - 2.3	(Konig *et al.*, 2017)
Surface water	MDA-kb2	<0.02 - 2.7	(Tousova *et al.*, 2017)
Surface water	MDA-kb2	<0.36 - 0.86	(Neale *et al.*, 2017b)
Surface water	MDA-kb2	<0.34*	(Escher *et al.*, 2014)
Surface water	YAS	<2.8*	(Escher *et al.*, 2014)
Surface water	YAS	<3.5 - 69	(French *et al.*, 2015)
Surface water	YAS	<2.5 - 46	(Huang *et al.*, 2016)
Surface water	YAS	<2.5 - 45	(Zhao *et al.*, 2011)
Riverbank filtrate	AR GeneBLAzer	<0.41*	(Albergamo *et al.*, 2020)
Ground water	AR CALUX	<30	(Jia *et al.*, 2015)
Drinking water	AR CALUX	<0.97*	(Escher *et al.*, 2014)
Drinking water	AR CALUX	<0.08 - 0.13	(Brand *et al.*, 2013)
Drinking water	AR GeneBLAzer	<0.41*	(Albergamo *et al.*, 2020)
Drinking water	AR GeneBLAzer	<2.0*	(Escher *et al.*, 2014)
Drinking water	AR GeneBLAzer	<0.41*	(Neale *et al.*, 2020b)
Drinking water	AR GeneBLAzer	<0.1	(Leusch *et al.*, 2018b)
Drinking water	MDA-kb2	<0.34*	(Escher *et al.*, 2014)
Drinking water	MDA-kb2	<0.77	(Medlock Kakaley *et al.*, 2020)
Drinking water	YAS	<2.8*	(Escher *et al.*, 2014)

**EC_{10} values converted to DHT EQ using EC_{10} values provided in Table 6.*

Table A7: Summary of reported anti-androgenic activity in units of µg/L flutamide EQ in different environmental water extracts.

NB: Only studies that have applied SPE or LLE are included.

Matrix	Assay	Anti-androgenic activity (µg/L Flutamide EQ)	Reference
Wastewater influent	Anti-AR CALUX	5.2 - 26	(Sauer *et al.*, 2018)
Wastewater influent	Anti-AR GeneBLAzer	Cytotoxic	(Nivala *et al.*, 2018)
Wastewater effluent	Anti-AR CALUX	40 - 105*	(Escher *et al.*, 2014)
Wastewater effluent	Anti-MDA-kb2	<4.4*	(Escher *et al.*, 2014)
Wastewater effluent	Anti-AR CALUX	3.5 - 8.9	(Sauer *et al.*, 2018)
Wastewater effluent	Anti-AR CALUX	<5.7 - 32	(Alygizakis *et al.*, 2019)
Wastewater effluent	Anti-AR CALUX	0.48 - 0.71	(Houtman *et al.*, 2018)
Wastewater effluent	Anti-AR CALUX	<300	(Jia *et al.*, 2015)
Wastewater effluent	Anti-AR CALUX	<8.8 - 360	(Gehrmann *et al.*, 2018)
Wastewater effluent	Anti-AR GeneBLAzer	Cytotoxic	(Nivala *et al.*, 2018)
Wastewater effluent	YAAS	16 - 178	(Stalter *et al.*, 2011)
Wastewater effluent	YAAS	195 - 367	(Fang *et al.*, 2012)
Wastewater effluent	YAAS	<460 - 3190	(Gehrmann *et al.*, 2018)
Wastewater effluent	Anti-AR GeneBLAzer	<15	(Leusch *et al.*, 2018b)
Recycled water (UV, UV/H_2O_2,O_3,O_3/UV)	Anti-AR CALUX	<300	(Jia *et al.*, 2015)
Recycled water (RO/AO)	Anti-AR CALUX	<20*	(Escher *et al.*, 2014)
Recycled water (RO/AO)	Anti-MDA-kb2	<1.9*	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	Anti-AR CALUX	<20*	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	Anti-MDA-kb2	<1.9*	(Escher *et al.*, 2014)
Surface water	Anti-AR GeneBLAzer	<1.5*	(Neale *et al.*, 2020b)
Surface water	Anti-AR CALUX	<LOD - 5.9	(Sauer *et al.*, 2018)
Surface water	Anti-AR CALUX	28*	(Escher *et al.*, 2014)
Surface water	Anti-AR CALUX	3.3 - 12	(Jia *et al.*, 2019)
Surface water	Anti-AR CALUX	<60 - 257	(Scott *et al.*, 2014)
Surface water	Anti-AR GeneBLAzer	<0.51 - 8.9*	(Konig *et al.*, 2017)
Surface water	Anti-AR GeneBLAzer	Cytotoxic	(Muller *et al.*, 2018)
Surface water	Anti-AR GeneBLAzer	73 - 90	(Scott *et al.*, 2018)
Surface water	Anti-AR GeneBLAzer	<2.9	(Leusch *et al.*, 2018b)
Surface water	Anti-MDA-kb2	0.27 - 0.31	(Konig *et al.*, 2017)
Surface water	Anti-MDA-kb2	<0.12	(Tousova *et al.*, 2017)
Surface water	Anti-MDA-kb2	9.2*	(Escher *et al.*, 2014)
Surface water	YAAS	20 - 935	(Zhao *et al.*, 2011)
Ground water	Anti-AR CALUX	<300	(Jia *et al.*, 2015)
Drinking water	Anti-AR CALUX	<20*	(Escher *et al.*, 2014)
Drinking water	Anti-MDA-kb2	<1.9*	(Escher *et al.*, 2014)
Drinking water	Anti-AR GeneBLAzer	<1.5*	(Neale *et al.*, 2020b)
Drinking water	Anti-AR GeneBLAzer	<0.7	(Leusch *et al.*, 2018b)

**$EC_{SR0.2}$ values converted to Flutamide EQ using $EC_{SR0.2}$ values provided in Table 7.*

Table A8: Summary of reported glucocorticoid activity in units of ng/L dexamethasone EQ in different environmental water extracts.

NB: Only studies that have applied SPE or LLE are included.

