Marine Pollution Bulletin 95 (2015) 590–597

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Widespread contamination of coastal sediments in the Transmanche Channel with anti-androgenic compounds

Diana Alvarez-Muñoz [a,1], Paolo Indiveri [a,1], Pawel Rostkowski [a], Julia Horwood [a], Emily Greer [a], Christophe Minier [b], Nick Pope [c], William J. Langston [c], Elizabeth M. Hill [a,*]

[a] School of Life Sciences, University of Sussex, Brighton BN1 9QG, United Kingdom
[b] Laboratory of Ecotoxicology, UPRES EA-3222, UFR de Sciences et Techniques, Université du Havre, 25 rue Philippe Lebon, BP 540, 76058 Le Havre Cedex, France
[c] Marine Biological Association, The Laboratory, Citadel Hill, Plymouth PL1 2PB, United Kingdom

ARTICLE INFO

Article history:
Available online 11 December 2014

Keywords:
Sediment
Effect-directed analysis
AR-CALUX
Clam
Anti-androgen
Endocrine disruption

ABSTRACT

This study analysed the levels of androgen receptor antagonist activity in extracts of coastal sediments sampled from estuaries in southern UK and northern France. Anti-androgenic (AA) activity varied between <0.2 and 224.3 ± 38.4 μg flutamide equivalents/g dry weight of sediment and was significantly correlated with the total organic carbon and silt content of samples. AA activity was detected in tissues extracts of clams, *Scrobicularia plana*, sampled from a contaminated estuary, some of which was due to uptake of a series of 4 or 5 ring polycyclic aromatic hydrocarbons (PAHs). Initial studies also indicated that fractionated extracts of male, but not female, clams also contained androgen receptor agonist activity due to the presence of dihydrotestosterone in tissues. This study reveals widespread contamination of coastal sediments of the Transmanche region with anti-androgenic compounds and these contaminants should be investigated for their potential to disrupt sexual differentiation in aquatic organisms.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Endocrine disrupting chemicals (EDCs) released into the environment can disrupt the sexual differentiation of wildlife and possibly humans too (Bergman et al., 2012; EEA, 2012; Kortenkamp et al., 2011). Many of these chemicals are released into the aquatic environment and include compounds that can interfere with sex steroid action with the potential to feminize or (de)masculinize organisms. Chemical contaminants with estrogen receptor agonist or androgen receptor antagonist activity are present in wastewater effluents (Hill et al., 2010; Sumpter and Jobling, 2013) and studies to date have indicated that the steroidal estrogens and possibly anti-androgens present in the receiving waters are causing the high levels of feminized fish present in some UK river sites (Jobling et al., 2006, 2009). Other EDCs such as organotins, which in the past were used in marine antifouling paints, have caused widespread masculinization of female gastropods and may act as ligands for different nuclear receptors involved in sexual differentiation rather than those for sex steroids (Pascoal et al., 2013; Titley-O'Neal et al., 2011).

High incidences of intersexuality has been reported in the estuarine clam *Scrobicularia plana* in some UK estuaries as well as those in Portugal and Northwest France (Chesman and Langston, 2006; Gomes et al., 2009; Langston et al., 2007; Tankoua et al., 2012). *S. plana* is normally thought to be gonochoristic but at some UK sites, two-thirds of the clam population was intersex and this condition appeared to arise from a feminization or demasculinization of the male clams. *S. plana* is an endobenthic deposit feeding mollusc and is likely to be exposed to a variety of EDCs associated with the surrounding sediments. There is little information as to whether EDCs which have the potential to disrupt the sex steroid signalling in vertebrate systems can act similarly to cause abnormal sexual differentiation in molluscan species. In part, this is due to our lack of understanding of molluscan endocrinology; the de novo synthesis of androgens and estrogens, and the presence of functional sex steroid receptors in these invertebrates have yet to be demonstrated (Scott, 2012, 2013). Nevertheless, it is important to identify bioavailable EDC mixtures in the aquatic environment that, either from in vitro or in vivo studies, demonstrate the potential to disrupt sexual differentiation in vertebrate systems; this will enable the relevant exposure studies to be undertaken to determine whether these contaminants cause intersex in *S. plana*. EDCs such as steroidal estrogens, and other estrogenic chemicals such as nonylphenol and bisphenol A are commonly detected in sediments (Koyama et al., 2013; Peck et al., 2004; Schmitt et al., 2012).

* Corresponding author. Tel.: +44 1273 678382; fax: +44 1273 877586.
E-mail address: E.M.Hill@sussex.ac.uk (E.M. Hill).
[1] Joint first authors.

http://dx.doi.org/10.1016/j.marpolbul.2014.11.014
0025-326X/© 2014 Elsevier Ltd. All rights reserved.

Laboratory experiments have revealed that exposure of sexually undifferentiated *S. plana* to sediment spiked with mixtures of steroidal estrogens and nonylphenol did induce significant levels of intersex in the mature adult, however exposure levels were far higher than those normally encountered in UK coastal sediments (Langston et al., 2007). There remains the possibility that benthic organisms are also exposed to other EDCs such as those with androgen receptor antagonist activity which may cause demasculinisation of clams and which either alone, or together with low amounts of estrogens, results in intersexuality. The levels of anti-androgenic (AA) activity in wastewater effluents can range between 0.2 and >1.0 mg flutamide equivalents/litre (Johnson et al., 2007) and predominant bioavailable anti-androgens present in wastewater effluents have been identified as chlorophene, triclosan and abietic acid (Rostkowski et al., 2011). Although AA activities have been detected in river (Creusot et al., 2013; Macikova et al., 2014; Weiss et al., 2009) and marine sediments from two polluted sites in Norway (Grung et al., 2011), there is little information on the levels of AA contamination in European coastal sediments and their effects on benthic organisms.

The aim of the present work was to investigate the levels of AA activity in extracts of coastal sediments sampled in the Transmanche Channel and any association of the levels of activity with sediment properties. In further work, the levels of bioavailable AA activity were determined in tissue extracts of *S. plana* sampled from the most contaminated estuarine site, and an initial study of the nature of androgen receptor antagonist and agonist chemicals detected in the clam was also undertaken.

