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

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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.

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).

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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