SUPPORTING INFORMATION

Bioassay- directed identification of novel antiandrogenic compounds in bile of fish exposed to wastewater effluents

Pawel Rostkowski[1], Julia Horwood[1], Janice Shears[2], Anke Lange[2], Francis Oladapo[1], Harrie T. Besselink[3], Charles R. Tyler[2] and Elizabeth M. Hill[1]*

[1]University of Sussex, Dept. of Biology and Environmental Science, BN1 9QG Brighton, UK

[2]School of Biosciences, University of Exeter, EX4 4PS Exeter, UK

[3]BioDetection Systems BV (BDS), Science Park 406, 1098 XH Amsterdam, Netherlands

*Corresponding author phone: 44-01273-67-8382; fax: 44-01273- 877586

E-mail: E.M.Hill@sussex.ac.uk.

This supporting information contains:

Materials

The Oasis HLB, MCX and WAX cartridges were purchased from Waters Corporation, Milford, MA, USA. Glass wool was purchased from Fisher Scientific, United Kingdom. All solvents (HPLC grade) were purchased from Rathburn Chemicals, Scotland. [2,3,16,16-2H4]estrone (E1-d4, > 98% D atom), testosterone-1,2-d2 (T2-d2, > 98% D atom) and 13C labelled 4,4'-DDE (ring 13C -12, 99%) were purchased from Cambridge Isotope Laboratories Inc. (MA, USA). Dichlorophene (DCP), vinclozolin (Vz), acetic acid (≥ 99% pure), triclosan (≥ 97% purity), L-histidine, L-adenine, L-valine and L-serine, iron(III)sulphate hydrate, potassium phosphate (monobasic), magnesium sulphate heptahydrate, flutamide, 5α-dihydrotestosterone, bisphenol-A, p,p'-dichloro-2,2-bis(p-chlorophenyl)ethylene (p,p'-DDE), 3,3',4,4'-tetrachlorobiphenyl (PCB-77) and 2,2',3,4,4',5-hexachlorobiphenyl (PCB-138), technical nonylphenol, estriol, 11-ketotestesterone, testosterone, estrone, 17β-estradiol, oxybenzone, 1-naphthol, 2-naphthol, chloroxylenol, 3-α-hydroxy-5-β-androstane-11,17-dione, 2,2'-dihydroxybiphenyl, pyridine, 4-chlorophenoxyphenol, methoxyamine hydrochloride, mixture of N,O-Bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA), bicalutamide, abietic acid, isopimaric acid and neoabietic acid were purchased from Sigma, Gillingham, UK. Chlorophene was purchased from Tokyo Chemical Industries (UK). Pimaric acid was purchased from Caltag Medsystems, UK. L-phenylalanine was purchased from MP Biomedicals, Cambridge, United Kingdom. All other amino acids (L-leucine, L-arginine-hydrochloride, L-methionine, L-tyrosine, L-isoleucine, L-lysine-hydrochloride, L-phenylalanine, L-glutamic acid,) were purchased from ICN, Aurora, Ohio, USA. Chlorophenol red-β-D-galactopyranoside (CPRG) was purchased from Roche Diagnostics Limited, Burgess Hill, West Sussex, United Kingdom. Deionised water was distilled from Elga UHQ Distiller System made by Elga Systems, Buckinghamshire, United Kingdom. Dimethyl sulfoxide (DMSO, 99.9% purity) was purchased from Fisher Scientific

UK Limited, Loughborough, United Kingdom. Polypropylene microtitre plates were purchased from Greiner Bio-One Ltd., Stonehouse, Gloucestershire, United Kingdom. The DNA recombinant yeast was provided by courtesy of Professor Sumpter, Brunel University, United Kingdom.

Extraction of bile and effluent samples by Oasis HLB.

Prior to extraction of AA compounds from bile samples, the Oasis HLB cartridge (6 cc/200 mg/30 µm) was preconditioned with 5 mL of hexane, 5 mL of dichloromethane, 5 mL of methanol and 5 mL of 1% acetic acid. Hydrolysed bile samples were passed through the cartridge which was then washed with HPLC water, dried under vacuum and eluted with 5 mL of methanol, 5 mL of dichloromethane and 5 mL of hexane. The same SPE protocol was used for wastewater samples except that Oasis HLB (20 cc/1 g/60 µm) cartridges were used which were conditioned and eluted with 10 mL of solvent. The solvent eluents from SPE were combined for further analysis. This efficiency of SPE was tested using trout bile spiked with standard androgens and antiandrogens (see below). 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 Oasis WAX (3 cc/60 mg/30 µm) (mixed mode weak anion exchange) followed by the Oasis MCX (3 cc/60 mg/30 µm) (mixed mode cation exchange) SPE (see below). 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.

Testing recoveries of standards from Oasis HLB.