2. Material and methods

2.1. Chemicals

Flutamide (FLU), dihydrotestosterone (DHT), phenanthrene-D10, benzo[a]anthracene-D12, benzo[a]pyrene-D12, bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), DMSO and anhydrous sodium sulphate were purchased from Sigma–Aldrich (Dorset, UK). [2,4,16,16-D4] estrone (E_1-D4, >98% D atom) was purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA), while [2,2,4,6,6, 17α,21,21,21-D9] progesterone (PG-D9, 98% D atom) and ^{13}C12-triclosan (^{13}C at 99%) were obtained respectively from CDN isotopes (Quebec, Canada) and LGC Standard (Teddington, UK). AR-CALUX cells® were obtained from Bio Detection Systems, Science Park 406, 1098 XH, Amsterdam, The Netherlands. Cellulose filters were supplied by Thames Restek UK Ltd. (Buckinghamshire, UK) and Ottawa sand from Fisher Scientific UK Ltd (Leicestershire, UK). All solvents were HPLC-grade and purchased from Rathburn (Walkerburn, UK).

2.2. Study area

Sediments were sampled along the Transmanche Channel between 2009 and 2012. Sites included the main estuaries and coastal regions from both sides and multiple sites were sampled in some estuaries, so that the overall survey encompassed a total of more than 100 sites in France and UK (Fig. 1). Sediments were sampled at 2–3 cm depth, and each sample comprised 20 subsamples taken in a 3 m^2 area. At the same time as the sediment collection, up to 30 clams (*S. plana)* were sampled from selected sites to determine the levels of AA activity within the tissues. Both the clam and sediments samples were stored at −80 °C until their analysis.

Sediments were sieved (2 mm) and the percentage of, sand (0.63 μm–2 mm), silt (2–63 μm), clay (1–2 μm) and colloids (<1 μm) were determined using a Partica LA-950 laser diffraction particle size distribution analyzer (Horiba UK, Ltd., Stanmore, Middlesex). The organic carbon content in sediment was determined by dichromate oxidation according to El Rayis (El Rayis, 1985).

2.3. Sediment and clam extraction

Sediment samples were analysed in triplicate for each site and were freeze dried and sieved (2 mm) prior to extraction. Sediments were extracted using an Accelerated Solvent Extraction unit (ASE 200 from Dionex). The extraction cells (22 mL) were prepared by inserting a disposable cellulose filter into the cell outlet followed by 5 g of sample plus Ottawa sand to improve the cell packing, and another cellulose filter on the top. The ASE conditions were pressure 1500 psi, temperature 120 °C, static time 5 min and 3 static cycles. The recovery of AA activity from sediments was initially tested using two sequential methanol/acetone (MeOH/Acetone 50/50, 30 mL) extractions followed by a third one with dichloromethane/hexane (50/50, 30 mL), and the results revealed that the 100% of the AA activity was recovered by the first two extractions (supplementary information Table S1). The two MeOH/acetone sediment extracts (30 mL each) were combined, brought to dryness using a CombiDancer (Zinsser Analytic), and re-dissolved in 1 mL of MeOH/acetone (50/50). Finally, an aliquot of 250 μL of each extract was dried down under nitrogen and re-dissolved in 60 μL of dimethyl sulfoxide (DMSO) for in vitro analyses of AA activity using the AR-CALUX assay.

Clam samples (male and female individuals of *S. plana*) from 5 different sites of the Southampton estuary (Northam, St. Denys, Woolston, Cracknore and Warsash) were extracted using ultrasonic probe assisted solvent extraction technique. Frozen clams were cut to 3–4 mm width pieces, transferred into test tubes and weighed. A ratio of 8 mL of acetone per gram of wet weight (ww) of clam was added and each sample was extracted for 1 min at high power on ice (2 cycles of 30 s each with, in between, a cooling down step of 30 s). Compared with an initial acetone extraction, further extractions of the tissue pellet with methanol/acetone or dichloromethane/hexane solvent mixtures (each at 8 mL/g ww clam tissue) extracted <5% additional AA activity. To promote protein precipitation, the sample was kept on ice for 10 min, vigorously shaken for 1 min in presence of 4 g of anhydrous sodium sulphate and centrifuged for 30 min at 2000 rpm. The supernatant was removed, and the extract dried under vacuum and re-dissolved in 100 μL of acetone per 0.5 g ww of extracted tissue, and an aliquot (50 μL) dried and re-dissolved in 20μL of DMSO to measure AA activity.

2.4. Reversed phase high performance liquid chromatography (HPLC) fractionation

In order to characterise bioavailable contaminants with AA activity, extracts from either male or female clams were fractionated by HPLC. Aliquots of extracts prepared above were combined from either female or male clams sampled at Northam, the most contaminated site with AA activity. Tissue extracts were dried under nitrogen, re-dissolved in 150 μL of ACN:water (90/10) and injected onto the HPLC. Samples were fractionated on a C18 SunFire analytical column (3.5 μm particle size; 4.6 × 150 mm, Waters, UK) equipped with a guard column (3.5 μm particle size; 4.6 × 10 mm). The mobile phase was water (0.2% acetic acid, 5% ACN) as solvent A, and ACN (0.2% acetic acid) as solvent B at a starting ratio 70:30. The separation was performed at room temperature (flow rate 1.0 mL/min) with a linear gradient program of 0.0–3.0 min (65/35, A:B), 3.0–9.0 min (45/55, A:B), 9.0–17.0 min (29/71, A:B), 17.0–24.0 min (0/100, A:B) and 100% B for up to 40 min. For each HPLC run the re-equilibration step was 30 min at the starting condition. Forty HPLC fractions of 1 mL each were collected, dried down and re-suspended in 300 μL of ethanol. An

