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COMMENTARY

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Commentary: Assessing the endocrine disrupting effects of chemicals on invertebrates in the European Union

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Abstract

Evidence from both laboratory and field studies has shown that currently used synthetic and naturally occurring chemical substances may potentially disrupt invertebrate endocrine systems, although the extent of this in field populations remains unclear. Translating concerns about potential endocrine disrupting chemicals (EDCs) into practical and effective regulatory action is currently hampered by the breadth of invertebrate endocrinology when compared to the better understood vertebrate systems, a lack of fundamental knowledge about the endocrinology of many invertebrate groups, and the resulting uncertainty when making regulatory decisions. This commentary (i) outlines the breadth of invertebrate endocrine pathways for which European Union regulation of potential EDCs may be relevant; (ii) reviews the extent to which current knowledge meets regulatory requirements for invertebrates, including an assessment of the suitability of current invertebrate test guidelines for detecting endocrine modes of action; and (iii) proposes a roadmap towards the regulation of potential EDCs with greater confidence, based on the Adverse Outcome Pathway (AOP) concept and a focus on identifying Molecular Initiating Events (MIEs) within AOPs. We conclude there are no validated tools to determine any invertebrate endocrine mode of action in vitro or in vivo. However, there are commonly used invertebrate toxicity tests which might capture adverse effects that could potentially result from an endocrine mode of action but would not identify the causal mechanisms. Therefore, EU regulatory requirements for the identification of EDCs cannot currently be satisfied for invertebrates, either in general or for the specific invertebrates used in standard ecotoxicological studies. We propose that the most important research need is compilation of a comprehensive list of endocrine-related MIEs across invertebrate taxa via use of high-throughput 'omics in combination with bioinformatics reverse engineered analyses. Although tractable, such an approach would require significant resource investment for development and implementation.

Keywords: Invertebrate, Endocrine disruption, Population, Adverse Outcome Pathway, Molecular Initiating Event, Reverse engineering

Introduction

Approximately 95% of all known animals are invertebrates [1], with an estimated 6.77 million invertebrate species worldwide [2] covering around 30 different phyla and spanning an enormous morphological and physiological range from sponges through to more complex arthropods (insects, crustaceans), molluscs, and tunicates [3]. In 2011, the International Union for the Conservation of Nature concluded that in Europe almost half of freshwater mollusc species and one-fifth of selected terrestrial mollusc species were threatened with extinction; 9% of European butterflies were threatened, with a further 10% considered near threatened; 11% of assessed saproxylic beetles were threatened, with

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a further 13% considered near threatened; and 15% of the 137 assessed (sub)species of European dragonflies were threatened, of which 2% were critically endangered, 4% endangered, and 9% vulnerable, with a further 11% considered near threatened [4]. Some of the major threats to invertebrate biodiversity include habitat fragmentation, intense agricultural practices, and climate change [5]. Exposure to toxic substances, including chemicals which affect the function of animal endocrine systems (e.g. tributyltin), have also been implicated in invertebrate population declines [6].

Invertebrate endocrine systems use a variety of hormones for regulation of growth, development, reproduction, metabolism, and other physiological processes [7, 8]. The insect endocrine system is the specific target of a class of chemicals used for pest control, the insect growth regulators (IGRs), which are utilised in veterinary medicine, public health, and agriculture [9, 10]. IGR insecticides based on juvenile hormone receptor agonists have the potential to affect a wide range of insect taxa [11]. However, for other IGRs (e.g. ecdysteroid receptor agonists) selectivity towards specific insect orders, such as Lepidoptera and Coleoptera, has been identified [12, 13]. Evidence from both laboratory and field studies has shown that certain other synthetic and naturally occurring chemical substances may also disrupt invertebrate endocrine systems. The iconic example of this is gastropod mollusc exposure to tributyltin (TBT) leading to imposex and large-scale population declines in the marine environment [6]. However, even in this well-known case the precise mechanism of action of TBT in gastropods has not been fully determined, the regulatory implication of which is reviewed in Lagadic et al. [14]. The effects of TBT on other invertebrate phyla, at concentrations lower than those causing imposex in gastropods, is also poorly understood and may have been overlooked. There is also other, less conclusive, evidence of endocrine disrupting (ED) effects associated with either measured or assumed exposure to other substances in field populations of aquatic crustaceans and bivalve molluscs [1, 15], and laboratory studies have shown that invertebrate endocrine receptors may be affected by chemical exposure in a variety of different ways [16]. As many invertebrate populations are in decline [17, 18], concerns about the possible contribution of endocrine-disrupting chemicals (EDCs) to this decline are justified, although the current extent of the problem remains unclear [19].