Six composite samples (100 µL volume each) were formed from bile of control 1 year old trout. Three of them were spiked with 500 ng of: p,p'-DDE, flutamide, dichlorophene (DCP), dihydrotesterone (DHT), bisphenol A (BPA) and testosterone (T2). Bile samples were then

deconjugated (as described elsewhere – see main text for reference) and extracted using Oasis HLB (as described above). After the extraction, samples were spiked with 500 ng of internal standards: ^{13}C- DDE, bicalutamide, 2,2'-dihydroxybiphenol, 4-chlorophenoxyphenol, deuterated testosterone (T2- *d2*). Then the samples were dried down, and first derivatized by the addition of 50 μL methoxyamine solution (2% in pyridine) and heating for 1 hour at 80 ^{o}C to protect ketonic groups present in steroidal compounds (DHT and T2) by creating their methyloxime esters. In the next step the solvent was removed under nitrogen. All samples were then silylated, as described below and analysed by the GC-MS. Quantitation was carried out by determining the response factor of the target analyte to its respective internal standard and comparing this to a standard curve. Concentrations of analytes in the spiked samples were corrected for any amounts detected in the non-spiked bile samples. Only T2 was detected in non-spiked bile samples at a concentration of 6.0 ± 0.8 ng / 100 μL of bile. Recoveries of test compounds are presented in Table S2.

Extraction of anionic or cationic antiandrogens in bile extracts using SPE with Oasis WAX and MCX

All loadings and washes from the extraction of bile samples with Oasis HLB (with the pH set to 4) were passed through the Oasis WAX first and the subsequent loadings were passed through OASIS MCX to extract any remaining ionic antiandrogens not retained by Oasis HLB. Both WAX and MCX cartridges were preconditioned with 4 mL of MeOH followed by 4 mL of water. After sample loading, the cartridges were washed with 2 mL of 2% formic acid, dried and eluted with 4 mL of methanol and 4 mL of 5% NH_4OH in methanol. Eluates were tested for AA activity in the anti-YAS. Using this extraction methodology, tests with model compounds revealed that recoveries of carbaryl, alachlor, 4-nitrophenyl beta-D-glucuronide, potassium 4-nitrophenyl sulphate, and (1S)-(+)-10-camphorsulfonic acid were between 72-90% on WAX SPE and recovery of atrazine on MCX was 102%

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 a Waters Ltd. System. This comprised a model 600 pump and controller, 717 autosampler and 996 photodiode array detector. Samples were fractionated on a Waters Sunfire C18 analytical column (3.5 µm particle size, 4.6 x 150 mm) and guard column (3.5 µm, 4.6 × 20 mm) with water (0.2% acetic acid) and acetonitrile (0.2% acetic acid). The solvent system (water: acetonitrile ratio) was operated on a gradient programme: 0 minute (90:10), 10 minutes (70:30), 65 minutes (0:100) and 80 minutes (0:100) at room temperature at a flow of 1 mL/min. The gradient programme was optimised to elute compounds with a wide range of polarities from log Kow 2.8 to 7.0 and retention times of model compounds were: β-estriol 17 min, 11-ketotestosterone 20 min, atrazine 25 min, testosterone 27 min, 17β-estradiol 27 min, bisphenol A 27 min, estrone 30 min, dichlorophene 35 min, technical 4-nonylphenol 52 min, PCB-77 and 4,4-DDE at 61 min. 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 mins) and retested in AntiYAS.

GC-MS analysis.

30 ng of an internal standard of deuterated E_1, [2,3,16,16-$^2H_4$]estrone, (Cambridge Isotope Laboratories, Andover, MA) was added to each of the HPLC fractions containing AA activity, and the samples were dried down under nitrogen. Fractions were then silylated by the addition of 30 µL bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 30 µL pyridine and

heating for 30 min at 65 ^{o}C. The sample was dried down under nitrogen up to the volume of 5 µL injected manually into the GC. Samples were analysed on a Trace GC (Thermoquest, Texas, USA) fitted with a 30 m Zebron ZB-5MS fused silica capillary column (30 m x 0.25 mm x 0.25 µm film thickness with 5 m guard column), connected to Polaris-Q ion trap mass spectrometer (Thermo, Texas, USA). The carrier gas was helium, at a constant flow rate of 1.5 mL/min, with the sample introduced using a 1 µL splitless injection. The injection port and the GC-MS transfer line were heated to 280 ^{o}C. The MS detector was used in full scan electron ionization mode with an ion range from 50 to 650 m/z. The source temperature was 250 ^{o}C and the electron energy of 70 eV. The oven programme was 70 ^{o}C for 2 min, increasing by 8 ^{o}C/min to 264 ^{o}C, held at 264 ^{o}C for 10 min, increasing by 10 ^{o}C/min to 300 ^{o}C and held at 300 ^{o}C for 5min. GC-MS spectra were analysed on Xcalibur v1.2 software (Thermoquest-Finnigan) first, then with IXCR (ACD Labs) spectra were deconvoluted and compared with the Wiley Registry of Mass Spectra 9th Edition, National Institute of Standards and Technology (NIST) MS library (version 2008) and custom made libraries of pure silylated standards. Identified compounds were quantified with a four point linear regression calibration curve using a ratio of internal standard and selected ions (most abundant in the spectrum) for each compound of interest.

Table S1. Details of WwTWs used in the studies.

