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Methodology for profiling anti-androgen mixtures in river water using multiple passive samplers and bioassay-directed analyses

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ABSTRACT

The identification of endocrine disrupting chemicals in surface waters is challenging as they comprise a variety of structures which are often present at nanomolar concentrations and are temporally highly variable. Hence, a holistic passive sampling approach can be an efficient technique to overcome these limitations. In this study, a combination of 4 different passive samplers used for sampling polar (POCIS A_{pharm} and POCIS $B_{pesticide}$) and apolar compounds (LDPE low density polyethylene membranes, and silicone strips) were used to profile anti-androgenic activity present in river water contaminated by a wastewater effluent. Extracts of passive samplers were analysed using HPLC fractionation in combination with an *in vitro* androgen receptor antagonist screen (YAS). Anti-androgenic activity was detected in extracts from silicone strips and POCIS A/B at (mean ± SD) 1.1 ± 0.1 and 0.55 ± 0.06 mg flutamide standard equivalents/sampler respectively, but was not detected in LDPE sampler extracts. POCIS samplers revealed higher selectivity for more polar anti-androgenic HPLC fractions compared with silicone strips. Over 31 contaminants were identified which showed inhibition of YAS activity and were potential anti-androgens, and these included fungicides, germicides, flame retardants and pharmaceuticals. This study reveals that passive sampling, using a combination of POCIS A and silicone samplers, is a promising tool for screening complex mixture of anti-androgenic contaminants present in surface waters, with the potential to identify new and emerging structures with endocrine disrupting activity.

1. Introduction

Contamination of natural waters is a major concern in many parts of the world, and there is a limited understanding of the toxicological consequences of pollution of surface waters through discharges of wastewater effluents. Many emerging contaminants originate from human use, and are still present in treated effluents from wastewater treatment plants (WWTPs). Aquatic monitoring is an on-going challenge and a key issue is to identify the most important biologically active compounds currently not covered by existing water-quality regulations, and which have the potential to cause

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deleterious health effects to aquatic biota (Snyder and Benotti, 2010; Soffker and Tyler, 2012). Amongst emerging pollutants, Endocrine Disrupting Compounds (EDCs) appear to be particularly prevalent in the aquatic environment, and some aquatic animals are highly susceptible to their effects as they can be continually exposed to these contaminants through discharges of WWTP effluents, and these exposures can be life-long (Jobling et al., 2006; Liney et al., 2006). Future concentrations of EDCs may increase in certain river catchments due to climate change resulting in changes in hydrology and high demands on limited water resources.

Thus far, the identification of EDCs in aquatic environments has been mostly focused on estrogenic compounds, but a recent UK survey study has revealed that the majority of the investigated WWTP effluents contained anti-androgenic (AA) as well as estrogenic activity. In addition, the observed feminisation of wild fish (roach, *Rutilus rutilus*) in downstream waters was correlated with exposure to both AA activity and estrogen levels or with AA activity alone (Jobling et al., 2009). Reports of AA activity in sediments, water and fish of European rivers have already been described suggesting their presence in the aquatic environment could be widespread (Hill et al., 2010; Urbatzka et al., 2007; Weiss et al., 2009), however in many cases the identities of AA structures still remain to be elucidated. Anti-androgens can bind to the androgen receptor (AR), but are unable to activate it (AR antagonism). The structures of chemicals containing androgen receptor antagonist properties can be extremely diverse (Rostkowski et al., 2011; Vinggaard et al., 2008) and it is therefore important to use methods which do not make any assumptions as to the nature of the chemicals involved. However, the identification of biologically active compounds in surface waters or treated effluents can be problematic, since they are present at ultra-trace levels (often 1–100 ng/L) and encompass a variety of chemical classes differing significantly in physical–chemical properties. Thus their identification may require sensitive analytical techniques, intensive sampling programs and large sample volumes (Focazio et al., 2008; Schultz et al., 2010). To overcome these limitations, the use of a holistic passive sampling approach to screen for AA contaminants in surface waters could be an efficient alternative to grab sampling. The use of a combination of different passive samplers would allow sampling of a wide range of chemical polarities with a significant pre-concentration of contaminants from surface waters (Mills et al., 2011; Tapie et al., 2011). Moreover, passive samplers can provide an integrative sample of mixtures of environmental contaminants over an exposure period and permit the sequestration of residues from episodic events that are not always detected with grab sampling. Currently available passive sampling devices are only able to efficiently sample a limited polarity range. Since AA compounds in effluents are a complex mixture of hydrophilic and lipophilic chemicals (Rostkowski et al., 2011), a combination of different passive samplers covering the broadest range of log K_{ow} (Vrana et al., 2005) must be used to guarantee an efficient sampling of the whole array of anti-androgens that are potentially present in the aquatic environment.

In this study, 4 different passive samplers were investigated for their ability to sample AA activity present in contaminated surface waters. Two types of Polar Organic Chemical Integrative Samplers (POCIS; POCIS A designed for pharmaceuticals and POCIS B for pesticides) were used for covering the polar log K_{ow} range, whilst silicone strips and low density polyethylene (LDPE) membranes were selected for sampling any apolar components contributing to AA activity. The POCIS samplers contain a sorbent phase sandwiched between two microporous polyethersulphone (PES) membranes. Chemicals diffuse from the water and adsorb onto the sorbent phase (i.e. OASIS HLB for POCIS A or a triphasic mixture for POCIS B) from which they can be extracted after deployment. The use of POCIS to investigate the presence of phenolic estrogens as well as a variety of pharmaceuticals in rivers or effluents is well established (Liscio et al., 2009; Morin et al., 2012; Rujiralai et al., 2011; Vallejo et al., 2013). LDPE and silicone are single phase samplers which allow the uptake of hydrophobic chemicals, where the driving force for analyte uptake by the sampler is the chemical activity gradient between the polymer and the sampled medium (Rusina et al., 2010b). Single phase LDPE and silicone material have largely replaced traditional semi-permeable membrane devices, and are widely used as passive sampling devices for assessing non-polar organic compounds (log $K_{ow} \geq 4$) in aquatic environments including chlorinated EDCs (Allan et al., 2009; Sacks and Lohmann, 2011).