Matrix	Assay	Glucocorticoid activity (ng/L Dexamethasone EQ)	Reference
Wastewater influent	GR CALUX	37 - 60	(Bain *et al.*, 2014)
Wastewater influent	GR CALUX	62 - 121	(Houtman *et al.*, 2018)
Wastewater influent	GR CALUX	66 – 85	(Roberts *et al.*, 2015)
Wastewater influent	GR GeneBLAzer	Cytotoxic	(Nivala *et al.*, 2018)
Wastewater influent	GR GeneBLAzer	<400	(Lee *et al.*, 2015)
Wastewater effluent	GR CALUX	285 - 628*	(Escher *et al.*, 2014)
Wastewater effluent	GR GeneBLAzer	<8.2*	(Escher *et al.*, 2014)
Wastewater effluent	GR Switchgear	21 - 24*	(Escher *et al.*, 2014)
Wastewater effluent	CV-1 GR	1.8 - 21	(Medlock Kakaley *et al.*, 2020)
Wastewater effluent	GR CALUX	11 - 38	(Van der Linden *et al.*, 2008)
Wastewater effluent	GR CALUX	up to 81	(Leusch *et al.*, 2014b)
Wastewater effluent	GR CALUX	<19 - 120	(Alygizakis *et al.*, 2019)
Wastewater effluent	GR CALUX	<LOD - 70	(Bain *et al.*, 2014)
Wastewater effluent	GR CALUX	45 - 151	(Houtman *et al.*, 2018)
Wastewater effluent	GR CALUX	<310	(Jia *et al.*, 2015)
Wastewater effluent	GR CALUX	31 - 33	(Roberts *et al.*, 2015)
Wastewater effluent	GR CALUX	<2500	(Leusch *et al.*, 2018b)
Wastewater effluent	GR GeneBLAzer	<22 - 392	(Mehinto *et al.*, 2016)
Wastewater effluent	GR GeneBLAzer	17 - 19	(Nivala *et al.*, 2018)
Wastewater effluent	GR GeneBLAzer	61 - 90	(Mehinto *et al.*, 2015)
Wastewater effluent	GR GeneBLAzer	188	(Chen *et al.*, 2016a)
Wastewater effluent	GR GeneBLAzer	<230	(Jia *et al.*, 2015)
Wastewater effluent	GR GeneBLAzer	39 - 155	(Jia *et al.*, 2016)
Wastewater effluent	GR GeneBLAzer	<120 - 130	(Leusch *et al.*, 2018b)
Wastewater effluent	GR Switchgear	19 - 24	(Jia *et al.*, 2015)
Recycled water (UV, UV/H_2O_2,O_3,O_3/UV)	GR CALUX	<310	(Jia *et al.*, 2015)
Recycled water (UV, UV/H_2O_2,O_3,O_3/UV)	GR GeneBLAzer	<230	(Jia *et al.*, 2015)
Recycled water (UV, UV/H_2O_2,O_3,O_3/UV)	GR Switchgear	<9.8 - 16	(Jia *et al.*, 2015)
Recycled water (RO/UV)	GR GeneBLAzer	<52 - 65	(Mehinto *et al.*, 2015)
Recycled water (RO/AO)	GR CALUX	<10*	(Escher *et al.*, 2014)
Recycled water (RO/AO)	GR GeneBLAzer	<4.1*	(Escher *et al.*, 2014)
Recycled water (RO/AO)	GR Switchgear	<9.8*	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	GR CALUX	<10*	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	GR GeneBLAzer	<4.1*	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	GR Switchgear	<9.8*	(Escher *et al.*, 2014)
Recycled water (after UV)	GR GeneBLAzer	<30	(Jia *et al.*, 2016)
Reclaimed water	GR GeneBLAzer	52	(Lee *et al.*, 2015)
Surface water	CV-1 GR	<6.8 - 43	(Conley *et al.*, 2017a)
Surface water	CV-1 GR	<1.2	(Medlock Kakaley *et al.*, 2020)
Surface water	GR CALUX	0.30 - 1.3	(Van der Linden *et al.*, 2008)
Surface water	GR CALUX	<0.4 - 31	(Tousova *et al.*, 2017)
Surface water	GR CALUX	<10*	(Escher *et al.*, 2014)
Surface water	GR CALUX	<8.0 - 34	(Roberts *et al.*, 2015)
Surface water	GR CALUX	<0.4 - 2.7	(Schriks *et al.*, 2013)
Surface water	GR CALUX	<500	(Leusch *et al.*, 2018b)

Matrix	Assay	Glucocorticoid activity (ng/L Dexamethasone EQ)	Reference
Surface water	GR CALUX	<2	(Brand *et al.*, 2013)
Surface water	GR GeneBLAzer	<1.4	(Neale *et al.*, 2020b)
Surface water	GR GeneBLAzer	<22 - 30	(Mehinto *et al.*, 2016)
Surface water	GR GeneBLAzer	<1.1	(Konig *et al.*, 2017)
Surface water	GR GeneBLAzer	<11	(Neale *et al.*, 2018a)
Surface water	GR GeneBLAzer	Cytotoxic	(Hashmi *et al.*, 2020)
Surface water	GR GeneBLAzer	<4.1*	(Escher *et al.*, 2014)
Surface water	GR GeneBLAzer	9 - 170	(Daniels *et al.*, 2018)
Surface water	GR GeneBLAzer	<20 - 37	(Mehinto *et al.*, 2017)
Surface water	GR GeneBLAzer	<1.8 - 44	(Muller *et al.*, 2018)
Surface water	GR GeneBLAzer	<23 - 96	(Leusch *et al.*, 2018b)
Surface water	GR Switchgear	<9.8*	(Escher *et al.*, 2014)
Riverbank filtrate	GR GeneBLAzer	<0.82	(Albergamo *et al.*, 2020)
Ground water	GR CALUX	<310	(Jia *et al.*, 2015)
Ground water	GR GeneBLAzer	<230	(Jia *et al.*, 2015)
Ground water	GR Switchgear	<9.8	(Jia *et al.*, 2015)
Ground water	GR GeneBLAzer	<25	(Lee *et al.*, 2015)
Drinking water	GR CALUX	<10*	(Escher *et al.*, 2014)
Drinking water	GR GeneBLAzer	<4.1*	(Escher *et al.*, 2014)
Drinking water	GR Switchgear	<9.8*	(Escher *et al.*, 2014)
Drinking water	CV-1 GR	<1.2	(Medlock Kakaley *et al.*, 2020)
Drinking water	GR CALUX	<2	(Brand *et al.*, 2013)
Drinking water	GR CALUX	<120	(Leusch *et al.*, 2018b)
Drinking water	GR GeneBLAzer	<0.82	(Albergamo *et al.*, 2020)
Drinking water	GR GeneBLAzer	<1.4	(Neale *et al.*, 2020b)
Drinking water	GR GeneBLAzer	<5.8	(Leusch *et al.*, 2018b)

**EC_{10} converted to dexamethasone EQ using EC_{10} values provided in Table 8.*

Table A9: Summary of reported anti-glucocorticoid activity in units of ng/L mifepristone EQ in different environmental water extracts.

NB: Only studies that have applied SPE or LLE are included.

Matrix	Assay	Anti-glucocorticoid activity (ng/L Mifepristone EQ)	Reference
Wastewater influent	Anti-GR GeneBLAzer	Cytotoxic	(Nivala *et al.*, 2018)
Wastewater effluent	Anti-GR CALUX	<623*	(Escher *et al.*, 2014)
Wastewater effluent	Anti-GR GeneBLAzer	<21*	(Escher *et al.*, 2014)
Wastewater effluent	Anti-GR CALUX	<1200	(Jia *et al.*, 2015)
Wastewater effluent	Anti-GR GeneBLAzer	<40	(Jia *et al.*, 2015)
Wastewater effluent	Anti-GR GeneBLAzer	Cytotoxic	(Nivala *et al.*, 2018)
Wastewater effluent	Anti-GR GeneBLAzer	<60	(Leusch *et al.*, 2018b)
Recycled water (UV, UV/H_2O_2,O_3,O_3/UV)	Anti-GR CALUX	<1200	(Jia *et al.*, 2015)
Recycled water (UV, UV/H_2O_2,O_3,O_3/UV)	Anti-GR GeneBLAzer	<40	(Jia *et al.*, 2015)
Recycled water (RO/AO)	Anti-GR CALUX	<1246*	(Escher *et al.*, 2014)
Recycled water (RO/AO)	Anti-GR GeneBLAzer	<43*	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	Anti-GR CALUX	<1246*	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	Anti-GR GeneBLAzer	<43*	(Escher *et al.*, 2014)
Surface water	Anti-GR GeneBLAzer	Cytotoxic	(Neale *et al.*, 2020b)
Surface water	Anti-GR CALUX	<1246*	(Escher *et al.*, 2014)
Surface water	Anti-GR CALUX	<50 - 610	(Jia *et al.*, 2019)
Surface water	Anti-GR GeneBLAzer	<5	(Daniels *et al.*, 2018)
Surface water	Anti-GR GeneBLAzer	<43*	(Escher *et al.*, 2014)
Surface water	Anti-GR GeneBLAzer	<0.2 - 2.5	(Konig *et al.*, 2017)
Surface water	Anti-GR GeneBLAzer	<12	(Leusch *et al.*, 2018b)
Ground water	Anti-GR CALUX	<1200	(Jia *et al.*, 2015)
Ground water	Anti-GR GeneBLAzer	<40	(Jia *et al.*, 2015)
Drinking water	Anti-GR CALUX	<1246*	(Escher *et al.*, 2014)
Drinking water	Anti-GR GeneBLAzer	<43*	(Escher *et al.*, 2014)
Drinking water	Anti-GR GeneBLAzer	<3	(Leusch *et al.*, 2018b)
Drinking water	Anti-GR GeneBLAzer	<0.49	(Neale *et al.*, 2020b)

**$EC_{SR0.2}$ values converted to mifepristone EQ using $EC_{SR0.2}$ values provided in Table 9.*

Table A10: Summary of reported progestagenic activity in units of ng/L levonorgestrel EQ in different environmental water extracts.