Translating reasonable concerns about potential EDCs into practical and effective regulatory action is hampered by several obstacles when considering invertebrates. The first of these is the breadth of invertebrate endocrine systems compared to the better understood vertebrate systems. Invertebrate hormones include steroids, proteins, terpenoids, and amides [3]. There are also some, such as ecdysteroids and juvenile hormones, that do not occur in vertebrates [20]. A second obstacle is the lack of fundamental knowledge about the endocrinology of many invertebrate groups [1].

The identification of a chemical as an endocrine disruptor relies upon the demonstration that an adverse effect in an intact organism is the consequence of an endocrine mode of action [21, 22], so the paucity of mechanistic data available on invertebrate endocrine pathways is a real hurdle to the use of these organisms in regulatory assessment of environmental EDs.

A coherent conceptual framework for addressing these obstacles does not currently exist, most likely because the focus of regulatory science to date has been on vertebrates, so existing regulations on EDCs may not currently be directly applicable to invertebrates. To start to understand and address these scientific and regulatory gaps and challenges, the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) convened a group of experts to:

1. Outline the breadth of invertebrate endocrine pathways for which regulation of potential EDCs may be relevant, with a focus on the European Union (EU) regulatory context;
2. Review the extent to which current knowledge meets regulatory requirements for invertebrates, including an assessment of the suitability of the internationally recognised Organisation for Economic Cooperation and Development (OECD) invertebrate test guidelines for detecting endocrine modes of action; and
3. Propose a pathway for regulation of potential EDCs in invertebrates with greater confidence, based on the Adverse Outcome Pathway (AOP) concept and a focus on identifying Molecular Initiating Events (MIEs) within AOPs.

For more than twenty years, the knowledge gap in relation to invertebrate biodiversity and endocrinology has been a common theme in the scientific and technical literature [1]. In this commentary we focus more on what is known and whether this knowledge is sufficient to construct a robust regulatory framework for identifying invertebrate EDCs. The commentary focuses primarily on direct exposure in aquatic systems because this is the environmental compartment and exposure route for which most information is currently available. However, we recognise that exposure of terrestrial invertebrates to potential EDCs, or exposure via food chains, may occur and also merits regulatory attention.

Invertebrate endocrine pathways

Invertebrate endocrine pathways are diverse and best understood for some arthropod groups (insects, crustaceans), and molluscs [3, 23], although less information is available for molluscs than for insects and crustaceans.

Three major classes of hormones are known in insects [24]:

- Peptide hormones, which are mainly produced in the central nervous system and midgut epithelium. For example, prothoracicotropic hormone which stimulates the prothoracic gland to produce ecdysone and adipokinetic hormone.
- Ecdysteroids, which are associated with moulting and metamorphosis and produced by the prothoracic gland in immature insects (usually as ecdysone, although some larval Lepidoptera secrete 3-dehydroecdysone, which is enzymatically converted to ecdysone in the haemolymph). Ecdysone, a prohormone, is then converted to the active hormone 20-hydroxyecdysone by a cytochrome P450 enzyme. In contrast, makisterone is the main ecdysteroid in the Hymenoptera (e.g. honeybees) and the Heteroptera (true bugs).
- Juvenile hormones modulate ecdysteroid action and are sesquiterpenes produced by the corpora allata. Juvenile Hormone III is the most common, although several different forms are known.

Three major classes of hormones are also known in crustaceans. Crustaceans and insects are closely related and belong to the same Clade (Pancrustacea), so some crustacean hormones are similar to those found in insects [25–27]:

- Peptide hormones, including:
- Crustacean hyperglycaemic hormones (CHHs), which are produced in the malacostracan X organ and stored and released from the sinus gland, both located in the eye stalk. Some CHHs regulate carbohydrate metabolism, while others regulate ecdysteroid synthesis, the secretion of methyl farnesoate, and gonadal maturation.
- Androgenic gland hormone, found so far in male isopod gamete ducts and which are responsible for male sexual differentiation. Insulin-like androgen gland hormone and Crustacean Female Sex Hormone are also found in decapods [28].
- Red pigment concentrating hormone and pigment dispersing hormone, which regulate colour change.
- Ecdysteroids, predominantly 20-hydroxyecdysone (as in insects).
- Methyl farnesoate, a terpenoid found in decapods, cirripedes, and anostracans, which has similar regulatory functions to insect Juvenile Hormone III, of which it is an unepoxidated form.

