Comparison of *in vitro* and *in vivo* bioassays to measure thyroid hormone disrupting activity in water extracts

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Abstract

Environmental chemicals can induce thyroid disruption through a number of mechanisms including altered thyroid hormone biosynthesis and transport, as well as activation and inhibition of the thyroid receptor. In the current study six *in vitro* bioassays indicative of different mechanisms of thyroid disruption and one whole animal *in vivo* assay were applied to 9 model compounds and 4 different water samples (treated wastewater, surface water, drinking water and ultra-pure lab water; both unspiked and spiked with model compounds) to determine their ability to detect thyroid active compounds. Most assays correctly identified and quantified the model compounds as agonists or antagonists, with the reporter gene assays being the most sensitive. However, the reporter gene assays did not detect significant thyroid activity in any of the water samples, suggesting that activation or inhibition of the thyroid hormone receptor is not a relevant mode of action for thyroid endocrine disruptors in water. The thyroperoxidase (TPO) inhibition assay and transthyretin (TTR) displacement assay (FITC) detected activity in the surface water and treated wastewater samples, but more work is required to assess if this activity is a true measure of thyroid activity or matrix interference. The whole animal Xenopus Embryonic Thyroid Assay (XETA) detected some activity in the unspiked surface water and treated wastewater extracts, but not in unspiked drinking water, and appears to be a suitable assay to detect thyroid activity in environmental waters.

Keywords: *in vitro*; *in vivo*; surface water; thyroid activity; wastewater

Abbreviations: AmiEQ: amiodarone equivalent concentrations; DOC: dissolved organic carbon; EC: effect concentration; ETU: ethylene thiourea; IC: inhibition concentration; MMI: methimazole; MMIEQ: methimazole equivalent concentrations; PCP: pentachlorophenol; REF: relative enrichment factor; SPE: solid-phase extraction; T3: triiodothyronine; T4: thyroxine; T4EQ: thyroxine equivalent concentration; TBG: thyroid binding globulin; TETRAC: tetraiodothyroacetic acid; TH: thyroid hormone; THBP: 2,2,4,4-tetrahydroxybenzophenone; TPO: thyroperoxidase; TR:

53 thyroid receptor; TRIAC: triiodothyroacetic acid; TTR: transthyretin; XETA: Xenopus Embryonic
54 Thyroid Assay

1. Introduction

The presence of endocrine disrupting chemicals in the environment has generated increasing attention over the last few decades due to the potential impacts on both wildlife and human health (Bergman et al., 2013). Given the diversity of potential endocrine disruptors in the aquatic environment and the fact that chemicals are often present in complex mixtures, *in vitro* bioassays indicative of hormone activity are often applied complementary to chemical analysis (Scott et al., 2014; Conley et al., 2017; König et al., 2017). However, the majority of research focuses on estrogenic and androgenic activity in environmental waters, with less known about other endocrine modes such as thyroid, progestagenic and glucocorticoid activity (Leusch et al., 2017). An improved understanding of thyroid hormone disrupting activity is required as commonly detected micropollutants, such as polychlorinated biphenyls, pesticides and plasticizers, have been shown to disrupt the hypothalamus–pituitary–thyroid axis in amphibians and fish (reviewed in Carr and Patino, 2011). Often there is limited information available on the mechanism of thyroid disruption *in vivo*, but *in vitro* bioassays indicative of specific biological processes have the potential to reveal more information about the mechanisms of thyroid disruption (Murk et al., 2013).

The mechanisms of thyroid disruption are varied and can include, for example, altered thyroid hormone (TH) biosynthesis, binding to transport proteins, TH metabolism and thyroid receptor (TR) activation and inhibition (Boas et al., 2006). Decreased TH biosynthesis can be caused by inhibition of enzyme thyroperoxidase (TPO) (Crofton, 2008) and a wide range of environmental chemicals can inhibit TPO, with Paul Friedman et al. (2016) finding that 29% of 1074 tested chemicals induced greater than 20% TPO inhibition. TH are transported through the body by binding to transport proteins, such as thyroid binding globulin (TBG) and transthyretin (TTR) (Boas et al., 2012). Previous studies have shown that environmental chemicals, such as perfluorinated compounds and flame retardants, can competitively bind to TTR (Hamers et al., 2008; Weiss et al., 2009; Weiss et al., 2015), which may alter TH bioavailability. Environmental chemicals can also

induce thyroid disruption through activating or inhibiting the TR (Zoeller, 2005). Of the 8306 chemicals analysed in the US EPA ToxCast database, 1815 (22%) were reported to be active in the stable TR reporter gene GH3.TRE-Luc assay in antagonist mode, with only 39 (0.5%) active in agonist mode (US EPA, 2015). In contrast to single chemicals, fewer studies have applied *in vitro* assays indicative of thyroid activity to environmental water samples (Ishihara et al., 2009; Jugan et al., 2009; Escher et al., 2014). In addition, early-life stage *in vivo* assays, such as the Xenopus Embryonic Thyroid Assay (XETA), have also being applied to environmental chemicals and water samples (Castillo et al., 2013; Fini et al., 2017), but it is currently unclear how *in vitro* responses correlate with *in vivo* effects for this endpoint.