Three questions were investigated in this study: a) Are there differences between the concentrations of AA activity sampled by the POCIS and single membrane passive sampling devices? b) How do the profiles of AA activity sampled by the different devices differ from each other and from a representative profile of AA activity present in grab samples of the water phase taken during the deployment period? c) Which of the passive sampling devices, or combinations thereof, are most suitable to screen the variety of contaminants with potential AA activity that are present in effluent-contaminated water?

In this study, four canisters, each of them containing all the four different sampling devices, were deployed for two weeks in river water 200 m downstream a domestic sewage effluent. Organic chemicals in extracts obtained from the passive samplers were analysed by a yeast recombinant androgen receptor transcription screen (YAS) to investigate the sampled amount of AA activity in each sampler type. The profiles and identification of some structures of potential anti-androgens were investigated using a bioassay-directed fractionation approach. Extracts of passive samplers were fractionated by HPLC and the fractions analysed by YAS. Contaminants present in fractions containing AA activity were identified by mass spectrometry techniques (GC–MS or LC-QTOFMS). Where available, commercial standards of putatively identified contaminants were tested for AA activity in YAS and used to confirm structural identity by comparison with retention time and mass spectral data.

2. Material and methods

2.1. Passive sampling devices

POCIS samplers were obtained from Environmental Sampling Technologies Inc, St. Joseph, USA. POCIS A contained 200 mg

of Oasis HLB as sorbent within two polyethersulfone (PES) membranes. The sorbent of POCIS B was 200 mg of a triphasic admixture; 80:20 (w/w) Isolute ENV+: Ambersorb 1500 carbon dispersed on S-X3 Bio Beads that was contained between two PES membranes. LDPE membranes (60 cm long; 2.5 cm wide) were prepared from lay flat tubing purchased from John Darvell Packaging Limited, Medmenham Marlow, UK. Silicone strips were made from AlteSil™ Silicone sheets obtained from Altec Products Limited, Bude, UK, and were of similar dimensions to LDPE membranes. The characteristics of the samplers are given in Table 1. Single-phase LDPE and silicone samplers were first precleaned by soaking them in ethyl acetate overnight in order to remove any background contamination before deployment. All prepared samplers were stored at −20 °C before transport to the field site. Field control samplers were prepared and transported in a similar way to exposed samplers and opened to the air during deployment and retrieval procedures. During deployment, controls were stored at −20 °C in closed containers.

2.2. Evaluation of extraction methodology of single-phase passive samplers

The extraction methodology for POCIS samplers is well established (Alvarez et al., 2008), however single phase passive samplers (LDPE membranes and silicone strips) have been used for targeted sampling of known hydrophobic compounds rather than for screening purposes. For this reason, the extraction methodology for single-phase devices was optimised in order to fulfil the requirements of this study. Solvent extraction methods were investigated for both LDPE membranes and silicone strips employing a standard mixture of selected compounds covering a wide range of polarities (Details are given in the Supplementary Information, SI, and Table S-1). Briefly, replicates of the two single-phase devices were spiked directly with the standard mixture in MeOH onto the sampler. Once the carrier solvent had dried overnight, the samplers were extracted with 25 mL of dichloromethane/hexane/ethyl acetate (DCM/Hex/EtAc 1:1:1 v/v), followed by a second extraction with 100% methanol (MeOH, 25 mL). The extracts were analysed by GC–MS, and revealed that recoveries of spiked compounds from both samplers in the DCM/Hex/EtAc solvent mixture ranged between 21 and 98%, depending on compound polarity, and an additional extraction with MeOH resulted in further recoveries of between 0 and 17% (Fig. S-1). Using both solvents, recoveries of all the test compounds with log K_{ow} > 2.0 from both sampler types were between 74% and 100%. Hence, extraction of these samplers after field deployment was performed with 25 mL of DCM/Hex/EtAc 1:1:1 (v/v), followed by a final extraction with 100% MeOH (25 mL).

2.3. Field site and sampling strategy

Passive samplers were deployed for a two week period between September and October 2010 at a river site situated 200 m downstream a WWTP effluent discharge in the South-East of England. The WWTP had an influent population equivalent of 107,250 and the influent source comprised 95% domestic inputs. The remaining industrial inputs came from

Table 1 – Properties of passive samplers and comparisons of concentrations of anti-androgenic activity detected in the different devices.

		Single-phase devices		*Biphasic devices*	
		Silicone ($n = 8$)	LDPE ($n = 8$)	POCIS A_{pharm} ($n = 4$)	POCIS $B_{pesticide}$ ($n = 2$)[d]
Membrane material		Silicone	LDPE	PES	PES
Polymer thickness		500 μm	127 μm	130 μm	130 μm
PES membrane pore size		–	–	0.1 μm	0.1 μm
Receiving phase material				OASIS HLB®	Triphasic mixture
Surface area (cm^2)		300	300	41	41
Absolute AA Activity (mg FEq/sampler)	Solvent 1	1.08 ± 0.10	<LOD	0.55 ± 0.06	0.55 ± 0.03
	Solvent 2	<LOD	<LOD	<LOD	<LOD
	Solvent 3	–	–	<LOD	<LOD
Total AA Activity per sampler area (μg FEq/cm^2)	Solvent 1	3.60 ± 0.33[a]	<LOD[b]	13.41 ± 1.76[c]	12.94 ± 0.70[c]
Limit of detection (LOD) (mg FEq/sampler)		0.084	0.084	0.084	0.084
LOD per sampler area (μg FEq/cm^2)		0.28	0.28	2.03	2.03

n = total replicates from the 4 canisters.
PES: polyethersulfone, Triphasic mixture: Isolute ENV+/Ambersorb 572/S-X3 Bio-Beads (200–400 mesh).
For Silicone strips and LDPE: Solvent 1: DCM/Hexane/Ethylacetate 1:1:1, additional extraction: Solvent 2: MeOH.
For POCIS A: Solvent 1: MeOH, additional extraction: Solvent 2: DCM and Solvent 3: Hexane.
For POCIS B: Solvent 1: DCM/MeOH/Toluene 8:1:1, additional extraction: Solvent 2: MeOH and Solvent 3: DCM/Hexane 1:4.
Total anti-androgenic activity reported as mean flutamide equivalents ± standard deviation. The mean and the standard deviation were calculated using the sampling replicates per sampling device.
[a,b,c] different letters indicate statistical significance at $P = 0.05$ calculated from Games-Howell post hoc test.
Field blanks were below LOD values.
[d] 2 sampling replicates as some extracts were lost during workup.

