

Review

Cite this article: Langston WJ (2020). Endocrine disruption and altered sexual development in aquatic organisms: an invertebrate perspective. *Journal of the Marine Biological Association of the United Kingdom* **100**, 495–515. <https://doi.org/10.1017/S0025315420000533>

Received: 2 March 2020
Revised: 13 May 2020
Accepted: 1 June 2020
First published online: 30 June 2020

Key words:

Contaminants; endocrine disruption; imposex; intersex; invertebrates; reproductive impairment

Author for correspondence:

W. J. Langston,
E-mail: wjl@mba.ac.uk

Endocrine disruption and altered sexual development in aquatic organisms: an invertebrate perspective

W. J. Langston 

Marine Biological Association of the United Kingdom, The Laboratory, Citadel Hill, Plymouth PL1 2PB, UK

Abstract

Contaminants causing sex-altering, endocrine disrupting-like (ED) effects, or otherwise influencing reproduction, have been of growing concern to humans for more than 50 years. They have also been a perturbing, though less well-studied, phenomenon in marine organisms, following the recognition of tributyltin (TBT)-induced imposex and population extinctions in (neo)gastropods in the 1970s. Whilst ED impacts in mammals and fish are characterized by mimicry or antagonism of endogenous hormones by environmental contaminants (acting through Nuclear Receptors which are present in all metazoans) much less is known regarding pathways to effects in invertebrates. Despite the absence of a defined steroidal/mechanistic component, the extent, severity and widespread nature of ED-like manifestations and altered sexual characteristics observed in marine invertebrates gives rise to comparable concerns, and have been a long-term component of the MBA's research remit. The manifestations seen in sensitive taxa such as molluscs and crustaceans confirm they are valuable indicators of environmental quality, and should be exploited in this capacity whilst we seek to understand the pervasiveness and underlying mechanisms. In so doing, invertebrate indicators address aims of organizations, such as the EEA, OECD, UNEP and WHO, charged with management and monitoring of chemicals and ensuring that adverse effects on humans and the environment are minimized (Bergman *et al.*, 2013). In view of the recent general declines in marine biodiversity, and the potential contribution of ED-like phenomena, safeguarding against deleterious effects through increased research which links pollutant exposure with reproductive dysfunction among invertebrates, is seen as a high priority.

Introduction

By strict definition, Endocrine Disrupting Chemicals (EDCs) are defined as (1) 'exogenous substances that cause adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine function' (EU, 1997), or as (2) 'exogenous agents that interfere with the production, release, transport, metabolism, binding and action or elimination of the natural hormones in the body responsible for the maintenance of homeostasis and regulation of developmental processes' (US EPA, 1997). Some EDCs can act directly on receptors, either as hormone mimics (agonists) or blockers (antagonists); others may act indirectly by acting on molecules and pathways that control delivery or action on its target tissue. Either way, sensitivity is often highest during a 'critical window' in early developmental stages. In laboratory-exposed mammals, effects on developing gonadal tissue include cryptorchidism (failure of testes to descend from the abdomen into the scrotum), hypospadias (impaired foetal development of the penis and urethral opening), altered testis pathology and low hormone/sperm count in males; ovarian pathologies, dysfunction and lowered fertility are analogous defects in females (EEA, 2012). There is reason to hypothesize that some of these effects could also be manifested in invertebrates living in contaminated environments.

The most commonly considered EDCs in higher organisms are natural hormones such as oestrogens (e.g. 17 β -oestradiol (E2), oestrone and progesterone) and testosterone (T) – all associated with domestic sewage. Synchronization of oestrous cycles in domestic animals (e.g. to ensure calving at the most appropriate time) may involve treatment with 17 β -oestradiol, progesterone or synthetics such as oestradiol benzoate which can reach the environment in excreta, resulting in disruptive effects in wildlife. In addition there are various hormone mimics present in domestic, industrial, shipping and agricultural wastes. These latter xeno-oestrogens and androgens include the contraceptive pill component 17 α -ethinyloestradiol (EE2), diethylstilbesterol (DES), phyto-oestrogens, bisphenol A (BPA, a synthetic oestrogen mimic previously used widely in food packaging), phthalates and other plastic additives, non-ionic surfactants such as the alkylphenols nonylphenol (NP) and octylphenol (OP), pesticides such as DDT, herbicides (e.g. atrazine, glyphosate), together with active ingredients in pharmaceuticals and personal care products. Various metals including arsenic, cadmium, lead, mercury and zinc (Lavicoli *et al.*, 2009) and a host of industrial chemicals, past and present, including polychlorinated biphenyls (PCBs), polybrominated flame retardants and the anti-foulant TBT can be added to this long list. Indeed, there are a growing number of chemicals



(perhaps of the order of 1000 according to Schug *et al.*, 2016) and environmental factors, which may cause perturbations in the development of marine invertebrates and should be considered alongside established EDCs (capable of interfering with hormone receptors) given that they may influence 'fitness' of populations in similar ways (i.e. 'chemicals toxic to reproduction' in EU REACH parlance – see EEA, 2012), despite alternative modes of action. Examples which reflect key research developments in relation to marine invertebrates (and, to a lesser extent, fish), and future research and testing needs to help monitor and maintain healthy environments, are included in this review.

Screening assays and threshold concentrations of EDCs

Threshold and No Observable Effects Concentrations (NOEC) have been derived for some of the more established EDCs (Table 1) and have been used effectively to monitor and manage environmental risk – notably in the case of TBT where a marine Environmental Quality Standard (EQS) of 2 ng l^{-1} was set based on a combination of effects levels in species such as oysters *Crassostrea gigas* (Thunberg, 1793) and dogwhelks *Nucella lapillus* (Linnaeus, 1758) and, pragmatically, on what could actually be measured in the environment. However, for many EDCs no such relevant thresholds exist, and may be dependent on exposure timing/duration (Oehlmann *et al.*, 2006, 2007; Kortenkamp & Faust, 2018), the nature of the receiving environment and the presence of synergetic or antagonistic agents. Furthermore, dose-response relationships may not follow linear form: activity may become relatively reduced at high (toxic) doses compared with highly sensitive chronic response thresholds. For example, embryo production in aquatic snails *Potamopyrgus antipodarum* (Gray, 1843) is increased by the oestrogenic sunblock component 3-benzylidene camphor at low, but not high concentrations (Table 1; Schlumpf *et al.*, 2004). Simple extrapolations are clearly not justified in such circumstances.

Faced with the potential for additive/interactive effects in environmental mixtures, chemical thresholds may be of questionable value as a regulatory tool in isolation without accompanying biological monitoring. In such circumstances procedures such as TIE (Toxicity, Identification and Evaluation) which combine chemical fractionation with bioassays to identify biologically active components, may (sometimes) be effective in assessing relevant EDCs and their thresholds in extracts of environmental samples (Schlenk, 2008). Methods may involve *in vivo* or, more frequently, *in vitro* bioassays including ER competitive ligand binding assays (Schwartz & Skafar, 1993) and oestrogen/androgen receptor-based cell-lines such as the yeast oestrogen/androgen screens (YES/YAS) described by Routledge & Sumpter (1996). In these, human ER/AR genes are coupled to a reporter gene initiating a colour change upon activation (in the analogous ER-CALUX assay described by Legler *et al.* (1999), reporter gene activation triggers a response in luminescence). An example of both types of response showing a relationship between oestrogenic activity in sediment extracts measured *in vitro* by the YES assay and in the *in vivo* response intersex (oocyte development in testis) in clams *Scrobicularia plana* (da Costa, 1788) from UK estuarine sites, is described later in this review.

Reporter-gene assays in yeast, fish or mammalian cells, along with competitive binding assays with the oestrogen and androgen receptor have been applied widely to screen for androgenic and oestrogenic stimuli. Establishing such relationships may add to the weight of evidence where ED-like effects are suspected. Nevertheless, although sensitive to changes in endocrine status from a range of potential EDC these *in vitro* assays are not proof of causality in nature and, if used in isolation, may be of limited value in predicting *in vivo* effects including altered sexual

characteristics, impaired development and reproductive function, and population-level consequences.

Endocrine disruption and reproductive effects in aquatic biota

Alongside suspicions of serious defects in humans (e.g. testicular cancers, low sperm counts, hypospadias and cryptorchidism), and the realization that ED-like chemicals impact a range of charismatic wildlife including mink, seabirds, alligators, turtles, frogs and toads (EEA, 2012), a variety of (mainly feminizing) effects, including vitellogenesis and intersex (ovotestis – occurrence of eggs in testis), have been reported to be widespread in many aquatic organisms, both freshwater and marine (Sumpter & Jobling, 1995; Jobling *et al.*, 2003; Matthiessen *et al.*, 2018).

At the population level, perturbations to normal sex ratios have been attributed to EDCs, both in nature and in the laboratory, along with impaired fertility rates (Langston *et al.*, 2005; Sumpter, 2005; Lewis & Ford, 2012). It is therefore not inconceivable that EDCs could contribute to species decline and diminishing wildlife diversity, particularly when combined with other stressors such as climate change, over-exploitation and habitat loss. Faced with such complexity, it is easy to see why it may be difficult to isolate and prove involvement of EDCs in such declines. Nevertheless, it is instructive to consider some of the key observations and trends in ED-like effects, highlighted by freshwater and marine fish, and in relatively under-studied invertebrate taxa.

Fish

Because of clear gender-related differences in both external and internal morphologies, coupled with a well-studied, steroidally based endocrine system, some of the strongest indications of reproductive impairment in aquatic ecosystems have involved fish species. As a result, ED in wild freshwater and marine fish is now well-characterized – often associated with exposure to effluent from waste water treatment works (WWTW) where steroidal oestrogens may be present at concentrations significantly above background (Table 1). It is possible that other modes of action contribute to effects in fish though emphasis of research to date has focused on the actions of steroidal oestrogens. In nature a complex mixture with xeno-oestrogens, androgens, anti-oestrogens and anti-androgens may add to the spectrum of observed effects which may, consequently, be difficult to interpret in terms of causality.

There are numerous ways in which fish reproduction can be disturbed by contaminants, and effects detected range from altered secondary sexual characteristics and gonad histopathology to disruption of subtle mechanisms of endocrine regulation. Examples at the biochemical level include: altered steroid titres; induction of synthesis of the egg-yolk precursor vitellogenin (VTG) in males (or inhibition in females in the case of trenbolone – a synthetic androgen agonist); and/or induction of chorionic proteins within male or juvenile animals. At higher levels of organization, pathological changes in reproductive organs include altered number/size of eggs, skewed sex ratios, increased frequency of intersexuality (ovotestis) and reductions in sperm density and motility. A resultant shift in the reproductive cycle may ultimately affect population size and diversity. For example, delayed hatching has been described in marine medaka *Oryzias melastigma* (McClelland, 1839) (and offspring) following exposure to the surfactant perfluorobutanesulfonate, primarily as a result of disruption to the thyroid system (Chen *et al.*, 2018). Other population-level effects are reviewed by Marty *et al.* (2017) and some are discussed later in this review.

Table 1. Effective concentrations of some known ED-like compounds to fish and aquatic invertebrates

Species	Substance	Response	Effects levels	Environmental levels (upper)	Reference
Dogwhelk <i>Nucella lapillus</i>	TBT	Imposex	<1 ng l ⁻¹ ; EQS set at 2 ng l ⁻¹	Up to µg l ⁻¹ levels prior to regulation	Bryan et al. (1986, 1987)
Oysters <i>Crassostrea gigas</i>	TBT	Shell thickening	2 ng l ⁻¹	Upto µg l ⁻¹ levels prior to regulation	Thain & Waldock (1986)
<i>Marisa cornuarietis</i> (FW snail) <i>N. lapillus</i>	bisphenol A (BPA) octylphenol (OP)	Superfemales induced, (enlarged sex organs and stimulation of oocyte production).	1 µg l ⁻¹ NOEC for superfemale induction in <i>M. cornuarietis</i> of 7.9 ng BPA l ⁻¹	13 µg l ⁻¹ OP	Oehlmann et al. (2000, 2007)
FW Snail <i>Potamopyrgus antipodarum</i> (Exposed to sediment)	BPA and OP	Increase in the number of embryos	LOEC 1 µg kg ⁻¹ dry wt (lowest concentration tested)	30–340 ng g ⁻¹ OP	(Duft et al., 2007)
	NP		LOEC of 10 µg kg ⁻¹	<100 up to 1700 ng g ⁻¹ dry wt	
	EE2		30 µg kg ⁻¹	<0.5–12 µg kg ⁻¹	
<i>P. antipodarum</i>	EE2 BPA OP	Increase in the number of embryos	LOEC 1 ng l ⁻¹ LOEC 1 µg l ⁻¹ LOEC 5 µg l ⁻¹	low ng l ⁻¹ 13 µg l ⁻¹	Jobling et al. (2004)
	Diluted oestrogenic sewage effluent.				
<i>P. antipodarum</i>	3-(4'-methylbenzylidene)-camphor (4-MBC) (sunblock component)	Embryo production	EC50 4.60 µM or 1.17 mg kg ⁻¹ dw in sediment		Schlumpf et al. (2004)
	EE2	Egg laying and embryo production	Stimulated at low ng l ⁻¹ range, inhibited at higher (>100 ng l ⁻¹) doses	low ng l ⁻¹	
FW snail <i>Lymnaea stagnalis</i>	EE2	Increased egg laying reduced growth, delayed hatching, and induction of a vitellin-like protein	500 ng l ⁻¹ concentrations down to 50 ng l ⁻¹	low ng l ⁻¹	Segner et al. (2003)
FW mussels <i>Dreissena polymorpha</i>	DDT/DDE	Altered timing gamete release. Oocyte malformation	Body burdens 1600 ng pp'DDE g ⁻¹ lipid; 100 ng op'DDT g ⁻¹ lipid,		Binelli et al. (2001, 2004)
Clams <i>Scrobicularia plana</i> (exposed to sediment)	E2, EE2; OP and NP individually or as mixtures	Induction of intersex in male clams	mixtures of 17β-oestradiol (E2), 17α ethinyloestradiol (EE2), at 100 µg kg ⁻¹ ; octylphenol (OP) and nonylphenol (NP) at 1000 µg kg ⁻¹ , or individually to E2, or NP	(E2) and (EE2) <0.5–4 µg kg ⁻¹ and <0.5–12 µg kg ⁻¹ , respectively	Langston et al. (2007a)
FW fish minnow <i>Pimaphales promelas</i>	EE2		Stimulated at low ng l ⁻¹ range, inhibited at higher (>100 ng l ⁻¹) doses	low ng l ⁻¹	Jobling et al. (2003)
	Nonylphenol			0.2–12 µg l ⁻¹ (to 330 µg l ⁻¹ exceptionally)	
FW prawn <i>Macrobrachium rosenbergii</i>	Chlordecone (OC)		0.2–20 µg l ⁻¹		Lafontaine et al. (2017)

Amongst the first and most significant examples of ED in wild fish populations was that seen in roach, *Rutilus rutilus* (Linnaeus, 1758), in UK rivers influenced by WWTW and industrial effluents (Sumpter & Jobling, 1995; Jobling *et al.*, 1998). Manifestations including VTG induction, gonadal intersex, lowered sperm counts in male fish and reduced fertility are now considered as classic indicators of environments containing high levels of sewage-derived oestrogens (notably the natural steroids 17 β -oestradiol (E2), oestrone (E1) and the synthetic hormone (EE2)) or anti-androgens. Controlled, lake-scale experiments with fathead minnows *Pimaphales promelas* (Rafinesque, 1820) exposed to EE2 have since confirmed potential for population collapse due to such EDCs (Kidd *et al.*, 2007, described later in this paper). Xeno-oestrogens – compounds that mimic or enhance the effects of endogenous oestrogens (Tyler & Routledge, 1998) – such as the surfactants nonylphenol NP and octylphenol OP, were also implicated in the feminization of fish populations (Sumpter & Jobling, 1995; Jobling *et al.*, 1998): though their comparative activity appears to be considerably lower than steroidal oestrogens, concentrations of these synthetic chemicals in detergent-rich effluent may be considerably higher than natural hormones (Table 1). Fortunately, recognition of their impact has led to regulations and voluntary restrictions by industry, which has lessened their threat, at least throughout Europe. (It is hoped that the UK's exit from EU legislation will not reverse such improvement.)

Given the large number of potentially active chemicals used by humans, which may enter waterways via WWTW and other inputs, causality of ED is likely to be site specific and dependent on the composition of the chemical cocktail present (EEA, 2012). Incidence and severity of ED is thus highly variable and although widespread in UK rivers (with some fish populations exhibiting high percentages of affected individuals), elsewhere only small proportions of wild populations may be affected. For example, a US survey by Hinck *et al.* (2009) found intersex in only 3% of the fish collected and in only 4 of 16 species examined at 34 of the 111 sites: intersex was most prevalent in SE USA, in largemouth bass *Micropterus salmoides* (Lacépède, 1802) (18% of males) and smallmouth bass *M. dolomieu* (Lacépède, 1802) (33% of males). Despite this variance, the potential threat of ED for aquatic species is virtually ubiquitous, as illustrated by stickleback (*Pungitius pungitius* Linnaeus, 1758) from remote rivers in the Arctic (St Lawrence Island, Alaska) where expression of VTG in male fish was consistent with exposure to oestrogenic PCBs – a legacy from previous military installations. Gene expression changes and DNA methylation in gonads were also allied to PCB exposure (von Hippel *et al.*, 2018). Higher in the food chain, terns from PCB-contaminated locations, feeding on fish and aquatic invertebrates such as molluscs and crustaceans, exhibited skewed sex ratios and elevated intersex frequencies – supporting evidence of ED and highlighting the potential threat of bioaccumulation and trophic transfer of EDCs (Hart *et al.*, 2003).