NB: Only studies that have applied SPE or LLE are included.

Matrix	Assay	Progestagenic activity (ng/L Levonorgestrel EQ)	Reference
Wastewater influent	PR CALUX	<0.32 - 3.2†	(Bain *et al.*, 2014)
Wastewater influent	PR CALUX	0.81 - 2.8#	(Houtman *et al.*, 2018)
Wastewater influent	PR CALUX	1.3 - 1.6†	(Roberts *et al.*, 2015)
Wastewater influent	PR GeneBLAzer	Cytotoxic	(Nivala *et al.*, 2018)
Wastewater effluent	PR CALUX	<0.36*	(Escher *et al.*, 2014)
Wastewater effluent	PR GeneBLAzer	<0.38*	(Escher *et al.*, 2014)
Wastewater effluent	PR CALUX	<0.32 - 2.7†	(Bain *et al.*, 2014)
Wastewater effluent	PR CALUX	0.43 - 5.3#	(Houtman *et al.*, 2018)
Wastewater effluent	PR CALUX	<90	(Jia *et al.*, 2015)
Wastewater effluent	PR CALUX	<0.01 - 5.4	(Leusch *et al.*, 2014b)
Wastewater effluent	PR CALUX	<33	(Leusch *et al.*, 2018b)
Wastewater effluent	PR CALUX	5.4 - 7.1†	(Roberts *et al.*, 2015)
Wastewater effluent	PR CALUX	1.7 - 1.8#	(Van der Linden *et al.*, 2008)
Wastewater effluent	PR GeneBLAzer	<2.5	(Leusch *et al.*, 2018b)
Wastewater effluent	PR GeneBLAzer	2.2 - 5.7	(Mehinto *et al.*, 2015)
Wastewater effluent	PR GeneBLAzer	Cytotoxic	(Nivala *et al.*, 2018)
Recycled water (UV, UV/H_2O_2,O_3,O_3/UV)	PR CALUX	<90	(Jia *et al.*, 2015)
Recycled water (RO/UV)	PR GeneBLAzer	<1.4	(Mehinto *et al.*, 2015)
Recycled water (RO/AO)	PR CALUX	<0.36*	(Escher *et al.*, 2014)
Recycled water (RO/AO)	PR GeneBLAzer	<0.19*	(Escher *et al.*, 2014)
Recycled water (RO)	PR CALUX	<0.01	(Leusch *et al.*, 2014b)
Recycled water (O_3/BAC)	PR CALUX	<0.36*	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	PR GeneBLAzer	<0.19*	(Escher *et al.*, 2014)
Surface water	PR CALUX	<0.36*	(Escher *et al.*, 2014)
Surface water	PR CALUX	<7	(Leusch *et al.*, 2018b)
Surface water	PR CALUX	<0.32 - 1.6†	(Roberts *et al.*, 2015)
Surface water	PR CALUX	<5	(Scott *et al.*, 2014)
Surface water	PR CALUX	<LOD - 9.6#	(Van der Linden *et al.*, 2008)
Surface water	PR CALUX	<0.11#	(Brand *et al.*, 2013)
Surface water	PR GeneBLAzer	<0.19*	(Escher *et al.*, 2014)
Surface water	PR GeneBLAzer	Cytotoxic	(Hashmi *et al.*, 2020)
Surface water	PR GeneBLAzer	Cytotoxic	(Konig *et al.*, 2017)
Surface water	PR GeneBLAzer	<0.50 - 1.1	(Leusch *et al.*, 2018b)
Surface water	PR GeneBLAzer	Cytotoxic	(Muller *et al.*, 2018)
Surface water	PR GeneBLAzer	<0.13*	(Neale *et al.*, 2018a)
Surface water	PR GeneBLAzer	<0.04*	(Neale *et al.*, 2020b)
Surface water	PR GeneBLAzer	<0.06 - 0.14	(Scott *et al.*, 2018)
Riverbank filtrate	PR GeneBLAzer	<0.04*	(Albergamo *et al.*, 2020)
Ground water	PR CALUX	<90	(Jia *et al.*, 2015)
Drinking water	PR CALUX	<0.36*	(Escher *et al.*, 2014)
Drinking water	PR CALUX	<0.11#	(Brand *et al.*, 2013)
Drinking water	PR CALUX	<2	(Leusch *et al.*, 2018b)
Drinking water	PR GeneBLAzer	<0.04*	(Albergamo *et al.*, 2020)
Drinking water	PR GeneBLAzer	<0.19*	(Escher *et al.*, 2014)
Drinking water	PR GeneBLAzer	<0.10	(Leusch *et al.*, 2018b)
Drinking water	PR GeneBLAzer	<0.04*	(Neale *et al.*, 2020b)

**EC_{10} values converted to levonorgestrel EQ using EC_{10} values provided in Table 10; †Results presented as progesterone EQ and converted to levonorgestrel EQ using relative effect potency of 0.16 (Sonneveld et al., 2011); # Results presented as Org2058 EQ and converted to levonorgestrel EQ using relative effect potency of 2.14 (Sonneveld et al., 2011)*

Table A11: Summary of reported anti-progestagenic activity in units of ng/L mifepristone EQ in different environmental water extracts.

NB: Only studies that have applied SPE or LLE are included.

Matrix	Assay	Anti-progestagenic activity (ng/L Mifepristone EQ)	Reference
Wastewater influent	Anti-PR GeneBLAzer	Cytotoxic	(Nivala *et al.*, 2018)
Wastewater effluent	Anti-PR CALUX	<4.3*	(Escher *et al.*, 2014)
Wastewater effluent	Anti-PR CALUX	<0.72 - 17	(Alygizakis *et al.*, 2019)
Wastewater effluent	Anti-PR CALUX	<8.6	(Jia *et al.*, 2015)
Wastewater effluent	Anti-PR GeneBLAzer	Cytotoxic	(Nivala *et al.*, 2018)
Wastewater effluent	Anti-PR GeneBLAzer	<2	(Leusch *et al.*, 2018b)
Recycled water (UV, UV/H_2O_2,O_3,O_3/UV)	Anti-PR CALUX	<8.6	(Jia *et al.*, 2015)
Recycled water (RO/AO)	Anti-PR CALUX	<8.6*	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	Anti-PR CALUX	<8.6*	(Escher *et al.*, 2014)
Surface water	Anti-PR GeneBLAzer	<1.3*	(Neale *et al.*, 2020b)
Surface water	Anti-PR CALUX	<8.6*	(Escher *et al.*, 2014)
Surface water	Anti-PR CALUX	<8 - 32000	(Scott *et al.*, 2014)
Surface water	Anti-PR GeneBLAzer	Cytotoxic	(Konig *et al.*, 2017)
Surface water	Anti-PR GeneBLAzer	<0.4	(Leusch *et al.*, 2018b)
Surface water	Anti-PR GeneBLAzer	Cytotoxic	(Muller *et al.*, 2018)
Surface water	Anti-PR GeneBLAzer	<1.8 - 4.2	(Scott *et al.*, 2018)
Ground water	Anti-PR CALUX	<8.6	(Jia *et al.*, 2015)
Drinking water	Anti-PR CALUX	<8.6*	(Escher *et al.*, 2014)
Drinking water	Anti-PR GeneBLAzer	<0.1	(Leusch *et al.*, 2018b)
Drinking water	Anti-PR GeneBLAzer	<1.3*	(Neale *et al.*, 2020b)

**$EC_{SR0.2}$ converted to Mifepristone EQ using $EC_{SR0.2}$ values provided in Table 11.*

Table A12: Summary of reported thyroid activity in units of ng/L triiodothyronine (T3) EQ in different environmental water extracts.