hospitals, a landfill site, electroplating, commercial vehicle washes, a brewery and a swimming pool. Discharge flow ranges were between 12,960 and 49,248 m^3/day and the average residence time in the WWTP was 12 h. The influent was treated by a primary treatment followed by a carbonaceous and then nitrifying biological aerated filter, a humus tank and sand filter. During this study, the values of pH, temperature, conductivity and flow velocity of the final effluent were in the range of 7.2–7.8, 15–18 °C, 555–829 μS, and 0.1–0.5 m/s, respectively. Samplers were mounted onto apposite holders in stainless steel canisters, and the four canisters, each of them containing all the four different devices, were deployed in line facing the river flow 200 m downstream of the effluent discharge. Detailed sampling configuration is provided in Fig. S-2 and the mean flow rate across all canisters over the 14 day period was 0.28 ± 0.06 m/s (Fig. S-3). Grab samples of ambient river water (2.5 L) were collected in solvent-rinsed glass containers at day 1, 4, 7, 9 and 14, respectively. On each sampling day, three replicates of water samples were taken across the river stream flowing in front of the samplers. Methanol (3%) and acetic acid (1%) were added to the water samples before storage overnight at 4 °C prior to processing.

2.4. Extraction of passive samplers and river water grab samples

At the end of the deployment period, the retrieved silicone strips and LDPE membranes were gently wiped with a damp paper tissue to remove biofilms. Samplers were extracted twice with 25 mL EtAc/Hex/DCM 1:1:1 (v/v) and the two extracts combined. An additional extraction with 25 mL MeOH was performed in order to estimate possible contribution of more polar compounds. Prior to processing, POCISs were allowed to reach room temperature and any remaining debris was rinsed away with deionised water. Each POCIS was carefully dismantled and deionised water was used to transfer the sorbent into a 1 cm i.d. glass syringe cartridge fitted with a Teflon frit and glass wool. POCIS A and B were extracted according manufacturer's instructions (50 mL of MeOH for POCIS A and DCM/Toluene/MeOH 8:1:1 for POCIS B, respectively). The adsorbent phases were further extracted with two different solvents (DCM followed by hexane for POCIS A and MeOH followed by DCM/Hex 1:4 for POCIS B) in order to confirm the recovery of the total AA activity from the POCIS. All extracts were dried down under nitrogen and recovered in 2 mL of the same initial extraction solvent before further investigation. Therefore similar solvents, or their mixtures, were used extract AA activity from the LDPE, silicone and POCIS samplers, but as the silicone and LDPE samplers favour extraction of hydrophobic compounds, then activity was extracted by non-polar solvents first followed by a polar solvent to ensure complete recovery of AA analytes. POCIS were first extracted by polar solvents followed by additional extractions with non-polar solvents to ensure all AA activity was recovered. Extraction of anti-androgens in river water grab samples was performed using two methods; by solid phase extraction (SPE) using Oasis HLB cartridges and by liquid–liquid extraction (LLE) to ensure extraction of the most apolar range of compounds. Details are described in the SI. Briefly, after loading with sample, SPE cartridges were eluted with MeOH, followed by DCM and Hex and the solvent eluents were combined prior to further analyses by YAS and HPLC fractionation. For LLE, 500 mL of water was extracted twice with 250 mL of ternary mixture DCM/Hex/EtAc 1:1:1, the same solvent used for extracting the single-phase devices. The organic phase was separated, and the extracts combined, evaporated to dryness and reconstituted in 1 mL of the extraction solvent.

2.5. RP-HPLC fractionation

Aliquots of standard mixtures and samples (passive sampling and grab sampling extracts) were dissolved in water:-acetonitrile (70:30 v/v) and filtered (0.2 μm) prior to injection. Using this sample preparation method, the recoveries of AA activity from sampler or water extracts were >90% which ensured that the majority of AA activity was injected onto the HPLC system. Samples (200 μL) were injected on a Waters Ltd. system comprising a model 600 pump and controller, 717 autosampler and 996 photodiode array detector. In reverse-phase mode, the system was equipped with a Kinetex C_{18} column (Phenomenex, 2.6 μm, 4.6 × 100 mm) and 2.6um Krud-Katcher Ultrafilter. The solvent system (water:acetonitrile ratio with 0.1% TFA) was operated with a gradient programme: 0 min (98:2), 2 min (98:2), 5 min (50: 50), 30 min (0: 100) and 50 min (0:100) at room temperature at a flow of 1 mL/min. HPLC fractions of sampler extracts were collected every minute, the solvent removed under vacuum and the residue reconstituted in 150 μL ethanol for further analysis. Some sampler extracts were also analysed in NP-HPLC mode, and the details of the chromatography methods are given in SI.

2.6. Analysis of anti-androgen activity

Androgenic and AA activities of sampler extracts and HPLC fractions were quantified using a recombinant yeast screen containing the human androgen receptor (YAS). Full details are given in SI. Briefly samples and pure standards were serially diluted in ethanol which was then evaporated to dryness before addition of culture media. To test for AA (i.e. receptor antagonist) activity, the agonist (5α-dihydrotestosterone, DHT) was added to the yeast medium at a concentration giving a 65% sub-maximal response of the assay. AA activity was quantified as flutamide standard equivalents (FEq) and androgenic activity as DHT standard equivalents (DHTEq). Samples showing toxicity which resulted in poor yeast growth (monitored at 620 nm in media without agonist, and in comparison with values from blank samples containing ethanol only) were serially diluted in order to allow quantification of any receptor antagonist activity.

