In vitro screening for endocrine disruptive activity in selected South African harbours and river mouths

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Various waterborne anthropogenic contaminants disrupt the endocrine systems of wildlife and humans, targeting reproductive pathways, among others. Very little is known, however, regarding the occurrence of endocrine disruptive activity in South African freshwater ecosystems, and coastal ecosystems have not been studied in this regard. In a first attempt to investigate endocrine disruptive activity in South African coastal waters, surface water samples collected from harbours, river mouths and estuaries in three metropolitan municipalities, eThekwini (which includes Durban), Nelson Mandela (specifically Port Elizabeth Harbour) and City of Cape Town, were screened for (anti) oestrogenicity and (anti)androgenicity using recombinant yeast bioassays. Moreover, levels of the female hormone 17β-(o)estradiol (E2) were determined in all samples, as well as a selection of hydrocarbons in the eThekwini samples. A high proportion of samples collected from eThekwini were oestrogenic, whereas none from Port Elizabeth Harbour and only a single river mouth sampled in the City of Cape Town were oestrogenic. E2 was detected in all the samples tested, but at higher concentrations at the eThekwini and City of Cape Town localities than Port Elizabeth Harbour. In addition, the recombinant yeast assays revealed that anti-androgenicity was widespread, being detected in the majority of samples screened apart from those representing Port Elizabeth Harbour. Conversely, no anti-oestrogenic or androgenic activity was detected. Anti-androgenicity did not associate with hydrocarbon loads, providing evidence that other anti-androgens were responsible for the observed activity. The present data suggest potential reproductive disruption in marine and estuarine fauna inhabiting the eThekwini and City of Cape Town regions.

Keywords: coastal ecosystem, endocrine disruptors, estuary, pollution

Online supplementary material: Supplementary material containing additional detail of sampling locations, hydrocarbon concentrations and methods can be found online at http://dx.doi.org/10.2989/1814232X.2015.1105296.

Introduction

Estuaries are the final phase of surface-water runoff (from drainage basins) into oceans and therefore act as repositories for a range of contaminants of agricultural, residential and industrial origin (Kennish 1994). Certain marine fish species use estuaries as nurseries; in particular, these fish spawn at sea and enter estuaries as juveniles, where they remain until just prior to sexual maturity. Other marine or freshwater fish occasionally enter estuaries, but this is not a feature of their life cycle (Whitfield 1999; Elliot et al. 2007). The estuarine ichthyofaunal assemblage, therefore, consists of marine and freshwater species and estuaries can be a contact point for pollutants to enter both marine and freshwater food webs. Various anthropogenic and natural chemicals have been identified as endocrine disruptors (i.e. endocrine disrupting chemicals [EDCs]) (Colborn et al. 1993). Reproductive impairment associated with EDC exposure has been observed in freshwater and marine organisms (Jobling et al. 1998; Allen et al. 1999; Ford et al. 2007; Kidd et al. 2007). The presence of EDCs that modulate the reproductive system may affect fecundity in marine fish species that use estuaries as nurseries, because these fish are exposed during the maturation of their reproductive organs. Crustaceans and molluscs that inhabit estuaries represent a further route of entry of EDCs, such as certain persistent organic pollutants (POPs), into food webs (Geyer et al. 2000; Vos et al. 2000). In fact, it has been shown that invertebrate hormone systems and other key physiological processes such as cellular metabolism and immunity, as well as embryonic development, can be affected by certain EDCs (Depledge and Billinghamurst 1999; Zhou et al. 2010, 2011). The evaluation of surface waters in estuarine habitats for endocrine disruptive activity is therefore important for risk assessment and holistic conservation efforts.

Harbours are important entrance points to the marine environment for pollutants (Young et al. 1979; Soclo et al. 2000), especially petrochemicals from vessel exhaust emissions and spillage (Soclo et al. 2000; Mestres et al. 2010). Certain petrochemicals are known endocrine disruptors (Tollefsen et al. 2007; Vrable et al. 2010; Wang et al. 2010) and organisms occurring in the proximity of harbours may therefore be affected.