NB: Only studies that have applied SPE or LLE are included.

Matrix	Assay	Thyroid activity (ng/L T3 EQ)	Reference
Wastewater influent	PC-DR-LUC	<20 - 25	(Jugan *et al.*, 2009)
Wastewater influent	T-Screen	190 - 204	(Kusk *et al.*, 2011)
Wastewater effluent	TRβ CALUX	<0.14*	(Escher *et al.*, 2014)
Wastewater effluent	T-Screen	<6.1*	(Escher *et al.*, 2014)
Wastewater effluent	GH3.TRE-Luc	<10†	(Leusch *et al.*, 2018a)
Wastewater effluent	GH3.TRE-Luc	<25	(Leusch *et al.*, 2018b)
Wastewater effluent	PC-DR-LUC	<20	(Jugan *et al.*, 2009)
Wastewater effluent	TRβ CALUX	<5.6	(Jia *et al.*, 2015)
Wastewater effluent	TRβ CALUX	<21†	(Leusch *et al.*, 2018a)
Wastewater effluent	TRβ GeneBLAzer	<2.3†	(Leusch *et al.*, 2018a)
Wastewater effluent	T-Screen	<5.5	(Jia *et al.*, 2015)
Wastewater effluent	XETA	<LOD - 1340	(Valitalo *et al.*, 2017)
Wastewater effluent	XETA	1100†	(Leusch *et al.*, 2018a)
Recycled water (UV, UV/H_2O_2,O_3,O_3/UV)	TRβ CALUX	<5.6	(Jia *et al.*, 2015)
Recycled water (UV, UV/H_2O_2,O_3,O_3/UV)	T-Screen	<5.5	(Jia *et al.*, 2015)
Recycled water (RO/AO)	TRβ CALUX	<0.14*	(Escher *et al.*, 2014)
Recycled water (RO/AO)	T-Screen	<6.1*	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	TRβ CALUX	<0.14*	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	T-Screen	<6.1*	(Escher *et al.*, 2014)
Surface water	GH3.TRE-Luc	<5.3†	(Leusch *et al.*, 2018a)
Surface water	GH3.TRE-Luc	<20	(Leusch *et al.*, 2018b)
Surface water	PC-DR-LUC	<20	(Jugan *et al.*, 2009)
Surface water	TRβ CALUX	<0.14*	(Escher *et al.*, 2014)
Surface water	TRβ CALUX	<3.5†	(Leusch *et al.*, 2018a)
Surface water	TRβ GeneBLAzer	<1.2†	(Leusch *et al.*, 2018a)
Surface water	T-Screen	<6.1*	(Escher *et al.*, 2014)
Surface water	XETA	960†	(Leusch *et al.*, 2018a)
Surface water	Yeast two-hybrid TR	<14 - 43	(Chinathamby *et al.*, 2013)
Ground water	TRβ CALUX	<5.6	(Jia *et al.*, 2015)
Ground water	T-Screen	<5.5	(Jia *et al.*, 2015)
Drinking water	TRβ CALUX	<0.14*	(Escher *et al.*, 2014)
Drinking water	T-Screen	<6.1*	(Escher *et al.*, 2014)
Drinking water	GH3.TRE-Luc	<0.05†	(Leusch *et al.*, 2018a)
Drinking water	GH3.TRE-Luc	<1.3	(Leusch *et al.*, 2018b)
Drinking water	PC-DR-LUC	<20	(Jugan *et al.*, 2009)
Drinking water	TRβ CALUX	<3.5†	(Leusch *et al.*, 2018a)
Drinking water	TRβ GeneBLAzer	<1.2†	(Leusch *et al.*, 2018a)
Drinking water	XETA	<300†	(Leusch *et al.*, 2018a)

**EC_{10} values converted to T3 EQ using EC_{10} values provided in Table 12; †Results presented as thyroxine (T4) EQ and converted to T3 EQ using relative effect potency from Leusch et al. (2018a)*

Table A13: Summary of reported anti-thyroid activity in units of µg/L amiodarone hydrochloride (AH) EQ in different environmental water extracts.

NB: Only studies that have applied SPE or LLE are included.

Matrix	Assay	Anti-thyroid activity (µg/L AH EQ)	Reference
Wastewater influent	Yeast two-hybrid	60 - 422	(Li *et al.*, 2011)
Wastewater effluent	GH3.TRE-Luc	<1700	(Leusch *et al.*, 2018a)
Wastewater effluent	TRβ GeneBLAzer	350	(Leusch *et al.*, 2018a)
Wastewater effluent	Yeast two-hybrid	13 - 35	(Li *et al.*, 2011)
Surface water	GH3.TRE-Luc	<870	(Leusch *et al.*, 2018a)
Surface water	TRβ GeneBLAzer	<28	(Leusch *et al.*, 2018a)
Surface water	Yeast two-hybrid	3.3 - 16	(Li *et al.*, 2011)
Drinking water	GH3.TRE-Luc	<87	(Leusch *et al.*, 2018a)
Drinking water	TRβ GeneBLAzer	<28	(Leusch *et al.*, 2018a)

Table A14: Summary of reported anti-mineralocorticoid activity in units of µg/L spironolactone EQ in different environmental water extracts.

NB: Only studies that have applied SPE or LLE are included.

Matrix	Assay	Anti-mineralocorticoid activity (µg/L spironolactone EQ)	Reference
Wastewater influent	HG5LN-hMR	1.3 - 2.3	(Bellet *et al.*, 2012)
Wastewater effluent	HG5LN-hMR	<3.1 - 3.1	(Leusch *et al.*, 2018b)
Surface water	HG5LN-hMR	<0.66 - 0.91	(Leusch *et al.*, 2018b)
Drinking water	HG5LN-hMR	<0.16	(Leusch *et al.*, 2018b)

Table A15: Summary of studies that have applied photosystem II (PSII) inhibition assays to different environmental water extracts. PSII inhibition expressed in units of µg/L diuron EQ.