2.7. Structural identification of compounds in HPLC fractions

The identity of chemicals in fractions showing significant AA activity was investigated by both GC–MS and LC-QTOFMS analysis (Details in SI). GC–MS spectra of identified compounds were investigated by Xcalibur v 2.1 software (Thermo

Scientific) first, then spectra were deconvoluted using IXCR macro in MS manager software (ACD Labs, Toronto, Canada) and compared with the Wiley Registry of Mass Spectra 9th Edition, National Institute of Standards and Technology (NIST) MS library (version 2011) and custom made libraries of pure silylated standards. In LC-QTOFMS analysis, the elemental composition of the peaks of interest was calculated from their accurate mass and isotopic fit using MassLynx V4.1 (Waters, UK) software. The molecular formulae of all putatively identified compounds were searched in a number of on-line databases in order to confirm the structural identity. The databases used in this study were KEGG LIGAND (http://www.genome.jp/ligand/), PubChem (http://pubchem.ncbi.nlm.nih.gov/), ChemSpider (http://www.chemspider.com/), and METLIN (http://metlin.scripps.edu/). For both GC–MS and LC-QTOFMS analyses, the chromatography profiles of aliquots of receptor active and neighbouring less active fractions were compared to guide in the identification of possible AA structures. Key peaks for structural identification were chosen if they were a predominant signal in the chromatogram of the receptor active fraction or showed a halogenated isotopic pattern, since biological activity has often been related to the presence of halogens in the molecular structure (Butt et al., 2011). Where available, pure standards of identified compounds were tested in YAS to assess their AA activity. Concentrations of identified AA structures in the fractions were quantified using internal standard calibration with 2,4,16,16-d_4 estrone, ^{13}C12 triclosan and 3,4,5,6-d_4 n-dioctylphthalate for GC–MS. Calibration curves were plotted of the ratio between the analyte peak area and the internal standard peak area versus absolute quantities of the analyte. In some instances, analytes were quantified by LC-QTOFMS using external calibration and internal standards of 2,2,4,6,6,17α,21,21,21-d_9 progesterone (positive mode) and 17 beta-estradiol-2,4,16,16-d_4 3-sulphate (negative mode) to monitor machine performance and any sample losses.

2.8. Statistical analyses

Statistically significant differences between sampler types, and within deployed canisters containing replicate membrane devices were investigated by ANOVA followed by the Games Howell post hoc test to examine between group differences (SPSS ver 18, IBM Corp, USA).

3. Results and discussion

3.1. Concentrations of total anti-androgenic activity in passive samplers and grab samples

The efficiency of passive sampling is positively related to the sampling area of the device, therefore in order to be able to compare sampling performances of different passive samplers the total AA activity was expressed as flutamide equivalents (FEq) per sampling area (cm^2). Both single-phase passive samplers were tested for AA activity during and after a pre-cleaning step with ethyl acetate to investigate possible contributions to AA activity from polymer additives used by the manufacturer (e.g. plasticisers). Extraction of LDPE membranes did not reveal any background AA activity (<LOD value of 280 ng Feq/cm^2 for single phase samplers). Extracts used to pre-clean the silicone strips revealed AA activity of 800 ± 0.2 ng FEq/cm^2 (mean ± SD), but subsequent extraction with the same solvents used to extract deployed samplers failed to detect any background AA activity. The AA activity of field blanks of POCIS were all below LOD levels of 2.0 μg Feq/cm^2. After deployment in river water downstream of the WWTP effluent, the highest AA activity in the sampler types was detected in POCIS samplers and was similar for both POCIS A and B at 13 μg Feq/cm^2 (Table 1, and Fig. S-4). AA activity was present in silicone strips at a concentration of 3.6 μg Feq/cm^2, whereas it was not detected in LDPE membranes. In the silicone and POCIS samplers, all the AA activity was extracted in the first solvent, and no further significant AA activity was detected with additional extractions using solvents with differing polarity (Table 1). The higher concentrations of AA activity detected in POCIS samplers maybe due to the efficient sampling of polar or moderately polar chemicals in the anti-androgen mixture which were efficiently adsorbed onto the receiving phase of the POCIS samplers which contain multiple binding sites. Any nonpolar anti-androgens were more likely to be sampled by silicone strips and LDPE membranes which can sample compounds with a log K_{ow} between 3 and 9 by the process of absorption into the polymeric membrane (Rusina et al., 2010b; Vrana et al., 2005). The rate of absorption, and therefore the sampling efficiency, is dependent on the diffusion coefficient (D) of the compound, which is dependent on its physico-chemical properties including the log K_{ow}^{27}. A low value of D in the polymer tends to reduce the uptake rate, and therefore, the sampling rate. The D values for many contaminants are generally 2–2.5 orders of magnitude lower in LDPE membranes compared with silicone strips (Rusina et al., 2010a) and this may account for the lack of AA activity detected in the LDPE sampler.

Silicone strips were deployed as replicates (n = 2) per canister, and so it was also possible to estimate the between- and within-canister variability for this sampler type. The ANOVA of AA activity revealed there were significant differences between the canisters ($p < 0.005$) with a relative standard deviation (RSD) of 9.4% in comparison with the within-canister variability which was 2.8%. Analysis of flow rates between the canister positions revealed no significance difference between the mean flow rates of each canister position (ANOVA $p < 0.4$, Fig. S-3) indicating that the sampling efficiency, which can be dependent on the water flow rate (Vrana et al., 2005), should be similar for each canister position during the deployment period. It was possible that the between-canister variability may reflect spatially heterogeneous contaminant levels in the ambient water in relation to the position of the canisters themselves, as well as the deployment of the silicone strips at different vertical positions within the 4 canisters (Fig. S-2).

