Analysis of endocrine activity in drinking water, surface water and treated wastewater from six countries

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Abstract

The aquatic environment can contain numerous micropollutants and there are concerns about endocrine activity in environmental waters and their potential impact on human and ecosystem health. In this study a complementary chemical analysis and bioanalysis approach was applied to evaluate endocrine activity in treated wastewater, surface water and drinking water samples from six countries (Germany, Australia, France, South Africa, the Netherlands and Spain). The bioassay test battery included assays indicative of seven endocrine pathways, while 58 different chemicals, including pesticides, pharmaceuticals and industrial compounds, were analysed by targeted chemical analysis. Overall, the endocrine activity in the studied water samples was low, with only some treated wastewater and surface water samples exhibiting estrogenic, glucocorticoid, progestagenic and anti-mineralocorticoid activity. Mixture toxicity modelling using the bioanalytical equivalent concentration (BEQ) approach was applied to predict the contribution of detected chemicals to the observed effect. Bioanalytical equivalent concentrations from chemical analysis (BEQ_{chem}) also indicated low effects, with many BEQ_{chem} values lower than the bioassay limit of detection. Detected endocrine activity was compared to available effect-based trigger values (EBT), with some of the wastewater and surface water samples exceeding the EBT for estrogenic and glucocorticoid activity, suggesting these environmental waters may pose a potential risk to ecosystem health. In contrast, the drinking water samples do not appear to pose a risk to human endocrine health, with all samples below relevant EBTs.

Keywords: bioanalytical equivalent concentration; endocrine activity; environmental waters; *in vitro*; mixture toxicity modelling

Introduction

The aquatic environment can contain a wide range of micropollutants, including pharmaceuticals, pesticides and industrial compounds, from sources such as wastewater effluent and agricultural run-off (Heeb et al., 2012; Eggen et al., 2014). The presence of micropollutants in environmental waters can potentially have adverse effects on both human and ecological health. For example, in the early 1990s estrogenic compounds in treated wastewater effluent were found to cause widespread endocrine disruption in fish in UK rivers (Purdom et al., 1994; Harries et al., 1996).

Targeted chemical analysis is commonly used to monitor micropollutant concentrations in water samples, but this approach alone has some limitations. For example, the aquatic environment can contain a complex mixture of micropollutants and their transformation products, often at low concentrations, and chemical analysis cannot account for the mixture effects that occur between these chemicals. For a more comprehensive assessment of water quality, chemical analysis can be complemented with *in vitro* bioassays. Bioassays can quantify the effect of all active known and unknown chemicals in a sample, account for mixture effects and are risk scaled, so chemicals that are more potent will have a greater effect (Escher and Leusch, 2012; Wernersson et al., 2015). Due to concerns about the adverse effects of endocrine disrupting chemicals, *in vitro* bioassays indicative of hormonal activity have been applied to wastewater (e.g. Rutishauser et al., 2004; Bain et al., 2014; Suzuki et al., 2015), surface water (e.g. Chinathamby et al., 2013; Scott et al., 2014) and drinking water (e.g. Brand et al., 2013; Conley et al., 2017). A recent review found that the majority of studies to date focus on estrogenic activity, with much less known about progestagenic, glucocorticoid and thyroid activity in environmental waters (Leusch et al., 2017). However, a range of hormonal pathways is essential for the maintenance of growth, development and metabolism, so a more comprehensive understanding of endocrine activity in the aquatic environment is required.

To address this knowledge gap, the current study aimed to quantify endocrine activity in treated wastewater, surface water and drinking water collected from six countries (Germany, Australia, France, South Africa, the Netherlands and Spain) in order to assess the potential risks to

ecological and human health. This was achieved using a comprehensive test battery of assays indicative of activation of the estrogen receptor (ER), androgen receptor (AR), glucocorticoid receptor (GR), progesterone receptor (PR), thyroid receptor (TR), retinoid X receptor (RXR), retinoid acid receptor (RAR) and mineralocorticoid receptor (MR). Some micropollutants can also act as antagonists (Sohoni and Sumpter, 1998; Ait-Aissa et al., 2010), so assays indicative of inhibition of ER, AR, GR, PR, RXR, RAR and MR were also applied.

Bioanalysis was complemented with chemical analysis of 58 micropollutants, including hormones, pharmaceuticals and personal care products, pesticides and industrial compounds. Mixture toxicity modelling was applied to determine the contribution of detected chemicals to the biological effect using the bioanalytical equivalent concentration (BEQ) approach (Neale et al., 2015). The BEQ approach assumes that chemicals are acting in a concentration additive manner and has successfully been applied to assays indicative of endocrine activity in a range of water matrices (e.g. Creusot et al., 2014; König et al., 2017; Neale et al., 2017). The endocrine activity detected in the current study was benchmarked against activity reported in previous studies and was also compared with available effect-based trigger values (EBT) to determine if the studied water samples posed a potential risk to human or ecological health.