WwTW	Date Sampled	Influent population equivalent	Discharge flow (m^3/day)	Average residence time (hours)	Influent characteristics	Level of Treatment
WwTW A	April/09	107,250	12,960-49,248	12	Influent is 95% domestic, other 5% (pharmaceuticals, landfill site, electroplating, commercial vehicle wash, brewery, swimming pool).	Primary treatment (2 units, 2 hours retention time) followed by a two stage Biological Aerated Filter: carbonaceous BAF (3 units) nitrifying BAF (3 units), humus tanks and sand filters (2 cells, 2.5 hours retention time).
WwTW B	April 2010	47,200	13,590-17,000	6	Influent is 92.5% domestic, and other 7.5% (commercial vehicle cleaning, pharmaceutical, fish processing, laundering).	Primary treatment (2 tanks with 2 hours retention time) followed by a two stage Biological Aerated Filters (BAF): carbonaceous BAF (1 tank with 2.3 hour retention time) followed by nitrifying BAF (1-tank, 0.8 hours) followed by a denitrification stage (4 tanks, for 0.13 hours).
WwTW C	July 2010	142,370	52,000-74,500	12	Influent is 99.6 % domestic, and 0.4% other (commercial vehicle cleaning, laundering, electroplating, plastic manufacturing, photographic development, meat processing and electronic circuit board manufacturing).	Primary treatment followed by secondary treatment processes which include two percolating filter works which treat 50% of the total flow, the other 50% is treated in fine diffused activated sludge plant. Final effluent is a mixture of two streams.

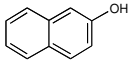
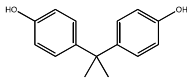
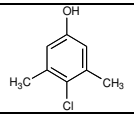
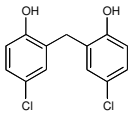
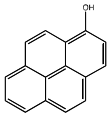
Table S2. Recoveries of standards spiked into control bile samples and extracted with Oasis HLB (%).

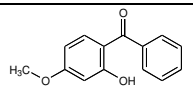
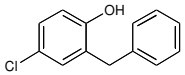
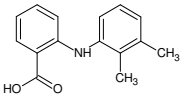
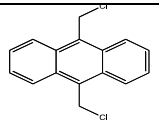
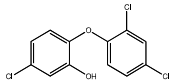
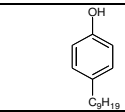
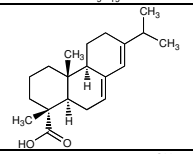
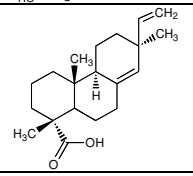
Sample name	DDE	DHT	T2	flutamide	BPA	DCP
bile replicate 1	90	81	86	91	84	72
bile replicate 2	89	82	89	85	82	76
bile replicate 3	85	84	85	92	78	75
mean	88	82	87	89	81	74
SD	3	2	2	4	3	2

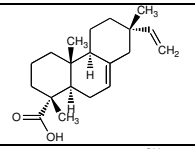
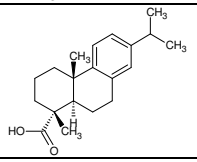
DDE: p,p'dichlorodiphenyldichloroethylene, DHT: dihydrotestosterone, T2: testosterone. BPA: bisphenol A, DCP: dichlorophene.

Recoveries of analytes in the spiked samples were calculated after subtraction for any amounts detected in the non-spiked bile samples. Only T2 was detected in non-spiked bile samples at a concentration of 6.0 ± 0.8 ng / 100 µL of bile.

Table S3. Antiandrogenic compounds and other xenobiotics identified in the bile of mature 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.

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)	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)	Contrib. of AA activity of the identified compound to total activity of the fraction (%)	Contrib. of the AA activity of the identified compound to total activity of the bile (%)
1-naphthol	27		216, 201, 185	1.24	41.59	0.15	0.186	0.45	0.01
	28			16.44	7.35	0.15	2.47	33.6	0.13
2-naphthol	27		216, 201, 185	1.7	41.59	0.32	0.544	1.3	0.03
	28			0.7	7.35	0.32	0.224	3.05	0.01
bisphenol A	27		372, 357, 207, 191, 73	1.05	41.59	0.60	0.63	1.5	0.03
	28			1.5	7.35	0.60	0.9	12.24	0.05
chloroxylenol	32		228, 213, 172	16.8	26.11	0.16	2.69	10.3	0.15
Isomer of chlorobisphenol A	32		406, 393, 391, 73	NM	26.11	SA*	-	-	-
dichlorophene	35		412,414,377,379, 73	0.2	36.35	4.70	0.94	2.59	0.05
	36			6	48.16	4.70	28.2	58.5	1.5
2 isomeric structures of dichlorobisphenol A	35		440, 425, 241, 73	NM	36.35	SA	-	-	-
1-hydroxypyrene	39		290, 275, 259	6.53	68.10	9.90**	64.65	94.9	3.52
Unknown hydroxypyrene isomer	39		290, 275	NM	68.10	SA	-		-

oxybenzone	40		300, 299, 283. 225, 73	1.1	521.10	0.42	0.462	0.08	0.02
	41			2.5	145.34	0.34	0.85	0.58	0.046
chlorophene	40		292, 290, 277, 275	37.33	521.10	13.00	485.29	93.13	26.42
	41			0.7	145.34	13.00	9.1	6.26	0.495
Mefenamic acid	41		313, 298, 223, 208, 180	1.45	145.34	Toxic in the anti-YAS	-	-	-
Diclosan like compound	42		330, 328, 326,315, 313, 311,278,276, 202, 200	NM	96.23	SA	-	-	-
Methoxy metabolite of chlorophene			320, 305, 290, 255	NM	96.23	SA	-	-	-
9,10-di(chloromethyl)anthracene	43		274, 276,239,241, 203, 204	10.42	45.40	2.5	26.05	57.38	1.42
triclosan	46		364, 362, 360, 349, 347, 310, 312, 345, 202, 200	58.9	264.20	4.80	282.72	107.0	15.4
4-nonylphenol	52		292,263, 221, 193, 179,73	6.41	2.15	0.30	1.923	89.44	0.1
abietic acid	59		374, 359, 256, 241	4.9	74.91	4.00	19.6	26.16	1.07
pimaric acid	59		374, 359, 256, 241	4.57	74.91	2.73	12.47	16.65	0.68

isopimaric acid	59		359, 256, 241	7.9	74.91	5.00	39.5	52.7	2.15
Dehydroabietic acid	59		372, 357, 239	NM	74.91	SA	-	-	-
Unknown isomer of resin acids	59		374, 359, 256, 241	NM	74.91	SA	-	-	-
Unknown isomer of resin acids	59		374, 359, 256, 241	NM	74.91	SA	-	-	-