NB: Only studies that have applied SPE or LLE are included

Matrix	Assay	PSII Inhibition (µg/L diuron EQ)	Reference
Wastewater influent	Combined algae assay (2 h IPAM)	0.26 - 2.2	(Macova *et al.*, 2011)
Wastewater influent	Combined algae assay (2 h IPAM)	0.07	(Tang and Escher, 2014)
Wastewater influent	Max-I-PAM*	0.04-0.23	(Leusch *et al.*, 2014a)
Wastewater effluent	Combined algae assay (2 h IPAM)	0.18 - 0.33	(Tang *et al.*, 2014)
Wastewater effluent	Combined algae assay (2 h IPAM)	0.16 - 0.28	(Escher *et al.*, 2008b)
Wastewater effluent	Combined algae assay (2 h IPAM)	0.27 - 0.83	(Jia *et al.*, 2015)
Wastewater effluent	Combined algae assay (2 h IPAM)	0.09 - 1.3	(Macova *et al.*, 2011)
Wastewater effluent	Combined algae assay (2 h IPAM)	0.07 - 0.26	(Neale *et al.*, 2017b)
Wastewater effluent	Combined algae assay (2 h IPAM)	0.05 - 0.22	(Reungoat *et al.*, 2010)
Wastewater effluent	Combined algae assay (2 h IPAM)	0.033	(Tang and Escher, 2014)
Wastewater effluent	Max-I-PAM*	<0.03-0.08	(Leusch *et al.*, 2014a)
Wastewater effluent	Max-I-PAM*	0.12	(Macova *et al.*, 2010)
Recycled water (UV, UV/H_2O_2,O_3,O_3/UV)	Combined algae assay (2 h IPAM)	0.54 - 1.3	(Jia *et al.*, 2015)
Recycled water (RO/UV)	Combined algae assay (2 h IPAM)	<0.004	(Tang and Escher, 2014)
Recycled water (RO/AO)	Combined algae assay (2 h IPAM)	0.05	(Macova *et al.*, 2011)
Recycled water (RO/AO)	Combined algae assay (2 h IPAM)	<0.01	(Tang *et al.*, 2014)
Recycled water (RO)	Max-I-PAM*	<0.03	(Leusch *et al.*, 2014a)
Recycled water (O_3/BAC)	Combined algae assay (2 h IPAM)	<0.01	(Tang *et al.*, 2014)
Recycled water (O_3)	Combined algae assay (2 h IPAM)	<0.01 - 0.05	(Reungoat *et al.*, 2010)
Recycled water (O_3)	Max-I-PAM*	0.02	(Macova *et al.*, 2010)
Surface water	Combined algae assay (2 h IPAM)	<0.13 - 1.3	(Allan *et al.*, 2017)
Surface water	Combined algae assay (2 h IPAM)	0.19 - 0.23	(Escher *et al.*, 2008b)
Surface water	Combined algae assay (2 h IPAM)	0.01-0.05	(Macova *et al.*, 2011)
Surface water	Combined algae assay (2 h IPAM)	0.01 - 0.07	(Neale *et al.*, 2017b)
Surface water	Combined algae assay (2 h IPAM)	<0.01	(Tang *et al.*, 2014)
Surface water	Max-I-PAM*	<0.03-0.06	(Leusch *et al.*, 2014a)
Ground water	Combined algae assay (2 h IPAM)	0.003-0.004	(Jia *et al.*, 2015)
Drinking Water	Combined algae assay (2 h IPAM)	0.05	(Macova *et al.*, 2011)
Drinking Water	Combined algae assay (2 h IPAM)	0.02	(Tang *et al.*, 2014)

**Max-I-PAM uses* Chlorella vulgaris*, while all other assays used* Raphidocelis subcapitata *(formerly* Selenastrum capricornutum *and* Pseudokirchneriella subcapitata*)*

Table A16: Summary of studies that have applied the enzymatic acetylcholinesterase (AChE) inhibition assay to different environmental water extracts. AChE inhibition expressed in units of µg/L parathion EQ.

NB: Only studies that have applied SPE or LLE are included.

Matrix	AChE Inhibition (µg/L parathion EQ)	Reference
Wastewater influent	4.4-6.0	(Macova *et al.*, 2011)
Wastewater effluent	1.6 - 2.8*	(Escher *et al.*, 2014)
Wastewater effluent	0.41 – 3.0	(Escher *et al.*, 2008b)
Wastewater effluent	<0.04 - 3.2†	(Leusch *et al.*, 2014b)
Wastewater effluent	0.67 - 1.5	(Macova *et al.*, 2011)
Wastewater effluent	2.4	(Neale and Escher, 2013)
Wastewater effluent	0.48 - 0.52	(Neale *et al.*, 2017b)
Wastewater effluent	2.8 - 3.9	(Reungoat *et al.*, 2010)
Wastewater effluent	3.2	(Macova *et al.*, 2010)
Surface water	0.21 - 0.27	(Escher *et al.*, 2008b)
Surface water	<2.6*	(Escher *et al.*, 2014)
Surface water	0.11 - 0.24	(Macova *et al.*, 2011)
Surface water	0.14	(Neale and Escher, 2013)
Surface water	0.11 - 0.23	(Neale *et al.*, 2017b)
Recycled water (RO/AO)	<2.6*	(Escher *et al.*, 2014)
Recycled water (RO/AO)	<0.06	(Macova *et al.*, 2011)
Recycled water (O_3/BAC)	<2.6*	(Escher *et al.*, 2014)
Recycled water (O_3)	<0.3-1.2	(Reungoat *et al.*, 2010)
Recycled water (O_3)	0.36	(Macova *et al.*, 2010)
Drinking water	<2.6*	(Escher *et al.*, 2014)
Drinking water	0.28	(Macova *et al.*, 2011)

**Parathion EQ calculated using the parathion EC_{10} in Neale et al. (2017b); †Chlorpyrifos EQ converted to Parathion EQ based on relative effect potency (REP) of 4.05 (Neale and Escher, 2013).*

Table A17: Summary of studies that have applied the umuC assay indicative of genotoxicity to different environmental water extracts. Genotoxicity with metabolic activation (+S9) is expressed in units of µg/L 2-aminoanthracene (2AA) EQ and genotoxicity without metabolic activation (+S9) is expressed in units of µg/L 4-nitroquinoline N-oxide (4NQO) EQ.