Recent reviews of endocrine endpoints identified a lack of knowledge regarding TH activity (Global Water Research Coalition, 2012; OECD, 2014). Therefore, in this study six *in vitro* assays indicative of distinct biological processes relevant for thyroid disruption including TH biosynthesis (TPO inhibition assay), TH transport (TTR displacement assays (FITC and ANSA)) and TR mediated action (reporter gene assays TRβ-CALUX, TRβ-GeneBLAzer, GH3.TRE-Luc) were applied to known (or suspected) thyroid agonists and antagonists. Further, water extracts, including treated wastewater, surface water and drinking water, were analysed. To complement the *in vitro* assays, a selection of model compounds and water extracts were run in the *in vivo* XETA.

2. Materials and Methods

2.1. Model Compounds

Nine model compounds (Table 1) were prepared in DMSO with a final concentration of 1 mM by the Water Technology Center (TZW), Germany. Triiodothyronine (T3) and pentachlorophenol (PCP) were purchased from LGC Standards GmbH (Wesel, Germany), while thyroxine (T4), triiodothyroacetic acid (TRIAC), tetraiodothyroacetic acid (TETRAC), amiodarone, ethylene thiourea (ETU), 2,2,4,4-tetrahydroxybenzophenone (THBP) and methimazole (MMI) were

purchased from Sigma-Aldrich (Steinheim, Germany). DMSO served as the solvent control. All chemicals and solvents were of analytical grade.

2.2. Water Extracts

Three environmental water samples, including surface water from the river Rhine at Karlsruhe, Germany (river kilometre 359.3), drinking water and treated domestic wastewater, as well as a laboratory blank (ultra-pure water), were tested in the bioassays. The samples were extracted using StrataX solid-phase extraction (SPE) cartridges (200 mg, Phenomenex). The cartridges were conditioned using methanol and ultrapure water (pH 2), with the water samples adjusted to pH 2 prior to extraction. A total of 20 L of surface water, drinking water and ultra-pure water (1 L per SPE cartridge) were extracted, while 10 L of treated wastewater (0.5 L per SPE cartridge) were extracted. After drying, the cartridges were eluted sequentially with methanol, acetonitrile and acetone, and the extracts combined for each water type, evaporated to dryness and reconstituted in 2 mL of methanol. This provides an enrichment factor of 10,000 for surface, drinking and ultra-pure water and an enrichment factor of 5,000 for treated wastewater. The extracts for each water type were split into two 1 mL aliquots, with one of the aliquots spiked with 5 µL of 20 mM T4 and 5 µL of 20 mM ETU, giving a final concentration of 100 µM T4 and 100 µM ETU in the spiked extract. The other aliquot was left unspiked.

2.3. Bioanalysis

The model compounds and water extracts were analysed in six *in vitro* assays, representing three different biological endpoints, as well as one *in vivo* assay (XETA). A summary of the studied bioassays can be found in Table 2, with further information provided in Section S1 of the Supplementary Information (SI). It should be noted that known agonist model compounds were not run in antagonist mode in the TRβ-CALUX and GH3.TRE-Luc reporter gene assays, while known antagonist model compounds were not run in agonist mode in GH3.TRE-Luc. In addition to the

model compounds, internal laboratory reference compounds were prepared by each participating laboratory to run in their assays.

2.4. Data Analysis

Log-logistic concentration-effect curves were used to determine the concentration causing 50% inhibition (IC_{50}) or 50% effect (EC_{50}) for the model compounds and water extracts. The EC_{50} and IC_{50} values for the model compounds were reported in nanomolar units, while the EC_{50} and IC_{50} values for the water extracts were reported in units of relative enrichment factor (REF), which considers both sample enrichment by SPE and dilution in the assay (Escher and Leusch, 2012). The effect in the water extracts were expressed as bioanalytical equivalent concentrations, which were calculated by dividing the EC_{50} or IC_{50} value of the reference compound by the EC_{50} or IC_{50} of the water extract. MMI equivalent concentrations (MMIEQ) were reported for the TPO inhibition assay and T4 equivalent concentrations (T4EQ) were reported for the other assays. Amiodarone equivalent concentrations (AmiEQ) were reported for the reporter gene assays run in antagonist mode.