Data is based on the analysis of a composite bile from trout exposed to effluent in one tank. The sum of all the compounds identified and tested in the anti-YAS accounted for 53.28% of the total AA activity measured in all the bile fractions. The LOD of anti-YAS = 7 µg FEq/mL of bile; SA – standard not available; NM – not measured

** Antiandrogenic potency possibly overestimated due to toxicity in anti-YAS.

Compounds in italics indicate that AA activity could not be tested due to lack of availability of commercial standards.

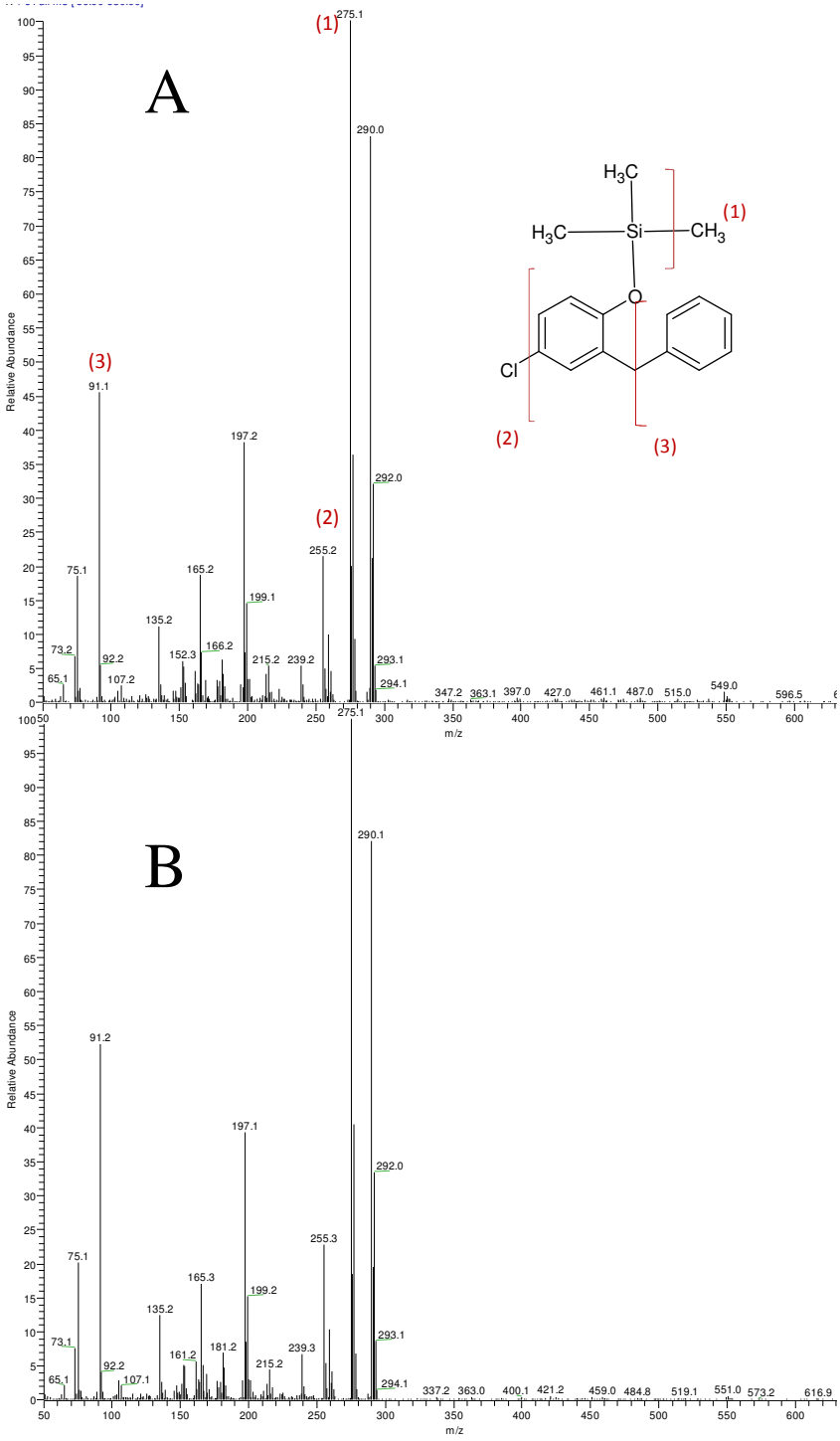


Figure S1A. A) Mass spectra of derivatized chlorophene-TMS identified in fraction 40 at the retention time of 19.32 min. B) Mass spectra of derivatized pure commercially available standard of chlorophene.