Marine fish appear to be equally sensitive to the threat of ED. One of the simplest manifestations is reduced gonad size, measured as the gonadosomal index (GSI – gonad weight expressed as a proportion of somatic weight), or in extreme cases immaturity (no mature gonad) as in Atlantic croaker *Micropogonias undulatus* (Linnaeus, 1766) exposed experimentally to naphthalene and water-soluble fractions of diesel oil (Thomas & Budiantara, 1995). PCBs (Thomas, 1988), pesticides (Ram & Sathyanesan, 1986) and some metals (Singh, 1989) have also been shown to influence GSI in the laboratory. In aquatic monitoring programmes poor gonad development has been observed in response to pollution in burbot *Lota lota* (Oken, 1817) from the Gulf of Bothnia, English sole *Parophrys vetulus* (Girard, 1854) in Puget Sound, and white sucker *Catostomus*

commersonii (Lacépède, 1803) in Canada (reviewed in Hansson *et al.*, 2017).

ED, manifested as low frequencies (<17%) of intersex (ovotestis) and elevated VTG concentrations (in males), occurs in marine fish such as flounders *Platichthys flesus* (Linnaeus, 1758), especially in estuaries subjected to EDC-containing WWTW and industrial effluents, which in the UK includes the Tyne, Tees, Wear and Mersey (Lye *et al.*, 1997; Kirby *et al.*, 2004). VTG induction and intersex levels in male flounder from the Mersey were found to be particularly elevated in the 1990s, raising concerns over links between endocrine disruption and environmental quality. The influence of hormone-containing sewage wastes and the presence of ubiquitous persistent organic compounds such as alkylphenols (considered by the EA as being at medium or high risk of EQS failure in the Mersey) were suggested as possible causes of ED. Since then, time-series monitoring indicates declining levels of VTG in male flounder (Kirby *et al.*, 2004) consistent with reduced concentrations of EDCs following industrial decline and large-scale efforts to clean up domestic sewage and trade inputs, implemented in the Mersey basin at the end of the last century. More recent fish monitoring under the UK Clean Seas environmental monitoring programme (Defra) indicates continuing improvements – with <10% of male flounder exhibiting egg-yolk protein levels above 1 $\mu\text{g ml}^{-1}$ in 2010, compared with >90% in 1996 (Defra 2009–2012).

Fish from more remote UK offshore sites, such as flounder and dab from the North Sea's Dogger Bank, also exhibit ovotestis and malformation of the male reproductive ducts (Allen *et al.*, 1999; Stentiford & Feist, 2005). Unfortunately, from a monitoring viewpoint, these fish migrate extensively, and there may be uncertainty over the exposure site and regime during the critical gonad developmental period. The use of fish in marine monitoring programmes is therefore circumspect, to an extent, although they undoubtedly highlight important regional differences in ED.

Similar, if less pronounced, oestrogenic effects have been detected in offshore species in the USA, Japan and the Mediterranean, in addition to the UK (EEA, 2012). Pleuronectids from PCB-/PAH-contaminated parts of Puget Sound exhibited ED-like effects in terms of reduced E2 titres in blood and reduced fecundity (Johnson *et al.*, 1988a, 1988b); in contrast, flounder exposed to harbour dredge spoil had elevated plasma E2 and VTG levels (Janssen *et al.*, 1997). Induction of both VTG and ovotestis (2%) were detected in contaminated male flounder *Pleuronectes yokohamae* (Günther, 1877) from Tokyo Bay (Hashimoto *et al.*, 2000). *In vivo* treatment of Atlantic salmon *Salmo salar* (Linnaeus, 1758) with nonylphenol has been shown to induce VTG and ER upregulation (along with a reduction of steroid metabolizing enzymes), signifying its oestrogen-mimicking properties, though no effects on reproduction were evident (Arukwe *et al.*, 1997). Direct suppression of hormones (testosterone and oestradiol) has also been observed in Atlantic croaker in response to laboratory exposure to PCB and PAH (Thomas, 1988).

Although oestrogenic effects predominate the ED literature on fish, a small number of masculinization events have been reported in several FW species (mosquitofish, killifish and catfish) in conjunction with exposure to pulp mill effluent (possibly due to androstenedione from bacterial degradation of plant sterols) and also from inputs of androgenic anabolic steroids from intensive cattle farming (Howell, 1999). Downstream, estuarine and coastal fauna could be vulnerable to these and other androgenic EDC (Alvarez-Munoz *et al.*, 2015), given that the androgen receptor (AR) in some marine species is known to bind contaminants such as organochlorines, albeit weakly, which may subsequently impair the gamete-maturation signalling pathway (Oberdorster & Cheek, 2001).

Invertebrates

In parallel with warning signs that reproductive effects such as intersex and VTG induction may occur in fish subjected to anthropogenic waste, there is evidence that chemicals can influence growth, development and reproduction in invertebrates, though the support for an endocrine disrupting mechanism is generally weak – with the possible exception of TBT-induced imposex (in neogastropod snails) and intersex in certain molluscs, crustaceans and rotifers. Paucity of evidence for ED in invertebrates is partly due to an absence of a defined endocrine-mediated mode of action (MOA) but is hindered, generally, by a lack of targeted pollution research on invertebrate populations – particularly marine species. This omission should be rectified, particularly given the importance of coastal and estuarine habitats for wildlife biodiversity and food chains, and the increase in coastal urbanization (and associated contamination) worldwide. The following examples of imposex and intersex phenomena in invertebrate taxa are now well established and illustrate why it is important to include diverse organisms in environmental assessment programmes even in the absence of an established endocrine MOA. Notably, in the case of TBT-induced imposex, without the development and application of relevant diagnostic molluscan indices (i.e. reliance on routine *in-vitro*/vertebrate testing), the damage inflicted on marine ecosystems would have been more extensive and remained ‘under the radar’ for much longer.

Imposex and effects of TBT in molluscs

Tributyltin-induced imposex, observed in certain neogastropod molluscs, represents the most significant and specific manifestation of ED in the marine environment. The rise of the organotin (OT) ‘problem’ stemmed from the widespread use of TBT and, to a lesser extent, triphenyltin (TPT), as biocides in antifouling preparations (used to protect boat hulls and fish cages). Over the four decades since the initial discovery of the pollution-related origin of imposex in female dogwhelks *Nucella lapillus* – at the MBA’s Plymouth Laboratory in the 1980s – more than 150 prosobranch species worldwide have been shown to exhibit similar effects. Specificity of the response to OT is matched by its sensitivity – initiated at concentrations of 1 ng TBT l⁻¹ and possibly lower. Details are described in numerous papers (e.g. Bryan *et al.*, 1986, 1987; Langston *et al.*, 1990, Matthiessen & Gibbs, 1998; Langston & Pope, 2009). A brief account of key features is warranted here to highlight the nature of the response, spatial and temporal trends, and the value of research in guiding regulatory action to remediate EDCs.

Imposex – the imposition of male characteristics (penis and vas deferens development) on females – was first noticed extensively in dogwhelks along the English Channel coast, and progresses along established indices of severity, culminating, at higher exposure levels, in the prevention of egg-laying and localized extinction of populations. The development of the Relative Penis Size Index (RPSI – penis size of females in a population compared with that of males) and Vas deferens Sequence Index (Figure 1) helped to quantify the extent and scale of impact and inform the development of TBT legislation (Bryan *et al.*, 1986, 1987; Langston *et al.*, 1990; Matthiessen & Gibbs, 1998).

Statutory measures invoked to halt damage from TBT to vulnerable marine species and ecosystems are detailed elsewhere (Champ & Seligman, 1996; Bray, 2006; Arai *et al.*, 2009). Studying the various aspects and timescales of recovery has provided a unique opportunity to understand the efficacy of TBT regulations. Two crucial pieces of legislation have influenced recovery from TBT pollution in the UK (with similar measures being adopted in many countries worldwide). The first of these measures, in 1987 in the UK, banned the use of TBT on vessels

<25 m (the leisure market), but allowed continued use on larger vessels (commercial and naval fleets), based on the assumption that, since they spend most time on the open seas, biocide leachates from large ships would be diluted, harmlessly. However, doubts over this hypothesis, based on long-term surveillance of TBT concentrations and biological effects near shipping routes and ports, led to comprehensive legislation to remove, or seal in, tin-based coatings on the global fleet during the 2000s (final adoption and ratification of the IMO antifouling (AF) treaty in 2008, along with incorporation in UK/EU law).

The physical manifestations of imposex are irreversible, and recovery of affected populations is therefore dependent on recruitment from unaffected individuals by inward migration, or rafting of eggs and juveniles. Even at relatively ‘clean’ coastal sites, dogwhelk populations exhibited moderate imposex during the 1980s (>50% at VDS stage 3&4) and surprisingly few females (<1%) were without imposex, leading to extinction of many populations of *N. lapillus* subjected to boating traffic and near major shipping lanes – such as Plymouth Sound and many other locations along the south coast of England. Despite this extensive impact, recovery from imposex in populations of *N. lapillus* (and other neogastropods worldwide), is now evident at open coastal sites (few females now affected; see examples at Widemouth, Renney and Bude, Figure 2; Langston *et al.*, 2015). Indeed, recruitment may be surprisingly rapid (~10 years), given that the species has no planktonic phase (Colson & Hughes, 2004; Bray *et al.*, 2012). However, near ports – for example the Fal Estuary, UK – recovery of dogwhelks has been delayed by shipping impacts (Falmouth Docks), including the legacy of TBT retained by fine sediments with the potential for re-suspension and re-release, especially during dredging (Langston *et al.*, 2015): enhanced VDSI and RPS were still present in *N. lapillus* near the port of Falmouth, 25 years after the height of the TBT problem, with >75% of females still affected (Figure 2). This represents limited improvement; higher upstream in the Fal Estuary, dogwhelks have still to recolonize previously inhabited sites.

Whilst reproductive traits in these gastropods are clearly highly sensitive to gender-influencing organotins, bivalves – particularly those in contact with, or feeding on, contaminated sediment – may be highly responsive to TBT through processes such as survival, growth and developmental effects. Notably, shell-thickening – observed in filter-feeding oysters *Crassostrea gigas* – is induced at comparable TBT concentrations to those causing imposex, and is due to interference with calcium channelling and deposition (Waldock & Thain, 1983). Steroidal involvement has not been linked with this particular response (unlike imposex in snails, as discussed later in this paper). However, the presence and influence of sex-hormones in bivalves is intriguing and a cause for debate, given that testosterone metabolism and steroid titres have been shown to be affected by TBT in clams *Ruditapes decussatus* (Linnaeus, 1758) (Morcillo *et al.*, 1998).

The long-term impacts of TBT have been evaluated in estuarine populations of the sediment-dwelling gonochoristic clams *S. plana*. As a deposit-feeder, *S. plana* is responsive to various sediment-associated contaminants, including TBT (Langston & Burt, 1991). At heavily TBT-contaminated sites, typified by the Solent area of the English Channel, clam numbers declined considerably during the 1980s, as TBT concentrations peaked (Figure 3A, reproduced from Langston *et al.*, 2015). The subsequent efficacy of TBT regulatory measures in achieving chemical and ecological improvements is also demonstrated in these long-term observations. As with TBT-impacted dogwhelks, clam recruitment patterns and abundance have recovered (slowly – over 25 years) as TBT concentrations fall in response to pollution control measures, confirming the close link between TBT and reproductive success in these bivalves. The underlying mode of action in these bivalves is unclear since, in contrast to

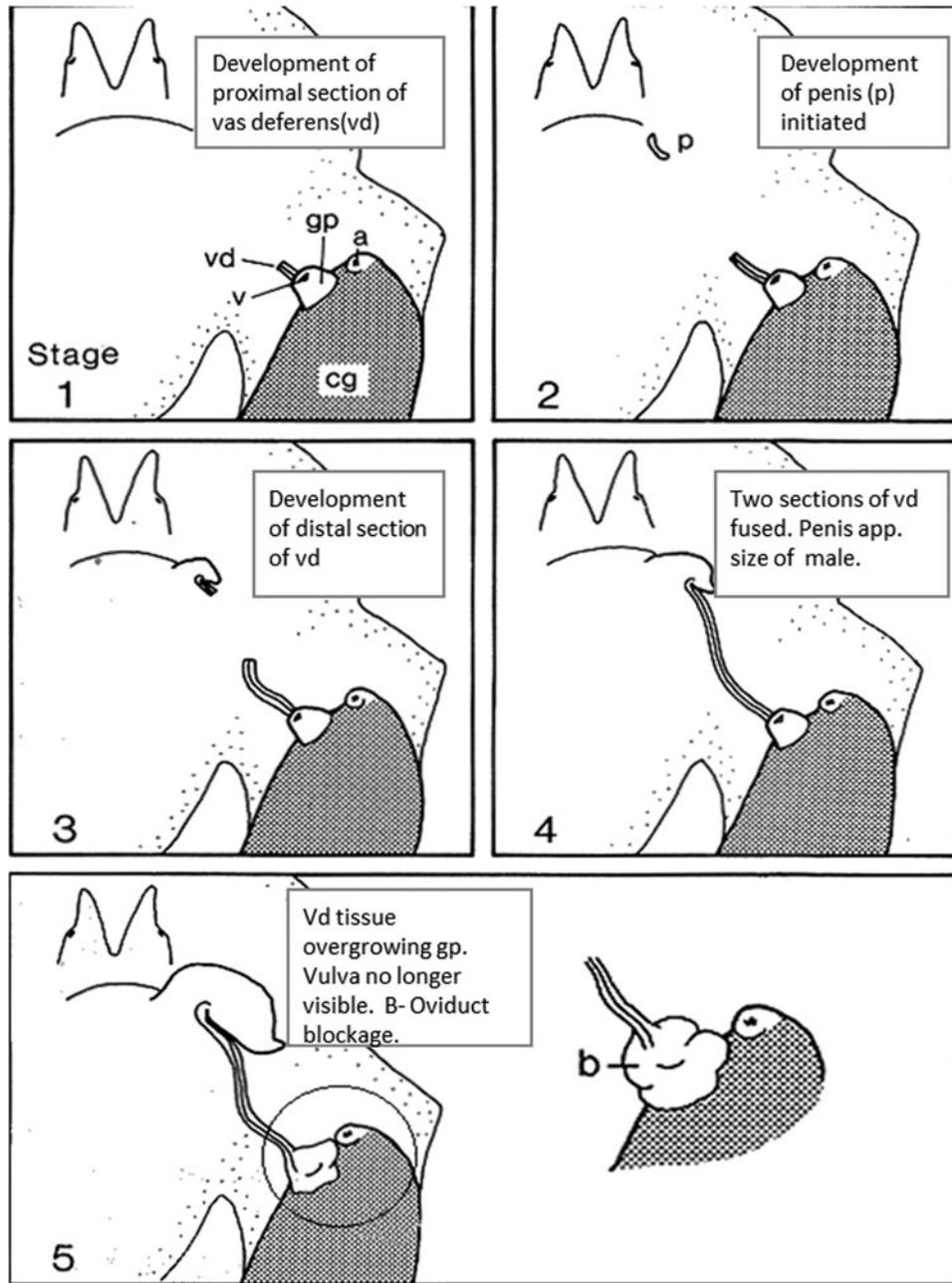


Fig. 1. *Nucella lapillus*. Development of imposex, based on the VDSI and RPSI (after Gibbs *et al.*, 1987, with permission).

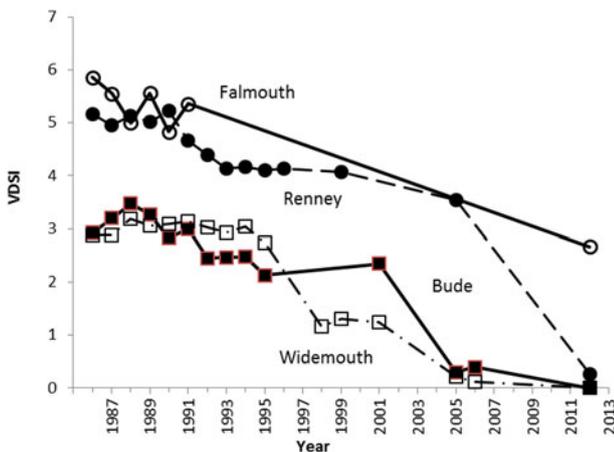


Fig. 2. *Nucella lapillus*. Changes in mean VDSI at four sites in SW England over a 25 year period following introduction of TBT regulations in 1987 (after Langston *et al.*, 2015, with permission).

TBT-induced imposex in neogastropods, there are no analogous reproductive structures (penis, vas deferens) in *S. plana* and therefore no comparable indices of masculinization. However, analysis of sex ratios in clams from the Solent area indicated deviation from the expected ratio of 1:1 (Langston *et al.*, 2015). At sites where TBT concentrations in sediments were highest, there was a significant bias towards males (2:1) compared with the Channel average (N = 107 sites) of 0.998:1 (Figure 3B). Influence of TBT on reproductive development and success in bivalves such as *S. plana* is therefore likely. Masculinization and reduced larval production was similarly thought to have accompanied TBT-related decline in UK native oysters *Ostrea edulis* (Linnaeus, 1758) (Thain & Waldoock, 1986).