3. Results and Discussion

3.1. Assay Performance

Representative concentration-effect curves for the internal laboratory assay reference compounds can be found in Figures S1 to S10. The IC_{50} and EC_{50} values for the internal laboratory assay reference compounds are provided in Table 2, along with the available literature IC_{50} and EC_{50} values. Generally, the IC_{50} and EC_{50} values were similar to those previously published, although in some cases our values were lower, suggesting that some assays, namely the TTR displacement assay (FITC), are more sensitive than previously reported (Ren and Guo, 2012), which was due to assay optimisation. Further information about the optimised method can be found in Section S1.

3.2. Model Compounds

The IC_{50} and EC_{50} values for the commonly prepared model compounds in the *in vitro* and *in vivo* assays are provided in Table 3, with all concentration-effect curves shown in Figures S11 to S20. The four agonist model compounds, T3, T4, TRIAC and TETRAC, induced a response in all assays (TETRAC was not tested in XETA), with the exception of the TPO inhibition assay. The TPO inhibition assay detects compounds which decrease TH biosynthesis; therefore, it is not surprising that the two thyroid hormones, T3 and T4, and the two thyroid hormone analogues, TRIAC and TETRAC, did not induce a response. The reporter gene assays indicative of TR mediated action, TRβ-CALUX, TRβ-GeneBLAzer and GH3.TRE-Luc assays, were highly sensitive to the thyroid agonists and showed the same trends in effect with the assays most sensitive to TRIAC, closely followed by T3, then T4 and TETRAC. The GH3.TRE-Luc was the most sensitive of the tested assays. The difference in sensitivity between the three reporter gene assays is related to a number of factors including the assay cell line and dilution factor in the assay. The GH3.TRE-Luc is based on the rat pituitary cell line GH3, making the assay particularly sensitive to thyroid hormones (Mengeling and Furlow, 2015). In contrast, the TRβ-CALUX and TRβ-GeneBLAzer are based on the human bone osteosarcoma U2OS cell line and the human embryonic kidney HEK 293T cell line, respectively. TRβ-GeneBLAzer was more sensitive than TRβ-CALUX and this can be attributed to the higher solvent tolerance and subsequent lower dilution factor in the assay (Leusch et al., 2017). The *in vivo* XETA was less sensitive to TR agonists as compared to the reporter gene assays, which is likely due to toxicokinetic factors, such as adsorption, distribution metabolism and excretion. The two TTR displacement assays were generally less sensitive compared to the reporter gene assays and XETA, and there was a 2 to 10 times difference in the corresponding agonist IC_{50} values for the FITC and ANSA assays.

Five model compounds previously reported to act as antagonists were run in all *in vitro* assays. Only the ultraviolet filter THBP and antithyroid pharmaceutical MMI had an effect in the TPO

inhibition assay. The pesticide PCP and industrial compound ETU were previously reported to be active in this assay in the US EPA ToxCast database, but only at concentrations 6.2 and 3.9 times higher than tested in the current study, respectively (US EPA, 2015). MMI is a potent TPO inhibitor and has been shown to induce thyroid disruption *in vivo* (Degitz et al., 2005), though no effects were observed in the other *in vitro* assays indicative of TH transport and TR mediated action. In contrast, both PCP and THBP were able to compete with T4 for binding sites in the two TTR displacement assays, though the FITC assay was again more sensitive than the ANSA assay. The antiarrhythmic drug amiodarone also showed a response in the TTR displacement assay (FITC), but did not induce 50% inhibition at the highest concentration tested. None of the five model antagonists showed an effect in the TRβ-CALUX and TRβ-GeneBLAzer assays in agonist mode (the model antagonists were not run in GH3.TRE-Luc in agonist mode given no effects were expected), but the three reporter gene assays were also run in antagonist mode in the presence of T3 at EC_{100} (TRβ-CALUX), EC_{80} (TRβ-GeneBLAzer) or EC_{50} (GH3.TRE-Luc) concentration. Despite similar trends for the thyroid agonists, a variable picture emerged in antagonist mode. Amiodarone was the antagonist reference compound in all three assays, though no IC_{50} value could be calculated for TRβ-CALUX and the IC_{50} value was close to reported cytotoxic concentrations for GH3.TRE-Luc (Freitas et al., 2011). Amiodarone has previously been shown to be a competitive antagonist at high concentrations and a non-competitive antagonist at low concentrations in TRβ (Drvota et al., 1995), and this may contribute to the variability observed between the different assays. PCP showed a response in all three reporter gene assays; though no IC_{50} value could be calculated for GH3.TRE-Luc, while IC_{50} values could be determined for ETU and THBP in the TRβ-GeneBLAzer assay. The background T3 agonist concentration used in antagonist mode can have implications for both assay sensitivity and robustness, with an agonist concentration of EC_{80} recommended (Neale and Leusch, 2015). Consequently, the observed variability may be partially related to the different T3 concentrations used in the reporter gene assays.