In a recent review of the status of marine pollution research in South Africa, Wepener and Degger (2012) highlighted the need for more studies on organic pollutants.
in South African coastal waters. Studies on EDCs in the South African coastal environment are extremely limited, and apart from Marshall and Rajkumar (2003), who observed imposex in the mollusc *Nassarius kraussianus*, report on contaminant loads only (Fatoki and Awofolu 2004; Bollmohr et al. 2007; Ogata et al. 2009; Ryan et al. 2012; Wepener and Degger 2012; La Guardia et al. 2013). It is difficult, however, to accurately predict endocrine disruptive (biological) activity based on chemical data due to mixture interactions (Kortenkamp 2007); bioassays, *in vivo* exposures or animal tissue are more reliable predictors.

The aim of this study was to screen surface water from selected South African rivers, harbours and estuarine environments in three metropolitan municipalities on the South African coastline, namely eThekwini (which includes Durban), Nelson Mandela (specifically Port Elizabeth Harbour) and the City of Cape Town, for endocrine modulation effects (hormone receptor interaction) and female reproductive hormone contamination. The specific objectives were to measure (anti)oestrogenic and (anti)androgenic activity in surface water samples using recombinant yeast bioassays (interaction with human steroid receptors), and environmental concentrations of the female hormone 17β-(o)estradiol (E2).

A further aim was to explore the association between hydrocarbon loads and endocrine disruptive activity in surface water samples. For this purpose the concentrations of a selection of hydrocarbons were determined in Durban Bay and selected rivers and estuaries in the greater eThekwini metropolitan area.

**Material and methods**

**Sample collection and extraction**

Surface water was collected at 10 localities in the eThekwini region in August 2012, and three and six locations were sampled in the Nelson Mandela (Port Elizabeth Harbour) and City of Cape Town regions, respectively, during October 2012 (Figure 1, Supplementary Table S1, available online). The samples were collected in 500 ml acid-cleaned, amber glass bottles with PTFE-lined caps, kept on ice and shipped to the laboratory within 24 h. The samples were subsequently filtered through 0.5 µm glass fibre filters (MN 85/90 Macherey-Nagel, Germany), and the pH adjusted to 3 using H₂SO₄. Non-polar and slightly polar compounds were extracted from 250 ml of water within 96 h of collection using 500 mg DSC-18 columns and a Visiprep® manifold system (~10 ml min⁻¹) (Sigma, South Africa). The columns were flushed with 50 ml of Milli-Q water directly after environmental samples were passed through, in order to remove salts. A Milli-Q negative control was included during each extraction event. The SPE columns were subsequently air-dried overnight, after which compounds were eluted from the column using a solvent mixture (40% hexane, 45% methanol and 15% 2-propanol), air-dried, reconstituted in absolute ethanol and stored at −20 °C.

**In vitro recombinant yeast assay**

Oestrogenic, anti-oestrogenic, androgenic and anti-androgenic activity of surface water C18 extracts (representing non-polar and slightly polar compounds) were evaluated using recombinant yeast bioassays (Routledge and Sumpter 1996; Sohoni and Sumpter 1998). The yeast culture and exposure was performed as described by Sohoni and Sumpter (1998) with slight modifications, described in more detail in the supplementary material, available online.

Samples were assayed in duplicate at a 10× and 5× concentrated state (1× denotes the state in nature) in two experiments performed using different yeast stocks and environmental sample loading to assay plates. Each assay...
plate contained a 12-point serial dilution of E2 (≥98% pure), tamoxifen (TAM) (≥99% pure), dihydrotestosterone (DHT) (≥97.5% pure) or flutamide (FLU) (Sigma, South Africa) as standards, and a solvent control. Oestrogen and androgen receptor antagonism were evaluated in the presence of 1.43 nM E2 and 7.13 nM DHT, respectively. Oestradiol-, dihydrotestosterone-, tamoxifen- and flutamide equivalents were calculated for environmental samples using standard curves generated during experiments (see supplementary material for more information). These equivalents express biological activity (i.e. hormone receptor agonism [E2 and DHT] or hormone receptor antagonism [FLU and TAM]) equivalent to the activity associated with the particular hormone or pharmaceutical standard represented. Cell densities were determined spectrophotometrically at 620 nm absorbance (Abs). Sample-containing wells with Abs620nm below a specific cell density/viability benchmark were calculated for environmental samples using standard deviation Abs620(solvent control) – 3[standard deviation Abs620(solvent control)] were not included in the analyses due to potential inhibitory effects.