2. Materials and Methods

2.1. Water samples

Treated wastewater (200 mL), surface water (1000 mL) and drinking water (2×2000 mL) grab samples were collected from six different countries (Germany, Australia, France, South Africa, the Netherlands, Spain). Ultrapure water was also included as a negative control (1000 mL). A range of water quality parameters, including temperature, pH, conductivity and total organic carbon, were measured for each water sample, with details provided in Table S1 of the Supplementary Information (SI), along with sample ID numbers. The water samples were enriched by partners in each participating country using StrataX solid phase extraction (SPE) cartridges (200 mg,

Phenomenex) following the same protocol. Briefly, water samples adjusted to pH 2 were added to conditioned SPE cartridges at a flow rate of approximately 7-10 mL/min. One cartridge was used for each water matrix, with the exception of drinking water where two cartridges were used (2000 mL per cartridge). After extraction, the SPE cartridge was dried under a gentle nitrogen stream and then sent to DVGW – Technologiezentrum Wasser (TZW) in Germany for elution with 3 mL methanol, 3 mL acetonitrile and 3 mL acetone. All solvents were of analytical grade. The eluate was blown to dryness under a gentle nitrogen stream and reconstituted in 1 mL of methanol, giving enrichment factors of 200, 1000 and 4000 for treated wastewater, surface water and drinking water, respectively. The control ultrapure water also had an enrichment factor of 1000. The extract was divided into 100 µL aliquots and sent to all participating laboratories for chemical and bioassay analysis.

2.2. Chemical analysis

Four different chemical analysis methods were applied to detect micropollutants in the water extracts. Forty six micropollutants, including pharmaceuticals, pesticides and industrial compounds, were analysed using liquid chromatography - tandem mass spectrometry (LC-MS/MS), while liquid chromatography - electrospray ionisation - high resolution mass spectrometry (LC-ESI-HRMS) was applied to detect five hormones, androsterone, cortisol, cortisone, epitestosterone and norethisterone. Further, volatile micropollutants were detected using gas chromatography - mass spectrometry (GC/MS). In total, 58 unique chemicals were analysed using these three methods. Further information about the applied methods can be found in Section S1 and Tables S2 to S3 of the SI, with the analytical limit of quantification (LOQ) in the different matrices provided in Table S4. Targeted chemical analysis was complemented with suspect screening using liquid chromatography - high resolution mass spectrometry (LC-HRMS) for over 2500 compounds in the instrument's database. Further information about the LC-HRMS method can be found in Table S5.

128 *2.3. Bioanalysis*

129 Ten bioassays covering 14 different endpoints were applied in the current study. A summary of the
130 studied assays is provided in Table 1. With the exception of RXR-CALUX, detailed descriptions of
131 all bioassays have been previously published, with the references provided in Table 1. RXR-
132 CALUX is a recently developed assay, but follows the same protocol of the other CALUX assays,
133 with further information about the CALUX protocol found in Piersma et al. (2013). To ensure
134 reliable results, all extracts were run in duplicate on each plate and tested on at least two separate
135 occasions. Further, each plate included a full reference compound concentration-effect curve,
136 solvent controls and media controls.

137

138 *2.4. Data evaluation*

139 The concentration causing 50% effect (EC_{50}) or 50% inhibition (IC_{50}) for the assay reference
140 compounds were derived from log-sigmoidal concentration-effect curves using Equation 1. The
141 minimum effect (min) was set to 0% and the maximum effect (max) was set to 100%, while the
142 slope was an adjustable parameter. The concentration-effect curves for the assay reference
143 compounds are provided in Figure S1.

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145

146

147 (1)

148

149 The LOQ for each bioassay was calculated as the baseline (i.e. the raw bioassay response with
150 negative control samples) plus 10× the standard deviation of the baseline. This was close to 10% for
151 most agonist assays, therefore the assay LOQ was set to the concentration causing 10% effect
152 (EC_{10}). The baseline was less variable in the ER-GeneBLAzer and PR-CALUX assays, so the LOQ
153 was set to the concentration causing 5% effect (EC_{05}). The LOQ in antagonist mode was set as the

concentration causing 20% inhibition (IC_{20}) due to the greater variability in the response of the negative control. The LOQ for each assay is provided in Table 1.

With exception of some extracts in the AR-GeneBLAzer assay, the responses in most bioassays were low, with often only the highest tested concentration above the LOQ. Therefore, it was not possible to derive an EC value from a linear or log-sigmoidal concentration-effect curve. Instead, the biological activity in the sample, expressed as either EC_{10} or IC_{20} in units of relative enrichment factor (REF), was calculated from the positive response using Equation 2, where EC_x or IC_x is the percent effect of the sample and REF_{sample} is the REF of the sample. REF was calculated based on the sample enrichment factor by SPE and the dilution factor in the assay (Escher and Leusch, 2012).

(2)

Sample EC_{10} or IC_{20} values in units of REF were converted to bioanalytical equivalent concentrations from bioassays (BEQ_{bio}) using Equation 3 in order to translate the effect of a sample to the concentration of a reference compound that would elicit the same response. For comparison with previous studies on endocrine activity in environmental waters, the equivalent concentrations for AR-GeneBLAzer were reported as 5α-dihydrotestosterone equivalents (DHTEQ) in agonist mode and flutamide equivalents (FluEQ) in antagonist mode using EC values from Leusch et al. (2017). Further, levonorgestrel equivalents (LevoEQ) were reported for PR-CALUX in agonist mode using the EC value from Leusch et al. (2017). These values are provided in the footnote to Table 1. BEQ_{bio} was expressed in units of ng/L.

