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Bioassay-Directed Identification of Novel Antiandrogenic Compounds in Bile of Fish Exposed to Wastewater Effluents

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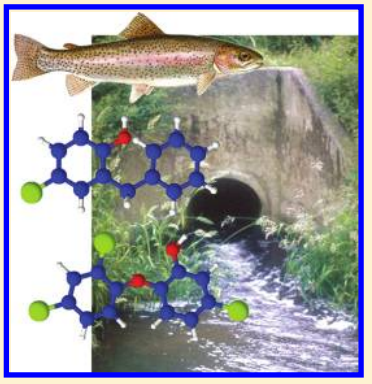
Supporting Information

ABSTRACT: The widespread occurrence of feminized male fish downstream of some UK Wastewater Treatment Works (WwTWs) has been associated with exposure to estrogenic and potentially antiandrogenic (AA) contaminants in the effluents. In this study, profiling of AA contaminants in WwTW effluents and fish was conducted using HPLC in combination with *in vitro* androgen receptor transcription screens. Analysis of extracts of wastewater effluents revealed complex profiles of AA activity comprising 21−53 HPLC fractions. Structures of bioavailable antiandrogens were identified by exposing rainbow trout to a WwTW effluent and profiling the bile for AA activity using yeast (anti-YAS) and mammalian-based (AR-CALUX) androgen receptor transcription screens. The predominant fractions with AA activity in both androgen receptor screens contained the germicides chlorophene and triclosan, and together these contaminants accounted for 51% of the total anti-YAS activity in the fish bile. Other AA compounds identified in bile included chloroxylenol, dichlorophene, resin acids, napthols, oxybenzone, 4-nonylphenol, and bisphenol A. Pure standards of these compounds were active in the androgen receptor screens at potencies relative to flutamide of between 0.1 and 13.0. Thus, we have identified, for the first time, a diverse range of AA chemicals in WwTWs that are bioavailable to fish and which need to be assessed for their risk to the reproductive health of these organisms and other aquatic biota.



INTRODUCTION

The widespread occurrence of feminized male fish downstream of discharge from UK wastewater treatment works (WwTWs) has led to substantial interest from environmental biologists, government organizations, and industry.[1,2] The phenomenon of feminized fish has further been observed in freshwater and marine environments throughout the world. Feminized responses include the formation of the egg-yolk precursor, vitellogenin, within male and juvenile animals as well as histopathological changes in reproductive organs such as testes-ova and reductions in sperm count and motility.[1,3] These effects have been attributed to exposure to environment estrogens, particularly steroidal estrogens present in WwTWs effluents.[4] However, a survey of UK WwTWs has revealed that the majority of the effluents sampled contain antiandrogenic (AA) activity (between 21.3−1231 μg of flutamide equivalents/L) as well as estrogenic activity (between 0.4−42.7 ng of estradiol (E2) equivalents/L).[5] Feminization of fish at river sites was correlated with their predicted exposure to both antiandrogens and estrogens or to antiandrogens alone.[6]

There is good evidence that exposure of fish to some antiandrogens in the laboratory can cause feminization and sexual disruption. For instance exposure of fish to either environmental or clinical antiandrogens induced intersexuality in male and ovarian atresia in female medaka[7] and resulted in a reduction of sperm count and male secondary sexual features in guppies and fathead minnows. It also inhibited androgen-induced spiggin production in the stickleback.[8] The widespread occurrence of antiandrogens in WwTW effluents may contribute (alongside estrogens) to the gonadal disruption of fish in UK rivers. Therefore, it is essential that antiandrogens present in wastewaters are identified and to allow for their possible associated risks to wildlife health to be properly established.

The nature of AA compounds in UK effluents is currently unknown. Structures of chemicals containing androgen receptor antagonist properties can be extremely diverse and include environmental contaminants such as insecticides or their metabolites (e.g., pp′-DDE and certain pyrethroids), fungicides (vinclozolin and procymidone), herbicides (linuron and prochloraz), components of sunscreen products, some industrial contaminants such as selected PCB congeners and pharmaceuticals (e.g., flutamide and cyproterone acetate).[2] It is unlikely that many of these environmental antiandrogens would be present in UK

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WwTW effluents which arise primarily from domestic (sewage) inputs. Therefore, analytical approaches such as bioassay-directed fractionation and analysis are needed to identify unknown compounds with AA activity in wastewater samples. Identification of key active compounds in wastewaters is difficult as they can often be present at subnanogram per liter concentrations. However, a wide range of xenobiotics can bioconcentrate in fish bile at concentrations tens of thousands greater than in the effluent itself, facilitating the structural identification of bioavailable contaminants present in the ambient environment.[9,10] Bioassay-directed fractionation of bile has proven to be effective in studies identifying estrogens,[9] androgens, and some antiandrogens taken up into fish from WWTW effluents.[11] However in that previous work, due to complexity of the bile fractions and the limited amount of bile available to purify and detect antiandrogens, only two antiandrogens were identified; dichlorophene and 9,10-di(chloromethyl)anthracene, and together these accounted for less than 5% of the AA activity in the bile samples.[11] In this study, we reveal the complex profiles of AA activity that are present in effluent samples from WwTWs, and we identify a comprehensive array of AA contaminants bioavailable (in bile) from trout exposed to a WwTW effluent using a bioassay-directed analytical approach. A composite bile sample from mature trout held in a tank and exposed to effluent was used to determine the structures of AAs in the fish, and an additional exposure, using juvenile trout, was used to confirm the identification of the key bioavailable AAs contributing to the AA activity in the bile. To identify AA structures, bile extracts were fractionated using reverse phase-high performance liquid chromatography (RP-HPLC) and the fractions interrogated for antiandrogen activity using two *in vitro* assays: a yeast and a mammalian cell-based androgen receptor transcription assay, anti-YAS and AR-CALUX, respectively. Fractions containing antiandrogen activity were analyzed by GC-MS to identify key structures.