Matrix	Strain	Genotoxicity +S9 (µg/L 2AA EQ)	Reference
Wastewater influent	TA1535/pSK1002	19*	(Lee *et al.*, 2015)
Wastewater influent	TA1535/pSK1002	2	(Tang *et al.*, 2014)
Wastewater influent	TA1535/pSK1002	<2.7 - 13†	(Macova *et al.*, 2011)
Wastewater effluent	TA1535/pSK 1002	1.6 - 4.6*	(Escher *et al.*, 2014)
Wastewater effluent	TA1535/pSK 1002	<1.6*	(Escher *et al.*, 2008b)
Wastewater effluent	TA1535/pSK 1002	1.6 - 2.2†	(Fang *et al.*, 2012)
Wastewater effluent	TA1535/pSK1002	0.5 - 1.8	(Jia *et al.*, 2015)
Wastewater effluent	TA1535/pSK1002	2.7*	(Macova *et al.*, 2010)
Wastewater effluent	TA1535/pSK1002	<0.2	(Tang *et al.*, 2014)
Wastewater effluent	TA1535/pSK1002	1.1 - 2.5†	(Macova *et al.*, 2011)
Recycled water (UV, UV/H_2O_2,O_3,O_3/UV)	TA1535/pSK1002	2.1 - 4.3	(Jia *et al.*, 2015)
Recycled water (RO/UV)	TA1535/pSK1002	<0.2	(Tang *et al.*, 2014)
Recycled water (RO/AO)	TA1535/pSK 1002	<1.6*	(Escher *et al.*, 2014)
Recycled water (RO/AO)	TA1535/pSK1002	<0.34†	(Macova *et al.*, 2011)
Recycled water (O_3/BAC)	TA1535/pSK 1002	<1.6*	(Escher *et al.*, 2014)
Recycled water (O_3)	TA1535/pSK1002	<0.64*	(Macova *et al.*, 2010)
Reclaimed water	TA1535/pSK1002	<1.9*	(Lee *et al.*, 2015)
Surface water	TA1535/pSK 1002	<0.32*	(Escher *et al.*, 2008b)
Surface water	TA1535/pSK 1002	<0.31 - 0.38*	(Farre *et al.*, 2013)
Surface water	TA1535/pSK 1002	<1.6*	(Escher *et al.*, 2014)
Surface water	TA1535/pSK1002	0.18	(Neale *et al.*, 2012)
Surface water	TA1535/pSK1002	<0.34†	(Macova *et al.*, 2011)
Ground water	TA1535/pSK1002	0.4 - 0.5	(Jia *et al.*, 2015)
Ground water	TA1535/pSK1002	<0.47*	(Lee *et al.*, 2015)
Drinking water	TA1535/pSK 1002	<1.6*	(Escher *et al.*, 2014)
Drinking water	TA1535/pSK1002	1.29	(Neale *et al.*, 2012)
Drinking water	TA1535/pSK1002	<0.34†	(Macova *et al.*, 2011)
Matrix	**Strain**	**Genotoxicity -S9 (µg/L 4NQO EQ)**	**Reference**
Wastewater influent	TA1535/pSK1002	6.9#	(Lee *et al.*, 2015)
Wastewater influent	TA1535/pSK1002	0.48 - 1.5	(Macova *et al.*, 2011)
Wastewater influent	TA1535/pSK1002	0.56	(Tang *et al.*, 2014)
Wastewater effluent	TA1535/pSK 1002	0.80 - 1.0#	(Escher *et al.*, 2014)
Wastewater effluent	TA1535/pSK 1002	1.8 - 2.6	(Fang *et al.*, 2012)
Wastewater effluent	TA1535/pSK1002	0.12 - 0.24	(Jia *et al.*, 2015)
Wastewater effluent	TA1535/pSK1002	3.5#	(Macova *et al.*, 2010)
Wastewater effluent	TA1535/pSK1002	0.17 - 0.24	(Macova *et al.*, 2011)
Wastewater effluent	TA1535/pSK1002	3.4 - 5.8#	Reungoat et al. 2010
Wastewater effluent	TA1535/pSK1002	0.24	(Tang *et al.*, 2014)
Recycled water (UV, UV/H_2O_2,O_3,O_3/UV)	TA1535/pSK1002	0.13 - 0.29	(Jia *et al.*, 2015)
Matrix	**Strain**	**Genotoxicity -S9 (µg/L 4NQO EQ)**	**Reference**
Recycled water (RO/UV)	TA1535/pSK1002	<0.1	(Tang *et al.*, 2014)
Recycled water (RO/AO)	TA1535/pSK 1002	<0.6#	(Escher *et al.*, 2014)
Recycled water (RO/AO)	TA1535/pSK1002	<0.05	(Macova *et al.*, 2011)
Recycled water (O_3/BAC)	TA1535/pSK 1002	<0.6#	(Escher *et al.*, 2014)
Recycled water (O_3)	TA1535/pSK1002	<0.26#	(Macova *et al.*, 2010)
Recycled water (O_3)	TA1535/pSK1002	<0.18 - 0.72#	Reungoat et al. 2010
Reclaimed water	TA1535/pSK1002	0.55#	(Lee *et al.*, 2015)
Surface water	TA1535/pSK 1002	0.25 - 0.69	(Sun *et al.*, 2017)
Surface water	TA1535/pSK 1002	0.01 - 0.03	(Han *et al.*, 2016)
Surface water	TA1535/pSK 1002	<0.12 - 0.17#	(Farre *et al.*, 2013)

Surface water	TA1535/pSK 1002	<0.6#	(Escher *et al.*, 2014)
Surface water	TA1535/pSK1002	<0.05	(Macova *et al.*, 2011)
Surface water	TA1535/pSK1002	0.05	(Neale *et al.*, 2012)
Ground water	TA1535/pSK1002	0.11	(Jia *et al.*, 2015)
Ground water	TA1535/pSK1002	<0.18#	(Lee *et al.*, 2015)
Drinking water	TA1535/pSK 1002	<0.6#	(Escher *et al.*, 2014)
Drinking water	TA1535/pSK1002	<0.05	(Macova *et al.*, 2011)
Drinking water	TA1535/pSK1002	0.43	(Neale *et al.*, 2012)
Drinking water	TA1535/pSK1002	0.28 - 0.61#	(Stalter *et al.*, 2016b)

**2-AA EQ calculated using 2-AA $EC_{IR1.5}$ of 46.7 ng/L (Tang et al., 2014); †Results presented as benzo(a)pyrene (BaP) EQ and converted to 2-AA EQ using relative effect potency from Macova et al. (2011); #4NQO EQ calculated using 4NQO $EC_{IR1.5}$ of 18 ng/L (Macova et al., 2011)*

Table A18: Summary of studies that have applied the Ames assay indicative of mutagenicity to different environmental water extracts. Mutagenicity is expressed as an effect concentration inducing a revertant ratio of 1.5 ($EC_{RR1.5}$) in units of relative enrichment factor (REF).

Matrix	Strain	$EC_{RR1.5}$ (REF) +S9	Reference
Wastewater effluent	TA98	4.1 - 5.6	(Escher *et al.*, 2014)
Wastewater effluent	TAmix	2.9 - 3	(Escher *et al.*, 2014)
Wastewater effluent	TA98	3.5 - >100	(Jia *et al.*, 2015)
Wastewater effluent	TAmix	29 - 66	(Jia *et al.*, 2015)
Recycled water (UV, UV/H_2O_2,O_3,O_3/UV)	TA98	73 - >100	(Jia *et al.*, 2015)
Recycled water (UV, UV/H_2O_2,O_3,O_3/UV))	TAmix	36 - >100	(Jia *et al.*, 2015)
Recycled water (RO/AO)	TA98	>30	(Escher *et al.*, 2014)
Recycled water (RO/AO)	TAmix	13.7	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	TA98	12.5	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	TAmix	13.7	(Escher *et al.*, 2014)
Surface water	TA98	4.5	(Escher *et al.*, 2014)
Surface water	TAmix	>30	(Escher *et al.*, 2014)
Ground water	TA98	>100	(Jia *et al.*, 2015)
Ground water	TAmix	84 ->100	(Jia *et al.*, 2015)
Drinking water	TA98	3.2	(Escher *et al.*, 2014)
Drinking water	TAmix	13.8	(Escher *et al.*, 2014)
Matrix	**Strain**	**$EC_{RR1.5}$ (REF) -S9**	**Reference**
Wastewater effluent	TA98	6.3	(Escher *et al.*, 2014)
Wastewater effluent	TAmix	6.9 - 21	(Escher *et al.*, 2014)
Wastewater effluent	TA100	5.4 - 16	(Escher *et al.*, 2014)
Wastewater effluent	TA98	30 - >100	(Jia *et al.*, 2015)
Wastewater effluent	TAmix	8.5 - 83	(Jia *et al.*, 2015)
Wastewater effluent	TA100	0.6 - 2.7	(Jia *et al.*, 2015)
Recycled water (UV, UV/H_2O_2,O_3,O_3/UV)	TA98	>100	(Jia *et al.*, 2015)
Recycled water (UV, UV/H_2O_2,O_3,O_3/UV)	TAmix	35 - 69	(Jia *et al.*, 2015)
Recycled water (UV, UV/H_2O_2,O_3,O_3/UV)	TA100	0.5 -0.7	(Jia *et al.*, 2015)
Recycled water (RO/AO)	TA98	>30	(Escher *et al.*, 2014)
Recycled water (RO/AO)	TAmix	>30	(Escher *et al.*, 2014)
Recycled water (RO/AO)	TA100	>30	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	TA98	>30	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	TAmix	>30	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	TA100	>30	(Escher *et al.*, 2014)
Surface water	TA98	14	(Escher *et al.*, 2014)
Surface water	TAmix	>30	(Escher *et al.*, 2014)
Surface water	TA100	25	(Escher *et al.*, 2014)
Ground water	TA98	>100	(Jia *et al.*, 2015)
Ground water	TAmix	>100	(Jia *et al.*, 2015)
Ground water	TA100	4.8 - >20	(Jia *et al.*, 2015)
Drinking water	TA98	4.6	(Escher *et al.*, 2014)
Drinking water	TAmix	4.9	(Escher *et al.*, 2014)
Drinking water	TA100	5.0	(Escher *et al.*, 2014)

Table A19: Summary of studies that have applied assays indicative of the oxidative stress response to different environmental water extracts. The oxidative stress response was expressed as the concentration causing an induction ratio of 1.5 ($EC_{IR1.5}$) in units of relative enrichment factor (REF).