(3)

The BEQ approach can be used to determine how well the detected chemical explain the observed effect by comparing BEQ_{bio} with the bioanalytical equivalent concentrations from chemical analysis (BEQ_{chem}) (Neale et al., 2015). BEQ_{chem} was calculated using the detected chemical concentration (C_i) in molar units and the relative effect potency (REP_i) (Equation 4). REP_i was calculated using Equation 5, with EC values for the detected chemicals collected from the US EPA ToxCast database (US EPA, 2015) and the peer reviewed literature. Data in the ToxCast database is provided as 50% activity concentrations (AC_{50}), so $EC_{50,absolute}$ values were calculated using the approach described in Neale et al. (2017).

(4)

(5)

The BEQ_{chem} was converted to ng/L using the molar weight of the assay reference compound for comparison with BEQ_{bio}.

3. Results and Discussion

3.1. Chemical Analysis

Of the 58 chemicals monitored by targeted chemical analysis, only 23 were detected above the LOQ in the water extracts (Tables S6-S9). Further, 149 compounds were qualitatively identified with medium to high confidence from a database of over 2500 compounds during suspect screening (Table S10). Many chemicals were detected in the same matrix in over half of the countries sampled, illustrating the widespread contamination of environmental waters by pharmaceuticals, pesticides, personal care products and industrial compounds (Tables S11-S12).

An overview of the detected chemicals by chemical class is shown in Figure 1. Unsurprisingly, treated wastewater had the highest sum chemical concentration, with pharmaceuticals the dominant detected chemical class, while drinking water and control ultrapure water had the lowest sum chemical concentrations, with mostly industrial compounds detected. The pharmaceuticals diclofenac, carbamazepine and atenolol were detected in the microgram per litre concentration range in treated wastewater, while personal care product triclosan, industrial compound bisphenol A and herbicides atrazine and diuron were also found at high concentrations. These chemicals were also detected in surface water, with the exception of atenolol, but at lower concentrations. The profile of chemicals in surface waters differed with the studied countries, suggesting varying sources of contamination (Figure 1). For example, pharmaceuticals dominated the surface water profile from Spain, suggesting the presence of wastewater contamination, while only pesticides (carbendazim, diuron and simazine) were detected in surface water from the Netherlands.

Nine compounds were detected at low levels in drinking water, with dibutyl phthalate detected in 5 out of 6 of the drinking water extracts. 4-*t*-Butylphenol was detected at a concentration of 460 ng/L in the drinking water extract from the Netherlands, but was not detected in drinking water extracts for any other country. In addition carbamazepine, atrazine, simazine, diuron and triclosan were all detected at low concentrations in drinking water. Further, industrial compounds 4-nonylphenol, bisphenol A and dibutyl phthalate were found in ultrapure water. The widespread

presence of plasticisers, particularly in the laboratory, means that they are often detected at low concentrations in ultrapure waters (Devier et al., 2013).

As the water samples are grab samples and each matrix was collected for one location per country, it is difficult to make any country-specific generalisations; however, some trends were apparent. For example, pesticides diazinon and chlorpyrifos, which are banned in Europe, were only detected in Australian water extracts. A more representative longer-term sampling campaign is required to better understand micropollutant contamination patterns in different geographic regions.

3.2. Bioanalysis

A summary of all bioassay results, expressed as BEQ_{bio}, is provided in Table 2. Despite the wide coverage of endpoints applied to four different water matrices from 6 countries, the observed effects in the extracts were generally low. None of the samples had a response in agonist mode for the GR-CALUX, PR-CALUX, GH3.TRE-Luc, RXR-CALUX, HELN-RARa-RXR or HG5LN-MR assays or in antagonist mode for the ER-GeneBLAzer, AR-GeneBLAzer, GR-GeneBLAzer, PR-GeneBLAzer or HELN-RARa-RXR assays. The surface water from Spain was the most active sample, with an estradiol equivalent concentration (EEQ) of 0.31 ng/L in ER-GeneBLAzer, a dexamethasone equivalent concentration (DexaEQ) of 96 ng/L in GR-GeneBLAzer, a LevoEQ of 1.1 ng/L in PR-GeneBLAzer and a spironolactone equivalent concentration (SpiroEQ) of 910 ng/L in HG5LN-MR. This water extract also had the highest sum chemical concentration of all surface water samples, with pharmaceuticals the main chemical class detected. Treated wastewater from Spain also had a response in the GR-GeneBLAzer (130 ng/L DexEQ) and HG5LN-MR (3100 ng/L SpiroEQ) assays. Both GeneBLAzer and CALUX assays were applied to assess activation of GR and PR, with responses observed in the GeneBLAzer assays, but not in the equivalent CALUX assays. As can be seen in Table 2, the assay LOQ was lower for the GeneBLAzer assays, which can be attributed to a combination of sensitivity and ability to tolerate higher solvent concentrations (Leusch et al., 2017).

Treated wastewater and surface water from France also had an effect in the ER-GeneBLAzer (0.78 ng/L EEQ) and HG5LN-MR (660 ng/L SpiroEQ) assays, respectively. Three samples from Germany, surface water, drinking water and ultrapure water, induced a response in the AR-GeneBLAzer assay in agonist mode, with no effects observed in the other extracts. This result is unusual and is likely due to sample contamination given that the activity was comparable in all three samples including the ultrapure water (3 to 5 ng/L DHTEQ), which is unlikely as they represent different matrices. Therefore, the activity in these samples was not considered to be representative of typical water samples.