Intersex and other effects in invertebrates

Intersex is a condition found in normally gonochoristic species (separate sexes) where both male and female gametes are present

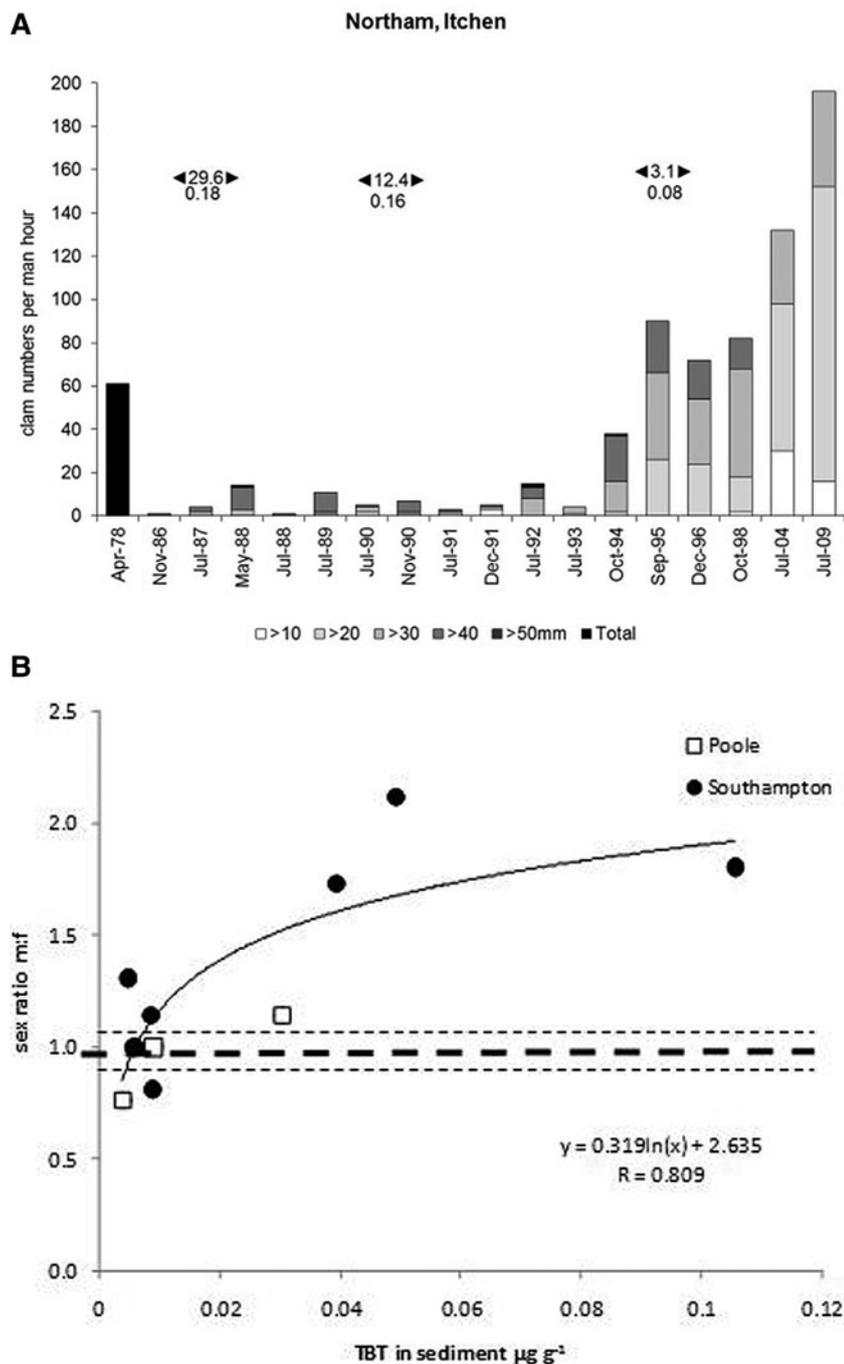


Fig. 3. *Scrobicularia plana*. (A) Abundance of clams (numbers collected per man hour) at Northam in the Itchen Estuary 1978–2009. Data in 1978 are total clam numbers; numbers of different sized clams (10 mm intervals) are indicated at other times. Upper numbers above bars are mean TBT seawater concentrations (ng l⁻¹ as Sn) for periods 1986–1988, 1990–1992, 2000–2009 (left to right). Lower numbers are mean sediment TBT concentrations (µg g⁻¹ as Sn) for the same periods. (B) Relationship between sex ratio in *S. plana* and TBT content of sediment, Solent Area, July 2009. Dashed line is mean (± SE) sex ratio for English Channel (2009–2012, N = 107 sites). (after Langston *et al.*, 2015, with permission).

in the same individual. In contrast to TBT-induced imposex, precise causality of intersex is seldom definite (European Environment Agency, 2012). The appearance of oocytes in the testes (ovotestis) is regarded as an endpoint of endocrine disruption in male fish and has been linked to the presence of endocrine disrupting compounds which are thought to mimic the actions of the female sex hormone 17β-oestradiol, and thus disrupt natural hormonal functioning (Bateman *et al.*, 2004). In contrast to fish, unequivocal confirmation of environmentally relevant ED effects on the reproductive systems of aquatic invertebrates is scarce because of the lack of basic knowledge of invertebrate endocrine systems. Another issue concerns frequency of intersex – which may be present in only a proportion of the population – and in some species is not much lower at perceived non-contaminated sites than in polluted areas. The presence of intersex at so-called pristine sites, in gonochoristic species, does not necessarily signify inherent hermaphroditism but could reflect that there may be few sites where

pollution is truly absent. Nevertheless, there are invertebrate examples which imply that the intersex condition is a response to environmental quality – one of which is the estuarine clam *S. plana* which, as discussed above, is sensitive to TBT.

Initial survey of *S. plana* populations in the Avon Estuary, SW England, chosen to represent a relatively uncontaminated reference site, showed unexpectedly that intersex, in the form of ovotestis, was present in varying degrees of severity in males (Chesman & Langston, 2006; Langston *et al.*, 2007a). This feature, together with the fact that these clams are intrinsically gonochoristic (Hughes, 1971; Ruiz, 1993), widely distributed, sedentary deposit-feeders make *S. plana* a potentially valuable monitoring species – prompting the development of an intersex index (Figure 4) to assess the severity of the condition, and offering potential for widespread screening and investigation.

Long-term observations in the Avon, a rural south Devon estuary with little industry or urbanization, revealed further

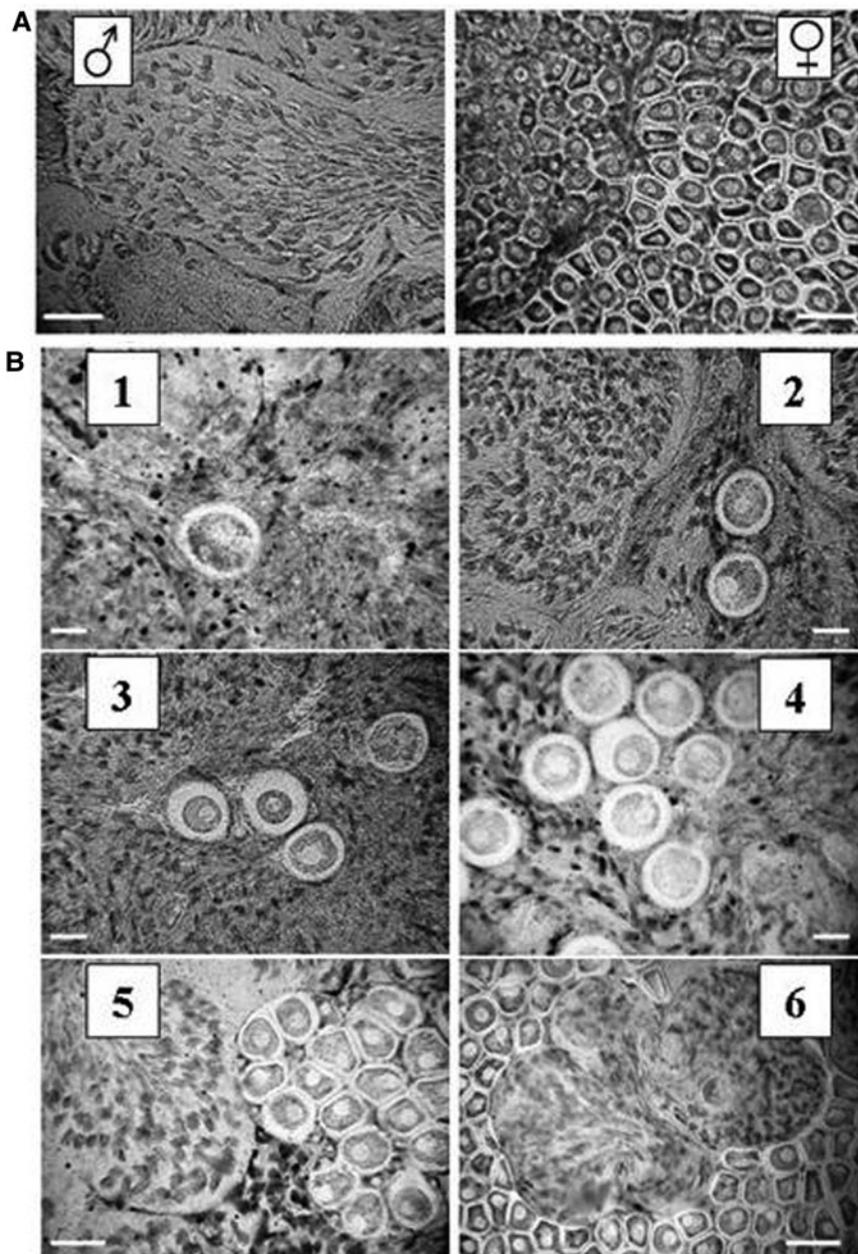


Fig. 4. *Scrobicularia plana*. (A) Gonadal follicles containing sperm or oocytes in normal male and female, respectively. Scale bars = 120 μm . (B) Oocyte distribution patterns in gonads of clams exhibiting increasing severity of ovotestis: examples illustrate the intersex index scores 1–6 (Scale bars 1–4 = 80 μm , Scale bars 5–6 = 120 μm) (after Chesman & Langston, 2006, with permission).

insights into the sexuality of these clams. Gametogenesis commences in April and by early July most of the adults have mature gonadal cells, which in many individuals were either sperm or oocytes (Figure 4A). However, ovotestis was manifested in a percentage of individuals (Figure 4B), increasing in frequency until spawning (late July) and subsequently receding, as the gonads regressed (Figure 5). The proportion of females in the sampled populations remained unchanged at ~50% throughout, whilst the proportion of unaffected males dropped, especially in July, implying that intersex represents feminization of males. The affected males exhibited varying degrees of ovotestis with an index of severity ranging from a single oocyte in otherwise normal testicular tissue, to large regions of mature ovarian tissue interspersed within testicular tissue (Figure 4B). The causes and consequences of the ovotestis condition in *S. plana* are speculative, though given its undeveloped catchment, one of the most likely causes of intersex in the Avon appears to be agriculture, notably livestock (cattle) which, at the time, were able to access the waterway unhindered.

Nearby commercial oyster beds in the Avon have been closed periodically because of high bacterial (*Escherichia coli*)

counts, and though there is ongoing debate as to the importance of wastewater and agricultural sources, bacterial DNA analysis points to bovine sources. The implication is that even 'undeveloped' estuaries can be impacted by a relatively moderate level of anthropogenic activity, including dairy farming. Annual trends in intersex in Avon clams, with respect to cattle statistics, add credence to this hypothesis; proportions of affected male clams have declined over several years since initial intersex observations in 2004 (Figure 5), coinciding with efforts by the farming community to prevent cattle directly accessing the waterway (by fencing off pastureland). Given that cows (especially pregnant females) may excrete milligram quantities of oestradiol during micturition, removal of this source could clearly have benefits to clams and other fauna inhabiting adjacent mudflats and which may accumulate exogenous oestrogens. Coinciding with this removal of local direct sources, and the decline in intersex, there has been an overall national reduction in the dairy herd (Figure 5). Agriculture may therefore represent a potential source of feminization, compounding the influence of domestic and industrial wastes where these are prevalent.

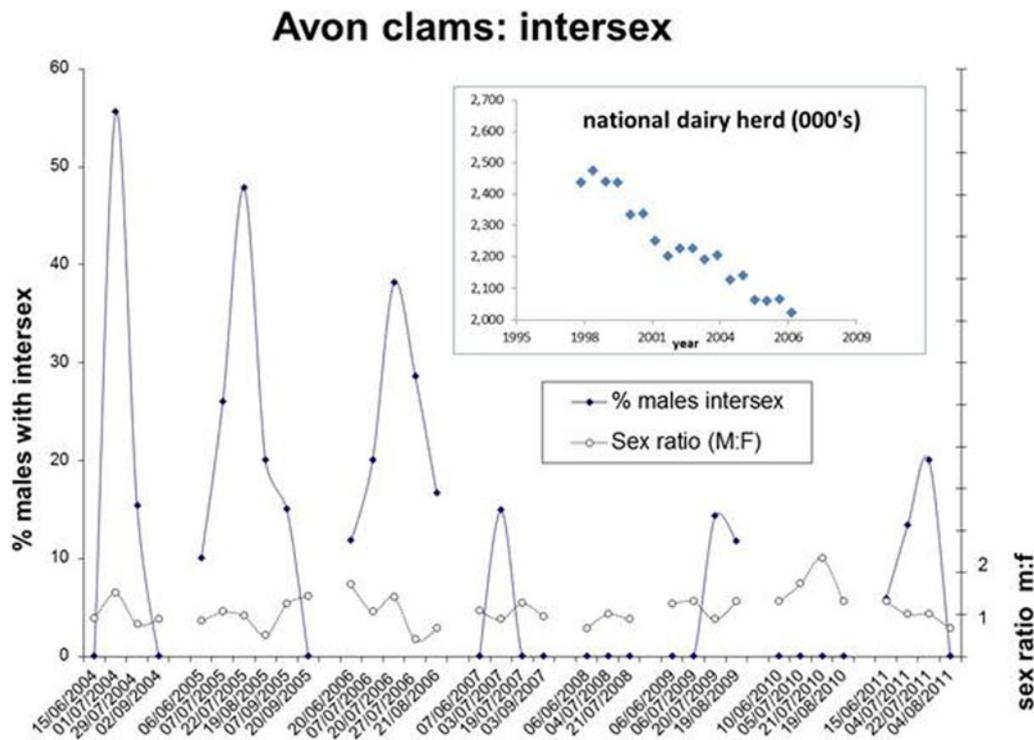


Fig. 5. *Scrobicularia plana*. Seasonal development and declining trends in intersex in the Avon estuary (SW England) coinciding with local efforts to fence off access to the waterway by cows. Inset: the decline in cattle numbers in the UK dairy herd.

Broader examination of populations of *S. plana* in SW England showed that disruption to the 'normal' gonadal development of male clams is widespread (17 out of 23 populations exhibited intersex with 60% of males from the Bristol Channel and Severn Estuary, the most impacted area surveyed, displaying the condition). Oocyte size was also higher in the Severn, notably at upstream sites (Langston *et al.*, 2007a). Subsequently, an extensive survey of more than 100 sites along English and French Channel coastlines in 2009–2014 returned a similar range of frequency and severity figures (Pope *et al.*, 2015); intersex was present in 58% of populations studied and proportions of affected males ranged from 0–53% (average 9%). The severity of intersex in Channel clams varied across the entire classification range (stages 1–6, Figure 4), though the majority of impacted individuals show only low severity (68% stage 1, 13% stage 2 and above). The overall mean M:F ratio was almost unity (1.007), indicating equal numbers of males and females, though sex ratios were significantly skewed at some sites: Nine populations were significantly biased towards females, six biased towards males – the latter including sites known to be affected by TBT (Pope *et al.*, 2015). Intersex frequencies varying between 1.5% and 71% have also been reported in *S. plana* from other estuarine locations in Europe, including Ireland (Raleigh & Keegan, 2006), Portugal (Gomes *et al.*, 2009) and NW France (Tankoua *et al.*, 2012).

It is possible that low levels of intersex (up to 10%) are the 'norm' for this species. Establishing a true baseline for the species is an important priority for the future, but may prove difficult since there are likely to be few, if any, estuaries that are completely free from any human impact, as demonstrated by the Avon. However, clam populations sampled in the Fowey, Exe and Axe estuaries (SW England) and, notably, the undeveloped NW of Scotland were found to be completely free of intersex (none found in 186 clams examined from Scottish west coast mainland sites, Mull, Skye and Harris in the Outer Hebrides). There was no departure from unity in male to female sex ratios in any of these populations, implying that zero intersex (strict gonochorism) is

probably the true reference condition in *S. plana* and that values above that reflect anthropogenic influences.

Some of the highest levels of intersex severity in *S. plana* have been observed in the Mersey Estuary (20% of clams exhibiting stage 6), consistent with elevated ED effects-levels in flounders from this highly industrialized and urbanized estuary (Lye *et al.*, 1997; Kirby *et al.*, 2004). This similarity strengthens the hypothesis linking intersex in *S. plana* with anthropogenic sources of EDC. Likewise, experimental exposures of *S. plana* to sediment spiked with mixtures of E2, EE2 (100 $\mu\text{g kg}^{-1}$), octylphenol (OP) and nonylphenol (NP) (1000 $\mu\text{g kg}^{-1}$), or individually to E2 or NP, indicate that oestrogens and xeno-oestrogens could be a contributory factor in the induction of intersex in male clams (up to 44% of exposed males exhibited ovotestis compared with 0–8% in controls; Langston *et al.*, 2007a, 2007b). Furthermore, a link between intersex frequency in *Scrobicularia* populations and oestrogenicity in sediments, as determined by YES assay, has been observed (Figure 6) adding to the weight of evidence that this species is susceptible to EDC. If, as suspected, oestrogenic activity resides principally in sediment (Peck *et al.*, 2004), as a deposit-feeder, *S. plana* might be considered sensitive to ensuing impacts from sediment-bound contaminants and appears to have ideal credentials as a sentinel species.