**Enzyme-linked immunosorbent assays (ELISAs)**

17β-oestradiol levels were measured in the C18 SPE extracts of water collected from the 19 localities using a commercially available ELISA kit (DRG International Inc., USA) according to the manufacturer’s instructions. The extracted samples in ethanol (2 000 × concentrated) were diluted 1/20 in a 0.1% w/v human serum albumin and 0.9% NaCl solution, and assayed (Swart and Pool 2007).

**Hydrocarbon analysis**

The concentrations of an array of hydrocarbons (the polycyclic aromatic hydrocarbon (PAH) isomers benzene, ethylbenzene, toluene, o-xylene, m′p′-xylene (collectively BTEX), naphthalene, acenaphthene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(g,h,i)perylene, indeno(1,2,3-c,d)pyrene, benzo(a)pyrene and dibenzo(a,h)anthracene), and total petroleum hydrocarbon (TPH) carbon equivalent ranges C10–C12, C12–C16, C16–C21, C21–C30, C30–C35 and C35–C40, were analysed in surface water samples collected in the eThekwini region. The analyses were performed by the commercial analytical laboratory Eurofins Analytico (Barneveld, Netherlands), using accredited methods that are proprietary. Briefly, internal standards were added to allquots of water samples. BTEX was analysed using a headspace gas chromatograph mass spectrometer. TPHs were analysed using large volume injection-gas chromatograph-flame ionisation detection with a heating profile between 175 and 575 °C. PAHs were extracted using dichloromethane, exchanged into acetonitrile, injected into a high pressure liquid chromatograph column, and eluted using a water/acetonitrile gradient. The PAHs were detected using ultraviolet absorbance. Quantification was performed against internal standards, but certified reference material was not analysed for purposes of quality assurance and control.

**Statistical analyses**

The correlation between E2 concentration and oestrogenicity was evaluated using the Spearman Rank test (Statistica 12, Statsoft, USA). Potential associations between hydrocarbons and anti-androgenic activity among sampling locations were evaluated using a principal component analysis bi-plot (Canoco 5, Microcomputer Power, USA). A p-value < 0.05 was considered significant.

**Results**

Oestradiol was detected in all samples analysed, although at concentrations <1 ng l–1 in Port Elizabeth and Cape Town harbours and in samples from the Lourens (LOU) and Palmiet (PAL) estuaries (Figure 2). The highest E2 concentration was detected in the mouth of the Salt River (SLT) in Cape Town (20.96 ng l–1), followed by the Isipingo Estuary (ISI) in the eThekwini region (17.41 ng l–1).

Oestrogenicity (binding to the human (o)estrogen receptor [ER]) was detected in seven of the 19 samples analysed (Figure 3). The eThekwini region had the highest proportion of oestrogenic samples, with activities ranging between 1.33 and 8.01 ng l–1 E2 equivalents (EEQs) (Figure 3). Conversely, no activity was detected in the Port Elizabeth Harbour samples. The sample collected at the mouth of Salt River in Cape Town was the most oestrogenically active, with an EEQ of 12.82 ng l–1. Although the EEQs (Figure 3) followed a similar trend as E2 concentrations (Figure 2), for example, being highest in the mouth of the Salt River, EEQs were generally lower than E2 concentrations measured using ELISA. There was a significant correlation between E2 concentrations and oestrogenic activity when the three study regions were evaluated collectively (Spearman rank r = 0.82, p < 0.05).