A limitation of the current study is that only one grab sample per water matrix was analysed for each country. Despite this, the results of the current study are in good agreement with previous findings (Table 3) and results can therefore be considered representative even if they provide only a snapshot. Higher estrogenic activity in treated wastewater and surface water, as well as higher anti-androgenic and anti-progestagenic activity in surface water, was reported in the literature, but generally the results from the current study were within an order of magnitude of previously detected hormonal activity. Very few studies have applied assays indicative of activation and inhibition of MR to water extracts. To our knowledge, the HG5LN-MR has only been applied to raw wastewater (Bellet et al., 2012) and surface water passive sampler extracts (Creusot et al., 2014). Anti-mineralocorticoid activity was detected in both treated wastewater and surface water in the current study, and the significance of this endpoint for environmental waters should be investigated further.

3.3. How well do the detected chemicals explain the detected endocrine activity?

Of the 23 detected chemicals, effect data were available for only 2 to 7 chemicals in ER-GeneBLAzer, AR-GeneBLAzer, GR-GeneBLAzer and HG5LN-MR (antagonist mode only) using the US EPA ToxCast database and the peer reviewed literature. All EC and REP_i values used to calculate BEQ_{chem} are provided in Table S13. While GH3.TRE-Luc was also included in the

ToxCast database, none of the detected chemicals were active, so BEQ_{chem} could not be calculated. The comparison between BEQ_{bio} and BEQ_{chem}, along with the fraction of effect that could be explained by detected chemicals, is provided in Table S14. Qualitatively, BEQ_{bio} and BEQ_{chem} gave a similar picture of the quality of the water samples, with wastewater and surface water exhibiting the greatest effects. However, BEQ_{chem} was generally much lower than the BEQ_{bio} LOQ for the studied assays. In the few cases where both BEQ_{bio} and BEQ_{chem} were available for a sample, the detected chemicals could only explain up to 2.3% of the effect in the ER-GeneBLAzer assay, with the fraction explained much lower for the GR-GeneBLAzer and HG5LN-MR assays. Natural and synthetic hormones are often able to explain the majority of the endocrine activity in water samples (Murk et al., 2002; Leusch et al., 2010). While most work has focused on estrogenicity, recent studies have also shown that detected chemicals explain much of the effect in wastewater for assays indicative of activation of GR (Schriks et al., 2010; Jia et al., 2016), while chemicals detected in untreated wastewater (Bellet et al., 2012) and surface water downstream of pharmaceutical factory (Creusot et al., 2014) were able to explain a large fraction of anti-mineralocorticoid activity. The main causative chemicals in surface water in the HG5LN-MR assay were identified as dexamethasone, spironolactone and 6-alpha-methylprednisolone (Creusot et al., 2014). While a number of hormones were targeted by chemical analysis (Table S4), only two, androstenedione and cortisone, were detected above the LOQ. The LOQ for some of the potent hormones, such as 17β-estradiol and 17α-ethinylestradiol, was in the high nanogram per litre range due to limited sample enrichment. This limited enrichment and the small sample volumes (100 µL aliquots) contribute to the low fraction of effect explained in the current study.

3.4. Significance of the detected endocrine activity

The sensitivity of *in vitro* bioassays means that effects can be detected even in clean samples after sufficient enrichment. In order to differentiate between an acceptable effect and an unacceptable effect in a bioassay, effect-based trigger values (EBT) for both human and ecological health have

been developed in the last few years (Brand et al., 2013; Jarosova et al., 2014; Escher et al., 2015; van der Oost et al., 2017). Available surface water and drinking water EBTs for estrogenic activity, androgenic and anti-androgenic activity, glucocorticoid activity and progestagenic activity are compared with BEQ_{bio} values from the current study and the literature in Table 4. EBTs are typically determined for a specific assay, rather than an endpoint; however, the current lack of EBTs meant that all available EBTs for a particular endpoint were considered in the current study. While estrogenic activity was low compared to previous studies (Table 3), the detected EEQ values in treated wastewater in France and surface water in Spain were within the same range as the available surface water EBTs, suggesting these environmental waters may pose a potential risk to ecosystem health. Similarly, glucocorticoid activity in treated wastewater from Spain also exceeded the proposed surface water EBT, though detected activity in surface water was below the EBT. All environmental samples were below the EBT for anti-androgenic activity in surface, though some FluEQ values from the literature greatly exceeded the EBT. All drinking water samples were below the relevant human health EBTs, suggesting that the studied drinking waters do not pose a risk to the endocrine health of humans.

While EBTs are useful tools to interpret bioassay results in a risk context, there is still a significant knowledge gap about what constitute a 'safe' effect for many bioassays. As can be seen from Table 4, the availability of EBTs is still rather limited, with very few EBTs available for antagonist activity and none for effects such as thyroid activity or mineralocorticoid activity. Therefore, further work on EBT development is required for a wide range of endocrine endpoints.