The major classes of hormones in molluscs are less well studied than those in insects and crustaceans, but the role of several neuropeptide hormones has been clearly demonstrated, particularly in the sea slug *Aplysia* and the pulmonate snail *Lymnaea* [29]. Lagadic et al. [30] summarised information on 11 different neuropeptides in *Lymnaea*, which regulate a wide range of behavioural, physiological, developmental, growth, and reproductive functions. Thyroid hormone receptors (THR), which are homologues to vertebrate THRs, have been identified in several molluscs [31, 32], but the role of thyroid hormones, although identified, is still unclear in molluscs [33] as is also the case for vertebrate-type steroidal hormones (see later).

Hormones reportedly found in other invertebrate phyla [34–39] include:

- Cnidaria
- Neuropeptides: glycine-leucine tryptophan amides involved in metamorphosis.
- Thyroids: thyroxine, involved in strobilation.

- Nematoda
- Ecdysteroids: reported but with a questionable functional role.
- Terpenoids: juvenile hormone-like hormones involved in growth.
- Neuropeptides: FMRFamide (function unknown).

- Annelida
- Ecdysteroids: ecdysone (function unknown).
- (Anti)diuretic neuropeptides: e.g. FMRFamide involved in neuromodulation.

- Echinodermata
- Steroids: progesterone, testosterone, 17-beta-estradiol, and estrone involved in vitellogenesis, oogenesis, spermatogenesis, and spawning.
- Neuropeptides: gonad-stimulating substance involved in spawning; and maturation-promoting factor involved in fertilisation.

- Tunicata
- Steroids: testosterone and 17-beta-estradiol, involved in oogenesis, spermatogenesis, and spawning.
- Neuropeptides: gonadotropin releasing hormone analogue involved in gonad development.
- Thyroids: thyroxine, probably involved in the tanning process during tunic formation.

The terminology used to name the hormones found in invertebrates has been constructed from the vertebrate hormonal system, but this does not necessarily imply that the molecular structures and physiological roles are the same in invertebrates (e.g. see [40, 41]). Therefore, the use of "steroid-like" or "thyroid-like" to designate invertebrate hormones which appear to be homologous to vertebrate hormones is usually preferable.

This brief summary illustrates the enormous breadth and diversity of invertebrate endocrine systems that might potentially be susceptible to an EDC, which is in sharp contrast to the current regulatory landscape that focuses on only four endocrine axes (Estrogen, Androgen, Thyroid, and Steroidogenesis; EATS) for vertebrates.

EU regulatory framework for assessing endocrine disruption in wildlife

In this section, we describe the EU regulatory context for classification of a substance as an EDC in wildlife (both vertebrate and invertebrate) and the OECD testing framework which underpins the EU regulations.

Current EU policy on potential EDCs is summarised in EC [21], which states that:

- There is broad consensus on the WHO-IPCS [22] definition of an EDC as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations". In this definition the term "(sub)population" is of relevance to humans, and for non-target organisms the term "population" is used synonymously in ECHA/EFSA [42]; see also [43].
- Test guidelines for non-vertebrates still require development or validation (with specific needs identified in [44]).
- When scientific evaluation of potential EDCs comes to uncertain conclusions the Commission will be guided by the precautionary principle [45].
- Specific provisions on how to address endocrine disruption are included in regulations for plant protection products, biocides, chemicals in general, medical devices and water. In the case of plant protection products and biocides the Commission has established criteria for identifying EDCs and will develop a "horizontal approach" based on these criteria across all EU legislation. More recently the Commission is working towards including identification of EDCs within the Classification and Labelling Regulation that would apply to substances across several regulations.

Criteria for identifying EDCs in plant protection products and biocides therefore appear as a key component in both current and future EU regulatory frameworks. These criteria for wildlife (a term that includes invertebrates and vertebrates) are [46]:

1. The substance shows an adverse effect in non-target organisms, which is a change in the morphology, physiology, growth, development, reproduction or life span of an organism, system or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other influences [22];
2. The substance has an endocrine mode of action, i.e. it alters the function(s) of the endocrine system; and
3. The adverse effect is a consequence of the endocrine mode of action.