No androgenic activity was detected during analyses of the 4 different passive samplers (LOD 0.07 μg DHTEq/sampler). However it was likely that any androgen receptor agonist activity, if present, was masked by the high concentrations of antagonist activity present in the extracts, an effect observed previously during the analysis of other environmental samples (Hill et al., 2010; Weiss et al., 2009).

Grab samples (1, 4, 7, 9 and 14 days of deployment) were also investigated in terms of total androgenic and AA activity. No androgenic activity was detected in the whole extracts of all grab samples. Total AA activity values were very variable and, depending on the sampling date, ranged from a mean of <l to 106 μg FEq/L with an average over the deployment period of 77 ± 43 μg FEq/L for samples extracted by liquid–liquid methods (LLE) (Table S-2). Daily values for samples extracted by SPE varied between 53–272 and, in one timepoint 1420 μg Feq/L, with a mean of 382 ± 586 μg FEq/L over the deployment period. The high day to day variability could be due to a number of factors including temporal variability in effluent quality and level of dilution, and spatial variability in effluent mixing. On day 1 and 14 of deployment, the grab samples extracted by SPE were many fold higher than samples extracted by LLE, indicating the possible presence of AA compound(s) predominantly extracted by SPE. The results highlight the variability of AA activity at this site and the utility of a passive sampling approach for sampling environmental contaminants in surface waters.

3.2. *Anti-androgenic profiles derived from HPLC fractionation*

The profiles of AA activity extracted by the POCIS and silicone passive samplers and the grab samples of ambient river water were compared after RP-HPLC fractionation of sample extracts (Fig. 1). The recoveries of AA activity after HPLC fractionation were estimated by comparing the AA activity of the sample prior to HPLC separation, and the sum of the fractions with AA activity after HPLC. Recoveries (mean ± SD) for the passive sampling extracts were 76% ± 5% (silicone strips), 80% ± 13% (POCIS B), and 69% ± 2% (POCIS A), and for grab sampling were 79% ± 15% (LLE) and 80% ± 4% (SPE) indicating that the majority of AA activity present in the different sample extracts was recovered after HPLC fractionation.

Three composite samples were prepared from river water which had been extracted either by LLE or by SPE after grab sampling at 1, 4, 7, 9 and 14 days during passive sampler deployment. Analysis of the composite river water samples gave an overview of the profiles of AA compounds that were present in the ambient water during deployment of passive samplers. In river water extracts, HPLC fractions with AA activity generally eluted between 2 and 31 min, however the profile from the SPE was dominated by a highly active fraction eluting at 15 min unlike the LLE profile where no one major fraction was apparent (Fig. 1). This indicated that there were some AA compound(s) that were more efficiently extracted by the SPE method, and this finding may account for the large differences in total AA activity extracted by SPE and LLE of grab samples of river water at some of the time points (Table S-2).

Extracts of the 3 passive samplers revealed a similar range of polar and apolar fractions as the grab water samples (Fig. 1). The profiles of AA activity from silicone strips were very similar to profiles of SPE water samples with a highly active fraction eluting at 16 min (standards used for comparison of retention times between batch analyses eluted 1 min later on

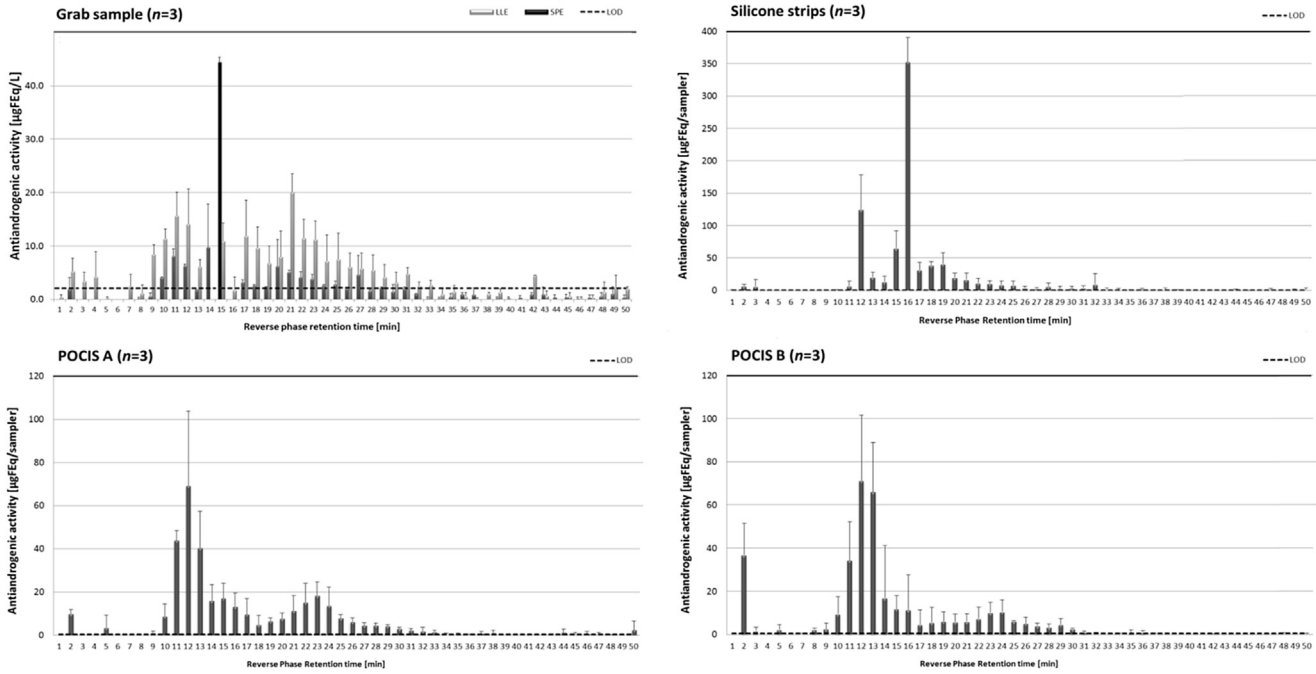

Fig. 1 – Comparison of the profiles of anti-androgenic activity in RP-HPLC fractions between extracts of contaminated river water grab samples and three different type of passive samplers. Grab samples of rivers water were extracted by liquid (LLE) or solid phase (SPE) methods and composite samples of the extracts profiled for AA activity. Profiles and SD error bars are representative of 3 replicate samples of water and passive sampler extracts. Dotted lines indicate limit of detection (LOD) values. Analysis of standards revealed that elution times on RP-HPLC were 1 min earlier with grab river water profiles compared with passive sampler profiles.