NB: Only studies that have applied SPE or LLE are included

Matrix	Assay	$EC_{IR1.5}$ (REF)	Reference
Wastewater influent	AREc32	0.28 - 0.30	(Nivala *et al.*, 2018)
Wastewater influent	AREc32	4.7	(Tang *et al.*, 2014)
Wastewater influent	AREc32	1.2	(Volker *et al.*, 2017)
Wastewater influent	AREc32	0.34 - 0.69	(Escher *et al.*, 2012)
Wastewater influent	Nrf2 reporter gene assay	8.1 - 30	(Lundqvist *et al.*, 2019b)
Wastewater effluent	ARE GeneBLAzer	8.9 - 17	(Neale *et al.*, 2017b)
Wastewater effluent	AREc32	1.6 - 4.4	(Escher *et al.*, 2012)
Wastewater effluent	AREc32	12 - 22	(Jia *et al.*, 2015)
Wastewater effluent	AREc32	1.5 - 1.9	(Nivala *et al.*, 2018)
Wastewater effluent	AREc32	8.4	(Tang *et al.*, 2014)
Wastewater effluent	AREc32	3.2 - 3.4	(Volker *et al.*, 2017)
Wastewater effluent	Nrf2 reporter gene assay	47 - >50	(Lundqvist *et al.*, 2019b)
Wastewater effluent	Nrf2-MDA-MB	>10	(Jia *et al.*, 2015)
Wastewater effluent	AREc32	1.7 - 2.0	(Escher *et al.*, 2013)
Wastewater effluent	Nrf2-CALUX	4.8	(Escher *et al.*, 2014)
Wastewater effluent	Nrf2-MDA-MB	>10	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	AREc32	4.2	(Escher *et al.*, 2012)
Recycled water (O_3/BAC)	AREc32	22	(Escher *et al.*, 2013)
Recycled water (O_3/BAC)	Nrf2-CALUX	4.8	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	Nrf2-MDA-MB	>20	(Escher *et al.*, 2014)
Recycled water (RO/AO)	AREc32	7.8	(Escher *et al.*, 2012)
Recycled water (RO/AO)	AREc32	94	(Escher *et al.*, 2013)
Recycled water (RO/AO)	Nrf2-CALUX	>30	(Escher *et al.*, 2014)
Recycled water (RO/AO)	Nrf2-MDA-MB (Nrf2-keap)	>20	(Escher *et al.*, 2014)
Recycled water (RO/UV)	AREc32	74	(Tang *et al.*, 2014)
Recycled water (UV, UV/H_2O_2, O_3, O_3/UV)	AREc32	30 - 67	(Jia *et al.*, 2015)
Recycled water (UV, UV/H_2O_2, O_3, O_3/UV)	Nrf2-MDA-MB	>10	(Jia *et al.*, 2015)
Surface water	ARE GeneBLAzer	6.9 - 31	(Konig *et al.*, 2017)
Surface water	ARE GeneBLAzer	16 - >490	(Neale *et al.*, 2015)
Surface water	ARE GeneBLAzer	21 - 60	(Neale *et al.*, 2017b)
Surface water	AREc32	35	(Neale *et al.*, 2012)
Surface water	AREc32	7.5 - 12	(Escher *et al.*, 2012)
Surface water	AREc32	17	(Escher *et al.*, 2013)
Surface water	AREc32	7.8 - 9.9	(Farre *et al.*, 2013)
Surface water	AREc32	12	(Hashmi *et al.*, 2018)
Surface water	AREc32	20 - >100	(Muller *et al.*, 2018)
Surface water	AREc32	23	(Neale *et al.*, 2018a)
Surface water	AREc32	43 - 63	(Neale *et al.*, 2020b)
Surface water	Nrf2 reporter gene assay	22	(Lundqvist *et al.*, 2019a)
Surface water	Nrf2-CALUX	6.9	(Escher *et al.*, 2014)
Surface water	Nrf2-MDA-MB	>20	(Escher *et al.*, 2014)
Ground water	Nrf2-MDA-MB	>10	(Jia *et al.*, 2015)
Ground water	AREc32	>160	(Jia *et al.*, 2015)
Riverbank filtrate	AREc32	6.5 - 6.7	(Albergamo *et al.*, 2020)
Drinking water	AREc32	5.3 - 9.6	(Escher *et al.*, 2012)
Drinking water	AREc32	16 - 97	(Hebert *et al.*, 2018)
Drinking water	AREc32	78 - >150	(Neale *et al.*, 2020b)
Drinking water	AREc32	2.7 - 19	(Stalter *et al.*, 2016b)
Drinking water	AREc32	>60	(Albergamo *et al.*, 2020)
Drinking water	AREc32	5.5	(Neale *et al.*, 2012)

Matrix	Assay	$EC_{IR1.5}$ (REF)	Reference
Drinking water	AREc32	5	(Escher *et al.*, 2013)
Drinking water	Nrf2 reporter gene assay	21 - 25	(Lundqvist *et al.*, 2019a)
Drinking water	Nrf2-CALUX	2.9	(Escher *et al.*, 2014)
Drinking water	Nrf2-MDA-MB	>20	(Escher *et al.*, 2014)

Table A20: Summary of studies that have applied assays indicative of the p53 response to different environmental water extracts. The p53 response was expressed as the concentration causing an induction ratio of 1.5 ($EC_{IR1.5}$) in units of relative enrichment factor (REF).

NB: Only studies that have applied SPE or LLE are included

Matrix	Assay	$EC_{IR1.5}$ (REF)	Equivalent Concentration	Reference
Wastewater influent	p53 CALUX +S9		61,000-6,200,000 ng/L Cyclophosphamide EQ	(Valitalo *et al.*, 2017)
Wastewater effluent	p53 CALUX	>30		(Escher *et al.*, 2014)
Wastewater effluent	p53 CALUX +S9	>30		(Escher *et al.*, 2014)
Wastewater effluent	p53 GeneBLAzer	>10		(Escher *et al.*, 2014)
Wastewater effluent	p53 CALUX +S9		<53,000 - 540,000 ng/L Cyclophosphamide EQ	(Valitalo *et al.*, 2017)
Wastewater effluent	p53 GeneBLAzer	Cytotoxic		(Neale *et al.*, 2017b)
Recycled water (RO/AO)	p53 CALUX	>30		(Escher *et al.*, 2014)
Recycled water (RO/AO)	p53 CALUX +S9	>30		(Escher *et al.*, 2014)
Recycled water (RO/AO)	p53 GeneBLAzer	>20		(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	p53 CALUX	>30		(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	p53 CALUX +S9	>30		(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	p53 GeneBLAzer	>30		(Escher *et al.*, 2014)
Surface water	p53 GeneBLAzer	Cytotoxic		(Neale *et al.*, 2020b)
Surface water	p53 CALUX	>30		(Escher *et al.*, 2014)
Surface water	p53 CALUX +S9	>30		(Escher *et al.*, 2014)
Surface water	p53 GeneBLAzer	>20		(Escher *et al.*, 2014)
Surface water	p53 GeneBLAzer	Cytotoxic		(Konig *et al.*, 2017)
Surface water	p53 GeneBLAzer	65 - >450	<34 - 235 ng/L Mitomycin EQ	(Neale *et al.*, 2015)
Surface water	p53 GeneBLAzer	Cytotoxic		(Neale *et al.*, 2017b)
Drinking Water	p53 CALUX	>30		(Escher *et al.*, 2014)
Drinking Water	p53 CALUX +S9	>30		(Escher *et al.*, 2014)
Drinking Water	p53 GeneBLAzer	>20		(Escher *et al.*, 2014)
Drinking water	p53 GeneBLAzer	Cytotoxic		(Hebert *et al.*, 2018)
Drinking water	p53 GeneBLAzer	>100		(Neale *et al.*, 2020b)

Table A21: Summary of studies that have applied assays indicative of the NF-κB response to different environmental water extracts. The NF-κB response was expressed as the concentration causing an induction ratio of 1.5 ($EC_{IR1.5}$) in units of relative enrichment factor (REF).