EC [46] further states that identification of a substance as an EDC must be based on:

1. All available relevant scientific data (in vivo, in vitro, and in silico) generated from internationally agreed study protocols or collected via a systematic review;
2. An assessment of the available relevant scientific data, based on a weight of evidence approach that considers:

a. Both positive and negative results, discriminating between taxonomic groups, where relevant;
b. The relevance of the study design for the assessment of the adverse effects plus its relevance at the (sub) population level and for assessment of an endocrine mode of action;
c. The adverse effects on reproduction, growth/development, and other relevant adverse effects which are likely to impact on (sub)populations;
d. Adequate, reliable, and representative field or monitoring data and results from population models, where available;
e. The quality and consistency of the data, considering the pattern and coherence of the results within and between studies of a similar design and across different taxonomic groups; and

f. the concept of the limit dose and international guidelines on maximum recommended doses and for assessing confounding effects of excessive toxicity.

3. Using a weight of evidence approach, the link between any adverse effect(s) and an endocrine mode of action is established based on the current understanding of biological plausibility; and
4. Adverse effects that are non-specific secondary consequences of other toxic effects are not used to identify a substance as an EDC.

These criteria, mandated by the European Commission, do not consider environmental exposure concentrations or potency, so are hazard-based (with limited options for derogation such as negligible exposure and essential use) and not risk-based. The treatment of endocrine disruption is therefore similar to the treatment of Category 1A or 1B hazardous properties to humans, such as carcinogenicity, mutagenicity, and reprotoxicity. However, this is a problematic approach for substances such as IGRs because the Plant Protection Regulation EC 1107/2009 [47], which incorporates EU 283/2013 [48] and EU 284/2013 [49], specifically identifies the need for *risk* assessment of pesticides relative to defined population-level protection goals. EC 1107/2009 also highlights that data generation should be designed appropriately to address the mode of action of IGRs in aquatic and terrestrial non-target arthropods (e.g. development and emergence of *Chironomus* larvae and honeybee broods). This means there is a disconnect in addressing both EC 1107/2009 and EC [46], because compliance with both regulatory demands requires an IGR pesticide *risk* assessment of non-target arthropod population-level protection goals for a chemical designed to target the endocrine system of the pest insect, whilst also confirming that there is no *hazard* to non-target insect species. The effect of this is highlighted by the EFSA conclusion for pyriproxyfen, a juvenile hormone analogue [50]. With respect to EC [46] the conclusion states that "no data and methods are available to further elucidate the specificity of the mode of action (MoA) for the target species and consequently possible endocrine-mediated effects on non-target invertebrates. According to point 3.8.2 of Annex II to Regulation (EC) No 1107/2009, as amended by Commission Regulation (EU) 2018/605, it can be concluded that pyriproxyfen is not an endocrine disruptor for non-target arthropods." However, it is unlikely that an IGR with an endocrine-directed mode of action would have effects limited only to the target pest species, even if it was designed to target a specific insect order. Thus, for these IGRs, even if the risk is identified as acceptable to non-target arthropods under EC 1107/2009, (e.g. due to low exposure), EC [46] considers that, as an EDC, the mode of action should be regarded as a hazard cut-off criterion, and even taxonomic order-specific insecticides should not be approved, with only limited options for derogation (negligible exposure and essential use).

Although, as shown above, hazard-based regulatory criteria can be contentious [42, 51–57], it is still possible to develop an operational, hazard-based regulatory framework for potential environmental EDCs (e.g. Crane 2019a) which might be applied to invertebrates. This might be based on an expansion of the tools available within the OECD's Conceptual Framework (CF) for the Testing and Assessment of Endocrine Disruptors.

The OECD CF was adopted in 2002 and subsequently updated in 2012, forming the technical foundation of Guidance Document 150 [58] and the EU's approach to ED identification. The OECD CF classifies environmental toxicity test information at five different levels from in silico through in vitro to in vivo:

- Level 1. Existing data and non-testing information (including in silico information);
- Level 2. In vitro assays which provide data about selected endocrine mechanism(s) and pathway(s);
- Level 3. In vivo assays which provide data about selected endocrine mechanism(s) and pathway(s);
- Level 4. In vivo assays which provide data about adverse effects on endocrine-relevant endpoints; and
- Level 5. In vivo assays which provide more comprehensive data about adverse effects on endocrine-relevant endpoints over extensive parts of the life cycle of an organism.