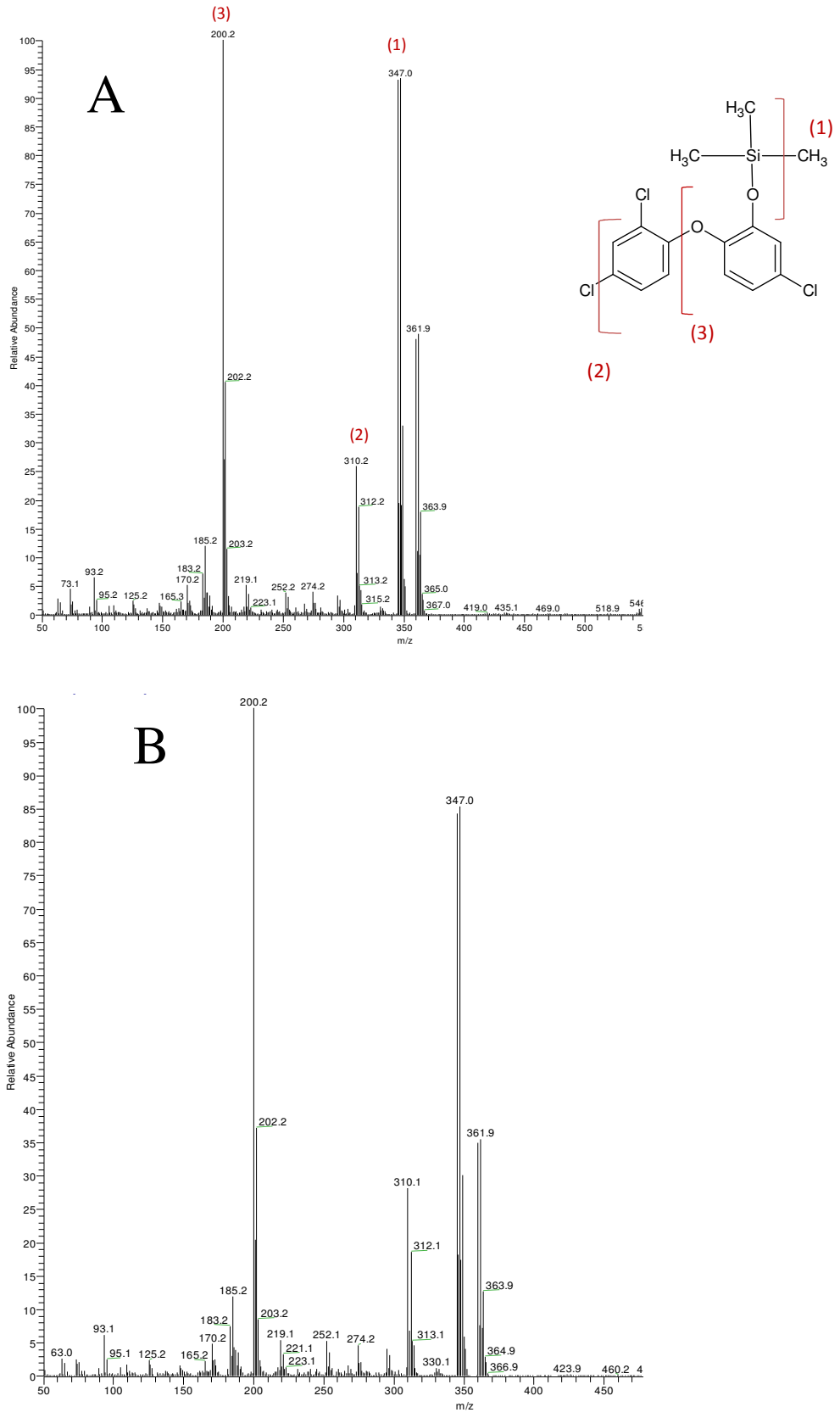
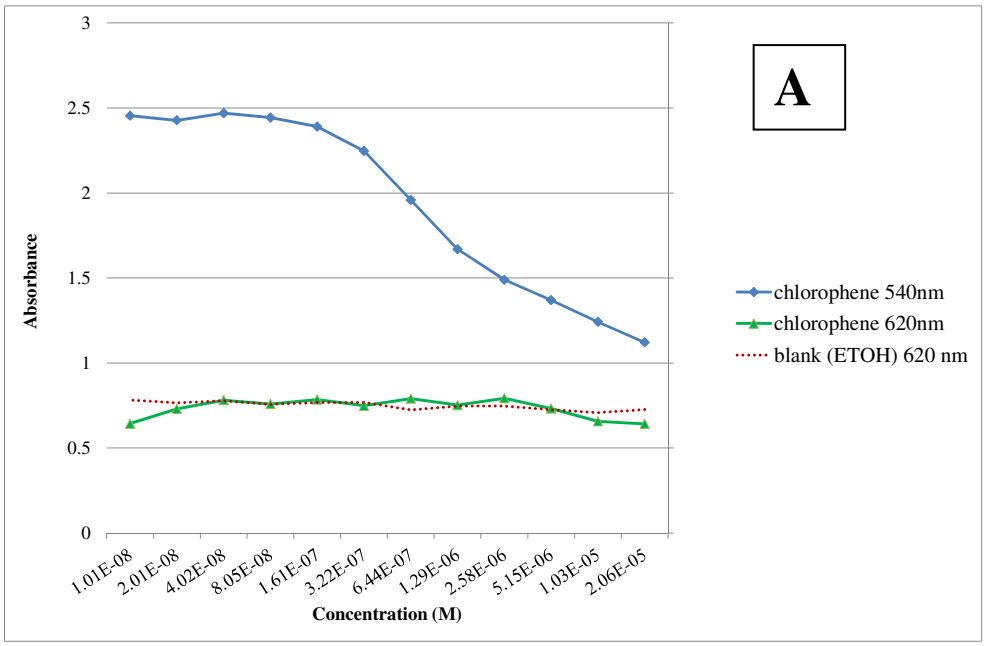