Mechanisms of the inducible feminizing effects in *S. plana* have yet to be determined and it is not yet confirmed that these observations indicate endocrine involvement. However, the absence of size-related differences in the sex of individuals denote that protandry or protogyny are not reproductive strategies for this species, further supporting the view that ovotestis is linked to anthropogenic influences. Endocrine disruption in a range of species has now been linked to a range of sources including exposure to WWTW effluents, general estuarine pollution (Skelly *et al.*, 2010; Schultz *et al.*, 2013), agriculture (McCoy *et al.*, 2008; Orton & Routledge, 2011), aquaculture (Ford *et al.*, 2007), pharmaceutical manufacture (Sanchez *et al.*, 2011) and paper mill effluents (Pollock *et al.*, 2010), or a combination

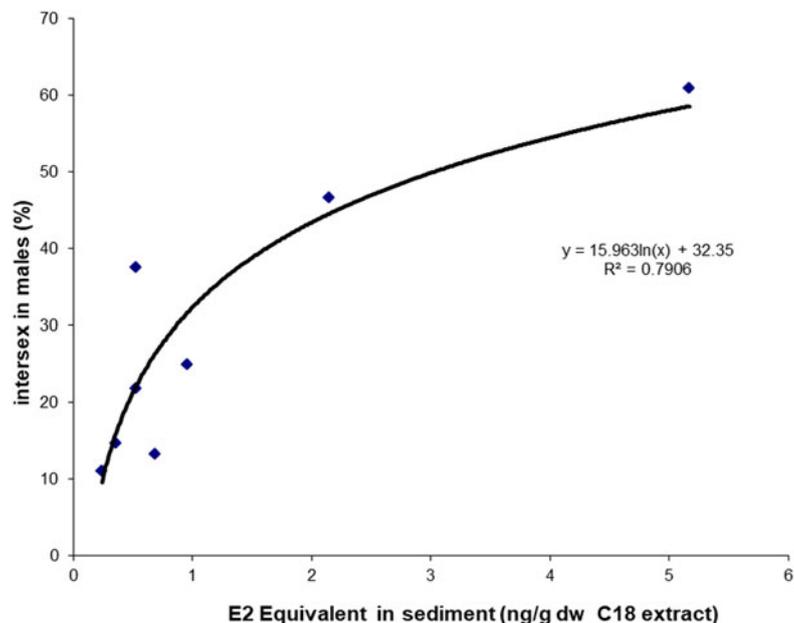


Fig. 6. *Scrobicularia plana*. Relationship between intersex and oestrogenic activity (YES assay) in sediments (SW estuaries): probability of the observed values was 0.027 suggesting that the E2 level might influence intersex. $R=0.588$ (unpublished observations, Langston and Routledge).

thereof. Whilst the relative importance of different sources has yet to be determined, *S. plana* clearly offers potential for large-scale screening and investigation of reproductive effects in the estuarine environment, helping to focus remedial strategy where it would be most beneficial.

Synergistic and antagonistic interactions between oestrogenic and androgenic EDC are feasible in nature and can cause complications in the interpretation of intersex levels and sex ratios – for example in *S. plana* collected at high TBT sites (discussed above), and in similarly exposed soft shell clams *Mya arenaria* (Linnaeus, 1758) (Gagné *et al.*, 2006) and mussels *Mytilus galloprovincialis* (Lamarck, 1819) (Ortiz-Zarragoitia & Cajaraville, 2010). Reproductive impairment (lower Gonadal Index), skewed sex ratios in favour of males, and elevated incidence of intersex (in both females and males) has also been observed in the venerid bivalve *Gamphina veneriformis* (Lamarck, 1818) as a result of prolonged exposure in the laboratory to elevated TBT levels (0.4–0.8 $\mu\text{g l}^{-1}$) during early gonadal development (Park *et al.*, 2015). Overall intersex frequencies in ‘control’ *G. veneriformis* were ~4%, rising to 31% at high TBT exposure (similar ranges to incidence levels in *S. plana* from UK estuaries). Measurement of steroid hormone levels in TBT-exposed gastropods and bivalves suggest an increase in testosterone and decrease in oestradiol (Horiguchi *et al.*, 1997; Morcillo & Porte, 2000; Abidli *et al.*, 2012); nevertheless, as with responses to other contaminants, the mode of action of TBT in many invertebrates is still tentative with regard to endocrine involvement. An alternative suggestion put forward by Hagger *et al.* (2006) proposes a causal association between imposex and genotoxicity, though this relationship has yet to be confirmed extensively.

Low levels of intersex are known to occur in other gonochoristic bivalves, worldwide, including mussels *Perumytilus purpuratus* (Lamarck, 1819) from Argentina (Villalobos *et al.*, 2010), razor clams *Tagelus plebius* (Lightfoot, 1786) and giant coquina *Iphigenia brasiliensis* (Lamarck, 1818) from Brazil (Ceuta *et al.*, 2007), *Ruditapes decussatus* (Linnaeus, 1758) from Galicia, Spain (Delgado & Camacho, 2002) and blood cockles *Anadara granosa* (Linnaeus, 1758) and *A. antiquata* (Linnaeus, 1758) from Indonesia (Afiati, 2007) – though in the latter two examples low incidences of ovotestis are believed to signify protandric hermaphroditism as opposed to exogenous response. Nevertheless, surveys of *R. decussatus* and cockles *Cerastoderma glaucum* (Bruguère, 1789) from areas receiving effluent from

waste water treatment plants in Tunisia imply more extensive frequencies of intersex due to pollution (Hamza-Chaffai, 2013). Experimentally, exposure of rock oysters *Saccostrea glomerata* Gould, 1850 to EE2 during early gonadogenesis has been shown to stimulate the transition of protandrous males to intersex and, eventually, females, though again the mechanism is uncertain (Andrew *et al.*, 2010).

Other observed reproductive effects in molluscs include enhanced embryo production at high dilutions of oestrogenic sewage effluent in freshwater mud-snails *Potamopyrgus antipodarum* (Gray, 1843), changing to inhibitory effects at low dilutions – a similar dose response to that following lab exposure of the same species to known oestrogenic chemicals (Jobling *et al.*, 2003). Enhanced oocyte production (along with proliferation of secondary sexual tissues) is also described in apple snails *Marisa cornuarietis* Linnaeus, 1758 and dogwhelks *N. lapillus* exposed to the xeno-oestrogens BPA and alkylphenol (Oehlmann *et al.*, 2000; Schulte-Oehlmann *et al.*, 2001). Reduced penis, prostate and development were observed in male *N. lapillus*. Interestingly, the responses of *Marisa* to BPA could not be reproduced in studies by Forbes *et al.* (2008), indicating that environmental conditions (tank sizes, design and configuration of animal exposures) can influence the outcomes of such experiments.

DDT exposure has been linked with altered germ cell development in freshwater mussels *Dreissena polymorpha* Pallas, 1771 in contaminated sections of Lake Maggiore: effects include oocyte degradation, and delayed sperm release, and may be symptomatic of neuroendocrine interference. The resultant asynchrony between sexes could compromise reproductive success (Binelli *et al.*, 2001, 2004). In contrast, E2 (and other sex steroids – testosterone and progesterone) stimulate both oogenesis and spermatogenesis in scallops *Mizuhopecten yessoensis* (Jay, 1857) and *Placopecten magellanicus* (Gmelin, 1791) leading to increased gonad weight and larger oocyte diameter (Varaksina & Varaksin, 1991; Varaksina *et al.*, 1992; Wang & Croll, 2004). Significantly, injections of E2 at early stages of sexual maturation of the oyster *C. gigas* have been shown to induce sex reversal from male to female (Mori *et al.*, 1969), illustrating the potential impact of non-endogenous sex steroids on bivalve sexuality.

Given this weight of evidence, not surprisingly, various measures of ‘endocrine disruption’ in molluscs, notably intersex, have been linked to sewage treatment works (WWTW) effluents (Moore & Stevenson, 1991; Chambers *et al.*, 1997; Gagné *et al.*,

2001; Gross *et al.*, 2001; Zulkosky *et al.*, 2002; Jobling *et al.*, 2003; Quinn *et al.*, 2004; Andrew-Priestley *et al.*, 2012), or known areas of anthropogenic contamination (Moore & Stevenson, 1991; Horiguchi *et al.*, 2000; Gagné *et al.*, 2002; Gauthier-Clerc *et al.*, 2002; Barbeau & Grecian, 2003; Lye *et al.*, 2005; Matozzo & Marin, 2007; Lee *et al.*, 2010).

There are additional changes in secondary sexual characteristics in marine arthropods (notably amphipods), which may be used to diagnose intersex. Intersex males possess rudimentary brood plates (female characteristics) and intersex females possess genital papillae (male characteristics) which may be used to assess the frequency and severity of intersexuality (number of papillae in females may denote degree of maleness). Intersexuality (and biased sex ratios) has been observed in the amphipod *Echinogammarus marinus* (Leach, 1815), frequency of which has been tentatively linked, partly, to feminizing microsporidian parasites. However, higher prevalence of intersex (affected males and females) was found at sites receiving industrial discharges in the Firth of Forth (~15%) when compared with reference sites (5–8%), suggesting parasitism is not the only cause of intersex and that pollution could be involved (Ford *et al.*, 2006). Analogous variability in intersex frequency (0.8–4.9%) is seen in freshwater *Gammarus pulex* (Linnaeus, 1758) from the Rivers Test and Itchen, with increasing levels found near WWTW, further implying a link to pollution (Love, 2017). *Gammarus* spp. have also been shown, experimentally, to be sensitive to EE2 and BPA, at least at comparatively high doses. Unlike fish, however, there is as yet no clear link in these amphipods to effects involving hormonal systems – though juvenile hormone mimics in crustaceans are known to have effects on vitellogenesis, intersex levels and sex ratios (EEA, 2012). Intersex frequency in *E. marinus*, as in *S. plana*, is seasonal and is lower in winter months, compared with warmer spring/summer months, as organisms mature. Other potential crustacean indicators include copepods (intersex levels found in sewage-exposed populations by Moore & Stevenson, 1991) and shore crab *Carcinus maenas* (Linnaeus, 1758), with male crabs displaying feminized abdomens and reduced claw depth in polluted UK estuaries (EDIT, 2003). Much of the information on endocrinology in crustaceans concerns the role of ecdysone and several peptide hormones in moulting and vitellogenesis, though the latter has yet to receive widespread attention as an ED biomarker in crustaceans. One exception is the induction of vitellogenin in grass shrimp *Palaeomonetes pugio* (Holthuis, 1949), following exposure to the PAH pyrene. In addition, the function of methyl farnesoate (a crustacean analogue of the insect growth hormone) may be compromised by insecticides designed to disrupt development (Oberdörster & Cheek, 2001).

In annelids, effects on development and reproductive output have been described in worms exposed experimentally to the xeno-oestrogen nonylphenol (Depledge & Billingham, 1999; Bettinetti *et al.*, 2002). Whilst an endocrine mode of action has not been established in these effects, various EDCs appear capable of agonistic and antagonistic activity, *in vitro*, on the annelid oestrogen receptor (Keay & Thornton, 2009). For these and the majority of invertebrate phyla however, our understanding of basic endocrinology, and hence true EDC-like effects, is sparse. For this level of analysis in aquatic environments we remain reliant on vertebrate responses, notably those described in fish.

Mechanisms

Vertebrates

The most obvious mechanism by which EDC can affect reproductive activity in vertebrates is through interference with the

function of nuclear receptors (NR, such as the Oestrogen, Androgen and Retinoid- X Receptors – ER, AR, RXR, respectively), resulting in altered gene expression. NRs are able to interact with endogenous ligands (e.g. hormones) or exogenous equivalents, including pollutants, which can perform either as agonists or antagonists. The development of transgenic mouse models and advances in genomics and proteomics has led to the expansion of *in vitro* assays, based on ER/AR potencies (including YES and ER-calux described earlier). These assays allow widespread screening of chemicals for their hormone activity and mode of action and are often used to guide the requirements for further animal testing. Relevance to lower organisms sometimes remains unclear and, ultimately, determination of ED impact and risk may depend upon identification of responses *in vivo* (as in the example of intersex in *S. plana*; Figure 6) since these effects usually integrate key processes such as bioaccumulation, elimination and metabolism.

Invertebrates

The most intensive work on mode of action in compromised invertebrate reproductive systems relates to TBT-induced imposex in marine snails. It is likely that TBT interacts with the endocrine system in these gastropod molluscs, though there are several hypotheses as to the precise mechanism. Suggestions for the main drivers of gender disruption include neuropeptides such as APGWamide, secreted by various ganglia of the central nervous system (Oberdörster & Cheek, 2001), and effects on steroid hormone titres. Methyltestosterone and the fungicide fenarimol have been shown to induce imposex in experimentally exposed snails, as does APGWamide, but in terms of the impacts of TBT in nature, the precise mode of action is still uncertain and may involve impeding conversion of testosterone to oestrogens in females (by inhibition of aromatase), targeting of nerves and ganglia associated with the neuro-endocrine system, or abnormal modulation of the retinoid-X receptor (Spooner *et al.*, 1991; Castro *et al.*, 2007; see below).

Even less certain is the mode of action in bivalves, but the TBT-related declines in UK native oyster (*Ostrea edulis*) and clam (*S. plana*) populations were thought to be associated with masculinization or failure to differentiate, and by reduced larval production and recruitment (Thain & Waldoock, 1986; Langston *et al.*, 2015), which may involve similar mechanisms to those seen in gastropods (Matthiessen, 2008).

In the freshwater prawn *Macrobrachium rosenbergii* (De Man, 1879), proteomic analysis has shown that proteins involved in the endocrine system and in the hormonal control of reproduction (including vitellogenin (VTG), farnesoic acid (FA), and o-methyltransferase (OMT)) may be deregulated by the organochlorine insecticide chlordecone (Lafontaine *et al.*, 2017), which is thus suggested to be an EDC for decapods, as in vertebrates (where it binds to ER). However, although a number of vertebrate-like sex steroids occur in phyla such as molluscs and crustacea, their origins, targets and mode of action in controlling reproduction (and identities of chemicals which interfere in this process) are often ambiguous. It is unlikely, for example, that upregulation of VTG, FA or OMT in the prawn *M. rosenbergii* is explainable by the interaction of chlordecone with endogenous oestrogen and ER pathways, as in vertebrates; rather it may involve signalling pathways of other hormones and neuroendocrine organs – though may still be manifested in similar endpoints, such as altered oocyte/VTG production or sex differentiation (Sumpter & Jobling, 1995; Lafontaine *et al.*, 2017).

Nevertheless, based on work with the scallop *M. yessoensis*, Osada and co-workers (Osada *et al.*, 2003; Thitiphuree *et al.*, 2019) have led the alternative viewpoint that the biosynthetic

pathway for sex steroids is similar to that of vertebrates and that vertebrate steroid-like receptors are functionally important in invertebrates, even if not classically similar to those in vertebrates, and the debate is still very much ongoing. A review of research on the identification and functioning of invertebrate receptors and pathways, in comparison to those in their vertebrate counterparts can be found in Lafont & Mathieu (2007). More recently Blalock *et al.* (2018) have reported evidence of a cholesterol side chain cleavage enzyme consistent with endogenous production of steroids and oestrogen signalling pathway, as a likely mechanism of action in mussels *M. edulis* exposed to 17- α -ethinylestradiol and 4-nonylphenol. A ligand-activated ER-like receptor has also been found in reproductive tissue of marine rotifers *Brachionus manjavacas* (Fontaneto, Giordani, Melone & Serra, 2007) which appears to bind human oestradiol and regulates reproductive output in females. This finding implies conservation of ER structure and function since the divergence of deuterostomes and proto-stomes 0.5 billion years ago (Jones *et al.*, 2017). However, among invertebrates active involvement of ER appears, on current evidence, to be the exception rather than the rule. Annelids may be another such exception, with ER apparently capable of binding oestradiol. In contrast, most invertebrate ER ligand-binding domains have occluded binding sites, preventing activation.

An orthologue of the vertebrate ER occurs in the molluscan genome but does not appear to bind to or be activated by vertebrate oestrogens (Bannister *et al.*, 2013) and no equivalent testosterone receptor has been identified. The term endocrine disruption is, therefore, ambiguous when applied to effects on such invertebrate reproductive systems. However, as indicated earlier, the involvement of the retinoid receptor RXR is implicated in molluscan imposex – in rock shell *Thais clavigera* (Küster, 1860) and *N. lapillus* – and activation and disruption of gene expression profiles has been demonstrated for both 9-Cis retinoic acid (a vitamin A derivative) and TBT (Castro *et al.*, 2007; Horiguchi *et al.*, 2008). Isolation of TBT-responsive nuclear receptors has been performed in other marine molluscs (and humans); Pacific oysters, *Crassostrea gigas* – also susceptible to TBT – host genes for a retinoic acid receptor orthologue (RAR) and a peroxisome proliferator-activated receptor orthologue (PPAR), as well as RXR (Castro *et al.*, 2007; Vogeler *et al.*, 2017). Computer predictions confirm the potential for RXR to bind 9-cis retinoic acid and TBT (with, it is hypothesized, corresponding effects on development of oyster larvae) supporting the concept that these NRs could be an important part of the mode of action of some EDC in invertebrates. PPAR and Gonadotrophin Releasing Hormone (and its receptor) are amongst other contenders for a role in imposex development in snails along with inhibition, by TBT, of testosterone-transforming aromatase (Spooner *et al.*, 1991; Castro *et al.*, 2007; Pascoal *et al.*, 2013).

Competitive interactions between EDCs add to the difficulty in interpreting modes of action. The anti-androgens cyproterone acetate and vinclozolin both appear capable of blocking the androgenic effects and activity of TBT in snails, whilst in *M. cornuarietis* it seems likely that activity of BPA (enhancing oocyte production) could be blocked by co-administration of other ER agonists (Oehlmann *et al.*, 2007). Furthermore, in higher (chordate) invertebrates, neurodevelopment and pigmentation in developing embryos seems to be impaired by EDCs such as BPA, through action on NRs, in a mechanism analogous to ED (Gomes *et al.*, 2019). This indicates susceptibility to EDCs, even though the mode of action does not directly involve ER or AR (which appear to have been lost in ascidians), nor does it appear to affect reproduction overtly. The PXR (Pregnane X Receptor) is a possible alternative candidate to bind EDCs in Ascidians.