NB: Only studies that have applied SPE or LLE are included

Matrix	Assay	$EC_{IR1.5}$ (REF)	Reference
Wastewater influent	NFκB reporter gene assay	0.30 - >50	(Lundqvist *et al.*, 2019b)
Wastewater influent	NFκB GeneBLAzer	0.05-0.36	(Nivala *et al.*, 2018)
Wastewater effluent	NFκB CALUX	>30	(Escher *et al.*, 2014)
Wastewater effluent	NFκB GeneBLAzer	17 - >20	(Escher *et al.*, 2014)
Wastewater effluent	NFκB reporter gene assay	>50	(Lundqvist *et al.*, 2019b)
Wastewater effluent	NFκB GeneBLAzer	0.91 - 1.9	(Neale *et al.*, 2017b)
Wastewater effluent	NFκB GeneBLAzer	0.09 - 0.32	(Nivala *et al.*, 2018)
Recycled water (RO/AO)	NFκB CALUX	>30	(Escher *et al.*, 2014)
Recycled water (RO/AO)	NFκB GeneBLAzer	>20	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	NFκB CALUX	>30	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	NFκB GeneBLAzer	>20	(Escher *et al.*, 2014)
Surface water	NFκB GeneBLAzer	5.1 - >100	(Neale *et al.*, 2020b)
Surface water	NFκB CALUX	>30	(Escher *et al.*, 2014)
Surface water	NFκB GeneBLAzer	>20	(Escher *et al.*, 2014)
Surface water	NFκB GeneBLAzer	5 - 79	(Konig *et al.*, 2017)
Surface water	NFκB GeneBLAzer	7.5 - >250	(Neale *et al.*, 2015)
Surface water	NFκB GeneBLAzer	0.1 - 4.2	(Neale *et al.*, 2017b)
Surface water	NFκB GeneBLAzer	1.0 - 4.2	(Neale *et al.*, 2018b)
Drinking water	NFκB CALUX	>30	(Escher *et al.*, 2014)
Drinking water	NFκB GeneBLAzer	>20	(Escher *et al.*, 2014)
Drinking water	NFκB GeneBLAzer	10 - >500	(Hebert *et al.*, 2018)
Drinking water	NFκB GeneBLAzer	16 - >100	(Neale *et al.*, 2020b)

Table A22: Summary of studies that have applied bacterial toxicity assays to different environmental water extracts. The effect was expressed as the concentration causing 50% effect (EC_{50}) in units of relative enrichment factor (REF).

NB: Only studies that have applied SPE or LLE are included

Matrix	Assay	EC_{50} (REF)	Reference
Wastewater influent	Microtox	3.6 - 17	(Escher *et al.*, 2012)
Wastewater influent	Microtox	1.5	(Volker *et al.*, 2017)
Wastewater influent	Microtox	0.47	(Tang *et al.*, 2014)
Wastewater influent	Microtox	0.48 - 1.3	(Macova *et al.*, 2011)
Wastewater effluent	Microtox	3.0 - 4.2	(Tang *et al.*, 2013)
Wastewater effluent	BLT-Screen	1.6	(van de Merwe and Leusch, 2015)
Wastewater effluent	Microtox	10 - 11	(Volker *et al.*, 2017)
Wastewater effluent	Microtox	7.3	(Tang *et al.*, 2014)
Wastewater effluent	Microtox	10 - 27	(Escher *et al.*, 2012)
Wastewater effluent	Microtox	5.6	(Macova *et al.*, 2010)
Wastewater effluent	Microtox	9.8 - 13	(Macova *et al.*, 2011)
Recycled water (RO/AO)	Microtox	51	(Tang *et al.*, 2013)
Recycled water (RO/AO)	Microtox	30	(Escher *et al.*, 2012)
Recycled water (RO/AO)	Microtox	102	(Macova *et al.*, 2011)
Recycled water (O_3/BAC)	Microtox	10	(Tang *et al.*, 2013)
Recycled water (O_3/BAC)	Microtox	27	(Escher *et al.*, 2012)
Recycled water (RO/UV)	Microtox	91	(Tang *et al.*, 2014)
Recycled water (O_3)	Microtox	29	(Macova *et al.*, 2010)
Surface water	BLT-Screen	12	(van de Merwe and Leusch, 2015)
Surface water	Microtox	13	(Tang *et al.*, 2013)
Surface water	Microtox	28 - 46	(Escher *et al.*, 2012)
Surface water	Microtox	8.2 - 14	(Farre *et al.*, 2013)
Surface water	Microtox	53 - 87	(Macova *et al.*, 2011)
Surface water	Microtox	17	(Neale *et al.*, 2012)
Drinking water	Microtox	3.2	(Neale *et al.*, 2012)
Drinking water	Microtox	3.4	(Tang *et al.*, 2013)
Drinking water	Microtox	7.3	(Macova *et al.*, 2011)
Drinking water	BLT-Screen	5.8	(van de Merwe and Leusch, 2015)
Drinking water	Microtox	8.7 - 40	(Escher *et al.*, 2012)
Drinking water	Microtox	10 - 31	(Stalter *et al.*, 2016b)

Table A23: Summary of studies that have applied algal growth inhibition assays to different environmental water extracts. The effect was expressed as the concentration causing 10% effect (EC_{10}) in units of relative enrichment factor (REF).

NB: Only studies that have applied SPE or LLE are included

Matrix	Assay	EC_{10} (REF)	Reference
Wastewater effluent	Combined algae assay (growth)	5.6 - 7.7	(Escher *et al.*, 2014)
Wastewater effluent	Combined algae assay (growth)	0.71 - 4.4	(Escher *et al.*, 2008b)
Wastewater effluent	Combined algae assay (growth)	1.0 - 3.2	(Jia *et al.*, 2015)
Wastewater effluent	Combined algae assay (growth)	2.4 - 13	(Neale *et al.*, 2017b)
Recycled water (UV, UV/H_2O_2,O_3,O_3/UV)	Combined algae assay (growth)	0.7 - 7.0	(Jia *et al.*, 2015)
Recycled water (RO/AO)	Combined algae assay (growth)	>20	(Escher *et al.*, 2014)
Recycled water (O_3/BAC)	Combined algae assay (growth)	>20	(Escher *et al.*, 2014)
Surface water	Algal growth inhibition	17 - >100*	(Tousova *et al.*, 2017)
Surface water	Combined algae assay (growth)	1.3 - 20	(Escher *et al.*, 2008b)
Surface water	Combined algae assay (growth)	>20	(Escher *et al.*, 2014)
Surface water	Combined algae assay (growth)	10 - >90	(Neale *et al.*, 2017b)
Ground water	Combined algae assay (growth)	>33	(Jia *et al.*, 2015)
Drinking Water	Combined algae assay (growth)	14.1	(Escher *et al.*, 2014)

**EC_{50} reported*