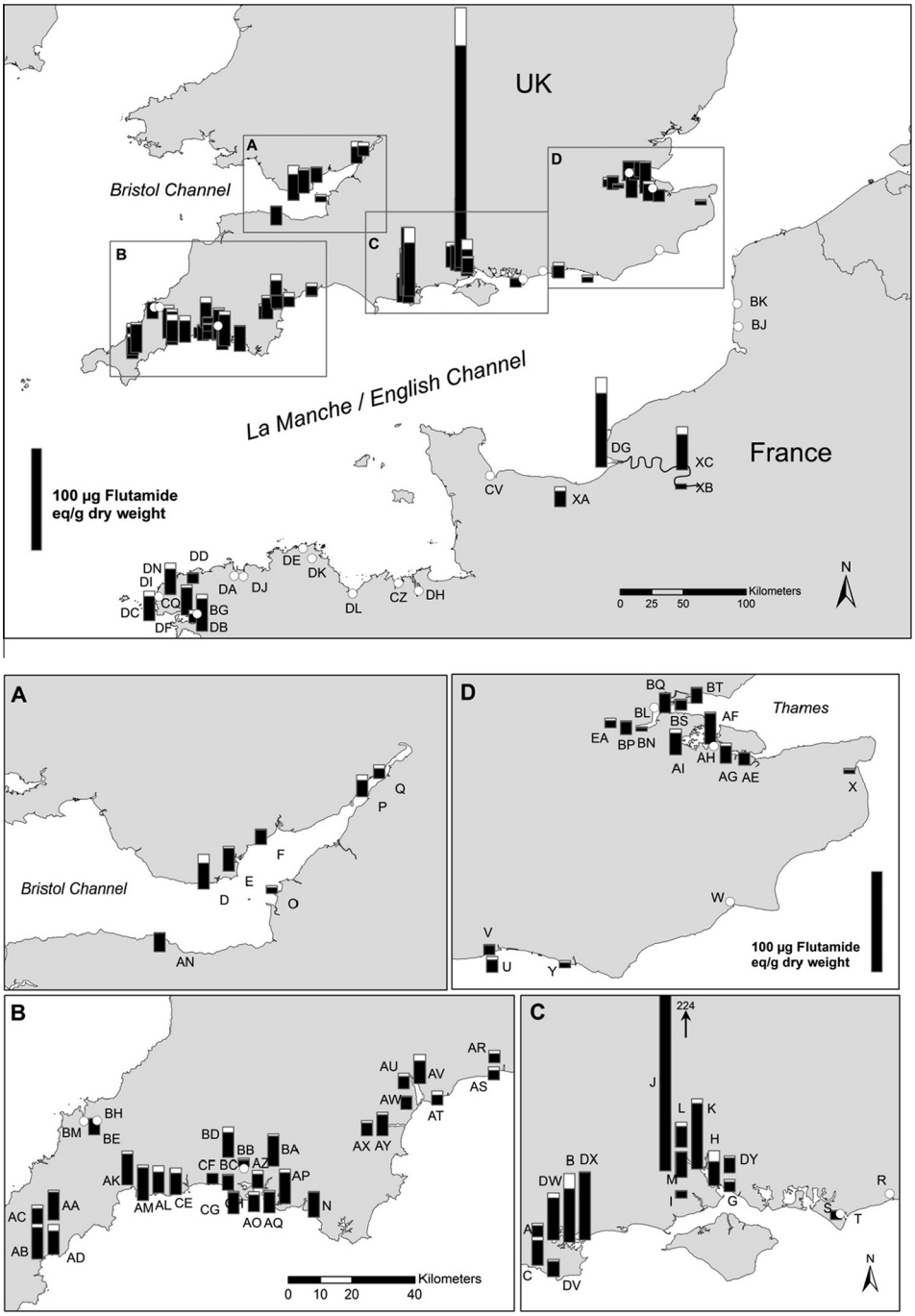

Fig. 1. Concentrations of anti-androgenic activity in extracts of sediments sampled in the Transmanche region. Figure shows overall and detail of anti-androgenic activities in the region. Black bars represent anti-androgenic activity and white areas the variability of duplicate measurements. Anti-androgenic activity was below the LOD (<0.2 µg FLU eq/g ww sediment) of lettered sites with no bars. Data values for each site are given in Table S2.

aliquot (50 µL) was kept for GC–MS analyses, and the remaining extract was dried down again, re-suspended in 10 µL of DMSO, and 0.2 µL tested in AR-CALUX for profiling the AA and androgenic activity of the fractionated clam extracts.

2.5. AR-CALUX assay

AA and androgenic activities of sediment, clam extracts and HPLC fractions were determined using AR CALUX® bioassay (BioDetection Systems, Amsterdam, The Netherlands). The AR CALUX bioassay is a reporter gene assay, comprising a human osteoblast cell line carrying a luciferase gene under transcriptional control of an androgen responsive element. The assay was performed according to published methods (Sonneveld et al., 2005; van der Burg et al., 2010), and androgen receptor activity responses were expressed as relative amounts of reference compounds equivalents; ng dihydrotestosterone (DHT) eq for agonist and µg flutamide (FLU) eq for antagonist activity expressed as per g of dry weight (dw) for sediment or g wet weight (ww) for clam samples. To measure antagonist activity, DHT (0.3 nM) was added to the culture medium. Aliquots of sediment or clam extracts in DMSO (0.2 µl) were dissolved in culture media (0.2 mL) and analysed in duplicate using a serial dilution of extracts. Controls comprised media with and without DHT, and sample response curves were normalised to the control responses. The toxicity of any samples extracts in the cells was determined by analysing serial dilutions in the MTT assay (Mosmann, 1983).

2.6. GC–MS analysis

Stable isotope internal standards (IS) E_1-D4, phenanthrene-D10, benzo[a]anthracene-D12, benzo[a]pyrene-D12 and triclosan-^{13}C (60 ng of each) were added to clam fractions containing AA receptor activity and chemicals in the fractions were derivatized to their trimethylsilyl ethers (TMS) prior to GC–MS. For the quantitation of androgenic steroids in clams, PG-D9 (100 ng) was added to the active fractions. An aliquot of the fraction was dried under a gentle stream of nitrogen (oxygen-free), dissolved in 60 μL of 1/1 pyridine and BSTFA (1% TMCS) and incubated for 30 min at 65 °C. The sample was then dried down again under nitrogen, and re-suspended in 20 μL of 1:1 pyridine and BSTFA. The sample (1 μL) was injected in splitless mode on a gas chromatograph (Trace GC Ultra, Thermo Scientific) linked to an ion trap mass spectrometer (ITQ1100, Thermo Scientific). Compounds were separated on an Agilent J&W DB5 (30 m × 0.25 mm i.d. and 0.25 μm film thickness) capillary column coated with 5% phenyl-95% polydimethylsiloxane copolymer phase (Agilent Technologies UK Limited, Stockport, UK), using helium as the carrier gas (99.996% purity) at a flow rate of 1.5 mL/min. The injector and transfer line were set at 270 °C and 300 °C respectively, the source at 250 °C. The oven temperature was 60 °C for 2 min, then at 12 °C/min to 200 °C, 7 °C/min to 270 °C (1 min hold time), 1 °C/min to 274 °C, (5 min hold time), and 5 °C/min to 300 °C, (7 min hold time). The MS was operated in 50–650 *m/z* full scan positive ionisation mode with electron ionisation at 70 eV and the dwell time was 25 ms. GC–MS spectra were analysed on Xcalibur v2.3 software (Thermoquest–Finningan) and compared with a combined MS library Wiley Registry of Mass Spectra (9th Edition) and NIST (National Institute of Standards and Technology, 2011) and a custom made library of pure silylated standards. Each identified compound in the fraction was confirmed by injecting an authentic standard onto the GC–MS to confirm retention time and MS spectra. The *m/z* ions with maximum intensity were used as quantifier ions and a calibration curve was used to calculate the absolute amount of each compound in the fraction in comparison with the IS response.