Work done on invertebrates therefore suggests that EDCs can exert impact in a number of different ways not necessarily akin to

ER/AR-based ED in vertebrates; as a result, effects of EDs, and susceptibility, may vary greatly among invertebrate species (Mintram *et al.*, 2018).

Indigenous or exogenous hormones and their mimics? Bioaccumulation via dissolved and particulate pathways

Sex steroids may play a functional role in reproduction in some molluscs, as demonstrated by sex reversal and induction of vitellin-like protein in E2-injected bivalves (Mori *et al.*, 1969; Blaise *et al.*, 1999; Osada *et al.*, 2003), correlations between steroid titres and gonad development in clams and mussels (Lafont & Mathieu, 2007), and penis development in testosterone-treated female snails (Bettin *et al.*, 1996). Indeed, the presence of steroid hormones (and other non-steroidal EDCs) in invertebrate field samples now seems incontrovertible (Janer & Porte, 2007; Scott, 2018), though evidence of an endogenous biosynthetic pathway, involvement in reproduction and their mode of action in terms of ED is equivocal: steroid receptors – present, for example, in molluscs – have still to be shown to be active in mediating steroid action (Matthiessen, 2008). There is, however, growing evidence that marine invertebrates are capable of accumulating hormones and their mimics from their surrounding milieu. It is therefore important to consider exposure sources, distribution behaviour and bioaccumulation pathways as part of the risk assessment process.

Partitioning of EDCs and the importance of sediments for marine invertebrates

Dominant sources of EDCs to the marine environment include industrial discharges, consumer products, treated and untreated wastewater, urban and agricultural runoff (animal waste and fertilizer/pesticide application). Concentrations are likely to be highest near points of release into aquatic environments, but may be dispersed by currents and tides and partitioned between liquid and solid phases, with differing bioavailabilities.

A significant knowledge gap relates to the possibility that much of the oestrogenicity in aquatic environments may reside in benthic sediments and, if bioavailable, may be transferred to benthic biota (and hence through the food chain). Because sediments may concentrate contaminants perhaps hundreds to thousands of times higher than overlying water, biological availability of only a small proportion of this particulate loading could result in significant uptake in filter-feeders and other benthic invertebrates which are in direct contact with sediments.

Previous observations regarding the bioavailability and effects of TBT have highlighted the role of sediments in the sequestration and concentration of EDCs in estuarine environments. TBT partition coefficients (K_d – the ratio of TBT in sediment relative to water) are relatively high – typically of the order of 10^5 in estuarine muds – with consequences for deposit-feeding organisms such as clams *S. plana* (Langston *et al.*, 1990; Langston & Burt, 1991; Langston & Pope, 1995). Investigations of sediment–water partitioning of other EDCs, including the hormone E2 and the alkylphenolic surfactant NP, show that sediments have an important role in their environmental behaviour also (Lai *et al.*, 2000; Bowman *et al.*, 2002; Langston *et al.*, 2005, 2007b).

Batch experiments using natural sediments, and ^{14}C -radiolabelled E2 and NP have been used to establish kinetic behaviour and K_d for these classes of EDC, enabling investigations into the influence of major estuarine variables (salinity, pH, suspended solid loading and sediment type) on sediment–water partitioning behaviour (Langston *et al.*, 2007b). Equilibration of both E2 and NP with natural sediments is achieved rapidly – largely within one hour. The molecular structure of the two substrates has a major influence on binding characteristics however, with

K_d values for NP ($0.2\text{--}1 \times 10^4$) nearly two orders of magnitude higher than E2 ($0.7\text{--}1.4 \times 10^2$). NP, with its long, linear non-polar (hydrophobic) 'tail' will pack around hydrophobic sites with greater density and strength than E2, which must present the hydrophobic regions of the ring structure as close to the sediment surface as possible and as a consequence the binding may not be particularly strong. These substrate binding results indicate, therefore, that E2 is much more hydrophilic, under typical estuarine conditions, than NP. Desorption behaviour for NP appears to be close to being completely reversible whilst a proportion of the sediment binding for E2 appears irreversible. The percentage binding to Avon Estuary surface sediment was 72% and 10% for NP and E2, respectively, though in other sediments proportions may vary (especially for E2), largely as a function of particulate organic matter content (Langston *et al.*, 2007b).

Other estuarine variables have subtle implications for particle-water interactions of NP and E2. Of these variables, substrate concentrations appear to have a relatively minor influence on partitioning. In contrast, suspended solids loadings potentially have a significant effect over the range of concentrations of suspended sediment encountered in UK estuaries – characterized by a decrease in K_d values for E2 and NP with increased suspended sediment load. However, the proportion of both E2 and NP bound to the sediment phase increases as a function of the suspended solids concentration, rising to ~25% and ~80% of the total budget, respectively, in conditions typical of turbid estuaries. Based on this evidence the transport of NP is likely to be determined by sediment dynamics to a greater extent than the more hydrophilic E2. Experiments with TBT, likewise, demonstrate the importance of turbidity in determining the fate of TBT (Langston & Pope, 1995).

Partitioning of EDCs is unlikely to be affected by pH in estuarine and coastal waters because of the buffering effect of seawater. Only near to industrial discharges would ambient pHs be likely to diverge significantly from circum-neutral and then differences in partitioning response may occur between classes of EDCs. For the alkylphenol NP an increase in binding to sediment occurs towards both ends of the pH range, implying reduced solubility. For E2, K_d rises gradually with increasing acidity (compared with that under neutral conditions) but decreases slightly with increasing alkalinity, due possibly to ionization of the phenol functional group at elevated pH. The more hydrophobic character of the NP molecule perhaps outweighs any such ionization effects at high pH. The effect of salinity on partitioning of both E2 and NP is typical of the salting-out effect on solubility common to many organic compounds. For NP, K_d values are lowest in fresh water and the binding of NP to the sediment is increased by even small amounts of ionic solutes in the water. E2 displays a more gradual overall trend towards increased K_d with rising salinity. The solubility of both compounds in the aqueous phase will clearly be reduced as a result of saline intrusion in estuaries, albeit to differing degrees.

Thus sediment type (particularly organic content), suspended solids loadings and salinity are likely to be major influences on partitioning behaviour of many EDCs, whilst pH and substrate concentration would not be expected to influence distributions in most estuaries. However, the chemical characteristics of the compounds themselves may account for the biggest variation in behaviour and distribution in estuaries: the alkylphenol NP is dominated by its association with particulates, unlike E2 which is preferentially associated with soluble fractions. This difference between compounds might intuitively be expected to be reflected in relative bioavailability to estuarine organisms and ultimately with their oestrogenic effects. However, whilst it is assumed that compounds which predominate in the dissolved phase, such as E2, are likely to have a stronger tendency for bioaccumulation this has rarely been put to the test. The potential for direct uptake from sediment has

thus largely been overlooked, though as described below, it is potentially important in infaunal deposit-feeders.

Bioaccumulation

The relative importance of dissolved and particulate vectors of EDC bioaccumulation has been investigated in the clam *S. plana*, using water-sediment systems equilibrated (in combination and separately) with ^{14}C -labelled E2 and NP (Langston *et al.*, 2007b). Average concentrations in water and sediment were 15 ng l^{-1} and 69 ng g^{-1} (dw), respectively for NP and 4 ng l^{-1} and 7 ng g^{-1} for E2 in the experiment depicted in Figure 7. Bioaccumulation from water was rapid and the principal source for clams in the short term, with steady-state concentrations of E2 reached after 24 h and NP after 36 h. Bioconcentration factors, relative to water, were 1517 for E2 and 1819 for NP under these conditions. Over the longer term, however, though displaying slower bioaccumulation kinetics, sediments increased in relative importance as sustained sources, particularly for NP. After 12 days exposure in equilibrated systems only 2% of the body burden of NP was accounted for by uptake from water (10% in the case of E2) and there was no indication of steady state in the sediment-exposed clams over this timescale. Clearly, however, bioaccumulation of exogenous EDC in both dissolved and particulate form is to be expected in aquatic invertebrates exposed to contamination in nature.

Mechanistic studies with radiolabelled steroidal hormones (E2, testosterone and progesterone) suggest that the uptake in mussels *Mytilus* spp. involves metabolism and conjugation of the parent compound to fatty acids, prior to bioaccumulation as highly lipophilic esters (Schwarz *et al.*, 2017a, 2017b, 2018). Though addressing the aqueous uptake route only, these results confirm the importance of exogenous sources of steroid hormones and the high accumulation potential of filter-feeding molluscs. As in clams, optimum uptake of dissolved E2 in mussels occurred within 24 h (Schwarz *et al.*, 2017a) and was mostly (80%) present in tissues in lipophilic, esterified form (remainder divided between free- or sulphated- E2, the latter presumably released back to water). Estimated elimination rates for E2 varied between 16% (over 10 days) in static tanks and 63% (20 days) in running seawater, with the largest reduction in the first 5 days, typical of hyperbolic decay (half-life 12.5 days in flow-through conditions).

Given the high rates of uptake of exogenous E2 it is easy to concede that this source may mask any endogenous concentrations, if these exist (Schwarz *et al.*, 2017a). Mussels exposed to tritiated testosterone also demonstrate a very high capacity for uptake, much of which becomes conjugated with fatty acid, notably as 5α -dihydrotestosterone (DHT) and 5α -androstane- $3\beta,17\beta$ -diol, but with little (<5%) as the parent compound. Molluscs are known to exhibit 5α -reductase activity – which mediates transformation into DHT (a potent inducer of male secondary sexual characteristics in higher vertebrates). Despite evidence of DHT presence in bivalves in the field, however, it is still not clear if endogenous pathways are involved, or that altered sexual characteristics ensue (Schwarz *et al.*, 2017b).

The levels of bioaccumulation observed in experiments with clams and mussels (Peck *et al.*, 2007; Langston *et al.*, 2007b; Schwarz *et al.*, 2018) imply that benthic invertebrates could be susceptible to increases in body burdens of both steroidal and non-steroidal EDCs near sources, hence, could be important vectors to higher members of the food chain. Under typical estuarine conditions (chronic contamination) sediments may represent a particularly important source for bioaccumulation over the lifetime of benthic organisms, given that K_d range from hundreds to thousands for EDCs such as E2 and NP, respectively. Similar findings of sediment-dominated partitioning and

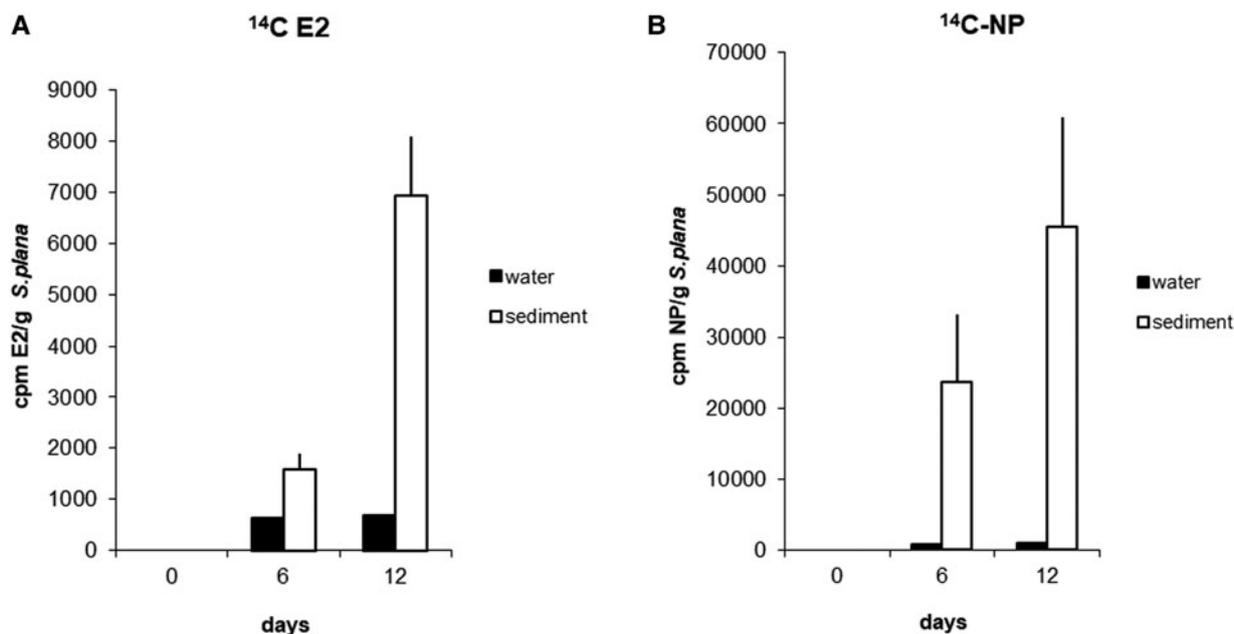


Fig. 7. *Scrobicularia plana*. Bioaccumulation of (A) ¹⁴C-oestradiol (E2) and (B) ¹⁴C-nonylphenol (NP) in individuals exposed via water or labelled sediment.

bioaccumulation are found in clams exposed to TBT, where ~90% may be derived from the particulate phase in equilibrated systems (Langston & Burt, 1991). These observations add to the weight of evidence that both steroidal and non-steroidal EDCs derived from various environmental compartments could be linked to disturbances in reproductive processes and altered sex ratios in molluscs and other invertebrates.

Reviewing the evidence from various taxa, Scott (2018) concluded that the ability to bioaccumulate steroids (including E2, testosterone and progesterone) was a more likely explanation for their presence and impact in invertebrate tissues than endogenous hormonal involvement in control of reproduction – questioning the possibility of invertebrates acting as surrogates of vertebrate-style endocrine disruption. The significance of steroidal burdens in invertebrates is further confounded by the possibility of transformations within both the organism itself or by other aquatic fauna and flora in the diet, including sediment. Therefore, it is perhaps not surprising that cycling of steroid hormones has only occasionally been observed to track reproductive cycles in invertebrates and evidence of biological activity is at best tenuous (Schwarz *et al.*, 2018). Apparent absence of the steroid metabolizing enzyme aromatase (catalysing metabolism of T to E2) in invertebrates was cited by Scott (2018) as a further reason for uncertainty over biosynthesis and mode of action of ED in invertebrates. However, whilst such mechanistic evidence is sparse and tentative, there are a number of reports suggesting aromatase involvement, and that correlations between steroids and reproductive cycles are not purely coincidental. These include an indication that aromatase activity (transformation of T to E2) may be blocked by TBT in female dogwhelks, leading to the initiation of imposex (Spooner *et al.*, 1991). However, as already discussed, this is not conclusive, since other possible explanations for imposex – involving RXR receptor or neuropeptide pathways – have also been proposed (Oberdörster & Cheek, 2001; Horiguchi *et al.*, 2008).

An additional factor in the debate over the nature and significance of the classic steroid-‘ED’ response model is illustrated by Schlenk (2008), who showed that *in vitro* responses (ER-ligand based bioassay) may sometimes underestimate *in vivo* oestrogenic activity (fish VTG, normalized to oestrogen equivalents) particularly in complex waste situations. Application of TIE fractionation schemes using biological responses to these composite wastes

failed to indicate any relationship to steroids (in contrast to simpler domestic waste), supporting the view that mechanisms other than direct ER binding may be important in the feminization of populations in nature, particularly those residing in habitats that receive mixed wastewaters or agricultural effluents. There would seem to be a case for additional mechanisms of disruption to be considered when determining causality of effects such as feminization. Thus, whilst screening assays for EDC based on interaction with ER/AR are rapid (and indicative of *potential* for hormone disruption), their relevance in terms of whole organism or population response may be uncertain, given the limited understanding of basic endocrinology in many invertebrates.

It is clearly plausible to put forward alternative hypotheses for the mode of action behind invertebrate ‘ED’ other than endogenous steroid pathways and control seen in most vertebrates. Nevertheless, the link between steroidal hormones and ED in invertebrates deserves further consideration (as do numerous other potential agonists and antagonists) in the search for appropriate explanations for reproductive disturbance. Moreover, the OECD has put forward strong ethical arguments recommending that invertebrate models should be sought to complement or replace vertebrate models in experimental testing systems. Invertebrates seem particularly well suited for testing issues such as the diversity and potency of EDC, synergism/antagonism, EDC metabolites and environmental transfer products, and no-effects levels – all of which involve testing with large numbers of individuals.

Experimental studies involving fish, molluscs and crustaceans have, to date, all contributed to our appreciation of the range of possible deleterious effects caused by EDCs. Ultimately, it is their ecological relevance which determines their value in impact assessment programmes. In this context, further examples where disruption to reproductive and development cycles permeate to the population level are discussed in the following section.

Consequences for populations

By definition the most useful models of ED ideally link an endocrine mode of action with a population-relevant deleterious effect. Impacts at the population level are often assumed, based on reproductive endpoints measured at the cellular, tissue or

individual level, though in reality these endpoints may not relate directly to population success or result from an endocrine mode of action. The case of tributyltin impacts on neogastropods and, to a lesser extent, bivalves, represents one of the few exceptions in marine systems where field evidence provides the link between impacts at the lower level of biological organization (molecular, cellular, tissue) and population responses (though endocrine involvement remains uncertain). The most convincing evidence of TBT-induced impact on reproduction in marine organisms has involved sensitive molluscan indicators, though a range of other invertebrate populations and communities have undoubtedly been damaged (Matthiessen, 2008). TBT has been shown to cause reproductive effects in fish, including testis histopathology, impaired embryo development and biased sex ratios, though at much higher concentrations ($>100 \text{ ng l}^{-1}$) than gastropods (Haubruge *et al.*, 2000; McAllister & Kime, 2003; Zhang *et al.*, 2009, 2011). Reduced sensitivity in fish probably reflects the presence of efficient detoxifying systems in vertebrates and evidence of TBT-induced effects in native fish populations in the field has not emerged. However sex ratio in fish can be affected by a number of EDCs and is almost certainly a population-relevant endpoint (Hazlerigg *et al.*, 2014).