4. Conclusions

The current study applied a battery of *in vitro* assays to quantify activity in seven endocrine pathways in treated wastewater, surface water and drinking water collected from six countries. Overall, the water samples had very low endocrine activity, with only estrogenic, glucocorticoid, progestagenic and anti-mineralocorticoid activity detected in some of the treated wastewater and

surface water samples, primarily from France and Spain. With the exception of a suspected contaminated sample, none of the drinking water extracts had a response in the bioassays. The observed low effects were confirmed by mixture toxicity modelling of the detected chemicals, with BEQ_{chem} generally lower than the quantification limit of the bioassays. This emphasises how the application of bioassays and chemical analysis for water quality monitoring can provide complementary information. All drinking water samples were below the available EBTs, suggesting that drinking water does not pose a risk to human endocrine health. Estrogenic and glucocorticoid activity in some surface and treated wastewater samples however exceeded surface water EBTs and wastewater discharges may pose a risk to aquatic organisms. A limited number of grab samples were analysed in the current study, with a more representative sampling campaign recommended to confirm the results. However, the findings of the study fit well with the current scientific consensus on endocrine effects in environmental and drinking waters.

Acknowledgments

This project, Global Water Research Coalition project # 2013-05, was funded by PUB (Singapore), the Foundation for Applied Water Research (STOWA), Water Research Australia, the Water Technology Center (TZW), the Water Research Foundation (WRF) and the Global Water Research Coalition (GWRC). In-kind support was kindly provided by Veolia - VERI, TZW, Suez – CIRSEE, KWR and Griffith University. The South African component of the project was funded by the Water Research Commission. Catherina van Zijl (University of Pretoria, South Africa) and Christin Kühnert (UFZ), Maria König (UFZ), Jason van de Merwe (Griffith University) and Erik Prochazka (Griffith University) are acknowledged for experimental assistance.

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Table 1: Summary of the applied bioassays with the concentration causing 50% effect (EC_{50}) or 50% inhibition (IC_{50}) and the assay limit of quantification (LOQ) (M).

Mode of action	Bioassay name	Mode	Reference compound	Reference	Reference compound EC_{50} (agonist) or IC_{50} (antagonist) (M)	Limit of quantification (LOQ) (M)*
Estrogen Receptor (ERα)	ER-GeneBLAzer	Agonist	17β-Estradiol (E2)	König et al. (2017)	3.4×10^{-11}	2.5×10^{-12}
		Antagonist	4-Hydroxytamoxifen	König et al. (2017)	4.7×10^{-8}	1.8×10^{-9}
Androgen Receptor (AR)	AR-GeneBLAzer	Agonist	R1881[a]	König et al. (2017)	6.5×10^{-10}	1.3×10^{-10}
		Antagonist	Cyproterone acetate[b]	König et al. (2017)	6.0×10^{-8}	1.6×10^{-8}
Glucocorticoid Receptor (GR)	GR-GeneBLAzer	Agonist	Dexamethasone	König et al. (2017)	5.9×10^{-10}	2.6×10^{-10}
		Antagonist	Mifepristone (RU486)	König et al. (2017)	3.7×10^{-10}	1.9×10^{-10}
	GR-CALUX	Agonist	Dexamethasone	Van der Linden et al. (2008)	4.4×10^{-9}	1.3×10^{-9}
Progesterone Receptor (PR)	PR-GeneBLAzer	Agonist	Levonorgestrel	König et al. (2017)	1.1×10^{-10}	2.5×10^{-12}
		Antagonist	Mifepristone (RU486)	König et al. (2017)	1.5×10^{-10}	7.6×10^{-12}
	PR-CALUX	Agonist	Org2058[c]	Van der Linden et al. (2008)	3.8×10^{-10}	7.3×10^{-11}
Thyroid Receptor (TR)	GH3.TRE-Luc	Agonist	Triiodothyronine (T3)	Freitas et al. (2011)	1.6×10^{-10}	3.8×10^{-11}
Retinoid X Receptor (RXR)	RXR-CALUX	Agonist	Trans retinoic acid (tRA)	N/A	2.4×10^{-7}	2.8×10^{-8}
Retinoid Acid Receptor (RARα) and Retinoid X Receptor (RXR)	HELN RARα-RXR	Agonist RAR/RXR	TTNPB	Balaguer et al. (2001)	4.8×10^{-9}	1.6×10^{-9}
		Antagonist RAR/RXR	BMS493	Balaguer et al. (2001)	2.7×10^{-9}	5.7×10^{-10}
Mineralocorticoid Receptor (MR)	HG5LN MR	Agonist	Aldosterone	Bellet et al. (2012)	9.8×10^{-10}	3.3×10^{-10}
		Antagonist	Spironolactone	Bellet et al. (2012)	4.0×10^{-9}	1.5×10^{-9}

[a]5α-dihydrotestosterone (DHT) with an EC_{50} of 2.20×10^{-9} M (Leusch et al., 2017) used for BEQ calculations; [b]flutamide with an EC_{50} of 2.20×10^{-6} M (Leusch et al., 2017) used for BEQ calculations; [c]levonorgestrel with an EC_{50} of 1.4×10^{-10} M (Leusch et al., 2017) used for BEQ calculations

475 *LOQ set as the concentration causing 10% effect (EC_{10}) in agonist mode or the concentration causing 20% inhibition (IC_{20}) in antagonist mode, with the exception

476 of ER-GeneBLAzer (agonist mode) and PR-CALUX where the LOQ was set as the concentration causing 5% effect (EC_{05})

477 TTNPB: [E]-4-[2-(5, 6, 7, 8-tetrahydro-5, 5, 8, 8-tetramethyl-2-naphthalenyl)propen-1-yl]benzoic acid

478

Table 2: Summary of bioanalytical equivalent concentrations from bioassays (BEQ_{bio}) for all water samples (WW = treated wastewater; DW = drinking; SW = surface; CW = control water, i.e. ultrapure water).