sampler profiles compared with the water profile). Profiles of AA activity from POCIS A and B samplers were almost identical and were dominated by a cluster of fractions eluting between 11 and 13 min, and also contained a very polar active fraction at 2 min (POCIS B). It would be expected that in comparison to POCIS, the silicone strips would sample non-polar compounds more efficiently than polar compounds, thus explaining the shift of the most active AA fractions towards the right of the chromatogram with silicone compared to POCIS profiles. The retention times of some lipophilic chemical standards tested on RP-HPLC were triclosan (log K_{ow} 5.2) 19.1 min, p′p DDE (log K_{ow} 6.1) 28.1 min, PCB 138 (log K_{ow} 6.8) 31.2 min, dioctylphthalate (log K_{ow} 8.1) 36.6 min (log K_{ow} values calculated from ChemSketch, ACD Labs, Toronto, Canada). The majority of AA fractions that were detected in either water or silicone extracts eluted from RP-HPLC prior to 28 min which indicated that most of the AA activity comprised polar or moderately hydrophobic compounds that were present in the ambient water. In order to check whether lipophilic compounds were not detected in the profiles due to high affinity with the RP-HPLC column, extracts of silicone strips were also profiled using NP-HPLC (Fig. S-5). An NP-HPLC profile of AA activity did not reveal the presence of a highly lipophilic fraction eluting at the solvent front which would co-elute with the lipophilic standards, and moreover the recoveries of AA activity after NP and RP fractionation were comparable (76% in RP versus 74% in the NP).

No androgen receptor agonist activity was detected during profiling of any of the passive sampler extracts, a finding that was likely due to the very high levels of AA activity co-eluting in the fractions. AA activity was always below the LOD of the assay (1 μg/mL) in fractions from the profiling of blank workup samples.

3.3. *General chemical profiling of the anti-androgenic fractions from passive samplers*

Fractions showing the highest values of AA activity were analysed by both GC–MS and LC-QTOFMS. When the compound was putatively identified, the commercial standard (if available) was purchased, and the identity of the compound was confirmed by comparison of retention times on HPLC and GC and the mass spectra. A list of all conclusively identified compounds present in the fractions containing AA activity are given in Table 2, and the GC–MS and LC-QTOFMS data of these compounds and those that were only putatively identified in the fractions are given in Table S-4. A number of emerging contaminants were identified in the fractions including pharmaceuticals used as anticlotting agents, calcium channel blockers, angiotensin II receptor antagonists, antibiotics, antifungals, analgesics, and anti-inflammatory agents. Many of these pharmaceuticals, such as clopidogrel, clothiapine, clozapine, bepridil, and amiodarone, are not usually recognised as common contaminants in wastewater effluents that are of primarily domestic origin. Other categories of emerging contaminants that were detected included personal care products (e.g. the agent sunscreen sulisobenzone), flame retardants (e.g tris-(2-chloropropanol)-phosphate TCPP, triphenylphosphate and tris(2-butoxyethyl)phosphate), pesticides (e.g. propiconazole, terbutryn, diazinon and piperonyl butoxide) and food products such as the artificial sweetener sucralose and the potato metabolite solanidine.

Chemicals identified in the early eluting fraction 2 from RP-HPLC had log K_{ow} values of $\leq$1 and were sampled by both POCIS A and B (Table 2). One of these compounds, sucralose, is an artificial sweetener (known mainly as Splenda or SucraPlus) which is an emerging contaminant now being found in fresh and marine waters and is recognized as persistent with a half-life up to several years[28]. In many instances, moderately hydrophilic compounds eluting in later HPLC fractions were sampled by silicone, as well as POCIS A and B devices. Some lipophilic compounds with a log K_{ow} > 3.0 (amiodarone, n-desethylamiodarone, miconazole, dehydrofelodipine, clopidogrel, and terbutryn) were sampled by silicone strips alone. However there was no clear relationship between the log K_{ow} value of the compounds and the sampler type, highlighting the importance of other physico-chemical properties in sampling efficiency of the different devices. For instance, some moderately lipophilic compounds such clopidogrel, terbutryn and diazinon (log K_{ow} 3.4–4.2) were only sampled by silicone and not POCIS devices, whereas other compounds of similar log K_{ow} values were sampled by POCIS A/B or all three samplers. POCIS A/B samplers contain a microporous hydrophilic PES membrane and certain chemicals (including diazinon) have been shown to accumulate in the PES membrane preventing efficient transfer to the POCIS receiving sorbent (Vermeirssen et al., 2012). Therefore the PES membrane should also be extracted to ensure complete recovery of chemicals sampled by the POCIS, and additionally a combination of different types of passive samplers maybe needed in order to fully encompass the whole array of diverse contaminants present in the aquatic environment.

Many compounds were sampled by both the POCIS A/B samplers, however some were sampled by POCIS A alone (Table 2, and S-4). This finding is in keeping with the design of the POCIS A configuration which contains a hydrophilic-lipophilic-balanced reversed-phase sorbent (OASIS HLB) with an affinity for weak acids and bases alongside neutral chemicals. POCIS B contains a triphasic stationary phase, also designed to sample a wide range of compounds. However some chemicals with multiple functional groups may be irreversibly bound to the Ambersorb carbonaceous component present in POCIS B and not easily recovered using standard solvent extraction techniques (Alvarez et al., 2007). For this reason, out of the two POCIS types, the POCIS A containing the OASIS phase would be considered the more universal sorbent for sampling a wide variety of contaminants, including AA chemicals, in water.