The implications of intersex (ovotestis) for the fecundity of invertebrates is unknown; however, the incidence of affected male *S. plana* in the field (up to 60%) is sometimes in excess of that observed in feminized fish populations whose reproductive success is compromised (Jobling *et al.*, 1998; Jobling & Tyler, 2003; Langston *et al.*, 2007a). It perhaps seems counterintuitive to anticipate population-level consequences in a broadcast spawner with planktotrophic larvae such as *S. plana*, nevertheless, although the clam is theoretically capable of substantial lateral migration, this may occur only rarely in nature (Casagrande & Boudouresque, 2005). Hence, faced with the prospect of elevated concentrations of endocrine disrupting chemicals and ensuing challenges to sexuality, failure to sustain clam populations, due to restriction in gamete/larval viability cannot be ruled out at some sites. Certainly, as depicted in Figure 3A, there is strong evidence that TBT has impacted populations of *S. plana*, through reductions in recruitment (Langston *et al.*, 2015). Wider decline and disappearance of *S. plana* populations, from undetermined causes, has also been reported from areas in the Danish and German Wadden Sea and the western half of the Dutch Wadden Sea (Essink *et al.*, 1991). It is not unconceivable, therefore, that populations of deposit-feeding species such as *S. plana* may be vulnerable to the wider pressure of endocrine disruption – a risk exacerbated by bioaccumulation of EDCs from sediment and meriting further investigation.

In the absence of convincing field data such as that provided by the TBT–mollusc example, models that link laboratory dose–effect relationships with consequences for dynamics of populations are often the best surrogate for predicting ecotoxicological risk. Nevertheless, caution has to be applied in the interpretation of such models given that dose–response relationships used in their derivation may extend beyond ranges which are relevant to wild populations (Marty *et al.*, 2017). Life history traits and behaviour, and their density-dependence, may also need to be factored in, as indicated in the modelling of the anti-androgenic effects of the pesticide fenitrothion on population dynamics of the stickleback *Gasterosteus aculeatus* Linnaeus, 1758 (Mintram *et al.*, 2018) which has freshwater and anadromous forms. Predictions of reductions in population size were parameterized from published data on disruption to male breeding behaviour (courtship, spiggin production and nest building) and suggested that effects were more marked following continuous, as opposed to intermittent, exposure and particularly influenced by altered sex-ratios. The value of these models lies in establishing endpoints which are likely to be most

relevant at the population level, and targeting surveillance at the most effective markers.

Ecological hazards have been predicted for invertebrate and vertebrate populations following exposure, experimentally, to a variety of EDC. For example, trenbolone, a synthetic anabolic compound, acts as an androgen agonist and may cause alterations in sexual differentiation in fish, notably altered sex-ratio – favouring males. Treatment of undifferentiated catfish fry (*Ictalurus punctatus*, Rafinesque, 1818) results in an all-male population incapable of producing viable offspring (Davis *et al.*, 2000). The effect of trenbolone at the population level has also been modelled from laboratory exposures in fathead minnow *P. promelas* (Miller & Ankley, 2004) and is an example of how such higher-level consequences of EDC can be simulated from robust experimental evidence. Given that it is not known whether ovotestis (and altered sex ratios) observed in crustaceans *Echinogammarus marinus* (Ford *et al.*, 2006) and molluscs *S. plana* (Langston *et al.*, 2007a) has an effect at the higher level, there is a strong argument for ecological modelling of invertebrate populations exhibiting varying degrees of intersex and testing predicted outcomes against survey data.

The synthetic oestrogen EE2 induces population effects in fish, alongside the lower level responses of vitellogenin induction, altered gonad histology, fertilization success and larval growth. Kidd *et al.* (2007) have shown reduced populations in fathead minnows *P. promelas*, as a result of feminization, in Canadian lake systems dosed with EE2 in the low ng l^{-1} range. The fungicide vinclozolin, an anti-androgen, has also been shown to alter male gonad histology and secondary sexual characteristics and to reduce fecundity in *P. promelas* (US EPA, 2013) though reproductive output was less affected in other species, including Japanese medaka *Oryzias latipes* (Temminck & Schlegel 1846). Correlative evidence of the effects of PCBs and pesticides (DDE) on fish reproduction is provided by Hansen *et al.* (1985) who showed that reduced hatching success in Baltic herring *Clupea harengus* (Linnaeus, 1758) coincided with elevated levels of these organochlorines in ovaries.

The study of recovery of affected populations in the field, following removal/reduction of EDC is crucial. These studies help understanding of timescales and benefits of pollution control – illustrated most graphically by TBT: the cases of *N. lapillus* and *S. plana* confirm it is not necessarily commercial species, or popular test organisms which demonstrate the true ecological significance of EDCs in nature, but rather readily sampled, stationary/territorial invertebrates. Such studies also reveal the nature of the recovery process, whether it is reversal of effects in individuals or immigration from unaffected populations. The relevance of other environmental factors (other contaminants, physico-chemical conditions, hydrography, mode of dispersal, proximity to recruitment reserves) may also become evident and be pertinent to the success of remedial efforts.

Another significant aspect of marine invertebrates lies in their role in the food chain transfer of bioaccumulative and bioactive compounds, including EDCs, to predators. For example, PCB concentrations in a declining population of killer whales *Orcina orca* (Linnaeus, 1758) were found to be comparable to, or higher than, those compromising immune activity in populations of other marine apex predators, harbour seals *Phoca vitulina* Linnaeus, 1758 (Noel *et al.*, 2009). Though causation is difficult to prove, the implication is that bioaccumulating organic contaminants and changes in steroid metabolism represent a threat to endocrine systems in this declining orca population – as with reduced pupping rates in ringed seals *Pusa hispida* (Schreber, 1775) and other pinniped species (reviewed in Oberdorster & Cheek, 2001; EEA, 2012), reduced gonadal size and productivity in polar bears *Ursus maritimus* Phipps, 1774 (Sonne *et al.*, 2006) and declines in otter *Lutra lutra* (Linnaeus, 1758) populations during the 1960s (attributed to PCBs, dieldrin and mercury

accumulated in food; Simpson *et al.*, 2000). Procurement of experimental evidence of ED is impractical in many of these protected species; however, reproductive failure (reduced progesterone-mediated embryo implantation) has been recorded in common seal *Phoca vitulina* fed PCB-enriched fish (Reijnders, 1986) and is relevant in the case of other mammals.

Given that trends in marine species and populations at all trophic levels mirror recent declining trends in wildlife biodiversity and abundance in terrestrial ecosystems, there seems a powerful case to consider ED as a possible contributory stressor, alongside the threats from global climate trends, plastics and overfishing.

Conclusions

There is evidence, from the field and in laboratory experiments, to suggest that chemical-induced effects on reproductive systems may occur in marine invertebrates and may be manifested at very low doses. Whilst not many examples have been proven to be endocrine-mediated (unlike some fish species), they are sufficient to raise concerns for biota in contaminated estuarine and coastal areas and promote the case for wider research into health trends in marine life. Despite the lack of basic knowledge concerning the invertebrate endocrine system, the diverse taxonomic groups within this branch of the animal kingdom (particularly marine representatives) offer much in terms of understanding comparative and ecological perspectives surrounding reproductive impairment. Strictly, such damage may not be a direct result of hormone disruption and should, as proposed by Oberdörster & Cheek (2000) be 'considered suggestive, but not conclusive, evidence of endocrine disruption': in-depth investigation is still required to provide this evidence.

Given that many newly developed chemicals enter the environment with little evaluation at the whole organism level, and considering the physiological diversity of invertebrate taxa, the need for a range of rapid (high-throughput) screening tests, along the lines of those developed to investigate imposex and intersex in molluscs, is evident. These assessments should supplement existing *in vitro* and *in vivo* testing protocols adopted by the US EPA and OECD – including those based on interaction with vertebrate nuclear receptors: the latter are most useful in identifying the *potential* rather than actual *consequences* for ED (not all *in vitro* screens translate into *in vivo* effects). Though mechanisms may not be fully understood yet, invertebrate taxa provide for a series of diverse but relevant endpoints on which to focus more intensive cause-effect investigations into mode of action, severity, sensitive windows of development, reversibility and need for regulatory action. At present many invertebrate groups are under-represented in risk assessment programmes. Tests with invertebrates will help to reduce the number of higher animals currently sacrificed in laboratory testing protocols, but will of course still require close ethical scrutiny.

Wider appreciation of the frequency and severity of suitable responses in nature, coupled with investigation of the mode of action of different classes of chemicals, should involve the use of strategic indicator species such as *Scrobicularia plana* in order to understand and manage future contaminant threats to the marine environment. The characteristics of intersex (ovotestis) in populations of *S. plana* and induction following exposure to mixtures of (xeno-)oestrogens appear to be similar to those in fish, where endocrine disruption, and causes thereof, have been established definitively (Tyler & Routledge, 1998; Jobling & Tyler, 2003). Intersex is a relatively widespread phenomenon in *S. plana* populations, with varying degrees of incidence and severity, which mirrors the extensive and variable nature of ovotestis in freshwater and estuarine fish from UK catchments – and implies that both may be symptomatic of reproductive modification caused by anthropogenic factors. It would be

informative to compare the responses in vertebrate and invertebrate indicators more widely across a range of sites.

There is a need for further research on vulnerability and resilience across the lifespan of invertebrates (windows of ED sensitivity), trans-generational effects, comparison of influence on gene expression and epigenetic factors, and synergism/antagonism between EDCs. Field monitoring which links effects at the individual and population levels should be added to this challenging list. Outcomes need to be incorporated into robust procedures, models and regulatory guidelines for the protection of marine habitats which are subjected to mixtures of many potentially harmful chemicals (EEA, 2012). Screening of each may be prohibitive in terms of resources; however, through careful targeting, prevention of the release of deleterious EDCs into water bodies will ultimately be preferable and more cost-effective than subsequent remediation. Such an approach should take into consideration not only the broad spectrum of chemical threats but also the taxonomic diversity of marine invertebrates.

Significant instances of ED in wildlife cannot always be predicted from conventional testing with standard organisms and may only become apparent after crucial field-based observations identify a problem – as in the case of TBT-impacted molluscs. Despite the limitations of established testing protocols, however, national and international action has proven to be effective in reducing risk from a number of EDCs including TBT, DDT, PCBs and other POPs over the last 20 years, and illustrates how reducing exposures can protect marine life from reproductive disorders. Interestingly, it is the study of recovery of populations – e.g. following the regulation of TBT inputs and reduction in sewage discharge to the marine environment – which is one of the most informative aspects of ED research and is one which deserves more attention in terms of learning how to handle future threats from EDCs. Improved spatial and temporal monitoring in relevant indicators is a particularly important requirement in this context. Given the global spread of contamination, an additional benefit from such surveillance will be the identification of 'pristine' locations which represent the true baseline condition – an essential conservation target for the protection of marine life.

Acknowledgements. Support from the Department for the Environment, Food and Rural Affairs (Defra) for research carried out under the EDAQ program (Project CTG0301) is gratefully acknowledged, and thanks are due to Dr Mike Roberts and Dr David Sheahan for their encouragement and helpful suggestions during that time.

Glossary

Androgen	Male sex hormone that is produced in the testes and responsible for typical male sexual characteristics.
Anti-androgen	Substance that mimics or blocks the natural male sex hormones.
Anti-oestrogen	Substance that mimics or blocks the natural female sex hormones.
Deposit feeder	Feeds on benthic sediment particles.
Endocrine disrupting chemicals (EDs/EDCs)	Defined as (1) 'exogenous substances that cause adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine function' (EU, 1997), or as (2) 'exogenous agents that interfere with the production, release, transport, metabolism, binding and action or elimination of the natural hormones in the body responsible for the maintenance of homeostasis and regulation of developmental processes' (US EPA, 1997).

Gonochoistic	Pertaining to a unisexual individual; remaining as the same sex throughout the life cycle.
Infauna	Category of organism whose natural habitat is within a soft substrate (sediment).
Oestrogen	Any of various natural steroids (e.g. oestradiol) that are formed from androgen precursors, that are secreted chiefly by the ovaries, adipose tissue and testes, and that stimulate the development of female secondary sex characteristics and promote the growth and maintenance of the female reproductive system.
Ovotestis	An 'intersex' condition whereby a gonad contains both testicular tubular elements and ovarian follicles.
Phyto-oestrogen	Natural chemicals found in plants, notably whole grains, fibres and soy products (resorcylic acid, lactones, isoflavones, coumestans, lignans).
Xeno-oestrogen	Any of various synthetic or semisynthetic steroids (such as ethinyl oestradiol) that mimic the physiological effect of natural oestrogens.

References

- Abidli S, Santos MM, Lahbib Y, Castro LFC, Reis-Henriques MA and El Menif NT (2012) Tributyltin (TBT) effects on *Hexaplex trunculus* and *Bolinus brandaris* (Gastropoda: Muricidae): imposex induction and sex hormone levels insights. *Ecological Indicators* **13**, 13–21.
- Afiati N (2007) Hermaphroditism in *Anadara granosa* (L.) and *Anadara antiquata* (L.) (BIVALVIA: ARCIDAE) from Central Java. *Journal of Coastal Development* **10**(3), 171–179.
- Allen Y, Scott A, Matthiessen P, Haworth S, Thain J and Feist S (1999) Survey of estrogenic activity in United Kingdom estuarine and coastal waters and its effects on gonadal development of the flounder *Platichthys flesus*. *Environmental Toxicology and Chemistry* **18**, 1791–1800.
- Alvarez-Muñoz D, Indiveri P, Rostkowski P, Horwood J, Greer E, Minier C, Pope N, Langston WJ and Hill EM (2015) Widespread contamination of coastal sediments in the Transmanche Channel with anti-androgenic compounds. *Marine Pollution Bulletin* **95**, 590–597.
- Andrew MN, O'Connor WA, Dunstan RH and Macfarlane GR (2010) Exposure to 17 alpha ethynylestradiol causes dose and temporally dependent changes in intersex, females and vitellogenin production in the Sydney rock oyster. *Ecotoxicology* **19**, 1440–1451.
- Andrew-Priestley MN, O'Connor WA, Dunstan RH, Van Zwieten L, Tyler T, Kumar A and MacFarlane GR (2012) Estrogen mediated effects in the Sydney rock oyster, *Saccostrea glomerata*, following field exposures to sewage effluent containing estrogenic compounds and activity. *Aquatic Toxicology* **120**, 99–108.
- Arai T, Harino H, Ohji M, Langston WJ (eds) (2009) *Ecotoxicology of Antifouling Biocides*. Berlin: Springer Verlag, 473 pp.
- Arukwe A, Forlin L and Goksoyr A (1997) Xenobiotic and steroid biotransformation enzymes in Atlantic Salmon (*Salmo salar*) liver treated with an estrogenic compound, 4-nonylphenol. *Environmental Toxicology and Chemistry* **16**, 2576–2583.
- Bannister R, Beresford N, Granger DW, Pounds NA, Rand-Weaver M, White R, Jobling S and Routledge EJ (2013) No substantial changes in estrogen receptor and estrogen-related receptor orthologue gene transcription in *Marisa cornuarietis* exposed to estrogenic chemicals. *Aquatic Toxicology* **140–141**, 19–26.
- Barbeau MA and Grecian LA (2003) Occurrence of intersexuality in the amphipod *Corophium volutator* (Pallas) in the upper Bay of Fundy, Canada. *Crustaceana* **76**, 665–679.
- Bateman KS, Stentiford GD and Feist SW (2004) A ranking system for the evaluation of intersex condition in european flounder (*Platichthys flesus*). *Environmental Toxicology and Chemistry* **23**, 2831–2836.
- Bergman A, Heindel JJ, Jobling S, Kidd KA and Zoelle RT (eds) (2013) *State of the Science of Endocrine Disrupting Chemicals – 2012. An Assessment of the State of the Science of Endocrine Disruptors Prepared by a Group of Experts for the United Nations Environment Programme and World Health Organization*. UNEP, 260 pp.
- Bettin C, Oehlmann J and Stroben E (1996) TBT-induced imposex in marine neogastropods is mediated by an increasing androgen level. *Helgolander Meeresunters* **50**, 299–317.
- Bettinetti R, Cuccato D, Galassi S and Provini A (2002) Toxicity of 4-nonylphenol in spiked sediment to three populations of *Chironomus riparius*. *Chemosphere* **46**, 201–207.
- Binelli A, Bacchetta R, Vailati G, Galassi S and Provini A (2001) DDT contamination in Lake Maggiore (N Italy) and effects on zebra mussel spawning. *Chemosphere* **45**, 409–415.
- Binelli A, Bacchetta R, Mantecca P, Ricciardi F, Provini A and Vailati G (2004) DDT in zebra mussels from Lake Maggiore (N. Italy): level of contamination and endocrine disruptions. *Aquatic Toxicology* **69**, 175–188.
- Blaise C, Gagné F, Pellerin J and Hansen PD (1999) Determination of vitellogenin-like properties in *Mya arenaria* hemolymph (Saguenay Fjord, Canada): a potential biomarker for endocrine disruption. *Environmental Toxicology* **14**, 455–465.
- Blalock BJ, Robinson WE, Loguinov A, Vulpe CD, Krick KS and Poynton HC (2018) Transcriptomic and network analyses reveal mechanistic-based biomarkers of endocrine disruption in the marine mussel, *Mytilus edulis*. *Environmental Science & Technology* **52**, 9419–9430.
- Bowman JC, Zhou JL and Readman JW (2002) Sediment-water interactions of natural oestrogens under estuarine conditions. *Marine Chemistry* **77**, 263–276.
- Bray S (2006) Tributyltin pollution on a global scale. An overview of relevant and recent research: impacts and issues. In Langston WJ (ed.), *Report to WWF UK*. Contract No. FND053998. Godalming: WWF.
- Bray S, McVean EC, Nelson A, Herbert R, Hawkins, S and Hudson, MD (2012) The regional recovery of *Nucella lapillus* populations from marine pollution, facilitated by man-made structures. *Journal of the Marine Biological Association of the United Kingdom* **92**, 1585–1594.
- Bryan GW, Gibbs PE, Hummerstone LG and Burt G (1986) The decline of the gastropod *Nucella lapillus* around south-west England: evidence for the effect of tributyltin from antifouling paints. *Journal of the Marine Biological Association of the United Kingdom* **66**, 611–640.
- Bryan GW, Gibbs PE, Burt GR and Hummerstone L (1987) The effects of tributyltin (TBT) accumulation on adult dog-whelks, *Nucella lapillus*: long-term field and laboratory experiments. *Journal of the Marine Biological Association of the United Kingdom* **67**, 525–544.
- Casagrande C and Boudouresque CF (2005) Abundance, population structure and production of *Scrobicularia plana* and *Abra tenuis* (Bivalvia: Scrobiculariidae) in a Mediterranean brackish lagoon, Lake Ichkeul, Tunisia. *International Review of Hydrobiology* **90**, 376–391.
- Castro LFC, Lima D, Machado A, Melo C, Hiromori Y, Nishikawa J, Nakanishi T, Reis-Henriques MA and Santos MM (2007) Imposex induction is mediated through the retinoid X receptor signalling pathway in the neogastropod *Nucella lapillus*. *Aquatic Toxicology* **85**, 57–66.
- Ceuta IO, Boehs G and Santos JDJB (2007) Registro de hermafroditas em duas espécies de bivalves dioicos – *Tagelus plebeius* (Lightfoot, 1786) e *Iphigenia brasiliiana* (Lamarck, 1818) no estuário do Rio Cachoeira, Ilheus (BA). In *Proceedings of the Anais do VIII Congresso de Ecologia do Brasil*, Caxambu, 23–28 September 2007.
- Chambers PA, Allard M, Walker SL, Marsalek J, Lawrence J, Servos M, Busnarda J, Munger KS, Adare K and Jefferson C (1997) Impacts of municipal wastewater effluents on Canadian waters: a review. *Water Quality Research Journal of Canada* **32**, 659–713.
- Champ MA and Seligman PF (eds) (1996) *Organotin: Environmental Fate and Effects*. London: Chapman & Hall, 664 pp.
- Chen L, Hu C, Tsui MMP, Wan T, Peterson DR, Shi Q, Lam PKS, Au DWT, Lam JCW and Zhou B (2018) Multigenerational disruption of the thyroid endocrine system in marine medaka after a life-cycle exposure to perfluorobutanesulfonate. *Environmental Science and Technology* **52**, 4432–4439.
- Chesman BS and Langston WJ (2006) Intersex in the clam *Scrobicularia plana*: a sign of endocrine disruption in estuaries? *Biology Letters* **2**, 420–422.