Anti-androgenicity (inhibition of androgen binding to the human androgen receptor [AR]) was more widespread than oestrogenicity and detected in 14 of the 19 samples analysed (Figure 4). All samples collected in the eThekwini region were anti-androgenic, with flutamide equivalents (FEQ) ranging between 89.10 and 604.44 µg l–1. None of the Port Elizabeth Harbour samples were anti-androgenic. Similarly, no inhibition of androgen binding to AR was observed in the Cape Town Harbour samples, whereas all four estuarine and river mouth samples collected in the City of Cape Town region were anti-androgenic, ranging between 59.60 and 196.86 µg FEQ l–1 (Figure 4).

No anti-oestrogenic or anti-androgenic activity was detected in any of the samples analysed.

A bi-plot of a principal component analysis of hydrocarbons and anti-androgenicity indicates three major groupings for sampling locations in the eThekwini region (Figure 5). In particular, locations AMA and ISI1 (see Figure 1) grouped together and were associated with the majority of TPHs analysed, PAH isomers pyrene, fluoranthene, anthracene and phenanthrene, and the sum of all PAH isomers. In addition, locations DBAY1, DBAY2, IVC, UML2, UMB and ISI2 grouped together and were more correlated with anti-androgenicity than other samples analysed.

Anti-androgenicity was not closely correlated to any of the hydrocarbons analysed (Figure 5). Hydrocarbon concentrations are presented in the supplementary material (Tables S2 and S3). All BTEX components in the eThekwini study area were below the method detection limit (Table S2). Conversely, the total petroleum hydrocarbon (TPH) analyses...
show the presence of TPHs at all the locations sampled, except for UMH and UML1 (Table S2). The highest diversity of PAHs was detected at the IVC locality in Durban Bay (total PAH: 0.22 µg l⁻¹), followed by AMA, which had the highest abundance of PAHs in the study area (total PAH: 0.28 µg l⁻¹) (Table S3). However, virtually no PAHs were detected at the other locations sampled (Table S3).

Discussion

Published records providing concentrations of organic pollutants such as pesticides and industrial chemicals in the South African coastal environment are extremely limited (Wepener and Degger 2012). Moreover, to our knowledge, no study of the South African coastal environment has described biological endpoints of endocrine disruptive activity, apart from Marshall and Rajkumar (2003), who observed imposex in a mollusc species.

The present study confirms oestrogenic and anti-androgenic activity in surface waters collected in Durban Bay and a number of rivers, river mouths and estuaries in the eThekwini and Cape Town regions. Conversely, no such activity was detected in the Cape Town and Port Elizabeth harbours, indicating good quality in terms of EDCs targeting...
oestrogen or androgen receptors. Durban Bay is a highly transformed estuarine embayment that receives inflows from three rivers and surface runoff from a multitude of stormwater outfalls and canals. Conversely, Port Elizabeth Harbour receives inflow from a single small river, historically an estuary, transformed to built-up land (Veldkornet et al. 2015), and subject to urban surface runoff. There is no riverine inflow to Cape Town Harbour. The higher
oestrogenic and anti-androgenic activity observed in Durban Bay may, therefore, be explained by the sheer number of anthropogenic sources of contaminants entering the bay via its large catchment, compared to the Cape Town and Port Elizabeth harbours.

The oestrogenic activity detected in the eThekwini and City of Cape Town regions (1.33–12.82 ng EEQ l−1) is within the range reported for Chinese rivers and estuaries (Zhao et al. 2011; Rao et al. 2013). Studies in other countries have typically reported lower activity, such as in the Rhine River, Germany (1.1–1.3 ng EEQ l−1) (Pawlowski et al. 2003), the Shannon River basin, Ireland (0.53–2.67 ng EEQ l−1) (Kelly et al. 2010), the Seine and Oise rivers, France (0.30–4.52 ng EEQ l−1) (Cargouet et al. 2004), and in a nationwide study in the Netherlands (0–0.17 ng EEQ l−1, n = 90 samples) which included estuaries (Vethaak et al. 2005). The E2 levels detected throughout the three study areas correlated well with oestrogenic activity, suggesting that the observed oestrogenicity was largely due to female hormone contamination and therefore likely linked to water treatment plant (WWTP) discharges.