2.7. UHPLC–TOFMS analysis

Further information on the identity of the androgenic chemical in an isolated fraction was obtained from ultraperformance liquid chromatography–time of flight mass spectrometry (UHPLC–TOF-MS). An aliquot of an androgenic clam fraction was injected onto a nanoAcquity UHPLC linked to a Xevo G2 TOFMS, equipped with a nanoESI source (Waters, Manchester, UK). The sample, containing PG-D9 internal standard (15 ng), was separated on a nanoAcquity UHPLC HSS T3 column (1.0 × 100 mm, particle size 1.8 μm and 100 Å pore size; Waters, Elstree, UK. Solvent A was 0.01% formic acid in water and solvent B 100% acetonitrile 0.01% formic acid with a gradient of 0–4.0 min from 10% to 30% B, 4–18 min from 30% to 50% B, 18–30 min 100% B. The MS was tuned to a mass resolution of 20,000 and samples were analysed in +ESI mode and acquisition was between 50 and 1000 *m/z*. The capillary voltage was 3.0 kV, collision gas argon, and the collision energy was cycled from 15 to 25 eV during acquisition. Nitrogen gas flow for the cone was 60 L/h and for desolvation 300 L/h, cone voltage was 30 V, and source temperature 120 °C. A leucine enkephalin lockspray standard in 1/1 MeOH/water (v/v) (2 ng/μl) was infused at 700 nL/min.

2.8. Statistical analyses

A Kolmogorov–Smirnov test revealed that sediment physico-chemical properities and the AA data all followed a non-normal distribution and, as consequence, Spearman correlation coefficients were used to examine correlations between AA activity and sediment type (SPSS ver 17). Significant differences between AA activities detected in clams sampled from different sites were determined by ANOVA using the Tukey post hoc test.

3. Results and discussion

3.1. Anti-androgenic activity concentrations in sediment extracts

AA activity was detected in extracts of sediments sampled in 92% of the UK and 45% of the French sites. Screening of extracts in the AR-CALUX revealed that only androgen receptor antagonist activity and no agonist activity was detected. However, any androgen receptor agonist activity present in low amounts in the sediment extracts would have been masked by the levels of antagonist activity which were present at ppm levels in many samples. The total AA activity generally ranged between below the limit of detection (LOD = 0.2 μg FLU eq/g dw sediment) to 74 μg FLU eq/g dw for the sediment samples collected along the south coast of England and northern France (Fig. 1 and Table S2). In the UK, the most contaminated estuary of the survey was Southampton, where AA activities of 224.3 ± 38.4 μg FLU eq/g dw sediment were measured at Northam, and levels close by at St Denys were 65.5 μg FLU eq/g dw. Two sites (Sterte and Parkston) in Poole estuary were also highly contaminated with AA activities >60 μg FLU eq/g dw. Other estuaries in the UK, including sites in the Severn and Thames, were contaminated at levels generally <30 μg FLU eq/g dw. Both the Poole and Southampton estuaries are associated with major shipping activities, including pleasure crafts, passenger and cargo shipping, as well as oil refining industries. In France, the most contaminated sites were the port of Le Havre where sediments contained AA activities of 74.5 ± 16.1 μg FLU eq/g dw sediment, and upstream on the Seine at Rouen where AA activity was 35.6 ± 8.0 μg FLU eq/g dw. AA activity was lower in most other sites in northern France which were less polluted with industrial or shipping activity. In other countries, AA values in coastal sediments in Oslo and the Grenland area in Norway have been measured at 2 and 557 μg FLU eq/g dw sediment respectively (Grung et al., 2011). Fractionated sediment samples from the River Lambro in Italy contained AA activity at between 46 and 82 μg FLU eq/g dw (Urbatzka et al., 2007) and similarly in the River Schijn, Belgium, AA activity was detected at up to 55 μg FLU eq/g dw sediment (Weiss et al., 2009). In other studies of river sediments in France, AA activity ranged 1.1–32.5 μg FLU eq/g dw sediment (Kinani et al., 2010). Therefore the range of AA contamination detected in coastal sediments in the Transmanche Channel was similar to the variety of levels detected in sediments from low, moderately or highly impacted sites in European rivers and in another coastal site in Norway.

The relationship between the levels of AA accumulation in the sampling sites in the Transmanche region and the physico-chemical properties of the sediments was also investigated. TOC values in sediments were between 0.4% and 4.8%, and sand content between 2% and 88%, silt 8% and 78%, and the clay between from 0% to 9% (Table S2). Spearman correlation analysis revealed that AA activity was strongly positively correlated with% TOC (r 0.463, p 6.4×10^{-6}) and also, to a lower extent, with % of silt content (r 0.264, p 0.014) and negatively correlated with % sand (Table S3, Fig. S1). In turn, TOC was strongly correlated with % silt (r .525, p 1.8×10^{-7}). This finding is in agreement with previous reports which indicate that many of the environmental contaminants with AA activity identified in effluents or sediment fractions are moderately hydrophobic and are likely to be associated with organic matter sorbed onto particulates (Rostkowski et al., 2011; Weiss et al., 2009). The relationship between TOC and finer particle sizes in sediments such as clay/silt is well established (Tyson, 1995) and

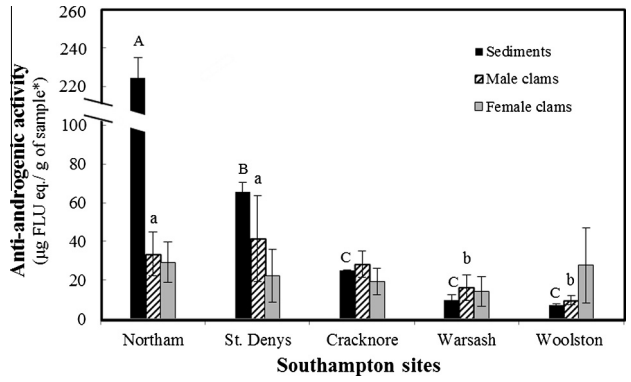

Fig. 2. Concentrations of anti-androgenic activity levels measured in sediments, male and female clams sampled from 5 sites in the Southampton estuary. *AA activity for sediments and clams is expressed as µg FLU eq adjusted for g of dry weight of sediment sample or wet weight of clam tissue. Significant differences in AA activity between sample groups analysed as a one way ANOVA ($p < 0.05$) and calculated from the Tukey post hoc test. A, B, C or a,b different letters indicate statistical significance at $P = 0.05$ for sediments or male clams respectively. No significant differences were detected between sites for female clams. Error bars refer to one SD for 3 analytical replicates of sediments, and between 6–14 replicates for either males or female clams at all sites except Cracknore which had 4 replicates for each sex.

in our study silt, rather than clay, dominated the fine particle fractions and therefore accounted for the majority of TOC content in the samples.