EXPERIMENTAL SECTION

Materials **see Supporting Information (SI).** *WwTW Sites.* Three grab samples (1−2.5 L) of final effluent from each of the 3 WwTWs (A, B, and C) were collected in solvent-rinsed glass containers. The population equivalents of the 3 WwTWs were between 47,200 and 142,370 and the influent consisted of primarily domestic input, and the effluent had been processed by both primary and secondary treatments (details on the treatment works are given in SI, Table S1). Methanol (final concentration 3%) and acetic acid (final concentration 1%) were added to the samples which were stored overnight at 4 °C prior to solid phase extraction (SPE) of the samples.

Fish Exposure. Trout were raised in the Hatherley Laboratories, University of Exeter, UK. The fish were exposed to undiluted effluent from only WwTW C in two tanks (1 m^3, flow rate of 10 L/min) for a period of 10 days. The exposure protocol consisted of two tanks: one tank contained 9 mature female rainbow trout *(Oncorhynchus mykiss)* that were 2^+ years old (in their third year of growth) (mean length ± SEM of 37.1 ± 0.6 cm and weight 465.3 ± 22.9 g) in order to obtain enough bile for method development and structural analyses of AAs. The other tank contained 15 smaller juvenile fish that were 1^+ year old (in their second year of growth) (length 26.0 ± 0.5 cm and weight of 121.1 ± 1.4 g, n = 15). The effluent exposures were not replicated; however, samples from these juvenile fish were used to obtain analytical replicates in order to confirm the identity of AA structures in the bile. A similar population of mature and juvenile trout were held in dechlorinated tap water in the laboratory for 10 days. Trout were fed daily on a commercial trout food until 2 days prior to the end of the experiment to maximize bile production. At the end of the exposure periods, fish were anaesthetized and sacrificed, and the bile sacs were removed for analysis of antiandrogens.

Extraction and Purification of AA Compounds. In order to prepare enough bile for preparation of semipurified fractions and unequivocal chemical identification of antiandrogenic structures, a composite sample (1.8 mL) was prepared from bile of mature trout exposed to effluent C. Aliquots of this sample were used for method development and for structural identification of AA compounds in the bile. Three different composite samples of bile were prepared from juvenile trout exposed to effluent C in another tank, and these were used to replicate the chemical analyses; each sample (200 μL volume) represented a composite from 5 different fish. Similar composite bile samples were prepared from control trout held in dechlorinated tap water. Metabolites in the composite bile samples were deconjugated as described elsewhere.[10,11] Extraction of wastewater and bile samples by SPE was similar to that described earlier[11] with the modification that samples were eluted sequentially from the cartridges with methanol, dichloromethane (instead of ethyl acetate used in previous work[11]), and hexane. The use of dichloromethane ensured that acceptable recoveries of chlorinated antiandrogens (identified previously[11]) were achieved from the SPE. The efficiency of SPE was tested using trout bile spiked with standard androgens and antiandrogens (for analytical details see the SI). In order to ensure all AA compounds in the bile were extracted by Oasis HLB, the solution eluting from the SPE cartridge during sample loading, as well as the washes, were collected from the cartridge and re-extracted on ion exchange SPE (details in the SI). The SPE eluents were dried down under vacuum and redissolved in ethanol for bioassay in the anti-YAS. After bioassay analysis, extracts were dried down and redissolved in acetonitrile:water (90:10, v:v) for RP-HPLC fractionation.

RP-HPLC Fractionation. Extracts of two replicate grab samples of each WwTW effluent, and analytical replicates of composite bile samples from mature fish (two replicates) and juvenile fish (three replicates) were fractionated by HPLC using an acetonitrile/water system (see the SI for details). HPLC fractions of bile or wastewater samples were collected every minute for analysis in receptor bioassays. With some bile fractions, where subsequent GC-MS analyses indicated they contained a complex mixture of xenobiotics, the fractions were repurified on HPLC using water: acetonitrile gradients between 70:30% to 10:90% (30 min) and retested in the anti-YAS.

GC-MS Analysis. The identities of chemicals in fractions with AA activity were investigated using gas chromatograph ion trap mass spectrometer (GC-MS) analyses (see the SI for details). Identified compounds were quantified with a four point linear regression calibration curve using a ratio of internal standard and selected ions for each compound of interest.