The anti-androgenic activity detected in the eThekwini and City of Cape Town regions exceeded 100 µg FEQ l−1 at 12 of the locations sampled, and although relatively high, they were similar to activities reported for surface waters elsewhere. Some examples include the Zhjiang River estuary, China (135 µg FEQ l−1; Zhao et al. 2011), the Lambro River, Italy (438.15 µg FEQ l−1; Urbatiska et al. 2007), and the Ray River, England (5–250 µg FEQ l−1; Grover et al. 2011). In a modelling study, FEQs of 0–100.12 µg l−1 were predicted for 30 rivers in the United Kingdom (Jobling et al. 2009).

Both oestrogenic and anti-androgenic chemicals may affect reproduction by altering sex organ development and function (Arukwe 2001), which may have severe consequences for fish populations (Kidd et al. 2007; Jobling et al. 2009). Oestriadiol concentrations as low as 4 ng l−1 induce the development of ovarian tissue in testes in Japanese medaka Orzias latipes (Metcalfe et al. 2001), and the predicted no-effect concentration (PNEC) for this compound in terms of reproductive impairment in fish has been estimated as 2 ng l−1 (Caldwell et al. 2012). Oestriadiol levels exceeding the PNEC for fish were detected in 10 of the 19 samples analysed in the present study. The pharmaceutical anti-androgen, flutamide, has been shown to disrupt the reproductive system of Asian catfish Clarias batrachus at 33 µg l−1 (Chakrabarty et al. 2012; Rajakumar et al. 2012), Murray rainbowfish Melanotaenia fluviatilis at 125 µg l−1 (Bhatia et al. 2014), and fathead minnow Pimephales promelas at 320 µg l−1 (Filby et al. 2007). The fish populations inhabiting a large proportion of the systems evaluated in the present study, including Durban Bay, are therefore at risk of endocrine disruption, potentially resulting in reproductive disorders such as intersex and impaired reproduction, due to the combined action of oestrogens and anti-androgens. Future studies evaluating phenotypic endpoints such as male plasma vitellogenin levels (Vethaak et al. 2005), the expression of marker genes integral to endocrine signalling (Truter et al. 2014), and histopathology of the gonads (Allen et al. 1999) are necessary, however, to confirm the presence of endocrine disruption. Moreover, seasonal investigations of potential endocrine disruptive activity will be of great value as baseline data and to aid conservation efforts.

Petrochemical pollution is an important component of contamination in harbours (Mestres et al. 2010). PAHs have been detected in sediment and mussels in the ports of Cape Town, Port Elizabeth and Durban, and in sediment in rivers and estuaries in the greater eThekwini and City of Cape Town areas (BKN, unpublished data; see also Degger et al. 2011; Kampire et al. 2015). The anti-androgenic activity observed in the present study may have been caused by petrochemicals, which are known to be potent androgen receptor antagonists (Kizu et al. 2003; Vrabie et al. 2010). The weak correlation observed between anti-androgenicity and hydrocarbon concentrations in the samples from the eThekwini region (Figure 5), however, provides some evidence that androgen receptor antagonists other than hydrocarbons were responsible. Environmental anti-androgens include pesticides, pharmaceuticals and industrial chemicals (Korner et al. 2004). Detailed chemical analyses are required to identify more potential anti-androgens in Durban Bay and the surrounding rivers that were sampled.

The present study is the first on endocrine disruptive activity in the South African coastal environment. It indicated that waters in Durban Harbour, at the Umlazi Canal mouth (UML) and in the Isipingo River estuary, and at the mouth of the Salt River in Cape Town, were oestrogenic. Moreover, widespread anti-androgenicity was observed in Durban Bay and selected rivers in the eThekwini region, and in three estuaries and a river mouth in the City of Cape Town region, although at a lower potency than in the eThekwini region. Conversely, no endocrine disruptive activity was detected in Port Elizabeth Harbour. The levels of oestrogenic and anti-androgenic activity observed are high enough to suggest an effect on the reproduction of certain fish species, and further investigation in this context is required.