3.2. Levels of anti-androgen activity in clams sampled at Southampton estuary

The AA levels in clams sampled from five sites in the Southampton estuary were measured. At these sites sediment AA activity ranged between background levels at 7.2 ± 0.5 to as high as 224 ± 38.4 µg FLU eq/g dw and populations of *S. plana* were also available for sampling. Sediments sampled from Northam contained significantly higher levels of AA activity compared with the St Denys site which in turn contained higher levels of activity compared with the Cracknore, Warsash and Woolston sites (Fig. 2). AA activity was detected in clam extracts and only quantified at dilutions which were not toxic in the MTT assay. There were significant increases in AA activity in male clams sampled at Northam (33.5 ± 11.4 µg FLU eq/g ww) and St Denys (41.5 ± 22.3 µg FLU eq/g ww) compared with those sampled at Warsash (16.3 ± 6.6 µg FLU eq/g) and Woolston (9.7 ± 2.4 µg FLU eq/g). No significant differences in AA activity were detected between female clams collected at these sites, however levels of AA activity in

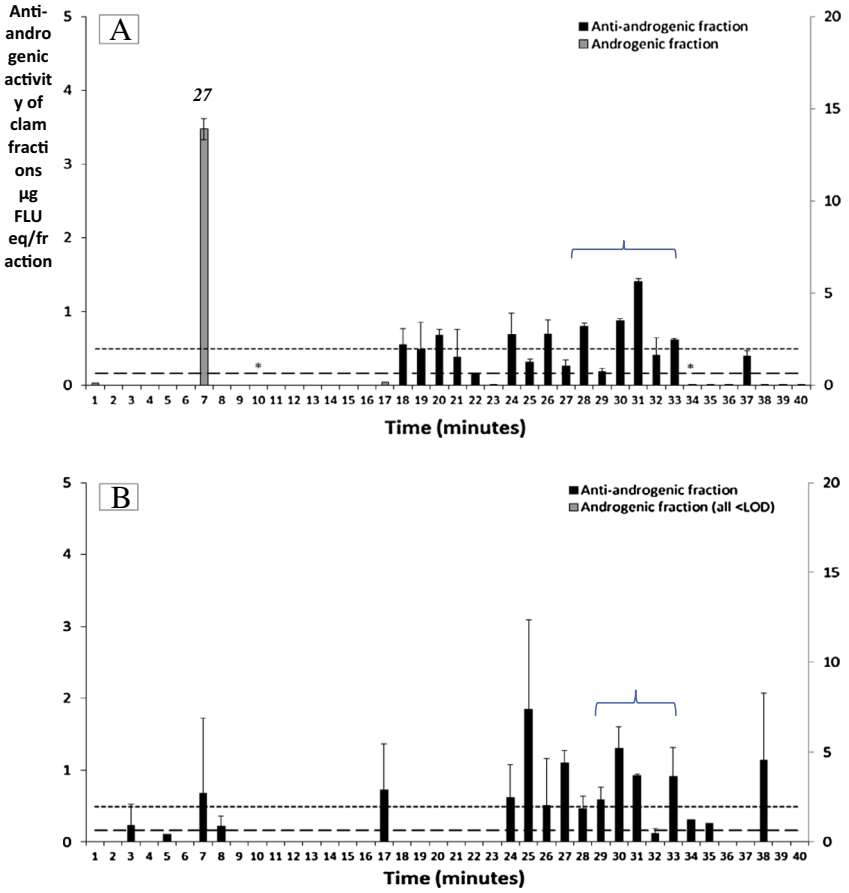

Fig. 3. Androgen receptor activity profiles of extracts of clams sampled at Northam, Southampton estuary. A composite sample of extracts from male or female clams extracts were fractionated by reverse phase HPLC and the fractions analysed for androgen receptor agonist and antagonist activity using a mammalian-cell based androgen receptor screen (AR-CALUX). (A) profiles from males; 7.6 µg FLU eq AA activity injected onto HPLC, (B) profiles from females; 19.2 µg FLU eq injected into HPLC. Numbers above fractions indicate fold dilution needed to either reduce toxicity to assay antagonist activity or to quantify agonist activity at non saturated concentrations. * = fractions 10 and 34 in male were still toxic after a 6 fold dilution. Results are given as mean ± range of two independent analyses per fraction. Dotted lines at 0.16 and 0.49 µg FLU eq/fraction represent respectively limit of detection and quantification for antagonistic activity; limit of detection and quantification for agonist activity were respectively 0.04 and 0.13 ng DHT eq/g fraction. Retention times of pure standards in RP-HPLC system were: methylparaben (6.3 min), bisphenol A (13.5 min), dichlorophene (18.6 min), chlorophene (20.6 min), triclosan (24.0 min), abietic acid (30.4 min), p-p′ dichlorodiphenyldichloroethylene (30.9 min).

Table 1
Androgen receptor active compounds identified in fractions of clam extracts.

HPLC fraction	Name of compound	Formula	CAS number	GC–MS ions (m/z)	GC–MS retention time (RT) min	Anti-Androgenic activity of HPLC fraction in CALUX assay (ng FLU eq/fraction)		ng of compound in clam fraction quantified by GC–MS		Relative potency to flutamide in AR-CALUX	Contribution to AA activity of fraction (%)	
						Male	*Female*	*Male*	*Female*		*Male*	*Female*
27	Fluoranthene	$C_{16}H_{10}$	206-44-0	101/**202**	17.60	261.1*	1103.7	219.4	357.9	0.955	80.2	31.0
28	Pyrene	$C_{16}H_{10}$	129-00-0	101/**202**	18.19	795.7	460.6*	188.6	ND	0.409	9.7	–
29–30	Unknown	–	–	108/216	18.88	1051.9	1891.5	–	–	–	–	–
	Unknown	–	–	108/216	19.74			–	–	–	–	–
	Benzo[b]fluorene	$C_{17}H_{12}$	243-17-4	108/**216**	19.47			1.3	2.2	1.87	0.2	0.2
	Benzo[a]anthracene	$C_{18}H_{12}$	56-55-3	101/113/202/**228**	21.83			729.1	1065.3	1.27	88.0	71.5
	Chrysene	$C_{18}H_{12}$	218-01-9	101/113/202/**228**	21.94			–	–	Not active	–	–
31	Benzo[k]fluoranthene	$C_{20}H_{12}$	207-08-9	113/125/224/**252**	25.31	1401.2	922.1	633.7**	1029.5**	2.57	116.2**	286.9**
	Mixture of unknown	$C_{20}H_{12}$	–	113/125/224/**252**	25.21			–	–	–	–	–
	Benzofluoranthene and Benzopyrene positional isomers		–	113/125/224/**252**	26.20			–	–	–	–	–
			–	113/125/224/**252**	26.41			–	–	–	–	–
			–	113/125/224/**252**	26.73			–	–	–	–	–
33	Indeno[1,2,3-*cd*]pyrene	$C_{22}H_{12}$	193-39-5	138/**276**	31.86	611.1	911.4	2.0	1.6	0.35	0.1	0.1
	Benzo[*ghi*]perylene	$C_{22}H_{12}$	191-24-2	138/**276**	33.89			–	–	Not active	–	–