Figure S1B. A) Mass spectra of derivatized triclosan-TMS identified in fraction 40 at the retention time of 21. min. B) Mass spectra of derivatized pure commercially available standard of triclosan.



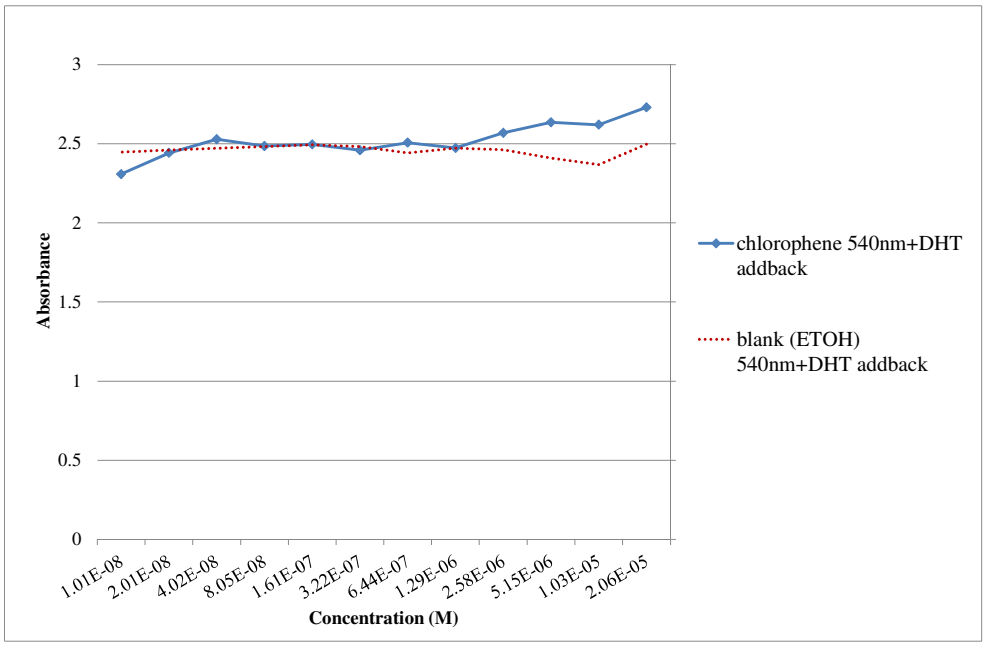


Figure S2A. Dose response curves of chlorophene the YAS: A) Response of pure analytical standard of chlorophene at 540 nm in the presence of DHT agonist (1.3 ng/mL) in the media, and at 620 nm without DHT. B) Response of pure analytical standard of chlorophene at 540 nm 30-hours after additional DHT agonist (27.4 ng/mL) added to incubations.