3.4. *Identification of some key anti-androgenic structures in passive sampler extracts*

Commercial standards of compounds identified in AA active fractions were tested for receptor antagonist activity in the YAS. Their relative potencies (RP) of AA activity compared to the flutamide standard are reported in Table 2 and details of their activity concentrations in the YAS in Table S-3. Many of the compounds tested in this study showed an RP comparable with flutamide and these included triclosan, clopidogrel, clothiapine, bepridil, desethylamiodatone and diclofenac amide.

Table 2 – Compounds identified in anti-androgenic HPLC fractions from passive sampler extracts.

RP-HPLC fraction number (mins)	Compound identity	CAS number	Use	Detected in passive sampler			Log K_{ow}[d]	Anti-androgenic potency relative to flutamide[a]	Activity of compound in the fraction ngFEq/sampler, sampler type[b] (% contribution to AA activity of the fraction)
				Silicone	POCIS A	POCIS B			
2	Lamotrigine	84057-84-1	Anticonvulsant		✓	✓	−0.19	0.01	nq
	Sucralose	56038-13-2	Sweetener		✓	✓	0.68	Not active	–
	Trimethoprim	738-70-5	Antibiotic		✓	✓	0.79	0.02	nq
	Codeine	76-57-3	Analgesic		✓	✓	1.20	Not active	–
10	Sulisobenzone	6628-37-1	Sunscreen		✓	✓	0.89	0.05	nq
11	Clopidogrel	113665-84-2	Antiplatelet clotting	✓			4.23	0.97	688 ± 206, Si (4%)
	Clothiapine	2058-52-8	Antipsychotic	✓	✓	✓	3.13	0.62	678 ± 180, Si (7%)
	Solanidine	80-78-4	Alkaloid potato metabolite	✓	✓		7.28	Not active	–
	Clozapine	5786-21-0	Antipsychotic	✓	✓	✓	2.36	0.13	528 ± 146, PA (1%); 216 ± 21, PB (1%)
	Venlafaxine	99300-78-4	Antidepressant	✓	✓	✓	2.91	0.01	nq
	Dipyridamole	58-32-2	Anticlotting	✓	✓	✓	−1.22	Not active	–
	Tramadol	46941-76-8	Analgesic	✓	✓	✓	2.51	Not active	–
11–12	Bepridil	74764-40-2	Calcium channel blocker		✓	✓	5.80	0.27	nq
	Chloroxylenol	88-04-0	Antimicrobial		✓	✓	3.35	0.16	nq
	Carboxyterbinafine (metabolite)	99473-14-0	Antifungal		✓	✓	5.72	0.07	nq
	Terbutryn	886-50-0	Pesticide	✓			3.44	0.06	nq
12	Diltiazem	33286-22-5	Calcium channel blocker	✓	✓	✓	3.63	0.01	3.7 ± 1.5, PA (<1%)
	Carbamazepine	298-46-4	Anticonvulsant	✓	✓	✓	2.67	0.01	106 ± 23, PA (1%); 37 ± 33, PB(1%)
	Escitalopram/Citalopram	128196-01-0	Antidepressant	✓	✓	✓	2.51	0.01	26 ± 14 PA (<1%)
12	Irbesartan	138402-11-6	Angiotensin II receptor antagonist		✓	✓	4.50	0.01	nq
	Telmisartan	144701-48-4	Angiotensin II receptor antagonist		✓	✓	7.73	Not active	–
	Terbinafine	78628-80-5	Antifungal	✓	✓	✓	6.61	0.05	15301 ± 7441, Si (5%); 41 ± 16, PA (1%); 18 ± 5 PB (1%)
13	Diethyltoluamide	134-62-3	Insect repellent	✓	✓	✓	1.96	0.05	nq
	Naproxen	22204-53-1	Anti-inflammatory		✓		3.00	Not active	–

(continued on next page)

Table 2 – (*continued*)

RP-HPLC fraction number (mins)	Compound identity	CAS number	Use	Detected in passive sampler			Log K_{ow}[d]	Anti-androgenic potency relative to flutamide[a]	Activity of compound in the fraction ngFEq/sampler, sampler type[b] (% contribution to AA activity of the fraction)
				Silicone	POCIS A	POCIS B			
13	Crotamiton	483-63-6	Anti-itching drug	✓	✓	✓	3.10	0.01	23 ± 19, Si (1%); 11 ± 6, PA (<1%)
14	Diclofenac	15307-86-5	Analgesic/anti-inflammatory		✓		4.06	0.02	nq
15	Miconazole	22916-47-8	Anti-fungal	✓			5.93	*(40)*[c]	*[1454 ± 862, Si (3%)]*
	TCPP (tris(1-chloro-2-propyl)phosphate)	13674-84-5	Flame retardant	✓	✓	✓	1.53	0.02	1058 ± 319, PA(10%); 344 ± 119, PB (4%)
	Diazinon	333-41-5	Insecticide	✓			3.81	0.17	nq
	Diclofenac amide	15307-86-5	Analgesic/anti-inflammatory		✓	✓	3.00	1.05	635 ± 221, PA (6%); 564 ± 301, PB (3%)
16	Propiconazole	60207-90-1	Antifungal	✓	✓	✓	3.88	*(126)*[c]	*[151507 ± 54644, Si (40%)]*
	N-Desethylamiodarone (metabolite)	96027-74-6	Antiarrhythmic agent	✓			7.86	1.68	nq
	Dehydrofelodipine (metabolite)	96382-71-7	Calcium channel blocker	✓			4.95	0.13	nq
	Amiodarone	1951-25-3	Antiarrhythmic agent	✓			8.89	0.05	nq
17	Triphenyl phosphate	115-86-6	Flame retardant	✓	✓		4.10	0.25	54 ± 33, Si (<1%)
18	Tris (2-butoxyethyl)phosphate	78-51-3	Flame retardant	✓	✓	✓	3.96	0.02	nq
19	Triclosan	3380-34-5	Antimicrobial	✓	✓	✓	5.17	4.8	20231 ± 4304, Si (52%)
	Piperonyl butoxide	51-03-6	Insecticide synergist	✓	✓		4.23	0.02	nq

[a] Determined from analysis of the pure standard in YAS.