HPLC fraction	Name of compound		CAS number	GC–MS ions after BSTFA derivatization	GC–MS RTmin	Androgenic activity of HPLC fraction in CALUX assay (ng DHT eq/fraction)		ng of compound in clam fraction		Relative potency to DHT	Contribution to agonist activity of fraction (%)	
						Male	*Female*	*Male*	*Female*		*Male*	*Female*
7	Dihydrotestosterone	$C_{19}H_{30}O_2$	521-18-6	**129**/257/272/319/347/362	23.41	13.9	Not active	4.9	ND	1	35.2	–

Bold characters for GC–MS data denote the quantifier ions used for the different chemicals.
* values below method LOQ of 490.0 ng FLU eq/fraction. ND = not detected and < LOD of 1.0 ng/fraction.
** values overestimated due to co-elution with isomeric compounds.

females were higher at Northam and St Denys (29.3 ± 10.6 and 22.2 ± 13.6 μg FLU eq/g respectively) compared with Cracknore and Warsash (19.4 ± 6.9 and 14.2 ± 7.7 μg FLU eq/g) whereas samples from Woolston were very variable (Fig. 2). All clams contained AA activity above the LOD of 2.2 μg FLU eq/g ww and no agonist activity was detected in any sample extract, possibly due to masking by the significant levels of AA activity present in the extracts.

3.3. Profiles and characterisation of androgen receptor active compounds in clam tissues

In order to characterise the bioavailable AA activity present in clam tissues, a composite sample of male or female clam extracts from the most contaminated site, Northam, was fractionated on HPLC and assayed using the AR CALUX assay in the presence of DHT agonist. Fractions were also analysed without DHT to detect any agonist activity which may have been masked in the previous analysis of the unfractionated extracts. Fractions showing androgen receptor activity were analysed by GC–MS to identify receptor active compounds. The antagonistic activity for extracts from male and female clams revealed similar AA profiles with the highest activity mostly concentrated between fractions 24 and 33, where mainly lipophilic chemicals elute on HPLC (Fig. 3). Summing the AA activity detected in fractions above the LOQ level, revealed that the recovery of AA activity after fractionation was the 111.7% and 60.3% of the total AA activity injected onto the HPLC system for male and female clams extracts respectively. It was possible that some losses in recovery of AA activity from the female extract may be due to incomplete solubilisation of hydrophobic chemicals in the HPLC injection solvent.

Fractions containing AA activity were analysed by GC–MS and those which contained contaminants that were tested for AA activity in the AR-CALUX are shown in Table 1. The main contaminants in the fractions were a series of polycyclic aromatic hydrocarbons (PAHs) which were identified on GC–MS in comparison with the retention time and fragmentation of pure standards. The potencies of the standards relative to flutamide were determined in the AR-CALUX. From their potency measurements, and the mass of PAH measured in the fraction, the contribution of the identified compound to the AA activity measured in the fraction can be determined. Fluoranthene was detected in fraction 27 and accounted for between 31% and 80% of the AA activity in this fraction from male and female clams. Pyrene detected in fraction 28 comprised <10% of AA activity in the fraction from male clams. A number of 4 ring PAHs were identified in fractions 29/30, of which benzo[a]anthracene accounted for between 72% and 88% of the activity of the fractions from male or female clams. Fraction 31 contained benzo[*k*]fluoranthene as well as a number of other benzofluoranthene and benzopyrene PAHs which were poorly separated by GC. The apparent concentrations of benzo[*k*]fluoranthene accounted for significant levels of AA activity in the fractions, but actual concentrations of this PAH in the fractions could not be determined accurately due to co-elution with other unidentified isomeric compounds. Higher molecular weight 6 ring PAHs were also identified in fraction 33 but these did not account for the AA activity in this fraction. This data reveals that some of the AA activity in clam fractions was due to PAH contaminants that may have been present in the sediments, pore and surface waters of the Northam site. The identified PAHs revealed a potency of between 0.3 and 2.6 in AR-CALUX in relation to flutamide indicating that some, such as benzo[*k*]fluoranthene, are potent androgen receptor antagonists in vitro. These contaminants may have dual mechanisms of toxicity as planar PAHs are normally associated with the activation of the aryl hydrocarbon rather than androgen receptor, and as a result have the potential to induce the neoplastic transformations in cells (Dietrich and Kaina, 2010). It is likely that the PAHs arose from exposure of clams to fossil oil spillage either from shipping or oil refining industries present in Southampton estuary, although a contribution from pyrogenic sources is also possible (Ghekiere et al., 2013; Manzetti, 2013; Nikolaou et al., 2009; Zemo, 2009). However, the significance of this finding of the bioaccumulation of AA contaminants in clam tissues is unclear. These contaminants were identified as antagonists in a human androgen receptor transcription screen, and to date there has been no evidence of an androgen receptor in molluscan species. In addition, due to metabolism and toxicokinetics, a demonstration of activity in vitro may not necessarily indicate similar activity in in vivo exposures. Further work is required to determine whether exposure to AA contaminants, either alone or in the presence of estrogens, can induce intersexuality in *S. plana.*

A strongly androgenic fraction eluting at 7 min was also detected in profiles of male but not in female clams (Fig. 3). This fraction contained high levels of agonist activity and had to be diluted 27-fold to allow quantification on the linear part of the response curve of the receptor assay. UHPLC–TOFMS analysis of the fraction in +ESI mode revealed it contained an ion of *m*/*z* 291.2308 corresponding to the $[M+H]^+$ ion of $C_{19}H_{30}O_2$ (mass accuracy of 3 ppm) and a compound with a C19 steroidal structure. High energy MS revealed a fragment at *m*/*z* 255.2112. Analysis of a pure standard of DHT revealed the same retention time, accurate mass of the parent ion and fragment for this steroid. An aliquot of the fraction was analysed by GC–MS and the silyl ether derivative also resulted in the same retention time and ion fragments as that of the pure standard of DHT. Quantification of DHT in fraction 7 from males, revealed that a concentration 4.9 ng/g was present in the clam fraction, which accounted for 35.2% of the total AA activity suggesting that other compounds with agonist activity could also present in this fraction (Table 1).