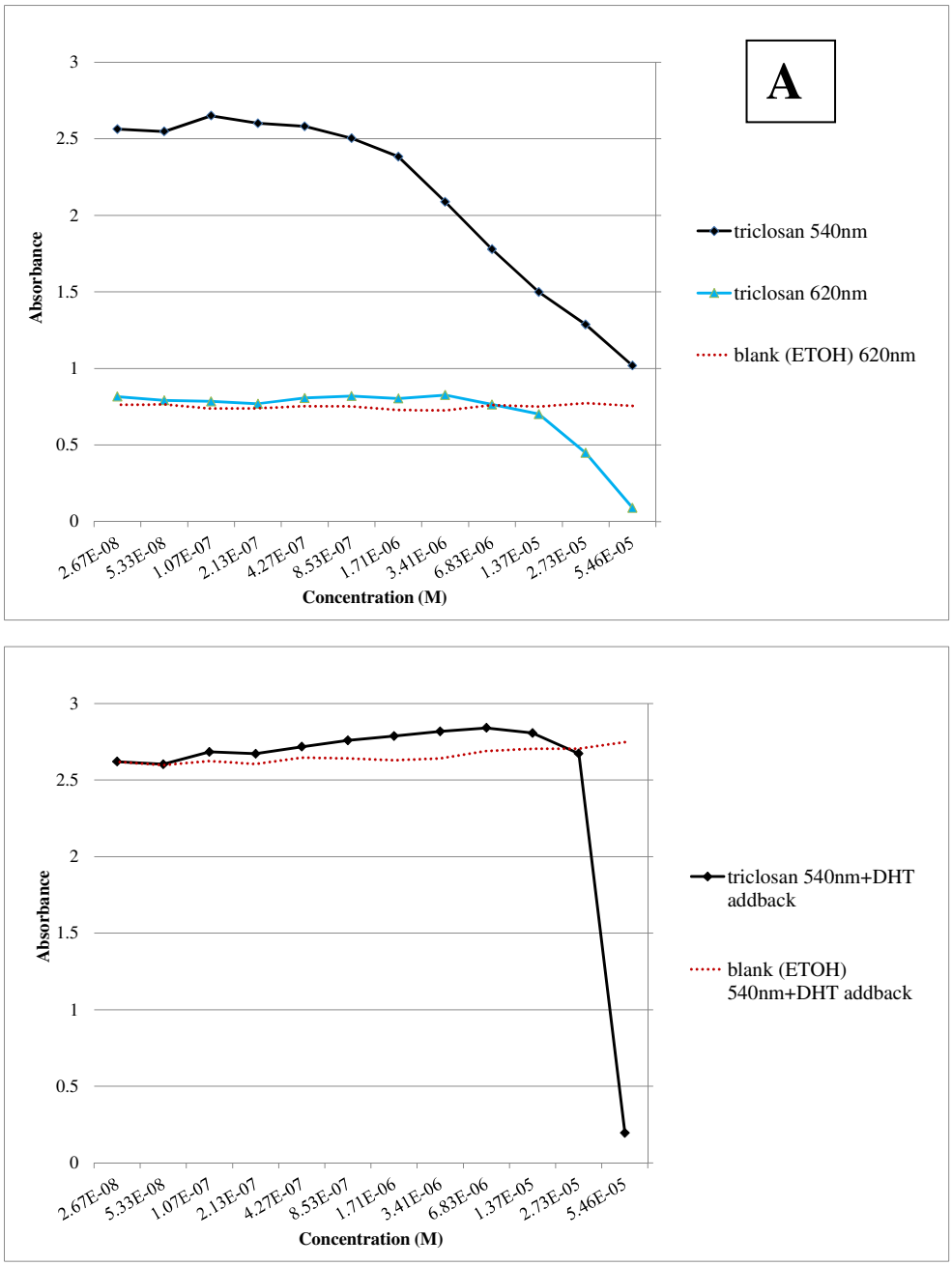


Figure S2B. Dose response curves of triclosan in the YAS: A) Response of pure analytical standard of triclosan at 540 nm in the presence of DHT agonist (1.3 ng/mL) in the media, and at 620 nm without DHT. B) Response of analytical standard of triclosan at 540 nm 30-hours after additional DHT agonist (27.4 ng/mL) added to incubations.

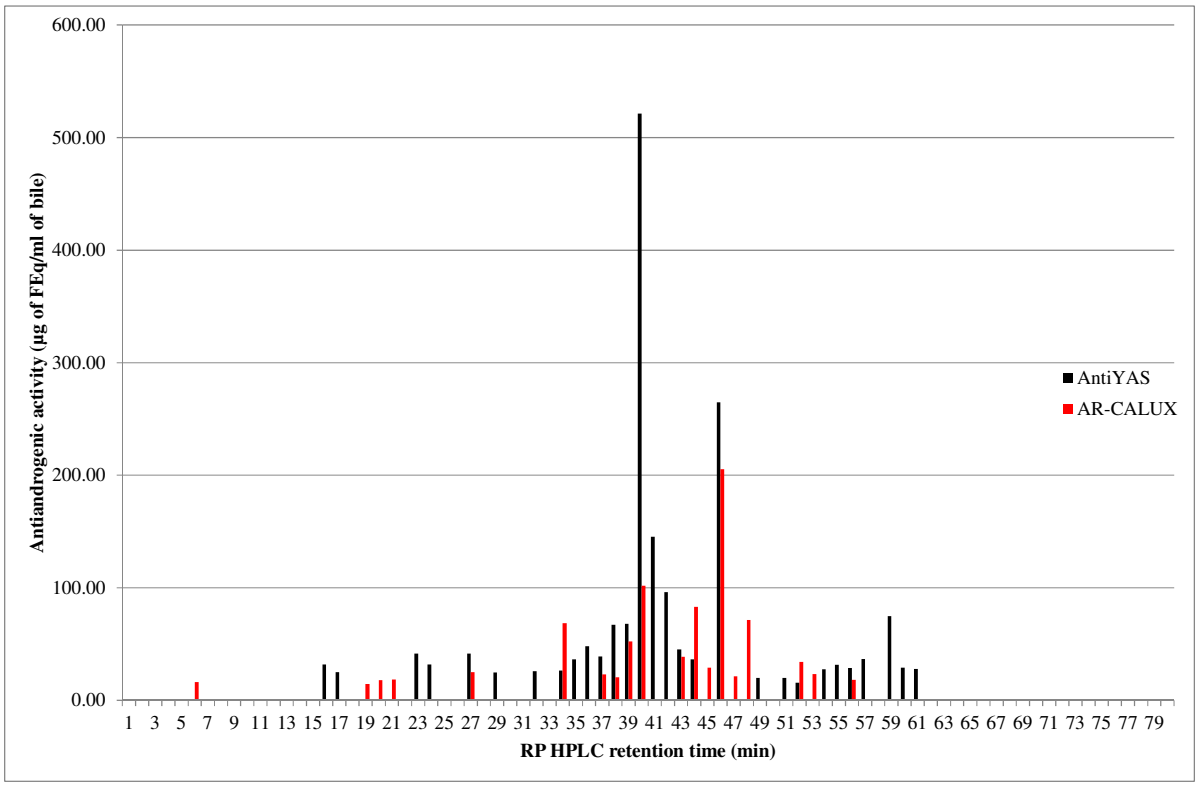


Figure S3. Representative profiles of the antiandrogenic activity present in HPLC fractions of bile extracts from WwTW C effluent-exposed 2 year old trout. Fractions detected in AR-CALUX only: 6, 19, 20, 34, 45, 47, 48, 52, 53, 56. Fractions detected in anti-YAS only: 16, 17, 23, 24, 29, 32, 35, 36, 41, 42, 49, 51, 54, 55, 57, 59, 60, 61.