It should be noted that there was a slight difference in the EC_{50} and IC_{50} values for the laboratory internal reference compounds in Table 2 and the corresponding EC_{50} and IC_{50} values of the model compounds in Table 3. For the majority of compounds, the difference was within a factor of 2 and could be attributed to differences in chemical purity, stock age or potential weighing errors. Further, the model compounds were prepared in DMSO, but DMSO alone was found to induce a response in antagonist mode in the TRβ-GeneBLAzer assay at concentrations as low as 0.06% (Figure S21). In contrast, methanol alone had no effect up to a concentration of 0.5% (Figure S21). Consequently, the chemical stocks were re-made in methanol and re-run in the TRβ-GeneBLAzer assay (results from methanol stock reported for TRβ-GeneBLAzer in Table 3 and Figures S16 and S17). A similar effect with DMSO was also observed for other reporter gene assays indicative of estrogenic activity and glucocorticoid activity (Leusch et al., 2017). Low concentrations of DMSO have been shown to affect gene expression and enzyme activity (Chauret et al., 1998; Sumida et al., 2011), which may explain the observed results. As a result, methanol was used for the water extracts in Section 3.3, except for the FITC assay, which was performed on samples solvent-exchanged into DMSO.

3.3. Water Extracts

The effects in the unspiked and spiked water extracts, expressed in bioanalytical equivalent concentrations, are shown in Table 4, with all concentration-effect curves provided in Figures S22 to S31.

3.3.1. Unspiked Water Extracts

In vitro bioassays indicative of different mechanisms of thyroid disruption showed varying responses to the unspiked water extracts. Surface water and treated wastewater caused TPO inhibition, while drinking water, surface water and treated wastewater all had a significant effect in the TTR displacement assay (FITC). The lab blank also induced a response at an REF of 100 in both the TTR displacement assays (0.35 to 0.64 µg/L T4EQ), suggesting possible contamination

during sample enrichment. In any case, effects in the environmental samples were observed at lower REFs in the TTR displacement assay (FITC). In contrast, the TTR displacement (ANSA) assay showed greater than 100% response in the assay when exposed to samples with an REF >1 for treated wastewater, >10 for surface water and >100 for drinking water. This is likely due to the autofluorescence of the water extracts, with this issue previously observed for sediment extracts in the assay (Montano et al., 2013). Given this limitation, the assay is not recommended for environmental water extracts and will not be considered further in this study.

None of the unspiked water extracts induced a response in agonist mode in the reporter gene assays, suggesting that the extracts do not contain chemicals that can activate the TR. The TRβ-GeneBLAzer and GH3.TRE-Luc were also run in antagonist mode, with the treated wastewater sample inducing a significant response in TRβ-GeneBLAzer, with an AmiEQ of 350 µg/L. The other water extracts caused less than 20% inhibition in TRβ-GeneBLAzer, with 20% inhibition typically considered the trigger for quantification of antagonism (Escher et al., 2014), while cytotoxicity masked any potential antagonism in GH3.TRE-Luc. Anti TR activity has previously been detected in wastewater effluent using the yeast two-hybrid assay, with AmiEQ values ranging from 13 to 96 µg/L (Li et al., 2011), which is lower than observed for TRβ-GeneBLAzer.

Treated wastewater and surface water both induced a response in the *in vivo* XETA, with 25 and 29 µg/L T4EQ, respectively. A number of environmental contaminants commonly found in surface water and wastewater, such as bisphenol A, metoprolol and perfluorooctanoic acid (Loos et al., 2009; Neale et al., 2017b), are active in the XETA (Neale et al., 2017a). Therefore, the presence of these chemicals may be contributing to the response observed in treated wastewater and surface water. The water extracts were also run in the presence of 5 nM T3 in the XETA, which increased the baseline fluorescence in the assay from 0% to 21% as the added T3 induced the THbZIP transcription factor (Figure S31). None of the water extracts decreased the baseline activity,

suggesting the water samples do not contain thyroid antagonists at high enough concentrations. The results confirm the XETA as a promising test to assess thyroid activity in environmental waters. A limitation of the assay is that it requires whole organisms, but in a legal context, the early-life stages used in the XETA do not fall under the scope of the European Union legislation (Directive 2010/63/EU) on the protection of animals used for scientific purposes.