			ER-GeneBLAzer		AR-GeneBLAzer		GR-CALUX		PR-GeneBLAzer			HG5LN-MR						
ID	**Type**	**Country**	**EEQ (ng/L)**	**OHTMXEQ (ng/L)**	**DHTEQ (ng/L)**	**FluEQ (ng/L)**	**DexaEQ (ng/L)**	**MifEQ (ng/L)**	**DexaEQ (ng/L)**	**LevoEQ (ng/L)**	**MifEQ (ng/L)**	**LevoEQ (ng/L)**	**T3EQ (ng/L)**	**tRAEQ (ng/L)**	**TTNPBEQ (ng/L)**	**BMS493EQ (ng/L)**	**AldoEQ (ng/L)**	**SpiroEQ (ng/L)**
1	**WW**	Germany	<0.6 *	<4200	<4	<15000	<120	<60	<2500	<2.5	<2	<33	<25	<2100	<2000	<1000	<600	<3100 *
5	**WW**	Australia	<0.6	<4200	<2	<22000	<120	<60	<2500	<2.5	<2	<33	<25	<2100	<2000	<1000	<600	<3100
9	**WW**	France	**0.78**	<4200	<2	<15000	<120	<60	<2500	<2.5	<2	<33	<25	<2100	<2000	<1000	<600	<3100 *
13	**WW**	South Africa	<0.6	<4200	<4	<15000	<120	<60	<2500	<2.5	<2	<33	<25	<2100	<2000	<1000	<600	<3100 *
17	**WW**	Netherlands	<0.6	<4200	<2	<15000	<120	<60	<2500	<2.5	<2	<33	<25	<2100	<2000	<1000	<600	<3100
21	**WW**	Spain	<0.6	<4200	<2	<15000	**130**	<60	<2500	<2.5	<2	<33	<25	<2100	<2000	<1000	<600	**3100**
3	**SW**	Germany	<0.1	<830	**5**	<2900	<23	<12	<500	<0.5	<0.4	<7	<20	<400	<400	<200	<120	<620 *
7	**SW**	Australia	<0.1	<830	<1	<4400	<23	<12	<500	<0.5	<0.4	<7	<20	<400	<400	<200	<120	<620
11	**SW**	France	<0.1	<830	<1	<5800	<23	<12	<500	<0.5	<0.4	<7	<20	<400	<400	<200	<120	**660**
15	**SW**	South Africa	<0.1	<830	<1	<5800	<23	<12	<500	<0.5	<0.4	<7	<20	<400	<400	<200	<120	<620
19	**SW**	Netherlands	<0.1	<830	<1	<4400	<23	<12	<500	<0.5	<0.4	<7	<20	<400	<400	<200	<120	<620
23	**SW**	Spain	**0.31**	<830	<1	<8900	**96**	<12	<500	**1.1**	<0.4	<2	<20	<400	<400	<200	<120	**910**

2	**DW**	Germany	<0.03	<210	**5**	<700	<5.8	<3	<120	<0.1	<0.1	<2	<1.3	<100	<90	<50	<30	<160
6	**DW**	Australia	<0.03	<210	<0.1	<4400	<5.8	<3	<120	<0.1	<0.1	<2	<1.3	<100	<90	<50	<30	<160
10	**DW**	France	<0.03	<210	<0.1	<700	<5.8	<3	<120	<0.1	<0.1	<2	<1.3	<100	<90	<50	<30	<160
14	**DW**	South Africa	<0.03	<210	<1	<2900	<5.8	<3	<120	<0.1	<0.1	<2	<1.3	<100	<90	<50	<30	<160
18	**DW**	Netherlands	<0.03	<210	<0.1	<700	<5.8	<3	<120	<0.1	<0.1	<2	<1.3	<100	<90	<50	<30	<160
22	**DW**	Spain	<0.03	<210	<0.4	<2200	<5.8	<3	<120	<0.1	<0.1	<2	<1.3	<100	<90	<50	<30	<160
4	**CW**	Germany	<0.1	<830	**3**	<1800	<23	<12	<500	<0.5	<0.4	<7	<5	<400	<400	<200	<120	<620
8	**CW**	Australia	<0.1	<830	<0.3	<1800	<23	<12	<500	<0.5	<0.4	<7	<5	<400	<400	<200	<120	<620
12	**CW**	France	<0.1	<830	<0.3	<1800	<23	<12	<500	<0.5	<0.4	<7	<5	<400	<400	<200	<120	<620
16	**CW**	South Africa	<0.1	<830	<0.3	<1800	<23	<12	<500	<0.5	<0.4	<7	<5	<400	<400	<200	<120	<620
20	**CW**	Netherlands	<0.1	<830	<0.3	<1800	<23	<12	<500	<0.5	<0.4	<7	<5	<400	<400	<200	<120	<620
24	**CW**	Spain	<0.1	<830	<0.3	<1800	<23	<12	<500	<0.5	<0.4	<7	<5	<400	<400	<200	<120	<620

Colour scheme: "*" and yellow background = slight activity detected but below the limit of quantification; bold and orange background = activity detected above quantification limit; bold and grey background = the androgenic activity in the German water samples is most likely the result of a contamination event and not a true representation of androgenic activity in those samples.