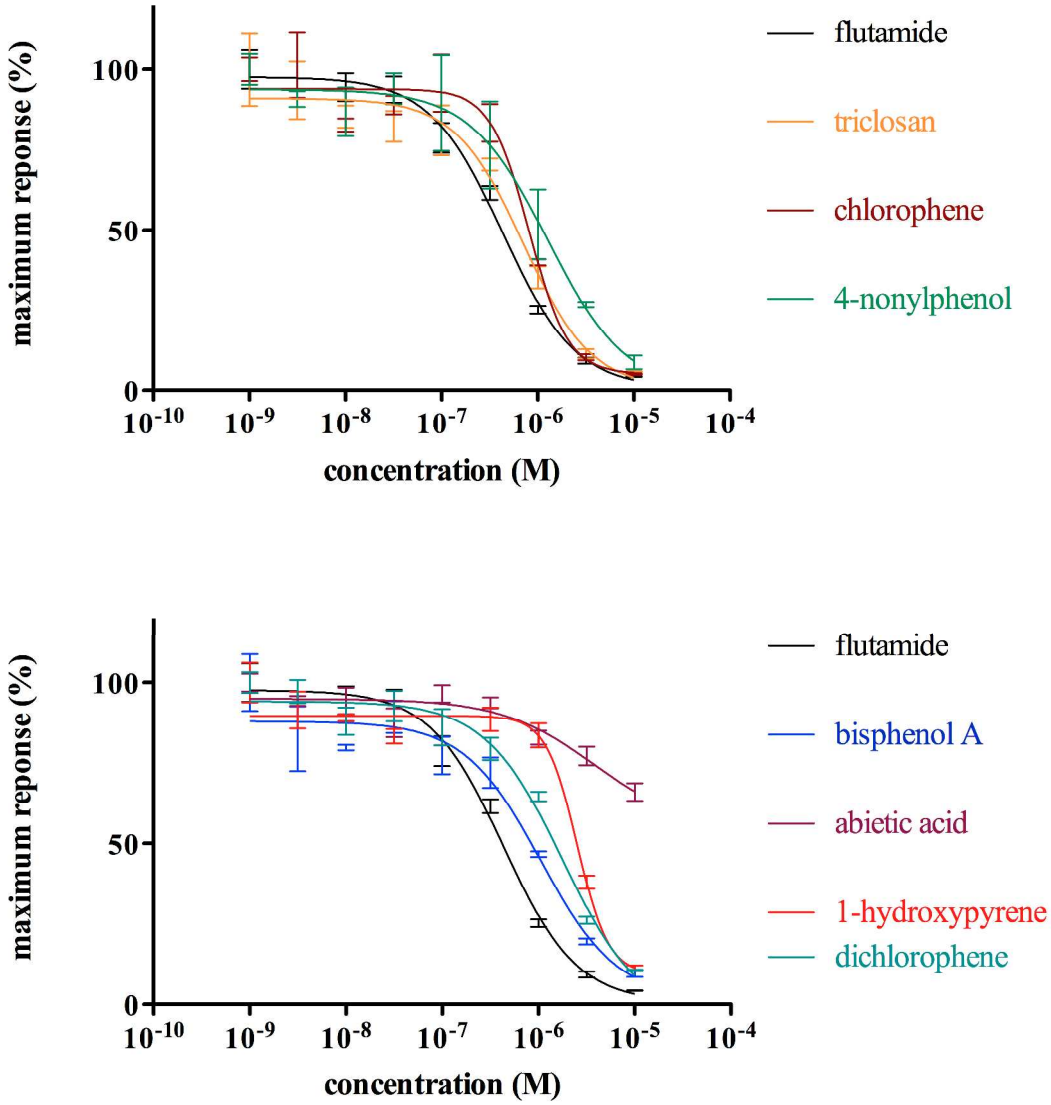


Figure S4. Dose-response curves of pure standard compounds tested in the AR-CALUX. Mean ± s.d IC_{50} values (n=3) of pure standard compounds are given in brackets (M): flutamide ($4.4 \times 10^{-7} \pm 1.8 \times 10^{-8}$), abietic acid ($5.3 \times 10^{-6} \pm 4.1 \times 10^{-7}$), triclosan ($7.2 \times 10^{-7} \pm 3.5 \times 10^{-8}$), hydroxypyrene ($2 \times 10^{-6} \pm 1 \times 10^{-7}$), chlorophene ($8.2 \times 10^{-7} \pm 2.4 \times 10^{-8}$), dichlorophene ($1.9 \times 10^{-6} \pm 1.5 \times 10^{-7}$), 4-nonylphenol ($1.3 \times 10^{-6} \pm 6.3 \times 10^{-8}$), bisphenol A ($1.1 \times 10^{-6} \pm 5.3 \times 10^{-8}$). None of the test chemicals exhibited toxicity in the AR-CALUX as determined by detachment of osteosarcoma cell cultures from the multiwell plate surface.