The different thyroid disruption mechanisms targeted by the *in vitro* assays could potentially explain the difference in effect of the unspiked water extracts. Surface water and wastewater may contain chemicals that inhibit TPO activity and displace T4 from TTR or alternatively dissolved organic carbon (DOC) in the water extracts may be interfering with the assays. DOC can be co-extracted during SPE and previous studies have shown that co-extracted DOC can interfere with naked enzyme assays (Neale and Escher, 2013), while DOC has negligible effects on cell-based assays, particularly those run in agonist mode (Neale and Escher, 2014). Therefore, it is possible that co-extracted DOC could cause TPO inhibition or bind T4 or other TTR binding compounds and thus prevent them from binding to TTR. To test whether DOC could have an effect, reference Suwannee River humic acid was run in the TTR displacement assay (FITC), with a significant response observed at 100 mgC/L (Figure 1). The highest concentration of DOC tested, 1,000 mgC/L, reduced the pH in the assay, potentially affecting binding of T4 to TTR. The DOC concentration of the water extracts was estimated by assuming 40% of DOC was co-extracted by SPE based on previous work with Oasis HLB cartridges (Neale and Escher, 2013). When plotted as a function of DOC concentration, the IC_{50} values for treated wastewater, surface water and drinking water were similar (8.0 to 11 mgC/L) and around an order of magnitude lower than Suwannee River humic acid. This suggests that DOC can interfere with the TTR displacement assay (FITC) at high concentrations. The effect of co-extracted DOC should also be further investigated for the TPO inhibition assay.

289 Finally, a number of previous studies have demonstrated that metabolic activation of environmental
290 chemicals is important for activation of thyroid hormone disrupting activity (Hamers et al., 2008;
291 Freitas et al., 2011), though most *in vitro* assays have limited metabolic capacity. To examine the
292 significance of metabolic activation, the unspiked water extracts were run in the presence and
293 absence of rat liver S9 fraction (final concentration 1%) with co-factors NADPH (0.05 mM) and
294 glucose-6-phosphate (0.25 mM) (Natsch and Haupt, 2013) in the TRβ-GeneBLAzer assay. While
295 there was a degree of interference with the assay at the S9 concentration used, as indicated by
296 reduced green fluorescence, there was no significant increase in the effect in the presence of S9
297 (data not shown). This suggests that thyroid active compounds in water are either not present at
298 high enough concentrations to produce an effect in TRβ-GeneBLAzer or were not bioactivated.
299
300 *3.3.2. Spiked Water Extracts*
301 The water extracts spiked with 5 µL of 20 mM of T4 and ETU were also analysed in the bioassay
302 test battery as a positive control. This gave a final concentration of 100 µM of T4 and ETU in the
303 extract, which was further diluted in the assays. With the exception of drinking water and ultra-pure
304 water in the TPO inhibition assay, all spiked extracts had a response in the *in vitro* and *in vivo*
305 assays. Both T4 and ETU did not induce TPO inhibition (Table 3), explaining the lack of effect in
306 spiked drinking water and ultra-pure water, and the presence of T4 and ETU only resulted in a small
307 increase in effect of surface water and wastewater in the TPO inhibition assay. While none of the
308 unspiked extracts had a response in the reporter gene assays in agonist mode, the presence of T4 in
309 the spiked extracts significantly increased the response in the assays. However, this increase was
310 less than expected for all samples for TRβ-CALUX (15-33% of expected increase) and GH3.TRE-
311 Luc (2-19% of expected increase) and for all samples except for ultra-pure for TRβ-GeneBLAzer
312 (17-36% of expected increase). Co-spiked ETU was a weak antagonist in TRβ-GeneBLAzer, but
313 had no effect in the other reporter gene assays, so this is unlikely to explain the difference.
314 Unspiked treated wastewater had an antagonistic effect in TRβ-CALUX and this may explain the

lower than expected recovery of T4 in spiked treated wastewater. Further, T4 is moderately hydrophobic, with an estimated octanol-water partition coefficient (log K_{OW}) of 4.12 (US EPA, 2012); therefore, it is possible that T4 could bind to DOC, potentially reducing the bioavailable concentration. This has been observed previously for estradiol in a reporter gene estrogen receptor assay (Neale et al., 2015) and could potentially explain the lower than expected increase observed for the environmental samples in TRβ-GeneBLAzer compared to ultra-pure water.