Steroid Receptor Screens. *Anti-YAS.* AA activities of bile or effluent samples were quantified using a recombinant yeast screen that contains androgen receptor (YAS). This assay has been validated for a range of AA contaminants.[12] Samples used for total AA activity measurements (before HPLC fractionation) and pure standards were serially diluted in ethanol, which was evaporated to dryness before addition of culture media. To test for receptor antagonist activity, the agonist (DHT) was added to

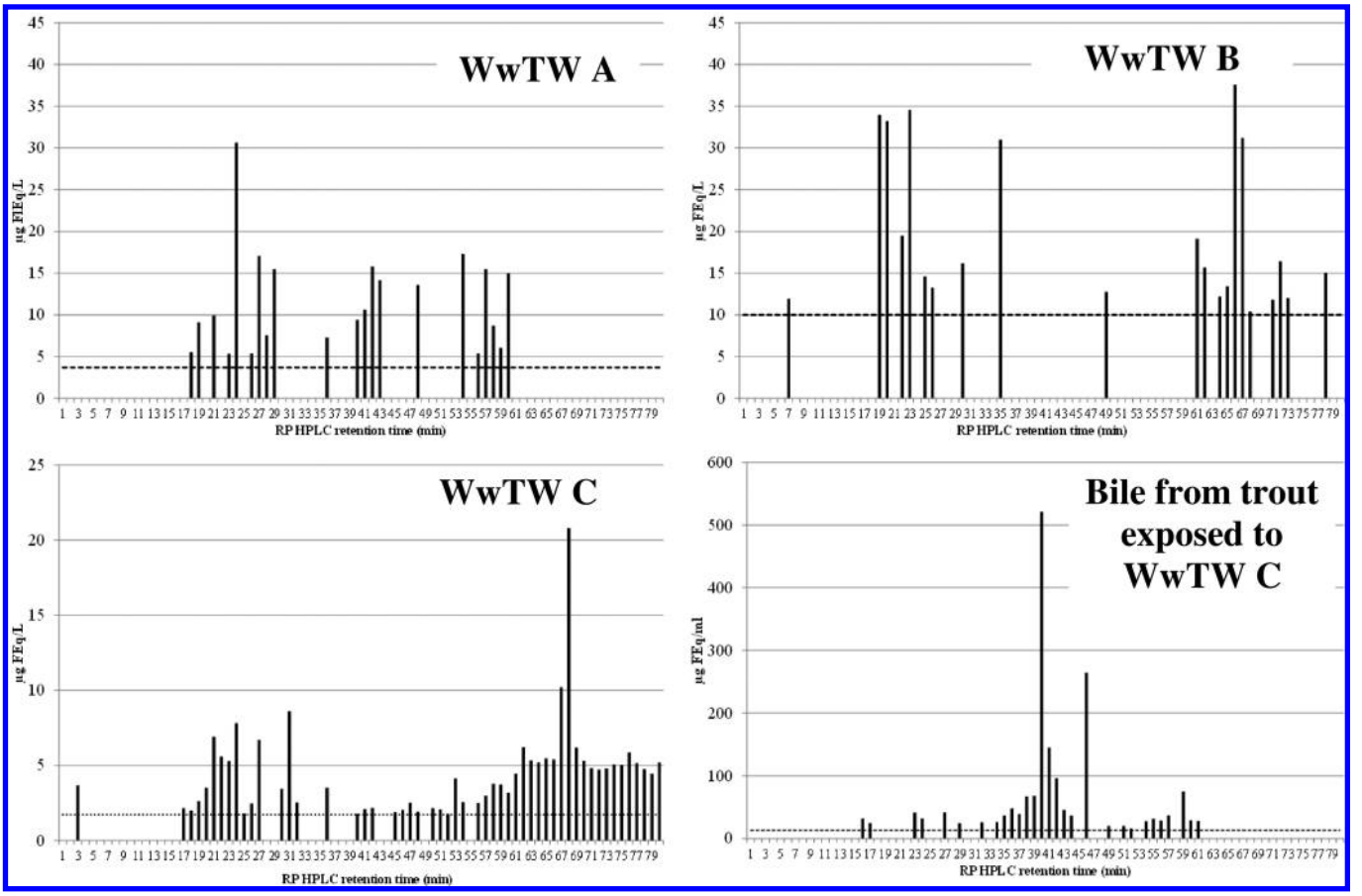


Figure 1. Profiles of the antiandrogenic activity in extracts of final effluent (μg FEq/L) from WwTW A, B, C and in extract of bile from trout exposed to effluent WwTW C (μg FEq/mL). Profiles are representative of 2 replicate grab samples of each of the wastewaters and 2 replicate analyses of one composite bile sample prepared from mature trout held in a tank exposed to WwTW C. No antiandrogenic activity was detected in blank workup samples of dechlorinated tap water or in profiles from bile of trout held in clean water. Dotted lines indicate limit of detection (LOD) values.

the yeast medium at a concentration giving a 65% submaximal response of the assay. AA activity was quantified as flutamide standard equivalents (FEq). Samples showing toxicity which resulted in poor yeast growth (monitored at 620 nm and in comparison with blank samples containing only ethanol and media) were not quantified. The toxicity of concentrations of standards of putative antiandrogens was also assessed by analysis of the 620 nm response in the YAS without addition of DHT to the media. In some cases, where toxicity of standard compound was observed only at the highest concentrations, then additional DHT (27.4 ng/mL) was added back to the wells containing cell incubations with the serial dilutions of test compound. This allowed the detection of any latent toxicity (measured as a reduced response to the DHT agonist) in the dilution curve.