Abbreviations: EEQ = 17β-Estradiol equivalent; OHTMXEQ = 4-hydroxytamoxifen equivalent; DHTEQ = 5α-dihydrotestosterone equivalent; FluEQ = flutamide equivalent; DexaEQ = dexamethasone equivalent; LevoEQ = levonorgestrel equivalent; MifEQ = mifepristone equivalent; T3EQ = triiodothyronine equivalent; tRAEQ = trans-retinoic acid equivalent; TTNPBEQ = TTNPB equivalent; BMS493EQ = BMS493 equivalent; AldoEQ = aldosterone equivalent; SpiroEQ = spironolactone equivalent.

Table 3: Bioactivity in water samples determined in the current study and compared with values previously reported in the literature.

		Treated wastewater		Surface water		Drinking water	
Activity	**Units**	*Current study*	*Literature*[a]	*Current study*	*Literature*[a]	*Current study*	*Literature*[a]
ER	EEQ (ng/L)	<0.6 – 0.78	<0.03 – 68	<0.1 – 0.31	<0.01 – 23	<0.03	<0.001 – 0.08
Anti-ER	OHTMXEQ (ng/L)	<4200	<500	<830	<260	<210	1.3 – 11.6
AR	DHTEQ (ng/L)	<4	<0.1 – 20	<1	<0.1 – 12	<1	<0.01 – 6.3
Anti-AR	FluEQ (ng/L)	<22000	Up to 36000	<8900	Up to 240000	<4400	<41 – 1000
GR	DexaEQ (ng/L)	<120 – 130	<3 -188	<23 – 96	<0.4 – 34	<5.8	<0.5 – 13
Anti-GR	MifEQ (ng/L)	<60	<8	<12	<15	<3	<15
PR	LevoEQ (ng/L)	<2.5	<0.01 – 7.1	<0.4 – 1.1	<0.1 – 8.7	<0.1	<0.01
Anti-PR	MifEQ (ng/L)	<2	<7	<0.4	<8 – 32000	<0.1	<13
TR	T3EQ (ng/L)	<25	<1.5	<20	<1.5	<1.3	<1.5
RAR/RXR	TTNPBEQ (ng/L)	<2000	+ in yeast assay[b]	<400	+ in yeast assay[b]	<90	+ in yeast assay[b]
Anti-RAR/RXR	BMS493EQ (ng/L)	<1000	+ in yeast assay[b]	<200	+ in yeast assay[b]	<50	+ in yeast assay[b]
MR	AldoEQ (ng/L)	<600	n/a	<120	n/a	<30	n/a
Anti-MR	SpiroEQ (ng/L)	<3100 - 3100	n/a	<620 – 910	n/a	<160	n/a

[a]reviewed in Leusch et al. (2017); [b]Inoue et al. (2011)

Colour scheme for the columns entitled "this study": green = no activity detected; orange = some activity detected. **Colour scheme for the columns entitled "literature":** green = no activity, in agreement with this study; orange = some activity within an order of magnitude of the activity detected in this study; red = strong bioactivity several orders of magnitude higher than detected in this study.

Abbreviations: EEQ = 17β-Estradiol equivalent; OHTMXEQ = 4-hydroxytamoxifen equivalent; DHTEQ = 5α-dihydrotestosterone equivalent; FluEQ = flutamide equivalent; DexaEQ = dexamethasone equivalent; LevoEQ = levonorgestrel equivalent; MifEQ = mifepristone equivalent; T3EQ = triiodothyronine equivalent; AmiEQ = amiodarone equivalent; TTNPBEQ = TTNPB equivalent; BMS493EQ = BMS493 equivalent; AldoEQ = aldosterone equivalent; SpiroEQ = spironolactone equivalent.

Table 4: Comparison of BEQ_{bio} values (ng/L) detected in this study and reported in the literature with available effects based trigger values (EBT) for surface waters (ecological health) and drinking waters (human health).