When run in antagonist mode, the spiked extracts resulted in a supramaximal response for ultra-pure, drinking and surface water in TRβ-GeneBLAzer, while the bioanalytical equivalent concentration for treated wastewater decreased from 350 to 110 µg/L AmiEQ due to the presence of T4. In contrast to the reporter gene assays, the spiked extracts were well aligned with the expected increase in effect of wastewater and surface water for the XETA (59-110% of expected increase). Further, spiked ultra-pure and surface water also aligned well with the expected increase for the TTR displacement assay (FITC), though the measured increase was considerably higher than expected (192-288%) for spiked drinking water and wastewater. This variability could be due to the limited number of replicates.

3.4. Thyroid Activity in Environmental Waters

In agreement with previous studies (reviewed in Leusch et al., 2017), the XETA detected thyroid activity in unspiked surface water and treated wastewater, but not in unspiked drinking water. The concentrations reported in the current study are higher than those in the literature, with 25 and 29 µg/L T4EQ in surface water and wastewater, respectively, compared to 1.8 µg/L T4EQ in both surface water and wastewater in the yeast two-hybrid assay (reported as 0.043 µg/L T3EQ in Inoue et al. (2011) (treated wastewater) and <0.014-0.043 µg/L T3EQ in Chinathamby et al. (2013) (surface water), but converted to T4EQ using the XETA relative potency of 41×). There is currently no trigger value for thyroid activity, and it is unclear whether this level of activity poses a risk to

ecosystem health. The EC_{10} value for T4 in the XETA (Figure S32) was used as a provisional trigger value and translates to approximately 12 μg/L T4EQ. The activity in surface water and wastewater samples is about double this provisional trigger value, suggesting that these waters may potentially induce a response in exposed amphibians, at least at the biochemical level. Further work is required to determine if any other adverse ecological effects could occur upon exposure to the reported concentrations of T4EQ.

4. Conclusions

In this study a suite of *in vitro* bioassays indicative of TH biosynthesis, TH transport and receptor mediated effects, as well as an early-life stage *in vivo* assay, were applied to model compounds and water extracts. The TPO inhibition assay and the TTR displacement assay (FITC) appear to be promising assays, though further work is recommended to assess the potential interference from co-extracted DOC. While the three reporter gene assays were sensitive to thyroid hormones and their analogues, none of the assays detected any activity in agonist mode in the unspiked water extracts, suggesting that either they do not target a relevant mode of action of thyroid disrupting chemicals or that the concentration of thyroid disrupting chemicals are below the assay detection limit. However, the latter seems unlikely given the response in the XETA, particularly for wastewater. The XETA assay was suitability sensitive to detect thyroid activity in surface water and wastewater. It has an advantage over the *in vitro* assays as it can incorporate toxicokinetic processes and appears to be a highly relevant assay for assessing the ecological impacts of wastewater discharges.

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548 **Table 1**: Model thyroid agonists (+) and antagonists (-) included in the current study.

Compound	CAS No.	Known biological target		
		Thyroperoxidase (TPO)	*Transthyretin (TTR)*	*Thyroid hormone receptor (TR)*
Triiodothyronine (T3)	6893-02-3		+ [a]	+ [b]
Thyroxine (T4)	51-48-9		+ [a]	+ [b]
Triiodothyroacetic acid (TRIAC)	51-24-1		+ [a]	+ [b]
Tetraiodothyroacetic acid (TETRAC)	67-30-1		+ [a]	+ [b]
Amiodarone	19774-82-4			- [b]
Pentachlorophenol (PCP)	87-86-5		+[c]	
Ethylene thiourea (ETU)	96-45-7	- [d]		
2,2,4,4-Tetrahydroxybenzophenone (THBP)	131-55-5	- [d]	+[e]	
Methimazole (MMI)	60-56-0	- [d]		

549 [a]OECD (2014); [b]Schriks et al. (2006); [c]van den Berg (1990); [d]Paul et al. (2014); [e]Zhang et al. (2015)

550 Note: TTR displacement assays cannot differentiate agonists or antagonists, thus '+' indicates binding to

551 TTR and '-' indicates no binding to TTR

552

Table 2: Summary of the studied *in vitro* and in *vivo* assays. Agonists and antagonists are indicated by (+) and (-), respectively. IC_{50} or EC_{50} values are for the laboratory internal reference compounds in the assay.