AR-CALUX. Bile fractions from HPLC analysis (mature fish) and pure standards of putative antiandrogens revealed by the anti-YAS and GC-MS analysis were retested in the AR-CALUX bioassay (BioDetection Systems, Netherlands) as a confirmation of androgen receptor antagonist activity. The AR-CALUX assay is a reporter gene assay consisting of a human osteoblast cell line that contains a luciferase gene under transcriptional control of an androgen responsive element. The assay was performed according to refs 13 and 14 using a 24 h exposure. Bile fractions in dimethyl sulfoxide (DMSO) were analyzed in triplicate in the presence of the EC_{50} of DHT (2×10^{-10} M). The amount of activity (FEq) was determined by interpolating the response of the sample into the concentration−response of the reference compound. Cell toxicity was identified if cell cultures were found to be detached from the multiwell plate surface.

RESULTS AND DISCUSSION

Profiles of Antiandrogen Activity (Anti-YAS) in Wastewaters and in Fish Bile. Anti-YAS analysis of AA activity of the SPE extracts of three replicate grab samples of each wastewater revealed that effluents A, B, and C contained 260 ± 40 μg, 468 ± 51 μg, and 214 ± 27 μg FEq/L, respectively. Extracts from two of the replicate grab samples from each WwTW effluent were fractionated by HPLC, and the fractions were analyzed for AA activity using the anti-YAS. This revealed that the profiles of AA activity in the three WwTW effluents were complex, with each sample containing clusters of polar, moderately polar, and nonpolar fractions (Figure 1). The effluent profiles contained between 21 (WwTWs A and B) and 53 (WwTW C) fractions with AA activity at or above the LOD value of the method. In contrast, previous studies have revealed that estrogenic profiles in wastewaters are dominated by far fewer active fractions.[11,15] In this study, the influent of the three WwTWs plants was primarily domestic origin and was treated by biological aerated filter and/or activated sludge processing (see Table S1). However the

effluent of WwTWs A and B were also subjected to additional treatments including sand filtering (WwTW A) or denitrification stages (WwTW B) which may account for the lower number of AA fractions detected in these effluents compared with WwTW C. The determination of the structures of the AA chemicals in an effluent extract could be challenging due to the possibility of low concentrations of such a complex number of AA chemicals. Therefore, the profiles of AA activity in bile from rainbow trout exposed to an effluent containing the most AA fractions (WwTW C) were analyzed.

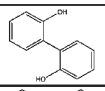
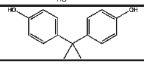
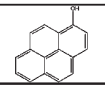
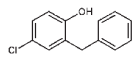
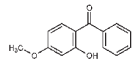
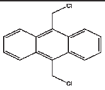
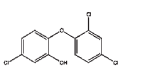
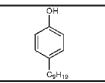
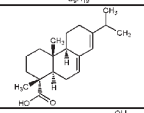
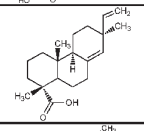
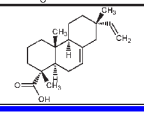
Development of Analytical Methods To Profile Antiandrogen Activity in Fish Bile. SPE methods were used to extract AA compounds in bile extracts. Using this methodology, standard androgens (dihydrotesterone and testosterone) and AA compounds (flutamide, p,p′-DDE, dichlorophene, bisphenol A) were spiked into bile samples from control fish which were extracted using Oasis HLB SPE. Analysis of the bile extracts by GC-MS revealed high SPE recoveries of the standards of between 74−89% (Table S2). Three analytical replicates from a composite bile sample prepared from effluent-exposed mature fish were used to determine the recoveries of AA activity from SPE. Recoveries of bile extracts were assessed by the comparison of the total AA activity of samples of bile (after hydrolysis) before and after Oasis HLB SPE and after additional serial extractions of the sample solution using two types of ion exchange SPEs. The majority of AA activity in the original bile sample was extracted with Oasis HLB cartridge (97.4 ± 1.6%,), with a very small part recovered with Oasis WAX and MCX SPE (0.6 ± 0.1% and 1.5 ± 0.1%, respectively). There was no detectable AA activity in blank workup samples that were carried over all steps of analytical procedure or in bile from the control population of trout held in dechlorinated tap water (LOD < 2.1 μg of FEq/mL of bile). However, the total AA activity in bile of effluent-exposed mature trout was 1840 ± 140 μg FEq/mL compared with 214 ± 27 μg FEq/L in the effluent, which indicated an average bioconcentration of AA chemicals (in bile) of 8,600 fold, similar to levels estimated in previous studies.[11] Analysis of bile extracts from the effluent-exposed mature trout revealed a simpler profile than in effluent WwTW C used in the exposures, with about 30 mainly moderately polar and nonpolar fractions eluting from the HPLC between 16 and 61 min (Figure 1). Differences between the bile and effluent profiles could be due to a number of factors; that either some of the polar or nonpolar AA chemicals that were present in the effluent were not bioavailable to fish or that some bioavailable AAs were not detected in the bile as they were metabolized and deactivated or excreted in the urine. Xenobiotics can be present in bile as phase 1 metabolites,[16] and these structures may have little or no AA activity compared to the parent structures. Similarly, although glucuronide and sulfate conjugates were hydrolyzed back to the parent structures, other phase II metabolites of AAs, such as glutathione conjugates, may not have been detected in the anti-YAS. Also polar or low molecular weight structures tend to be excreted in urine rather than the bile.[17] Nevertheless, this study indicates that a variety of xenobiotic compounds bioconcentrate in the bile of the fish allowing for the identification of bioavailable AA structures arising from exposure to the effluent.