Acknowledgements — We thank the South African National Research Foundation, the South African Water Research Commission (Grant No. K5/1977), and the Ernst and Ethel Eriksen Trust for providing funding for this study. We also thank the South African Environmental Observation Network (SAEON) for assistance with sample collection, and Prof. JP Sumpter for providing recombinant yeast strains. We also thank two anonymous reviewers for helpful comments that improved the quality of this article.

References
Allen Y, Matthiessen P, Scott AP, Haworth S, Feist S, Thain JE. 1999. The extent of oestrogenic contamination in the UK estuarine and marine environments – further surveys of flounder. Science of the Total Environment 233: 5–20.
Arukwe A. 2001. Cellular and molecular responses to endocrine-modulators and the impact on fish reproduction. Marine Pollution Bulletin 42: 643–655.
Bhatia H, Kumar A, Ogino Y, Du J, Gregg A, Chapman J et al. 2014. Effects of the commercial antiandrogen flutamide on the biomarkers of reproduction in male Murray rainbowfish (Melanotaenia fluviatilis). Environmental Toxicology and Chemistry 33: 1098–1107.
Bollmohr S, Day JA, Schulz R. 2007. Temporal variability in...
particle-associated pesticide exposure in a temporarily open estuary, Western Cape, South Africa. *Chemosphere* 68: 479–488.

Caldwell DJ, Mastrocco F, Anderson PD, Laenger R, Sumpter JP. 2012. Predicted-no-effect concentrations for the steroid estrogens estrone, 17 beta-estradiol, estradiol, and 17 alpha-ethyl estradiol. *Environmental Toxicology and Chemistry* 31: 1396–1406.

Cargouet M, Perdz D, Mouatassim-Souali A, Tamisier-Karolak S, Levi Y. 2004. Assessment of river contamination by estrogenic compounds in Paris area (France). *Science of the Total Environment* 324: 55–66.

Chakrabarty S, Rajakumar A, Raghuveer K, Sridevi P, Mohanachary A, Prathibha Y et al. 2012. Endosulfan and flutamide, alone and in combination, target ovarian growth in juvenile catfish, *Clarias batrachus*. *Comparative Biochemistry and Physiology C* 155: 491–497.

Colborn T, Saal FSV, Soto AM. 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environmental Health Perspectives* 101: 378–384.

Degger N, Wepener V, Richardson BJ, Wu RSS. 2011. Brown mussels (*Perna perna*) and semi-permeable membrane devices (SPMDs) as indicators of organic pollutants in the South African marine environment. *Marine Pollution Bulletin* 63: 91–97.

Depledge MH, Billinghurst Z. 1999. Ecological significance of endocrine disruption in marine invertebrates. *Marine Pollution Bulletin* 39: 32–38.

Elliott M, Whitleaf AK, Potter IC, Blaber JM, Cyrus DP, Nordlie FG, Harrison TD. 2007. The guild approach to categorizing estuarine fish assemblages: a global review. *Journal of Fish Biology* 8: 241–268.

Fatoki OS, Awofolu OR. 2004. Levels of organochlorine pesticide residues in marine-, surface-, ground- and drinking waters from the Eastern Cape Province of South Africa. *Journal of Environmental Science and Health Part B* 39: 101–114.

Filby AL, Thorpe KL, Maack G, Tyler CR. 2007. Gene expression profiles revealing the mechanisms of anti-androgen- and estrogen-induced feminization in fish. *Aquatic Toxicology 81*: 219–231.

Ford AT, Martins I, Fernandes TF. 2007. Population level effects of intersexuality in the marine environment. *Science of the Total Environment* 374: 102–111.

Geyer HJ, Rimkus GG, Scheunert I, Kaune A, Schramm KW, Kettrup A et al. 2000. Bioaccumulation and occurrence of endocrine-disrupting chemicals (EDCs), persistent organic pollutants (POPs), and other organic compounds in fish and other organisms including humans. *The Handbook of Environmental Chemistry* 2: 1–166.