Endpoint	Bioassay	Method Reference	Reference compound	IC_{50}/EC_{50} (nM) (current study)	IC_{50}/EC_{50} (nM) (literature)[a]
Hormone synthesis	TPO inhibition assay	Paul et al. (2014)	Methimazole (MMI) (-)	69 ± 5.9 (n=2) (+)	93 (-)
Hormone transport	TTR displacement assay (FITC)	Ren and Guo (2012), with modifications	T4 (+)	95 ± 9.2 (n=5) (+)	260 (+)
	TTR displacement assay (ANSA)	Montano et al. (2012)	T4 (+)	310 ± 64 (n=3) (+)	260 (+)
Thyroid receptor mediated action	TRβ-CALUX	Piersma et al. (2013)	T3 (+) Amiodarone (-)	1.1 ± 0.16 (n=8) (+) 7200 (n=1) (-) †	0.69[b] (+) -
	TRβ-GeneBLAzer	Huang et al. (2011), with modifications	T3 (+) Amiodarone (-)	0.27 ± 0.02 (n=5) (+) 7300 ± 800 (n=5) (-)	0.30[c] (+) -
	GH3.TRE-Luc	Freitas et al. (2011)	T3 (+) Amiodarone (-)	0.06 (n=1) (+) 8400 (n = 1) (-)	0.10 (+) -
In vivo bioassay	Xenopus Embryonic Thyroid Assay (XETA)	Fini et al. (2007)	T3 (+)	4.5 (n = 1)* (+)	-

n = number of independent runs; FITC: fluorescein 5-isothiocyanate; ANSA: 8-anilino-1-naphthalenesulphonic acid ammonium salt

†cytotoxicity observed at higher concentrations

*One pool of 3 independent runs (60 larvae in total)

[a]IC_{50}/EC_{50} values are taken from the method reference studies; [b]Leusch et al. (2014); [c]Invitrogen (2010)

559 **Table 3**: Concentration (nM) of the different model compounds tested required to produce 50% effect/inhibition (EC_{50}/IC_{50}) in all bioassays. Numbers

560 in brackets indicate that the number is extrapolated from a bioassay response between 20-50%. All numbers are rounded to 2 significant figures.

Endpoint	Bioassay	Parameter	T3	T4	TRIAC	TETRAC	Amiodarone	PCP	ETU	THBP	MMI
Hormone synthesis	TPO inhibition assay	IC_{50}	>2000	>2000	>2000	>2000	>2000	>2000	>2000	**510**	**35**
Hormone transport	TTR displacement assay (FITC)	IC_{50}	**370**	**57**	**20**	**29**	**(1900)**	**19**	>10,000	**150**	>10,000
	TTR displacement assay (ANSA)	IC_{50}	**780**	**420**	**280**	**280**	>24,000	**370**	>24,000	**470**	>24,000
Thyroid receptor mediated action	TRβ-CALUX assay (agonist)	EC_{50}	**0.73**	**23**	**0.27**	**28**	>1000	>1000	>1000	>1000	>1000
	TRβ-CALUX assay (antagonist)	IC_{50}	[ago]	[ago]	[ago]	[ago]	**(47,000)**	**770**	>7000	**(9900)**	>7000
	TRβ-GeneBLAzer assay (agonist)	EC_{50}	**0.18**	**10**	**0.11**	**16**	[anta]	[anta]	[anta]	[anta]	>88,000
	TRβ-GeneBLAzer assay (antagonist)	IC_{50}	[ago]	[ago]	[ago]	[ago]	**1900**	**40,000**	**3600**	**130,000**	>7000
	GH3.TRE-Luc assay (agonist)	EC_{50}	**0.0075**	**1.7**	**0.0012**	**2.2**	[anta]	[anta]	n.a.	n.a.	n.a.
	GH3.TRE-Luc assay (antagonist)	IC_{50}	[ago]	[ago]	[ago]	[ago]	**8400**	**(2600)**	>2500	>2500	n.a.
In vivo bioassay	XETA (agonist)	EC_{50}	**4.6**	**120**	**21**	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	XETA (antagonist)	IC_{50}	n.a.	[ago]	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

561 **Abbreviations**: n.a. = not available; ago = agonist; anta = antagonist.

562 **Chemicals:** T3 = Triiodothyronine; T4 = Thyroxine; TRIAC = triiodothyroacetic acid; TETRAC = tetraiodothyroacetic acid; PCP = Pentachlorophenol; ETU =

563 Ethylene thiourea; THBP = 2,2,4,4-tetrahydroxybenzophenone; MMI = Methimazole.

564 **Note:** The background cell colour is a visual "heat-map" indicator of potency, ranging from red (most potent) to yellow (least potent, but still calculable EC_{50}/IC_{50}).