The most active AA fractions in bile of mature fish eluted at retention times of 40, 41, and 46 min (Figure 1). Similar profiles of AA activity were observed in bile samples obtained from exposure of juvenile fish to WwTW C where some fractions eluted one min earlier than profiles of mature fish. (Table 1). The total AA in bile of juvenile fish was 2210 ± 230 μg FEq/mL, and the recovery of AA activity after HPLC profiling of the bile was 119.0 ± 11.2% (n = 3 analytical replicates). Together, these studies indicated that all the AA activity present in fish bile was recovered using the analytical methodologies of HLB SPE and RP-HPLC fractionation. Our studies indicate that steroidal androgens, if present in the bile extracts, would have been extracted by SPE. The presence of any androgen receptor agonists may have reduced the estimation of total AA activity in the bile extracts as HPLC fractionation did increase the amount of total AA activity estimated from the summed fractions.

Identification of Key Antiandrogen Structures in Fish Bile. GC-MS analyses of HPLC fractions with antiandrogen activity derived from a composite bile sample from mature fish resulted in detection of a variety of contaminants with AA activity (Table S3). The identities of the antiandrogens were also confirmed by analysis of corresponding fractions with AA activity in bile from juvenile fish (Table 1). The antiandrogens identified in the bile fractions included the following: biocides such as triclosan, chlorophene, dichlorophene, and chloroxylenol. Isomers of resin acids (abietic acid, pimaric acid, isopimaric acid, neoabietic acid) were also identified as AA as well as bisphenol A (a plastic monomer), a chlorinated anthracene compound and metabolites of PAHs (hydroxypyrene, naphthols). Other compounds with AA activity included 4-nonylphenol (a surfactant product), oxybenzone, an ingredient used in sunscreen filters, and (in juvenile fish) dihydroxybiphenyl. These compounds had the same retention times on HPLC and GC-MS and the same mass spectra as the commercially available standards (see examples in Figure S1A, B). Additional contaminants were tentatively identified in other fractions containing AA activity, and these were positional isomers of hydroxypyrene, chlorinated and dichlorinated derivatives of bisphenol A, a methoxy metabolite of chlorophene, additional isomers of resin acids, and a triclosan analogue − diclosan. However, due to lack of available commercial standards for these compounds, it was not possible to confirm their exact structure or their AA activity in the androgen receptor screens. Mefenamic acid was also detected in bile fractions from juvenile and mature fish but the pure standard was toxic in the anti-YAS.

Analysis of pure commercial standards of the identified structures in the fish bile confirmed that the compounds were antiandrogenic in the anti-YAS bioassay. Examples of the dose−response curves are shown in Figure 2 and potency values relative to the flutamide standard are given in Tables 1 and S3. To exclude the possibility that toxicity was responsible for the inhibition in the anti-YAS, two tests were performed. First, yeast growth in the YAS (without DHT coexposure) was monitored during incubation with the standard compounds using absorbance values at 620 nm to measure turbidity. If toxicity was observed at the highest concentrations, standards were measured again in YAS in the presence of an excess of DHT. That led to an increase absorbance (measured at the wavelength of 540 nm), and these values were compared with those of a blank positive control (ethanol only plus media with DHT). This allowed detection of the inhibition in the original anti-YAS which could be due to toxicity rather than receptor antagonist activity (examples shown in Figure S2). Using this approach, none of the standards except for 1-hydroxypyrene showed toxicity in the YAS at concentrations up to 10-fold higher than the corresponding IC_{50} antagonist values in the anti-YAS. In the case of 1-hydroxypyrene, reduction in yeast growth and in the response

Table 1. Antiandrogenic Compounds Identified in the Bile of Juvenile Trout Exposed to Wastewater Effluent from WwTW C and Their Contribution to the Total Antiandrogenic Activity in the Bile As Measured by the Anti-YAS[a,b]