The presence and role of androgenic sex steroids in marine molluscs is currently unclear and it has been suggested that, in the case of vertebrate sex steroids, they are accumulated from the external environment due to contamination with human waste rather than biosynthesised in situ by the mollusc (see reviews by Scott, 2012, 2013). However, in this study the DHT was not detected in workup blank samples nor from the corresponding fraction from female clams which had been collected in the same sample area. A number of studies have identified androgenic steroids such as testosterone in molluscs, however to date there is little evidence date that DHT is present as a major androgen in these invertebrates, or that androgens can be synthesized de novo in molluscs (Scott, 2012). There is much evidence that vertebrate type sex-steroids are readily metabolised in molluscs to long chain fatty acid esters (Labadie et al., 2007; Peck et al., 2007). In addition, the 5-alpha reductase enzyme which catalyses metabolism of androstenedione or testosterone to DHT has been reported in mollusc tissues (Janer et al., 2005). Sex differences in the metabolism of androstenedione were detected in the gastropod *Bolinus brandaris* and were attributed to higher 5 alpha-reductase activity in males (Lyssimachou et al., 2009). It is possible that the sex differences in levels of DHT detected in S. plana in this study were due to differences in esterification or other metabolisms of androgens that had accumulated in tissues. Clearly further studies are needed to determine whether *S. plana* do biosynthesize androgenic steroids rather than accumulate them from contaminated environments.

4. Conclusions

The contamination of sediments in a number of coastal habitats in the Transmanche Channel with high levels of AA activity raises concerns as to whether benthic organisms such as molluscs are susceptible to contaminants that have the potential to disrupt androgen signalling in vertebrate systems. In freshwaters, bioavailable anti-androgens present in fish tissues have been associated with

chlorinated contaminants such as germicides present in wastewater effluents. However, the identity of bioavailable anti-androgens accumulating in clams from contaminated sites appears to be PAH molecules from petrogenic sources. This suggests a potential additional mechanism of toxicity for these common contaminants other than that mediated by the aryl hydrocarbon receptor. In vivo investigations are needed to determine whether exposure to PAHs induce intersexuality in *S. plana* and studies are ongoing to identify the key mixtures of AA PAHs present in marine sediments. In addition, investigations on the identity and source of sex steroids in the clam are needed to determine their potential susceptibility to anti-androgen or estrogen exposure. To date, studies investigating the effects of chemical causes of sexual dysfunction in aquatic organisms often utilise mammalian-based sex steroid receptor assays and there is a need to develop mollusc-based receptor screening tools in order to determine the risk of exposure to endocrine disrupting chemicals in the aquatic environment.

Acknowledgments

This work funded by the European Regional Development Fund in the framework of the INTERREG IV A France (Channel) – England programme (DIESE project number 4040).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.marpolbul.2014.11.014.

References

Bergman, A., Heindel, J., Jobling, S., KA, K., Zoeller, R.T., 2012. State of the Science of Endocrine Disrupting Chemicals 2012. WHO, UNEP.

Chesman, B.S., Langston, W.J., 2006. Intersex in the clam *Scrobicularia plana*: a sign of endocrine disruption in estuaries? Biol. Lett. 2, 420–422.

Creusot, N., Tapie, N., Piccini, B., Balaguer, P., Porcher, J.M., Budzinski, H., Ait-Aissa, S., 2013. Distribution of steroid- and dioxin-like activities between sediments, POCIS and SPMD in a French river subject to mixed pressures. Environ. Sci. Pollut. Res. 20, 2784–2794.

Dietrich, C., Kaina, B., 2010. The aryl hydrocarbon receptor (AhR) in the regulation of cell–cell contact and tumor growth. Carcinogenesis 31, 1319–1328.

EEA, 2012. The impacts of endocrine disrupters on wildlife, people and their environments. The Weybridge+15 (1996–2011) report. EEA Technical Report 2, 1–112.

El Rayis, O.A., 1985. Re-assessment of the titration method for the determination of organic carbon in recent sediments. Rapp. Comm. Int. Mer. Medit. 29, 45–47.

Ghekiere, A., Verdonck, F., Claessens, M., Monteyne, E., Roose, P., Wille, K., Goffin, A., Rappe, K., Janssen, C.R., 2013. Monitoring micropollutants in marine waters, can quality standards be met? Mar. Pollut. Bull. 69, 243–250.

Gomes, T., Gonzalez-Rey, M., Bebianno, M.J., 2009. Incidence of intersex in male clams *Scrobicularia plana* in the Guadiana Estuary (Portugal). Ecotoxicology 18, 1104–1109.

Grung, M., Naes, K., Fogelberg, O., Nilsen, A.J., Brack, W., Lubcke-von Varel, U., Thomas, K.V., 2011. Effects-directed analysis of sediments from polluted marine sites in Norway. J. Toxicol. Environ. Health Part A 74, 439–454.

Hill, E.M., Evans, K.L., Horwood, J., Rostkowski, P., Oladapo, F.O., Gibson, R., Shears, J.A., Tyler, C.R., 2010. Profiles and some initial identifications of (anti)androgenic compounds in fish exposed to wastewater treatment works effluents. Environ. Sci. Technol. 44, 1137–1143.

Janer, G., LeBlanc, G.A., Porte, C., 2005. A comparative study on androgen metabolism in three invertebrate species. Gen. Comp. Endocrinol. 143, 211–221.

Jobling, S., Williams, R., Johnson, A., Taylor, A., Gross-Sorokin, M., Nolan, M., Tyler, C.R., van Aerle, R., Santos, E., Brighty, G., 2006. Predicted exposures to steroid estrogens in UK Rivers correlate with widespread sexual disruption in wild fish populations. Environ. Health Perspect. 114, 32–39.

Jobling, S., Burn, R.W., Thorpe, K., Williams, R., Tyler, C., 2009. Statistical modeling suggests that antiandrogens in effluents from wastewater treatment works contribute to widespread sexual disruption in fish living in English rivers. Environ. Health Perspect. 117, 797–802.

Johnson, I., Hetheridge, M., Tyler, C.R., 2007. Assessment of (anti-)oestrogenic and (anti-androgenic activities of final effluents from sewage treatment works. Science Report-SCO20118/SR Environment Agency.