- Colson I and Hughes RN (2004) Rapid recovery of genetic diversity of dogwhelk (*Nucella lapillus* L.) populations after local extinction and recolonization contradicts predictions from life-history characteristics. *Molecular Ecology* **13**, 2223–2233.
- Davis KB, Morrison J and Galvez JI (2000) Reproductive characteristics of adult channel catfish treated with trenbolone acetate during the phenocritical period of sex differentiation. *Aquaculture* **189**, 351–360.
- Defra (2009–2012). *ME5203: Monitoring and Assessment of Contaminant-related Effects in the Marine Environment (ACE)*. Available at http://randd.defra.gov.uk/Document.aspx?Document=12018_ME5203_Twopage_Final.pdf (Accessed 23 September 2018).
- Delgado M and Camacho AP (2002) Hermaphroditism in *Ruditapes decussatus* (L.) (Bivalvia) from the Galician coast (Spain). *Scientia; Rivista Di Scienza* **66**, 183–185.
- Depledge MH and Billingham Z (1999) Ecological significance of endocrine disruption in marine invertebrates. *Marine Pollution Bulletin* **39**, 32–38.
- Duft M, Schmitt C, Bachmann J, Brandelik C, Schulte-Oehlmann U and Oehlmann J (2007) Prosobranch snails as test organisms for the assessment of endocrine active chemicals: an overview and a guideline proposal for a reproduction test with the freshwater mudsnail *Potamopyrgus antipodarum*. *Ecotoxicology* **16**, 169–182.
- EU (1997) *European Workshop on the Impact of Endocrine Disruptors on Human Health and the Environment*. Environment and Climate Research Programme, DG XII, European Commission, Report EUR 17549, 1997.
- European Environment Agency (2012) *The Impacts of Endocrine Disruptors on Wildlife, People and Their Environments*. EEA Technical report No 2/2012. 112 pp. doi:10.2800/41462.
- EDIT (2003) *Endocrine Disruption in Invertebrates and Top Predators: A Programme of Research*. Newsletter no. 2. London: Department for Environment Food and Rural Affairs (Defra), 6 pp.
- Essink K, Beukema JJ, Coosen J, Craeymeersch JA, Ducrotoy J-R, Michaelis H and Robineau B (1991) Population dynamics of the bivalve mollusc *Scrobicularia plana* da Costa: comparisons in time and space. In Elliott M and Ducrotoy J-P (eds), *Estuaries and Coasts: Spatial and Temporal Intercomparisons*. 19th European Symposium on Marine Biology. Fredensborg: Olsen & Olsen, pp. 167–172.
- Forbes VE, Warbritton R, Aufderheide J, Van Der Hoeven N and Caspers N (2008) Effects of bisphenol a on fecundity, egg hatchability, and juvenile growth of *Marisa cornuarietis*. *Environmental Toxicology and Chemistry* **27**, 2332–2340.
- Ford A, Fernandes TF, Robinson CD, Davies IM and Read PA (2006) Can industrial pollution cause intersexuality in the amphipod, *Echinogammarus marinus*? *Marine Pollution Bulletin* **53**, 100–106.
- Ford AT, Read PA, Jones TL, Michino F, Pang Y and Fernandes TF (2007) An investigation into intersex amphipods and a possible association with aquaculture. *Marine Environmental Research* **64**, 443–455.
- Gagné F, Blaise C, Salazar M, Salazar S and Hansen PD (2001) Evaluation of estrogenic effects of municipal effluents to the freshwater mussel *Elliptio complanata*. *Comparative Biochemistry and Physiology* **128C**, 213–225.
- Gagné F, Blaise C, Pellerin J and Gauthier-Clerc S (2002) Alteration of the biochemical properties of female gonads and vitellins in the clam *Mya arenaria* at contaminated sites in the Saguenay Fjord. *Marine Environmental Research* **53**, 295–310.
- Gagné F, Blaise C, Pellerin J, Pelletier E and Strand J (2006) Health status of *Mya arenaria* bivalves collected from contaminated sites in Canada (Saguenay Fjord) and Denmark (Odense Fjord) during their reproductive period. *Ecotoxicology and Environmental Safety* **64**, 48–361.
- Gauthier-Clerc S, Pellerin J, Blaise C and Gagné F (2002) Delayed gametogenesis of *Mya arenaria* in the Saguenay fjord (Canada): a consequence of endocrine disruptors? *Comparative Biochemistry and Physiology* **131C**, 457–467.
- Gibbs PE, Bryan GW, Pascoe PL and Burt GR (1987) The use of the dogwhelk, *Nucella lapillus*, as an indicator of tributyltin (TBT) contamination. *Journal of the Marine Biological Association of the United Kingdom* **67**, 507–523.
- Gomes IDL, Gazo I, Besnardeau L, Hebras C, McDougall A and Dumollard R (2019) Potential roles of nuclear receptors in mediating neurodevelopmental toxicity of known endocrine-disrupting chemicals in ascidian embryos. *Molecular Reproduction and Development* **86**, 1333–1347.
- Gomes T, Gonzalez-Rey M and Bebianno MJ (2009) Incidence of intersex in male clams *Scrobicularia plana* in the Guadiana Estuary (Portugal). *Ecotoxicology* **18**, 1104–1109.
- Gross MY, Maycock DS, Thorndyke MC, Morrith D and Crane M (2001) Abnormalities in sexual development of the amphipod *Gammarus pulex* (L.) found below sewage treatment works. *Environmental Toxicology and Chemistry* **20**, 1792–1797.
- Hagger JA, Depledge MH, Oehlmann J, Jobling S and Galloway TS (2006) Is there a causal association between genotoxicity and the imposex effect? *Environmental Health Perspectives* **114**, 20–26.
- Hamza-Chaffai A (2013) Estrogenic endocrine disruptors and their possible deleterious effects on marine organisms: use of a novel monitoring bioassay. *International Journal of Biotechnology for Wellness Industries* **2**, 1–9.
- Hansen PD, vonWesternhagen H and Rosenthal H (1985) Chlorinated hydrocarbons and hatching success in Baltic herring spring spawners. *Marine Environmental Research* **15**, 59–76.
- Hansson T, Thain J, Martínez-Gómez C, Hylland K, Gubbins M and Balk L (2017) Supporting variables for biological effects measurements in fish and blue mussel. *ICES Techniques in Marine Environmental Sciences* **60**, 22 pp.
- Hart CA, Nisbet ICT, Kennedy SW and Hahn ME (2003) Gonadal feminization and halogenated environmental contaminants in common terns (*Sterna hirundo*): evidence that ovaries in male embryos do not persist to the pre fledgling stage. *Ecotoxicology* **12**, 125–140.
- Hashimoto S, Bessho H, Hara A, Nakamura M, Iguchi T and Fujita K (2000) Elevated serum vitellogenin levels and gonadal abnormalities in wild male flounder (*Pleuronectes yokohamae*) from Tokyo Bay, Japan. *Marine Environmental Research* **49**, 37–53.
- Haubruege E, Petit F and Gage MJ (2000) Reduced sperm counts in guppies (*Poecilia reticulata*) following exposure to low levels of tributyltin and bisphenol A. *Proceedings of the Royal Society of London B: Biological Sciences* **267**, 2333–2333.
- Hazlerigg CRE, Tyler CR, Lorenzen K, Wheeler JR and Thorbek P (2014) Population relevance of toxicant mediated changes in sex ratio in fish: an assessment using an individual-based zebrafish (*Danio rerio*) model. *Ecological Modelling* **280**, 76–88.
- Hinck JE, Blazer VS, Schmitt CJ, Papoulias DM and Tillitt DE (2009) Widespread occurrence of intersex in black basses (*Micropterus* spp.) from U.S. rivers, 1995–2004. *Aquatic Toxicology* **95**, 60–70.
- Horiguchi T, Shiraishi H, Shimizu M and Morita M (1997) Imposex in sea snails, caused by organotin (tributyltin and triphenyltin) pollution in Japan: a survey. *Applied Organometallic Chemistry* **11**, 451–455.
- Horiguchi T, Takiguchi N, Cho HS, Kojima M, Kaya M, Shiraishi H, Morita M, Hirose H and Shimizu M (2000) Ovo-testis and disturbed reproductive cycle in the giant abalone, *Haliotis madaka*: possible linkage with organotin contamination in a site of population decline. *Marine Environmental Research* **50**, 223–229.
- Horiguchi T, Ohta Y, Nishikawa T, Shiraishi F, Shiraishi H and Morita M (2008) Exposure to 9-cis retinoic acid induces penis and vas deferens development in the female rock shell, *Thais clavigera*. *Cell Biology and Toxicology* **24**, 553–562.
- Howell M (1999) Environmental formation of androgens and fish masculinization. In *Proceedings, Environmental Hormones: Past, Present, and Future*. Tulane-Xavier Center for Bioenvironmental Research, New Orleans, LA, USA, 18–21 October, p. 17.
- Hughes RN (1971) Reproduction of *Scrobicularia plana* Da Costa (Pelecypoda: Semelidae) in North Wales. *Veliger* **14**, 77–81.
- Janer G and Porte C (2007) Sex steroids and potential mechanisms of non-genomic endocrine disruption in invertebrates. *Ecotoxicology* **16**, 145–160.
- Janssen PAH, Lambert JGD, Vethaak AD and Goos HJT (1997) Environmental pollution caused elevated concentrations of oestradiol and vitellogenin in the female flounder, *Platichthys flesus* (L.). *Aquatic Toxicology* **39**, 195–214.
- Jobling S and Tyler CR (2003) Endocrine disruption in wild freshwater fish. *Pure and Applied Chemistry* **75**, 2219–2234.
- Jobling S, Nolan M, Tyler CR, Brighty G and Sumpter J (1998) Widespread sexual disruption in wild fish. *Environmental Science and Technology* **32**, 2498–2506.
- Jobling S, Casey D, Rodgers-Gray T, Oehlmann J, Schulte-Oehlmann U, Pawlowski S, Baunbeck T, Turner AP and Tyler CR (2004) Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent. *Aquatic Toxicology* **65**, 205–220.
- Johnson LL, Casillas E, Collier TK, McCain BB and Varanasi U (1988a) Contaminant effects on ovarian development in English sole (*Parophrys vetulus*) from Puget Sound, Washington. *Canadian Journal of Fisheries and Aquatic Sciences* **45**, 2133–2146.