Grover DP, Balaam J, Pacitto S, Readman JW, White S, Zhou JL. 2011. Endocrine disrupting activities in sewage effluent and river water determined by chemical analysis and *in vitro* assay in the context of granular activated carbon upgrade. *Chemosphere* 84: 1512–1520.

Jobling S, Nolan M, Tyler CR, Brighty G, Sumpter JP. 1998. Widespread sexual disruption in wild fish. *Environmental Science and Technology* 32: 2498–2506.

Jobling S, Burn RW, Thorpe K, Williams R, Tyler C. 2009. Statistical modelling suggests that antiandrogens in effluents from wastewater treatment works contribute to widespread sexual disruption in fish living in English rivers. *Environmental Health Perspectives* 117: 797–802.

Kampire E, Rubidge G, Adams JB. 2015. Distribution of polychlorinated biphenyl residues in sediments and blue mussels (*Mytilus galloprovincialis*) from Port Elizabeth Harbour, South Africa. *Marine Pollution Bulletin* 91: 173–179.

Kelly MA, Reid AM, Quinn-Hosey KM, Fogarty AM, Roche JJ, Brougham CA. 2010. Investigation of the estrogenic risk to feral male brown trout (*Salmo trutta*) in the Shannon International River Basin District of Ireland. *Ecotoxicology and Environmental Safety* 73: 1658–1665.

Kennis M. 1994. Pollution in estuaries and coastal marine waters. *Journal of Coastal Research* 12: 27–49.

Kidd KA, Blanchfield PJ, Mills KH, Palace VP, Evans RE, Lazorchak JM, Flick RW. 2007. Collapse of a fish population after exposure to a synthetic estrogen. *Proceedings of the National Academy of Sciences USA* 104: 8897–8901.

Kizu R, Okamura K, Toriba A, Mizokami A, Burnstein KL, Klinge CM, Hayakawa K. 2003. Antiandrogenic activities of diesel exhaust particle extracts in PC3/AR human prostate carcinoma cells. *Toxicological Sciences* 76: 299–309.

Korner W, Vinggaard AM, Terouanne B, Ma RS, Wieloch C, Schlumpf M et al. 2004. Interlaboratory comparison of four *in vitro* assays for assessing androgenic and antiandrogenic activity of environmental chemicals. *Environmental Health Perspectives* 112: 695–702.

Kortenkamp A. 2007. Ten years of mixing cocktails: a review of combination effects of endocrine-disrupting chemicals. *Environmental Health Perspectives* 115: 98–105.

La Guardia MJ, Hale RC, Newman B. 2013. Brominated flame retardants in Sub-Saharan Africa: burdens in inland and coastal sediments in the eThekwini Metropolitan Municipality, South Africa. *Environmental Science and Technology 47*: 9643–9650.

Marshall DJ, Rajkumar A. 2003. Imposex in the indigenous *Nassarius kraussianus* (Mollusca: Neogastropoda) from South African harbours. *Marine Pollution Bulletin* 46: 1150–1155.

Mestres M, Sierra JP, Mosso C, Sanchez-Arcilla A. 2010. Sources of contamination and modelled pollutant trajectories in a Mediterranean harbour (Tarragona, Spain). *Marine Pollution Bulletin* 60: 898–907.

Metcalfe CD, Metcalfe TL, Kiparisis Y, Koenig BG, Khan C, Hughes RJ, Crole TR, March RE, Potter T. 2001. Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by *in vivo* assays with Japanese medaka (*Oryzias latipes*). *Environmental Toxicology and Chemistry* 20: 297–308.

Ogata Y, Takada H, Mizukawa K, Hirai H, Iwasa S, Endo S et al. 2009. International Pellet Watch: global monitoring of persistent organic pollutants (POPs) in coastal waters. 1. Initial phase data on PCBs, DDTs, and HCHs. *Marine Pollution Bulletin* 58: 1437–1446.

Pawlowski S, Ternes T, Bonerz M, Kluczka T, van der Burg B, Nau H et al. 2003. Combined in *in situ* and *in vitro* assessment of the estrogenic activity of sewage and surface water samples. *Toxicological Sciences* 75: 57–65.