565 **Table 4**: Bioanalytical equivalent concentrations for the water extracts in all tested bioassays.
566 Numbers in brackets indicate that the number is extrapolated from a bioassay response between 20-
567 50%. All numbers are rounded to 2 significant figures.

	Unspiked	Spiked (100 µM T4 + 100 µM ETU)	Expected increase due to addition of spike [(a)]	Measured increase between spiked and unspiked
		TPO inhibition assay (in µg/L MMIEQ)		
Surface water	**(0.24)**	(0.29)	0	+0.050
Drinking water	<0.16	<0.16	0	0
Treated wastewater	**5.4**	6.8	0	+1.4
Ultra-pure water	<0.16	<0.16	0	0
		TTR displacement assay (FITC) (in µg/L T4EQ)		
Surface water	**4.5**	13	+7.8	+8.1
Drinking water	**2.7**	17	+7.8	+15
Treated wastewater	**50**	96	+16	+46
Ultra-pure water	**(0.64)**	9.5	+7.8	+8.9
		TTR displacement assay (ANSA) (in µg/L T4EQ)		
Surface water	n.a.	n.a.	+7.8	n.a.
Drinking water	n.a.	n.a.	+7.8	n.a.
Treated wastewater	n.a.	n.a.	+16	n.a.
Ultra-pure water	**0.35**	1.2	+7.8	+0.84
		TRβ-CALUX assay (agonist) (in µg/L T4EQ)		
Surface water	<0.11	2.2	+7.8	+2.1 to +2.2
Drinking water	<0.11	2.6	+7.8	+2.4 to +2.6
Treated wastewater	<0.67	3.6	+16	+2.9 to +3.6
Ultra-pure water	<0.11	1.4	+7.8	+1.2 to +1.4
		TRβ-GeneBLAzer assay (agonist) (in µg/L T4EQ)		
Surface water	<0.066	2.4	+7.8	+2.3 to +2.4
Drinking water	<0.066	1.4	+7.8	+1.3 to +1.4
Treated wastewater	<0.13	5.7	+16	+5.5 to +5.7
Ultra-pure water	<0.066	5.6	+7.8	+5.5 to +5.6
		TRβ-GeneBLAzer assay (antagonist) (in µg/L AmiEQ)		
Surface water	<28	<28	+3.4	0
Drinking water	<28	<28	+3.4	0
Treated wastewater	**350**	110	+6.8	-240
Ultra-pure water	<28	<28	+3.4	0
		GH3.TRE-Luc assay (agonist) (in µg/L T4EQ)		
Surface water	<1.2	1.5	+7.8	+0.3 to +1.5
Drinking water	<0.012	0.18	+7.8	+0.17 to +0.18
Treated wastewater	<2.3	3.0	+16	+0.66 to +3.0
Ultra-pure water	<0.012	0.81	+7.8	+0.80 to +0.81
		GH3.TRE-Luc assay (antagonist) (in µg/L AmiEQ)		
Surface water	<870	<870	0	0
Drinking water	<87	<87	0	0
Treated wastewater	<1700	<1700	0	0
Ultra-pure water	<87	<87	0	0
		XETA (unspiked mode) (in µg/L T4EQ)		
Surface water	**25**	29	+7.8	+4.6
Drinking water	<7.8	9.3	+7.8	+1.5 to +9.3
Treated wastewater	**29**	47	+16	+18
Ultra-pure water	<7.8	<7.8	+7.8	0

Abbreviations used: "AmiEQ" = amiodarone equivalent concentration; "MMIEQ" = methimazole equivalent concentration; "n.a." = not available due to interference in the assay; "T4EQ" = thyroxine equivalent concentration. **Notes**: [(a)] Expected increase due to the addition of the spike was calculated by combining the concentration of the chemicals spiked in the assay (100 μM in the concentrated aliquot for both T4 and ETU) and their respective potencies in that particular assay. **Colour**: The background colour indicates how close the measured increase is to the predicted increase, ranging from most accurate dark green (80-120%), to light green (50-150%), to orange (10-190%), to least accurate light red (<10 or >190%). Light blue indicates that no accurate prediction on the change could be made for this endpoint/assay.

Figure 1: Concentration-effect curves for the unspiked water extracts and Suwannee River humic acid as a function of dissolved organic carbon (DOC) concentration in the assay. The DOC concentration of the water extracts in the assay was calculated based on the DOC concentration of the unenriched extracts (0.67 mgC/L drinking water; 1.4 mgC/L surface water; 11 mgC/L wastewater), the relative enrichment factor (REF) in the assay and assuming that 40% of DOC is co-extracted by the solid-phase extraction (SPE) cartridges.