[b] Calculation of the anti-androgenic activity of the compound was determined from the mass in the fraction and the relative potency and is expressed as mean ± one standard deviation flutamide equivalents per sampler; ($n = 3$ as sampling canister replicates for silicone and POCIS A, 2 replicates for POCIS B). nq = the chemical was present in fraction but the mass could not be quantified from the internal standard calibrant.

[c] Apparent high relative potency values in the YAS could not be confirmed in the AR-CALUX assay. Figures in italics indicate apparent potency, apparent activity and apparent % contribution of the compound to the AA activity in the fraction

[d] Log K_{ow} calculated by ChemSketch, ACD Labs (ACD, Toronto, Canada).

In the case of the antiarrhythmic agent amiodarone, both the parent compound and the metabolite desethylamiodarone were detected in fractions from silicone strips. However, the AA potency of the metabolite (RP = 1.68) was 34 times higher than amiodarone (RP 0.05) itself indicating that, in some cases, metabolism may increase the androgen receptor antagonist activity of a compound. The anti-fungal agents propiconazole and miconazole were also identified in AA active fractions. They are currently used worldwide as agricultural fungicides (propiconazole) or in pharmaceuticals (miconazole) and are present in WWTP effluents at concentrations between 1 and 100 ng/L (Kahle et al., 2008; Van de Steene et al., 2010). Propiconazole and miconazole had an apparent high RP of 126 and 40 respectively in the YAS. However, in our laboratory when these compounds were tested in another *in vitro* assay for AA activity based on a mammalian cell line, the AR-CALUX (Legler et al., 1999), their RP values were 0.24 (propiconazole) and 0.62 (micoconazole) (J Horwood pers comm). This suggests that these compounds are weak anti-androgens, a finding that is in agreement with studies using reporter gene assays based on other mammalian cell lines (Ait-Aissa et al., 2010; Kjaerstad et al., 2010; Kjeldsen et al., 2013). The conazole fungicides inhibit ergosterol synthesis which is essential for formation of the fungal and yeast cell wall membranes (Kjaerstad et al., 2010). Although no effects on yeast growth were detected at their EC_{50} values corresponding to AA activity in the YAS, it is possible that there were effects on yeast cell function which were not relevant to the AR-CALUX which is an assay based on a mammalian cell line. Therefore the finding of their apparent high AA activity in the YAS model should be treated with caution. They are also thought to act as EDCs by disruption of sex steroid biosynthesis in vertebrate systems (Kjaerstad et al., 2010).

Wherever possible, the concentrations of AA activity of some of the compounds identified in the sampler fractions were estimated from the mass of the compound (from GC–MS or LC-QTOFMS analyses) and the RP of the compound in the YAS (Table 2). Compounds were selected that either had RP levels comparable with flutamide, or that were highly abundant in a fraction from a sampler type. However, concentrations of some compounds could not be accurately estimated from the three internal standard calibrants used in this study. Of the identified anti-androgens in the samplers, the concentrations of AA activity of triclosan (20 ± 4 μgFEq) and terbinafine were the highest. Although not a potent anti-androgen, terbinafine was present at high enough chemical concentrations (306 ± 148 μg/fraction) to give an estimated AA activity of 15 ± 7 μgFEq in silicone strips. The identification of triclosan as a significant AA contaminant in the passive samplers is in agreement with a recent study in which it was reported as a key structure with AA activity present in bile of fish exposed to wastewater effluents (Rostkowski et al., 2011). Furthermore, triclosan has shown AA activity in a number of *in vitro* androgen receptor screens (Ahn et al., 2008; Chen et al., 2007; Gee et al., 2008; Rostkowski et al., 2011) and also in an in vivo study (Kumar et al., 2009). One *in vitro* study has also reported a lack of androgen receptor antagonist activity but weak agonist activity instead for triclosan revealing potential inconsistency between different biological assays (Christen et al., 2010).

The concentrations of triclosan accounted for 52% of the total AA activity present in fraction 19 from silicone strips extracts. However, the concentrations of many of the other AA compounds that were identified in the different passive samplers only accounted for 1–10% of the total AA activity present in the fractions that they were detected in. Although 38 compounds were tested for activity in YAS, a further 25 compounds were not assayed, either because their structures were not conclusively identified or because pure standards were not commercially available for testing in YAS (see Table S-4). In addition, multiple compounds were present that could contribute to the AA activity of any one fraction. In receptor assays, mixtures of AA chemicals can act by the concentration addition model which can result in a higher measured concentration of AA activity in the mixture compared with a summation of the activity measured from individual components (Ermler et al., 2011). Finally it should be emphasised that further studies using androgen receptor transcription screens based on mammalian cell lines, receptor binding and in vivo studies are required to confirm the AA activity of compounds identified in this study.

4. Conclusions

This study reveals that passive sampling can be a very promising tool for screening of mixtures of contaminants such as anti-androgens which are present at ultra-trace yet toxicologically relevant concentrations in surface waters. Both POCIS configurations containing sorbents with multiple binding sites, as well as a silicone single membrane passive sampling device, sampled a diverse range of AA contaminants in river water. In contrast, no AA activity was detected in an LDPE passive sampling device, possibly due to poor diffusion of AA structures in this polymer. Analysis of RP-HPLC profiles of AA activity revealed that a combination of POCIS A and silicone samplers (rather than any one sampler alone) was the best method to screen for AA chemicals in the ambient water. Over 31 contaminants were identified which inhibited YAS activity and were potential anti-androgens and these included fungicides, germicides, flame retardants and pharmaceuticals. Using a combined passive sampling and *in vitro* assay approach will allow the identification of AA contaminants present in surface waters that could be a risk to the reproductive health of aquatic organisms.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2014.03.039.

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