Kinani, S., Bouchonnet, S., Creusot, N., Bourcier, S., Balaguer, P., Porcher, J.M., Ait-Aissa, S., 2010. Bioanalytical characterisation of multiple endocrine- and dioxin-like activities in sediments from reference and impacted small rivers. Environ. Pollut. 158, 74–83.

Kortenkamp, A., Martin, O., Faust, M., Evans, R., McKinlay, R., Orton, F., Rosivatz, E., 2011. State of the art assessment of endocrine disrupters. 2011 European Commission, DG Environment (Final Report Published: 2011).

Koyama, J., Kitoh, A., Nakai, M., Kohno, K., Tanaka, H., Uno, S., 2013. Relative contribution of endocrine-disrupting chemicals to the estrogenic potency of marine sediments of Osaka Bay, Japan. Water Air Soil Pollut. 224, 1570–1579.

Labadie, P., Peck, M., Minier, C., Hill, E.M., 2007. Identification of the steroid fatty acid ester conjugates formed in vivo in *Mytilus edulis* as a result of exposure to estrogens. Steroids 72, 41–49.

Langston, W.J., Burt, G.R., Chesman, B.S., 2007. Feminisation of male clams *Scrobicularia plana* from estuaries in Southwest UK and its induction by endocrine-disrupting chemicals. Mar. Ecol.-Prog. Ser. 333, 173–184.

Lyssimachou, A., Ramon, M., Porte, C., 2009. Comparative study on the metabolism of the androgen precursor androstenedione in two gastropod species: in vitro alterations by TBT and TPT. Comp. Biochem. Phys. C 149, 409–413.

Macikova, P., Kalabova, T., Klanova, J., Kukucka, P., Giesy, J.P., Hilscherova, K., 2014. Longer-term and short-term variability in pollution of fluvial sediments by dioxin-like and endocrine disruptive compounds. Environ. Sci. Pollut. Res. 21, 5007–5022.

Manzetti, S., 2013. Polycyclic aromatic hydrocarbons in the environment: environmental fate and transformation. Polycycl. Aromat. Compd. 33, 311–330.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival – application to proliferation and cyto-toxicity assays. J. Immunol. Methods 65, 55–63.

Nikolaou, A., Kostopoulou, M., Lofrano, G., Meric, S., 2009. Determination of PAHs in marine sediments: analytical methods and environmental concerns. Glob. Nest. J. 11, 391–405.

Pascoal, S., Carvalho, G., Vasieva, O., Hughes, R., Cossins, A., Fang, Y.X., Ashelford, K., Olohan, L., Barroso, C., Mendo, S., Creer, S., 2013. Transcriptomics and in vivo tests reveal novel mechanisms underlying endocrine disruption in an ecological sentinel, *Nucella lapillus*. Mol. Ecol. 22, 1589–1608.

Peck, M., Gibson, R.W., Kortenkamp, A., Hill, E.M., 2004. Sediments are major sinks of steroidal estrogens in two United Kingdom rivers. Environ. Toxicol. Chem. 23, 945–952.

Peck, M.R., Labadie, P., Minier, C., Hill, E.M., 2007. Profiles of environmental and endogenous estrogens in the zebra mussel *Dreissena polymorpha*. Chemosphere 69, 1–8.

Rostkowski, P., Horwood, J., Shears, J.A., Lange, A., Oladapo, F.O., Besselink, H.T., Tyler, C.R., Hill, E.M., 2011. Bioassay-directed identification of novel antiandrogenic compounds in bile of fish exposed to wastewater effluents. Environ. Sci. Technol. 45, 10660–10667.

Schmitt, S., Reifferscheid, G., Claus, E., Schlusener, M., Buchinger, S., 2012. Effect directed analysis and mixture effects of estrogenic compounds in a sediment of the river Elbe. Environ. Sci. Pollut. Res. 19, 3350–3361.

Scott, A.P., 2012. Do mollusks use vertebrate sex steroids as reproductive hormones? Part I: Critical appraisal of the evidence for the presence, biosynthesis and uptake of steroids. Steroids 77, 1450–1468.

Scott, A.P., 2013. Do mollusks use vertebrate sex steroids as reproductive hormones? II. Critical review of the evidence that steroids have biological effects. Steroids 78, 268–281.

Sonneveld, E., Jansen, H.J., Riteco, J.A., Brouwer, A., van der Burg, B., 2005. Development of androgen- and estrogen-responsive bioassays, members of a panel of human cell line-based highly selective steroid-responsive bioassays. Toxicol. Sci. 83, 136–148.

Sumpter, J.P., Jobling, S., 2013. The occurrence, causes, and consequences of estrogens in the aquatic environment. Environ. Toxicol. Chem. 32, 249–251.

Tankoua, O.F., Amiard-Triquet, C., Denis, F., Minier, C., Mouneyrac, C., Berthet, B., 2012. Physiological status and intersex in the endobenthic bivalve *Scrobicularia plana* from thirteen estuaries in northwest France. Environ. Pollut. 167, 70–77.

Titley-O'Neal, C.P., Munkittrick, K.R., MacDonald, B.A., 2011. The effects of organotin on female gastropods. J. Environ. Monit. 13, 2360–2388.

Tyson, R.V., 1995. Sedimentary Organic Matter: Organic Facies and Palynofacies. Chapman & Hall, London, New York.

Urbatzka, R., van Cauwenberge, A., Maggioni, S., Vigano, L., Mandich, A., Benfenati, E., Lutz, I., Kloas, W., 2007. Androgenic and antiandrogenic activities in water and sediment samples from the river Lambro, Italy, detected by yeast androgen screen and chemical analyses. Chemosphere 67, 1080–1087.

van der Burg, B., Winter, R., Man, H.Y., Vangenechten, C., Berckmans, P., Weimer, M., Witters, H., van der Linden, S., 2010. Optimization and prevalidation of the in vitro AR CALUX method to test androgenic and antiandrogenic activity of compounds. Reprod. Toxicol. 30, 18–24.

Weiss, J.M., Hamers, T., Thomas, K.V., van der Linden, S., Leonards, P.E., Lamoree, M.H., 2009. Masking effect of anti-androgens on androgenic activity in European river sediment unveiled by effect-directed analysis. Anal. Bioanal. Chem. 394, 1385–1397.

Zemo, D.A., 2009. Use of parent polycyclic aromatic hydrocarbon (PAH) proportions to attribute PAH sources in sediments: a case study from the Pacific Northwest. Environ. For. 10, 229–239.