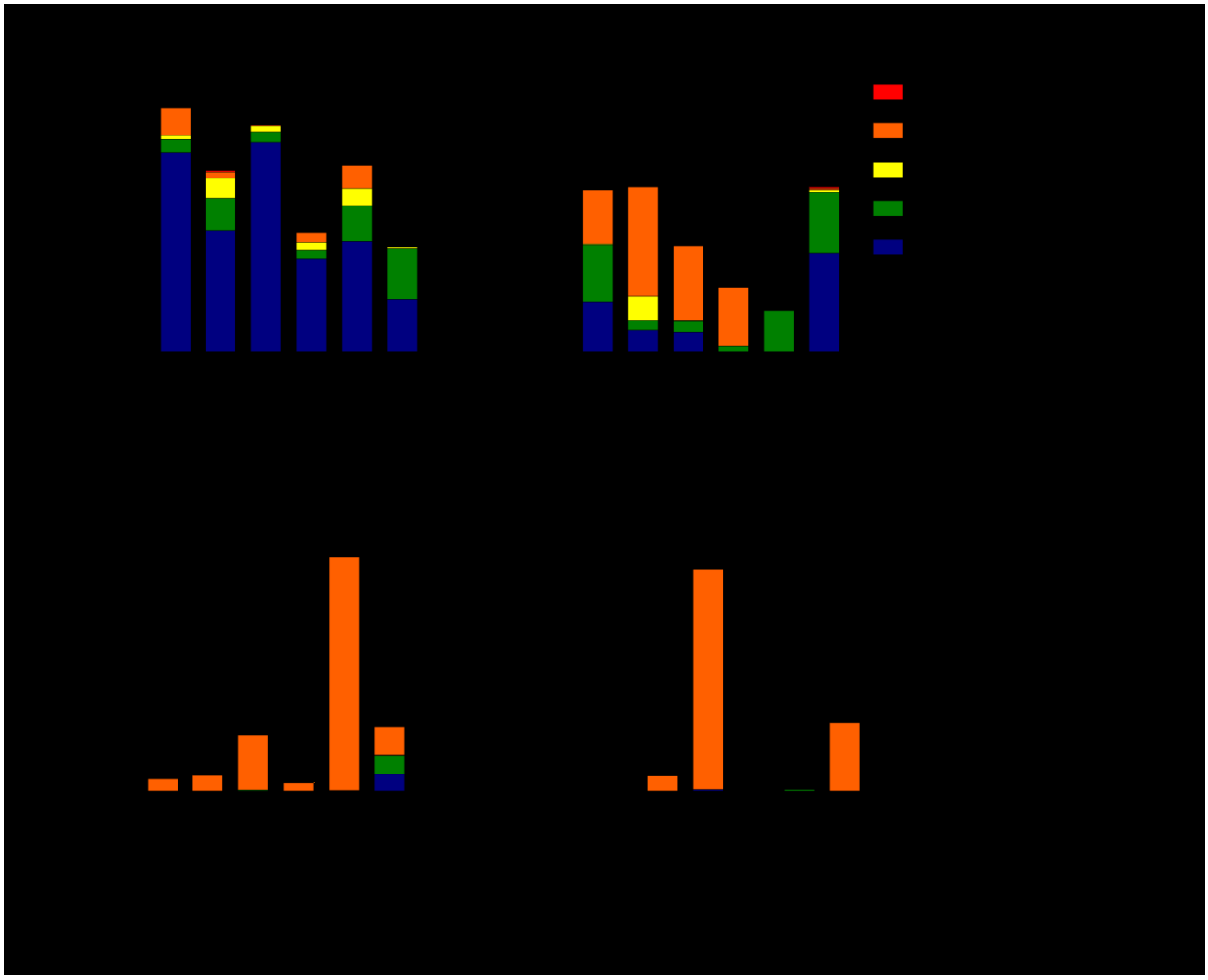
Activity	Units	Surface water EBT	Wastewater		Surface water		Drinking water EBT	Drinking water	
			Current study	*Literature*	*Current study*	*Literature*		*Current study*	*Literature*
ER	EEQ (ng/L)	**0.1 – 0.5**[a,b]	<0.6 – 0.78	<0.03 – 68	<0.1 – 0.31	<0.01 – 23	**0.2 – 3.8**[c,d]	<0.03	<0.001 – 0.08
AR	DHTEQ (ng/L)	**n/a**	<4	<0.1 – 20	<1	<0.1 – 12	**11**[c]	<1	<0.01 – 6.3
Anti-AR	FluEQ (ng/L)	**25000**[b]	<22000	Up to 36000	<8900	Up to 240000	**n/a**	<4400	<41 – 1000
GR	DexaEQ (ng/L)	**100**[b]	<120 – 130	<3 -188	<23 – 96	<0.4 – 34	**21-150**[c,d]	<5.8	<0.5 – 13
PR	LevoEQ (ng/L)	**n/a**	<2.5	<0.01 – 7.1	<0.5 – 1.1	<0.1 – 8.7	**730**[c]*	<0.1	<0.01

[a]EBT calculated for YES, MELN, ER-CALUX, E-SCREEN and MVLN (Jarosova et al., 2014); [b]EBT calculated for ER-CALUX, AR-CALUX (antagonist mode) and GR-CALUX (van der Oost et al., 2017); [c]EBT calculated for ER-CALUX, AR-CALUX, GR-CALUX and PR-CALUX (Brand et al., 2013); [d]EBT calculated for ER-GeneBLAzer, ER-CALUX, E-Screen, hERa-HeLa-9903 and GR-CALUX (Escher et al., 2015).

*Calculated from 333 ng/L Org2058 equivalent in the original reference divided by the relative potency of levonorgestrel in the PR-CALUX of 0.46 compared to Org2058 based on the EC_{50} values for levonorgestrel from Leusch et al. (2017) and Org2058 from Van der Linden et al. (2008)

Abbreviations: EEQ = 17β-Estradiol equivalent; DHTEQ = 5α-dihydrotestosterone equivalent; FluEQ = flutamide equivalent; DexaEQ = dexamethasone equivalent; LevoEQ = levonorgestrel equivalent; n/a = not available. **Colour scheme:** green = detected activity below available EBT; yellow = activity detected by no EBT available; orange = activity detected slightly above the EBT; red = activity detected >10× above the EBT; grey = no activity detected and no EBT.

509 Figure 1: Sum chemical concentration detected in each country (nM) by chemical class for treated
510 wastewater, surface water, drinking water and ultrapure water. Note different y-axis scales in each
511 figure.



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