Compound identified in the bile fraction	RP HPLC retention time of active fraction	Structure of non-derivatized compound	Characteristic ions from GC-MS analysis after derivatization	Concentration detected by GC-MS (μg/mL of bile)	AA activity of the fraction (μg FEq/mL of bile)	Potency relative to flutamide standard in anti-YAS.	Estimated AA activity of chemical in fraction based on GC-MS quantification and potency (μg FEq/mL of bile)	Contribution of AA activity of the identified compound to total activity of the fraction (%)	Contribution of the AA activity of the identified compound to total activity of the bile (%)
2-naphthol	25		216, 201, 185	5.06 ± 2.95	31.60 ± 7.97	0.32	1.62 ± 0.94	5.11 ± 1.68	0.06 ± 0.03
	26		216, 201, 185	8.42 ± 5.06	21.20 ± 0.10	0.32	2.69 ± 1.62	12.69 ± 7.64	0.10 ± 0.07
2,2'-dihydroxybiphenyl	25		330, 315, 227, 199, 147, 73	1.36 ± 0.21	31.66 ± 7.97	0.35	0.48 ± 0.07	1.52 ± 0.41	0.02 ± 0.00
	26			2.75 ± 0.22	20.40 ± 0.69	0.35	0.96 ± 0.08	4.71 ± 0.48	0.04 ± 0.00
bisphenol A	26		372, 357, 207, 191, 73	0.49 ± 0.33	20.80 ± 0.69	0.60	0.29 ± 0.22	1.30 ± 1.04	0.01 ± 0.01
chloroxylenol	31		228, 213, 172	119.4 ± 142.7	31.00 ± 15.72	0.16	19.10 ± 22.82	61.61 ± 37.95	0.73 ± 0.82
	32		228, 213, 172	107.6 ± 117.5	32.89 ± 16.06	0.16	17.22 ± 18.81	52.36 ± 39.07	0.66 ± 0.68
dichlorophene	36		412, 412,414,377,379, 73414,377,379, 73	4.52 ± 2.10	56.84 ± 26.74	4.70	21.25 ± 9.89	37.38 ± 8.57	0.81 ± 0.34
	37			5.01 ± 2.45	62.41 ± 22.21	4.70	23.53 ± 11.51	37.70 ± 30.85	0.90 ± 0.44
1-hydroxypyrene	39		290, 275, 259	0.81 ± 0.15	339.4 ± 84.0	9.90**	8.02 ± 1.49	2.36 ± 0.78	0.31 ± 0.06
chlorophene	39		292, 290, 277, 275	25.3 ± 6.00	339.4 ± 84.0	13.0	328.9 ± 77.98	96.90 ± 1.85	12.58 ± 3.57
	40		292, 290, 277, 275	29.5 ± 6.34	390.1 ± 81.6	13.0	383.5 ± 82.38	98.31 ± 1.18	14.67 ± 3.83
oxybenzone	41		300, 299, 283. 225, 73	1.78 ± 1.03	47.90 ± 3.76	0.34	0.61 ± 0.35	1.27 ± 0.61	0.02 ± 0.01
9,10-di(chloromethyl)anthracene	43		274, 276,239,241, 203, 204	17.73 ± 7.13	102.8 ± 55.0	2.50	44.33 ± 17.82	43.12 ± 11.97	1.70 ± 0.60
triclosan	45		364, 362, 360, 349, 347, 310, 312, 345, 202, 200	101.1 ± 15.7	494.3 ± 66.8	4.80	485.3 ± 75.5	98.18 ± 1.94	18.56 ± 2.33
	46			29.26 ± 6.97	160.6 ± 58.61	4.80	140.5 ± 33.5	87.48 ± 14.66	5.37 ± 1.16
4-nonylphenol	52		292,263, 221, 193, 179,73	55.14 ± 6.59	41.98 ± 3.88	0.30	16.54 ± 1.98	39.40 ± 1.34	0.63 ± 0.09
abietic acid	58		374, 359, 256, 241	12.59 ± 2.96	95.26 ± 18.49	4.00	50.36 ± 11.83	52.87 ± 1.25	1.93 ± 5.41
pimaric acid	58		374, 359, 256, 241	3.90 ± 3.23	95.26 ± 18.49	2.73	10.65 ± 8.83	11.18 ± 8.53	0.41 ± 0.36
isopimaric acid	58		359, 256, 241	4.28 ± 2.76	95.26 ± 18.49	5.00	21.40 ± 13.81	22.47± 11.39	0.82 ± 0.54

[a] Two asterisks denote the following: antiandrogenic potency possibly overestimated due to toxicity in the anti-YAS. [b] Data are mean ± s.d. values from 3 analytical replicates, each prepared from composite samples of different fish held in one exposure tank. In this HPLC analysis, active fractions and AA standards eluted 1 min earlier compared to profiles presented in Figure 1 and Table S3. The sum of all the compounds identified and tested in the anti-YAS accounted for 60.3 ± 5.7% of the total antiandrogenic activity measured in all the bile fractions. The LOD of the anti-YAS = 20 ± 7 μg FEq/mL of bile. Additional compounds (diclosan and methoxy metabolite of chlorophene) were identified in fraction 42, but standards were not available to test in the anti-YAS. Mefenamic acid was identified in fractions 40, 41, but a pure standard was toxic in the anti-YAS.

to addition of excess DHT was detected at concentrations ≥1.4 × 10^{-6} M which were 2-fold higher than the IC_{50} values in the anti-YAS. Therefore, the androgen receptor antagonist activity of this compound in the yeast screen cannot be verified.