- Johnson LL, Misitano D, Sol SY, Nelson GM, French B, Ylitalo GM and Hom T (1998b) Contaminant effects on ovarian development and spawning success in rock sole from Puget Sound, Washington. *Transactions of the American Fisheries Society* **127**, 375–392.
- Jones BL, Walker C, Azizi B, Tolbert L, Williams LD and Snell TW (2017) Conservation of estrogen receptor function in invertebrate reproduction. *BMC Evolutionary Biology* **17**, 65.
- Keay J and Thornton JW (2009) Hormone-activated estrogen receptors in annelid invertebrates: implications for evolution and endocrine disruption. *Endocrinology* **150**, 1731–1738.
- Kidd KA, Blanchfield PJ, Mills KH, Palace VP, Evans RE, Lazorchak JM and 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.
- Kirby MF, Allen YT, Dyer RA, Feist SW, Katsiadaki I, Matthiessen P, Scott AP, Smith A, Stentiford GD, Thain JE, Thomas K V, Tolhurst L and Waldock MJ (2004) Surveys of plasma vitellogenin and intersex in male flounder (*Platichthys flesus*) as measures of endocrine disruption by estrogenic contamination in United Kingdom estuaries: temporal trends, 1996 to 2001. *Environmental Toxicology and Chemistry* **23**, 748–758.
- Kortenkamp A and Faust M (2018) Regulate to reduce chemical mixture risk. *Science* **20**, 224–226.
- Lafont R and Mathieu M (2007) Steroids in aquatic invertebrates. *Ecotoxicology* **16**, 109–130.
- Lafontaine A, Baiwir D, Joaquim-Justo C, De Pauw E, Lemoine S, Boulangé-Lecomte S, Forget-Leray J, Thomé J-P and Gismondi E (2017) Proteomic response of *Macrobrychium rosenbergii* hepatopancreas exposed to chlordecone: identification of endocrine disruption biomarkers? *Ecotoxicology and Environmental Safety* **141**, 306–314.
- Lai KM, Johnson KL, Scrimshaw MD and Lester JN (2000) Binding of waterborne steroid estrogens to solid phases in river and estuarine systems. *Environmental Science and Technology* **34**, 3890–3894.
- Langston WJ and Burt GR (1991) Bioavailability and effects of sediment-bound TBT in deposit-feeding clams, *Scrobicularia plana*. *Marine Environmental Research* **32**, 61–77.
- Langston WJ and Pope ND (1995) Determinants of TBT adsorption and desorption in estuarine sediments. *Marine Pollution Bulletin* **31**, 32–43.
- Langston WJ and Pope ND (2009) Molluscs. In Arai T, Harino H, Ohji M and Langston WJ (eds), *Ecotoxicology of Antifouling Biocides*. Tokyo: Springer, pp. 271–289.
- Langston W, Bryan G, Burt G and Gibbs P (1990) Assessing the impact of tin and TBT in estuaries and coastal regions. *Functional Ecology* **4**, 433–443.
- Langston WJ, Burt GR, Chesman BS and Vane CH (2005) Partitioning, bioavailability and effects of oestrogens and xeno-oestrogens in the aquatic environment. *Journal of the Marine Biological Association of the United Kingdom* **85**, 1–31.
- Langston WJ, Burt GR and Chesman BS (2007a) Feminisation of male clams *Scrobicularia plana* from estuaries in South West UK and its induction by endocrine-disrupting chemicals. *Marine Ecology Progress Series* **333**, 173–184.
- Langston WJ, Chesman BS and Vane C (2007b) *Partitioning, Bioavailability and Effects of Oestrogens and Xeno-Oestrogens in Estuarine Sediment*. Final Report on EDAQ program (Defra). Project CTG0301. 100 pp.
- Langston WJ, Pope ND, Davey M, Langston KM, O'Hara S, Gibbs PE and Pascoe PL (2015) Recovery from TBT pollution in English Channel environments: a problem solved? *Marine Pollution Bulletin* **95**, 551–564.
- Lavicoli I, Fontana L and Bergamaschi A (2009) The effects of metals as endocrine disruptors. *Journal of Toxicology and Environmental Health Part B* **12**, 206–223.
- Lee JS, Lee YG, Kang SW, Park JS, Lee DG, Jeon MA and Jual SM (2010) Intersexuality of *Crassostrea gigas* and *Ruditapes philippinarum* in southern coastal waters of Korea. *Environmental Health and Toxicology* **25**, 287–294.
- Legler J, van den Brink CE, Brouwer A, Murk AJ, van der Saag PT, Vethaak AD and van der Burg B (1999) Development of a stably transfected estrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line. *Toxicological Sciences* **48**, 55–66.
- Lewis C and Ford AT (2012) Infertility in male aquatic invertebrates: a review. *Aquatic Toxicology* **120–121**, 79–89.
- Love AC (2017) *The Sub-lethal Impacts of Waste Water Effluent And Its Components on Gammarus pulex* (PhD thesis). University of Portsmouth, Portsmouth, UK.
- Lye CM, Frid CLJ, Gill ME and McCormick D (1997) Abnormalities in the reproductive health of flounder (*Platichthys flesus*) exposed to effluent from a sewage treatment works. *Marine Pollution Bulletin* **34**, 34–41.
- Lye CM, Bentley MG, Clare AS and Sefton EM (2005) Endocrine disruption in the shore crab *Carcinus maenas* – a biomarker for benthic marine invertebrates? *Marine Ecology Progress Series* **288**, 221–232.
- Marty MS, Blankinship A, Chambers J, Constantine L, Kloas W, Kumar A, Lagadic L, Meador J, Pickford D, Schwarz T and Verslycke T (2017) Population-relevant endpoints in the evaluation of endocrine-active substances (EAS) for ecotoxicological hazard and risk assessment. *Integrated Environmental Assessment and Management* **13**, 317–330.
- Matozzo V and Marin MG (2007) First evidence of altered vitellogenin-like protein levels in clam *Tapes philippinarum* and in cockle *Cerastoderma glaucum* from the Lagoon of Venice. *Marine Pollution Bulletin* **55**, 494–504.
- Matthiessen P (2008) An assessment of endocrine disruption in mollusks and the potential for developing internationally standardized mollusk life cycle test guidelines. *Integrated Environmental Assessment and Management* **4**, 274–284.
- Matthiessen P and Gibbs PE (1998) Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in mollusks. *Environmental Toxicology and Chemistry* **17**, 37–43.
- Matthiessen P, Wheeler JR and Weltje L (2018) A review of the evidence for endocrine disrupting effects of current-use chemicals on wildlife populations. *Critical Reviews in Toxicology* **48**, 195–216.
- McAllister BG and Kime DE (2003) Early life exposure to environmental levels of the aromatase inhibitor tributyltin causes masculinisation and irreversible sperm damage in zebrafish (*Danio rerio*). *Aquatic Toxicology* **65**, 309–316.
- McCoy KA, Bortnick LJ, Campbell CM, Hamlin HJ, Guillette Jr LJ and St Mary CM (2008) Agriculture alters gonadal form and function in the toad *Bufo marinus*. *Environmental Health Perspectives* **116**, 1526–1532.
- Miller DH and Ankley GT (2004) Modeling impacts on populations: fathead minnow (*Pimephales promelas*) exposure to the endocrine disruptor 17β-trenbolone as a case study. *Ecotoxicology and Environmental Safety* **59**, 1–9.
- Mintram KS, Brown AR, Maynard SK, Liu C, Parker S-J, Tyler CR and Thorbek P (2018) Assessing population impacts of toxicant-induced disruption of breeding behaviours using an individual-based model for the three-spined stickleback. *Ecological Modelling* **387**, 107–117.
- Moore CG and Stevenson JM (1991) The occurrence of intersexuality in harpacticoid copepods and its relationship with pollution. *Marine Pollution Bulletin* **22**, 72–74.
- Morcillo Y and Porte C (2000) Evidence of endocrine disruption in clams – *Ruditapes decussata* – transplanted to a tributyltin-polluted environment. *Environmental Pollution* **107**, 47–52.
- Morcillo Y, Ronis MJJ and Porte C (1998) Effects of tributyltin on the phase I testosterone metabolism and steroid titres of the clam *Ruditapes decussata*. *Aquatic Toxicology* **42**, 1–13.
- Mori K, Muramatsu T and Nakamura Y (1969) Effect of steroid: III. Sex reversal from male to female in *Crassostrea gigas* by estradiol 17β. *Bulletin of the Japanese Society of Scientific Fisheries* **35**, 1072–1076.
- Noel M, Barrett-Lennard L, Guinet C, Dangerfield N and Ross PS (2009) Persistent organic pollutants (POPs) in killer whales (*Orcinus orca*) from the Crozet Archipelago, southern Indian Ocean. *Marine Environmental Research* **68**, 196–202.
- Oberdörster E and Cheek AO (2001) Gender benders at the beach: endocrine disruption in marine and estuarine organisms. *Environmental Toxicology and Chemistry* **20**, 23–36.
- Oehlmann J, Schulte-Oehlmann U, Tillmann M and Markert B (2000) Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory, Part I: Bisphenol A and octylphenol as xeno-estrogens. *Ecotoxicology* **9**, 383–397.
- Oehlmann J, Schulte-Oehlmann U, Bachmann J, Oetken M, Lutz I, Kloas W and Ternes TA (2006) Bisphenol A induces superfeminization in the ramshorn snail (Gastropoda:Prosobranchia) at environmentally relevant concentrations. *Environmental Health Perspectives* **114**, 127–133.
- Oehlmann J, Di Benedetto P, Tillmann M, Duft M, Oetken M and Schulte-Oehlmann U (2007) Endocrine disruption in prosobranch molluscs: evidence and ecological significance. *Ecotoxicology* **16**, 29–43.
- Orton F and Routledge E (2011) Agricultural intensity *in ovo* affects growth, metamorphic development and sexual differentiation in the Common toad (*Bufo bufo*). *Ecotoxicology* **20**, 901–911.

- Ortiz-Zarragoitia M and Cajaraville MP (2010) Intersex and oocyte atresia in a mussel population from the Biosphere's Reserve of Urdaibai (Bay of Biscay). *Ecotoxicology and Environmental Safety* **73**, 693–701.
- Osada M, Takamura T, Sato H and Mori K (2003) Vitellogenin synthesis in the ovary of scallop, *Patinopecten yessoensis*: control by estradiol-17 β and the central nervous system. *Journal of Experimental Zoology* **299A**, 172–179.
- Park JT, Shin YK, Hung SSO, Romano N, Cheon Y-P and Kim JW (2015) Reproductive impairment and intersexuality in *Gomphina veneriformis* (Bivalvia: Veneridae) by the tributyltin compound. *Animal Cells and Systems* **19**, 61–68.
- Pascoal S, Carvalho G, Vasieva O, Hughes R, Cossins A, Fang Y-X, Ashelford K, Olohan L, Barroso C, Mendo S and Creer S (2013) Transcriptomics and *in vivo* tests reveal novel mechanisms underlying endocrine disruption in an ecological sentinel, *Nucella lapillus*. *Molecular Ecology* **22**, 1589–1608.
- Peck M, Gibson RW, Kortenkamp A and Hill EM (2004) Sediments are major sinks of steroidal estrogens in two United Kingdom rivers. *Environmental Toxicology and Chemistry* **23**, 945–952.
- Peck MR, Labadie P, Minier C and Hill EM (2007) Profiles of environmental and endogenous estrogens in the zebra mussel *Dreissena polymorpha*. *Chemosphere* **69**, 1–8.
- Pollock MS, Dube MG and Schryer R (2010) Investigating the link between pulp mill effluent and endocrine disruption: attempts to explain the presence of intersex fish in the Wabigoon River, Ontario, Canada. *Environmental Toxicology and Chemistry* **29**, 952–965.
- Pope ND, Childs K, Dang C, Davey M, O'Hara SCM, Langston K, Minier C, Pascoe PL, Shortridge E and Langston WJ (2015) Intersex in the clam *Scrobicularia plana* (Da Costa): widespread occurrence in English Channel estuaries and surrounding areas. *Marine Pollution Bulletin* **95**, 598–609.
- Quinn B, Gagné F, Costello M, McKenzie C, Wilson J and Mothersill C (2004) The endocrine disrupting effect of municipal effluent on the zebra mussel (*Dreissena polymorpha*). *Aquatic Toxicology* **66**, 279–292.
- Raleigh J and Keegan BF (2006) The gametogenic cycle of *Scrobicularia plana* (Mollusca: Bivalvia) in Mweeloon Bay (Galway, west coast of Ireland). *Journal of the Marine Biological Association of the United Kingdom* **86**, 1157–1162.
- Ram RN and Sathyanesan AG (1986) Effect of a mercurial fungicide on the gonadal development of the teleostean fish *Channa punctatus* (Bloch). *Ecotoxicology and Environmental Safety* **11**, 352–360.
- Reijnders PJ (1986) Reproductive failure in common seals feeding on fish from polluted coastal waters. *Nature* **324**, 456–457.
- Routledge EJ and Sumpter JP (1996) Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environmental Toxicology and Chemistry* **15**, 241–248.
- Ruiz JM (1993) Metallic Pollution in Estuaries, With Special Reference to the Effects of Tributyltin (TBT) and Copper on the Early Life Stages of *Scrobicularia plana* (Mollusca: Bivalvia) PhD thesis. University of Plymouth, Plymouth, UK.
- Sanchez W, Sremski W, Piccini B, Palluel O, Maillot-Maréchal E, Betoulle S, Jaffal A, Ait-Aïssa S, Brion F, Thybaud E, Hinfrey N and Porcher J-M (2011) Adverse effects in wild fish living downstream from pharmaceutical manufacture discharges. *Environment International* **37**, 1342–1348.
- Schlenk D (2008) Are steroids really the cause for fish feminization? A mini-review of *in vitro* and *in vivo* guided TIEs. *Marine Pollution Bulletin* **57**, 250–254.
- Schlumpf M, Lichtensteiger W, Jarry H, Seidlova-Wuttke D, Wuttke W, Oetken M, Bachmann J, Schulte-Oehlmann U and Oehlmann J (2004) Effects of UV filters on aquatic wildlife. *CREDO Newsletter* **3**, 1–3.
- Schug TT, Johnson AF, Birnbaum LS, Colborn T, Guillette LJ, Crews DP, Collins T, Soto AM, vom Saal FS, McLachlan JA, Sonnenschein C and Heindel JJ (2016) Minireview: endocrine disruptors: past lessons and future directions. *Molecular Endocrinology* **30**, 833–847.
- Schulte-Oehlmann U, Tillmann M, Casey D, Duff M, Markert B and Oehlmann J (2001) Östrogenartige wirkungen von bisphenol a auf vorderkiemerschnecken (Mollusca: Gastropoda: Prosobranchia). *Untertitel Zeitschrift für Umwelchemie und Ökotoxikologie* **13**, 319–333.
- Schultz MM, Minarik TA, Martinovic-Weigelt D, Curran EM, Bartell SE and Schoenfuss HL (2013) Environmental estrogens in an urban aquatic ecosystem: II. Biological effects. *Environment International* **61**, 138–149.
- Schwartz JA and Skafar DF (1993) Ligand-mediated modulation of estrogen receptor confirmation by estradiol analogs. *Biochemistry* **32**, 10109–10115.
- Schwarz TI, Katsiadaki I, Maskrey BH and Scott AP (2017a) Mussels (*Mytilus* spp.) display an ability for rapid and high capacity uptake of the vertebrate steroid, estradiol-17 β from water. *Journal of Steroid Biochemistry and Molecular Biology* **165**, 407–420.
- Schwarz TI, Katsiadaki I, Maskrey BH and Scott AP (2017b) Rapid uptake, biotransformation, esterification and lack of depuration of testosterone and its metabolites by the common mussel, *Mytilus* spp. *Journal of Steroid Biochemistry and Molecular Biology* **171**, 54–65.
- Schwarz T, Katsiadaki I, Maskrey BH and Scott AP (2018) Uptake and metabolism of water-borne progesterone by the mussel, *Mytilus* spp. (Mollusca). *Journal of Steroid Biochemistry and Molecular Biology* **178**, 13–21.
- Scott AP (2018) Is there any value in measuring vertebrate steroids in invertebrates? *General and Comparative Endocrinology* **265**, 77–82.
- Segner H, Caroll K, Fenske M, Janssen CR, Maack G, Pascoe D, Schäfers C, Vandenbergh GF, Watts M and Wenzel A (2003) Identification of endocrine-disrupting effects in aquatic vertebrates and invertebrates: report from the European IDEA project. *Ecotoxicology and Environmental Safety* **54**, 302–314.
- Simpson VR, Bain MS, Brown R, Brown BF and Lacey RF (2000) A long-term study of vitamin A and polychlorinated hydrocarbon levels in otters (*Lutra lutra*) in south west England. *Environmental Pollution* **110**, 267–275.
- Singh H (1989) Interaction of xenobiotics with reproductive endocrine functions in a protogynous teleost, *Monopterus albus*. *Marine Environmental Research* **28**, 285–289.
- Skelly DK, Bolden SR and Dion KB (2010) Intersex frogs concentrated in suburban and urban landscapes. *EcoHealth* **7**, 374–379.
- Sonne C, Leifsson PS, Dietz R, Born EW, Letcher RJ, Hyldstrup L, Riget FF, Kirkegaard M and Muir DCG (2006) Xenoendocrine pollutants may reduce size of sexual organs in East Greenland polar bears (*Ursus maritimus*). *Environmental Science and Technology* **40**, 5668–5674.
- Spooner N, Gibbs PE, Bryan GW and Goad LJ (1991) The effect of tributyltin upon steroid titers in the female dogwhelk, *Nucella lapillus*, and the development of imposex. *Marine Environmental Research* **32**, 37–49.
- Stentiford GD and Feist SW (2005) First reported cases of intersex (ovotestis) in the flatfish species dab *Limanda limanda*: Dogger Bank, North Sea. *Marine Ecology Progress Series* **301**, 307–310.
- Sumpter JP (2005) Endocrine disruptors in the aquatic environment: an overview. *Acta Hydrochimica et Hydrobiologica* **33**, 9–16.
- Sumpter JP and Jobling S (1995) Vitellogenin as a biomarker of exposure to oestrogens. *Environmental Health Perspectives* **103**(Suppl. 7), 173–178.
- Tankoua OF, Amiard-Triquet C, Denis F, Minier C, Mouneyrac C and Berthet B (2012) Physiological status and intersex in the endobenthic bivalve *Scrobicularia plana* from thirteen estuaries in northwest France. *Environmental Pollution* **167**, 70–77.
- Thain JE and Waldock MJ (1986) The impact of tributyltin (TBT) antifouling paints on molluscan fisheries. *Water Science and Technology* **18**, 193–202.
- Thitiphuree T, Nagasawa K and Osada M (2019) Molecular identification of steroidogenesis-related genes in scallops and their potential roles in gametogenesis. *Journal of Steroid Biochemistry and Molecular Biology* **186**, 22–33.
- Thomas P (1988) Reproductive endocrine function in female Atlantic croaker exposed to pollutants. *Marine Environmental Research* **24**, 179–183.
- Thomas P and Budiantara L (1995) Reproductive life history stages sensitive to oil and naphthalene in Atlantic croaker. *Marine Environmental Research* **39**, 147–150.
- Tyler CR and Routledge EJ (1998) Oestrogenic effects in fish in English rivers with evidence of their causation. *Pure and Applied Chemistry* **70**, 1795–1804.
- US EPA (1997) *Special Report on Environmental Endocrine Disruption: An Effects Assessment and Analysis*. Office of Research and Development, EPA/630/R-96/012.
- US EPA (2013) *Validation of the Medaka Multigeneration Test. Integrated Summary Report*. Washington, DC: Endocrine Disruptor Screening Program.
- Varaksina GS and Varaksin AA (1991) Effect of estradiol, progesterone, and testosterone on oogenesis of Yezo scallop. *Russian Journal of Marine Biology* **17**, 162–167.
- Varaksina GS, Varaksin AA and Maslennikova LA (1992) The role of gonadal steroid hormones in the spermatogenesis of the scallop *Mizuhopecten yessoensis*. *Russian Journal of Marine Biology* **18**, 77–83.

- Villalobos DM, Paz AO and Gonzalez MT** (2010) Hermaphroditism in marine mussel *Perumytilus purpuratus* (Lamarck, 1819), (Mollusca: Mytilidae). *International Journal of Morphology* **28**, 569–573.
- Vogeler S, Galloway TS, Isupov M and Bean TP** (2017) Cloning retinoid and peroxisome proliferator-activated nuclear receptors of the Pacific oyster and *in silico* binding to environmental chemicals. *PLoS ONE* **12**, e0176024.
- von Hippel FA, Miller PK, Carpenter DO, Dillon D, Smayda L, Katsiadaki I, Titus TA, Batzel P, Postlethwait JH and Buck CL** (2018) Endocrine disruption and differential gene expression in sentinel fish on St. Lawrence Island, Alaska: health implications for indigenous residents. *Environmental Pollution* **234**, 279–287.
- Waldock MJ and Thain JE** (1983) Shell thickening in *Crassostrea gigas*: organotin antifouling or sediment induced? *Marine Pollution Bulletin* **14**, 411–415.
- Wang C and Croll RP** (2004) Effects of sex steroids on gonadal development and gender determination in the sea scallop, *Placopecten magellanicus*. *Aquaculture* **238**, 483–498.
- Zhang JL, Zuo ZH, He CY, Cai JL, Wang YQ, Chen YX and Wang CG** (2009) Effect of tributyltin on testicular development in *Sebastes marmoratus* and the mechanism involved. *Environmental Toxicology and Chemistry* **28**, 1528–1535.
- Zhang J, Zuo Z, Wang Y, Yu A, Chen Y and Wang C** (2011) Tributyltin chloride results in dorsal curvature in embryo development of *Sebastes marmoratus* via apoptosis pathway. *Chemosphere* **82**, 437–442.
- Zulkosky AM, Ferguson PL and McElroy AE** (2002) Effects of sewage-impacted sediment on reproduction in the benthic crustacean *Leptocheirus plumulosus*. *Marine Environmental Research* **54**, 615–619.