Rajakumar A, Singh R, Chakrabarty S, Murugananthkumar R, Lakdinsangi C, Prathibha Y et al. 2012. Endosulfan and flutamide impair testicular development in the juvenile Asian catfish, *Clarias batrachus*. *Aquatic Toxicology* 110: 123–132.

Rao K, Lei B, Li N, Ma M, Wang Z. 2013. Determination of estrogens and estrogenic activities in water from three rivers in Tianjin, China. *Journal of Environmental Sciences-China* 25: 1164–1171.

Routledge E, Sumpter J. 1996. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast strain. *Environmental Toxicology and Chemistry* 15: 241–248.

Ryan PG, Bouwman H, Moloney CL, Yuyama M, Takada H. 2012. Long-term decreases in persistent organic pollutants in South African coastal waters detected from beached polyethylene pellets. *Marine Pollution Bulletin* 64: 2756–2760.

Socol HH, Garrigues P, Ewald M. 2000. Origin of polycyclic aromatic hydrocarbons (PAHs) in coastal marine sediments: case studies in Colonou (Benin) and Aquitaine (France) areas. *Marine Pollution Bulletin* 40: 387–396.

Sohoni P, Sumpter JP. 1998. Several environmental oestrogens are also anti-androgens. *Journal of Endocrinology* 158: 327–339.
Swart N, Pool E. 2007. Rapid detection of selected steroid hormones from sewage effluents using an ELISA in the Kuils River water catchment area, South Africa. Journal of Immunoassay and Immunochemistry 28: 395–408.

Tollefsen K, Harman C, Smith A, Thomas KV. 2007. Estrogen receptor (ER) agonists and androgen receptor (AR) antagonists in effluents from Norwegian North Sea oil production platforms. Marine Pollution Bulletin 54: 277–283.

Truter JC, van Wyk JH, Oberholster PJ, Botha A. 2014. The impacts of neutralized acid mine drainage contaminated water on the expression of selected endocrine-linked genes in juvenile Mozambique tilapia, Oreochromis mossambicus, exposed in vivo. Ecotoxicology and Environmental Safety 100: 209–217.

Veldkornet DA, Adams JB, van Niekerk L. 2015. Characteristics and landcover of estuarine boundaries: implications for the delineation of the South African estuarine functional zone. African Journal of Marine Science 37: 313–323.

Vos JG, Dybing E, Greim HA, Ladefoged O, Lambre C, Tarazona JV et al. 2000. Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European situation. Critical Reviews in Toxicology 30: 71–133.

Vrabie CM, Candido A, van Duursen MBM, Jonker MTO. 2010. Specific in vitro toxicity of crude and refined petroleum products: II. Estrogen (alpha and beta) and androgen receptor-mediated responses in yeast assays. Environmental Toxicology and Chemistry 29: 1529–1536.

Wang X, Shi W, Wu J, Hao Y, Hu G, Liu H et al. 2010. Reproductive toxicity of organic extracts from petrochemical plant effluents discharged to the Yangtze River, China. Journal of Environmental Sciences 22: 297–303.

Wepener V, Degger N. 2012. Status of marine pollution research in South Africa (1960–present). Marine Pollution Bulletin 64: 1508–1512.

Whitfield AK. 1999. Ichthyofaunal assemblages in estuaries: a South African case study. Reviews in Fish Biology and Fisheries 9: 151–186.

Young DR, Alexander GV, McDermott-Ehrlich D. 1979. Vessel-related contamination of Southern California harbours by copper and other metals. Marine Pollution Bulletin 10: 50–56.

Zhao J, Ying G, Yang B, Liu S, Zhou L, Chen Z, Lai H. 2011. Screening of multiple hormonal activities in surface water and sediment from the Pearl River system, South China, using effect-directed in vitro bioassays. Environmental Toxicology and Chemistry 30: 2208–2215.

Zhou J, Cai Z, Xing K. 2011 Potential mechanisms of phthalate ester embryotoxicity in the abalone Haliotis diversicolor supertexta. Environmental Pollution 159: 1114–1122.