AR-CALUX Analysis of Bile Antiandrogens. Due to the limited permeable nature of the yeast cell wall which comprises complex polysaccharides, there was the possibility that not all of the AA xenobiotics present in the bile of effluent-exposed trout

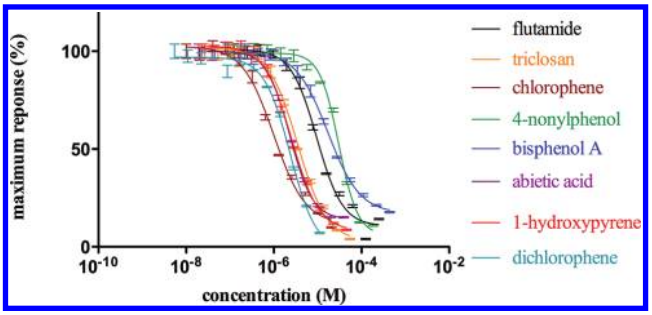


Figure 2. Dose−response curves of pure standard compounds tested in the anti-YAS Mean ± s.d. IC_{50} values (n = 3) of pure standard compounds are given in brackets (M): flutamide ($7.07 \times 10^{-6} \pm 9.47 \times 10^{-9}$), abietic acid ($1.77 \times 10^{-6} \pm 1.06 \times 10^{-7}$), triclosan ($1.47 \times 10^{-6} \pm 9.89 \times 10^{-8}$), 1-hydroxypyrene ($7.14 \times 10^{-7} \pm 5.61 \times 10^{-8}$), chlorophene ($5.43 \times 10^{-7} \pm 3.15 \times 10^{-8}$), dichlorophene ($1.50 \times 10^{-6} \pm 9.09 \times 10^{-8}$), 4-nonylphenol ($2.36 \times 10^{-5} \pm 2.483 \times 10^{-6}$), bisphenol A ($1.18 \times 10^{-5} \pm 5.58 \times 10^{-7}$). Yeast cell toxicity was detected at $\geq 1.4 \times 10^{-6}$ M concentrations of 1-hydroxypyrene.

were detected in the anti-YAS. Therefore, bile fractions from the composite sample from mature fish and pure standards of the identified AA compounds were retested in the AR-CALUX assay which is based on an osteosarcoma cell line. Both *in vitro* assays resulted in a similar profile of AA activity in the bile, and the two predominant fractions with AA activity in the anti-YAS were also principal AA fractions in the AR-CALUX (Figure S3). However, there were a number of minor antiandrogen fractions that were only detected in one of the assays. This observation may be due to the compound specific differences in sensitivity between the two screens. The AR-CALUX is 10-fold more sensitive to flutamide than the anti-YAS (The IC_{50} of flutamide in the anti-YAS is 1.2−1.8 mg/L, whereas in the AR-CALUX it is 0.09−0.15 mg/L.). Commercial standards of all the antiandrogens previously identified in the anti-YAS analysis of bile were also AA in the AR-CALUX, and none (including hydroxypyrene) showed toxicity in the AR-CALUX (Figure S4). However, although the potencies (relative to flutamide) of 4-nonylphenol and bisphenol A were similar in both screens, the potencies of triclosan, chlorophene, dichlorophene, and abietic acid were all higher in the anti-YAS compared with the AR-CALUX. The difference in potencies between the two assays for certain chemicals may be due to a number of factors, including metabolic transformation in the osteosarcoma cell line used in the AR-CALUX. It is unlikely that the difference in potencies is due to the solubility of the chemicals in different carrier solvents. Work in our laboratory revealed that regardless of the solvent used in the anti-YAS, the potencies of the chemicals relative to flutamide were similar: 4.8 ± 0.3 and 4.1 ± 0.8 for triclosan (mean ± s.d., n = 3) and 13.0 ± 0.3 and 15.8 ± 1.2 for chlorophene in ethanol and DMSO, respectively. Therefore, even using the DMSO as the same carrier solvent, there were still differences in potency for these compounds between the two assays. The permeability of the cell wall or membrane could also be a factor, but studies have indicated that the yeast cell wall is permeable to small (<600 g/mol) polar or nonpolar chemicals.[18] Our findings highlight that two widely used *in vitro* assays for screening antiandrogenic activity, although producing similar qualitative analyses, can produce results that differ quantitatively, and this is something that warrants further investigations to enable harmonization of data across studies using the different screens.

Contribution of Identified Structures to the Total AA Activity in Fish Bile. The contribution of the antiandrogens identified in effluent-exposed juvenile or mature fish to the AA activity in the HPLC fractions and in the bile extracts were estimated from their concentrations determined by GC-MS and their relative potency values to flutamide in the yeast screen (Table 1 and S3). In addition, the corresponding fraction in the effluent extracts from WwTWs A, B, and C were analyzed by GC-MS to determine the presence of the antiandrogen in the effluent samples.

Chlorophene contributed to 27% of the total AA activity in bile from juvenile or mature trout and was the most potent antiandrogen detected in the bile with a potency relative to flutamide of 13. It was also detected in the analogous HPLC fraction from profiles of the three WwTWs effluents at concentrations between 32 and 311 ng/L. Chlorophene is a germicide that is widely used in disinfectant products, and concentrations of 14−2850 ng/L have been reported in final WwTW effluents in the UK and Spain.[19,20] To our knowledge it is the first report in which its AA properties have been recognized. Triclosan, another germicide, contributed 24% and 15% to the total AA activity in the bile of juvenile and mature fish, respectively, and was detected in all three effluents at concentrations between 50 and 100 ng/L. Triclosan is used in cosmetics and other personal care products and is widely detected in environmental samples.[21] Triclosan has been reported previously as an androgen antagonist *in vitro*[22] and also to disrupt thyroid hormone signaling and decrease serum thyroxine in male juvenile rats.[23] The analysis of HPLC fractions of the effluent extracts revealed that although all three WwTWs contained chlorophene and triclosan, the concentrations detected in both compounds would have been below or close to the LOD of the profiling methods used for analyses of the effluent extracts (Figure 1).

A number of other contaminants were identified in bile from effluent-exposed trout which individually contributed <4% to the total AA activity in the bile (Table 1). These included other chlorinated phenolics, such as dichlorophene, an antimicrobial agent reported previously,[11] and chloroxylenol (para-chloro-meta-xylenol) which is actively used in disinfectants and antiseptics and has been used as a preservative in pharmaceuticals and cosmetic products. Both these compounds were detected in the three WwTW effluents at concentrations of 10−450 ng/L (dichlorophene) and 19−140 ng/L (chloroxylenol). The AA activity of chloroxylenol has not been reported previously; however, together with chlorophene and triclosan, these germicides have been detected in the bile of bream (*Abramis brama*) collected from rivers in The Netherlands.[24]

Hydroxypyrene is recognized as a biomarker of exposure to polycyclic aromatic hydrocarbons (PAHs) and is commonly detected in fish bile.[25] It was not detected in HPLC fractions of the WwTWs effluents indicating that it was a product of fish metabolism. Although 1-hydroxypyrene was toxic in the anti-YAS, it appeared to be a possible androgen antagonist in the AR-CALUX screen. We also identified for the first time naphthol isomers as other weakly active PAH metabolites in the anti-YAS assay.

A number of xenobiotics with known estrogenic activity were also identified contributing to the androgen receptor antagonist activity of the bile. Oxybenzone is the chemical found in most commercial sunscreens as well as other cosmetics and plastic mixtures and was detected in effluent of WwTW C at concentrations of 8 ng/L. *In vitro* studies have shown that oxybenzone is

estrogenic as well antiestrogenic and antiandrogenic.[26] Bisphenol A, a component of polycarbonate plastics and food packaging, contributed <1% to the AA activity of the bile and was detected in the WwTWs effluents at concentrations between <2.5 to 25 ng/L although concentrations of up to 7 μg/L have been reported at times for some WwTWs.[27] Similarly the contribution of 4-nonylphenol to the AA activity of the bile was insignificant. 4-Nonylphenol can be derived from degradation of 4-nonylphenol ethoxylates surfactants in WwTW, and it was detected in effluent from WwTW C at concentration of 0.42 μg/L. The levels of 4-nonylphenol in the environment have declined since the use of such compounds have been banned or strictly monitored in many countries, although it can still be found at concentrations of up to 4 μg/L in some river waters.[28] Both bisphenol A and 4-nonylphenol have been reported to be estrogen receptor agonists and androgen receptor antagonists when tested in steroid receptor transcription screens.[12,29]

A mixture of resin acids, including abietic acid and its analogues, were detected in HPLC fractions of bile and also in the effluent of WwTW C at a concentration of 5−10 ng/L. Resin acids are tricyclic diterpenes that occur naturally in the resin of tree wood and bark and are transferred to process waters during pulping operations. In paper mill effluents they have been detected at concentrations between 0.02 to 12 mg/L,[30] and the detection of abietic acid in the WwTW effluent in this study indicates that they are also present in effluent from domestic sources. We report for the first time that the three resin acid structures tested were androgen receptor antagonists in both the anti-YAS and AR-CALUX screens, although they did not give a full response in the latter.

Using androgen receptor bioassay-directed analysis, this study has revealed that wastewater effluents of primarily domestic origin contain a complex mixture of compounds with AA activity. The germicides chlorophene and triclosan were identified as the predominant androgen receptor antagonists bioconcentrating in bile from fish exposed to one of the effluents, and these compounds are currently being investigated for antiandrogenic properties in further *in vivo* tests in fish. Further work is needed to replicate this study using independent exposures with this and other WwTW effluents. Antiandrogens can affect sexual development and function, and there is a reasonable likelihood that exposure to the mixtures of antiandrogens taken up into fish may result in disruption of sexual differentiation or exacerbate the feminizing effects of estrogen exposure in male fish. This would have significant implications for risk assessment of wastewater effluents on aquatic wildlife, particularly as many (UK) WwTW effluents contain AA activity.[5] In this study germicidal chemicals were the predominant bioavailable antiandrogens identified in fish exposed to a domestic wastewater; however, it is likely that the profile of bioavailable AA contaminants will differ in other effluent types. As an example, the resin acids detected in our study are likely to contribute significantly to AA activity in fish exposed to paper mill effluents. Recent work has revealed that the principal contaminants with AA activity in effluents from oil production platforms were napthenic acids, PAHs, and alkylphenols.[31] Thus the nature of bioavailable antiandrogens present in contaminated fish are likely to be complex and variable reflecting the nature and sources of the effluent. Adding to concern about antiandrogens, a growing number of environmental chemicals are being recognized to demonstrate androgen receptor antagonist activity.[32,33] It should also be highlighted that to date bioassay-directed analytical studies used to identify environmental antiandrogens have relied on human androgen receptor transcription screens, and the parity between the fish and the human androgen receptor in affinity for environmental chemicals still remains to be investigated. Our work demonstrates the importance of establishing the full spectrum of bioavailable endocrine disrupting chemicals present in the environment, and highlights further the need for testing environmentally relevant mixtures for establishing their potential for deleterious effects on fish reproductive health.

■ ASSOCIATED CONTENT

Supporting Information. Additional information available as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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