Coady et al. [59] reviewed available invertebrate test guidelines (as compiled in [58]) and concluded that there were none specifically designed for characterising endocrine activity (i.e. none that can identify mechanisms). However, they identified several apical endpoints in level 4 and 5 tests that *may* indicate adverse effects potentially related to endocrine dysfunction (Table 1). Therefore, there are no internationally validated invertebrate toxicity test protocols providing mechanistic information on the mode of action of test substances, so they are unable on their own to fulfil EC (2018c) criteria for identifying a substance as an EDC. ECETOC [60] also noted that for invertebrates there are few mechanistic in silico, in vitro, and in vivo assays because invertebrate testing has focussed on capturing apical endpoints. This means that adverse outcomes in arthropods are well described, but the underlying mechanisms are often poorly understood.

Table 1 Internationally accepted invertebrate toxicity test guidelines. OECD CF level refers to the OECD Conceptual Framework [58]

Invertebrate group	Test guideline	OECD CF level	Endpoints	Comment
Annelida	OECD TG 220: Enchytraeid Reproduction Test	4	Mortality No. juveniles / adult	
	OECD TG 222: Earthworm Reproduction Test (*Eisenia fetida; Eisenia andrei*)	4	Mortality Growth (weight) No. juveniles/adult	
	OECD TG 225: Sediment–Water *Lumbriculus* Toxicity Test Using Spiked Sediment	4	Mortality Biomass Reproduction (total No. or increase in No.)	
Chelicerata	OECD TG 226: Predatory mite (*Hypoaspis* (*Geolaelaps*) *aculeifer*) reproduction test in soil	4	Mortality Reproduction (total No. of juveniles)	
Collembola	OECD TG 232: Collembolan Reproduction Test in Soil	4	Mortality Reproduction (total No. of juveniles)	
Crustacea	Short-Term Juvenile Hormone Activity Screening Assay using *Daphnia magna* (SJHASA) (draft OECD TG)	3	**Production of male neonates**	Under development
	OECD TG 211/OCSPP 850.1300: *Daphnia magna* Reproduction Test (with potential male induction assessed)	4	Mortality No. live offspring/parent Time to first brood Parental growth (length and body weight; optional) Presence of ephippia or **male neonates**	Male production is known in response to juvenile hormone mimics. However, males are also produced under changing environmental and stressful conditions
	OPPTS 850.1350: Mysid Chronic Toxicity Test	Not listed	Mortality Time to appearance of secondary sexual characters Time to first brood Growth (length and weight) Reproduction (No. young/female)	
	Mysid Life Cycle Toxicity Test (when OPPTS 850.1350 is finalised as an OCSPP guideline)	5	Mortality Time to maturity Time to 1st & 2nd brood release Inter-brood duration Number of young per female Percentage females reproductively active Total reproduction days Growth (length and weight)	OECD declined to validate this assay
	OECD GD 201: New Guidance Document on Harpacticoid Copepod Development and Reproduction Test with *Amphiascus*	5	Mortality Time to release of first and second clutch Number of necrotic/infertile eggs per clutch **Morphological abnormalities** **Intersexuality** Population growth rate **Sex ratio**	

Table 1 (continued)

Invertebrate group	Test guideline	OECD CF level	Endpoints	Comment
	Daphnia Multigeneration Assay (when TG is available)	5	Mortality Age at first reproduction Clutch size Intrinsic population growth rate Number of dead/aborted embryos/neonates **Sex ratio**	
Insecta	OECD TG 218: Sediment–Water Chironomid Toxicity Test Using Spiked Sediment OECD TG 219: Sediment–Water Chironomid Toxicity Test Using Spiked Water	4	Mortality (including development and emergence) Time to emergence (males and females) Growth (weight) **Sex ratio**	*Chironomus* are protandrous so care must be taken when interpreting sex ratio changes
	OECD GD 239 Guidance Document on Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure	4	Larval mortalities from day 3 to day 8 Pupal mortalities from day 8 to day 15 Emergence rate on day 22	
	OECD TG 228: Determination of Developmental Toxicity to Dipteran Dung Flies (*Scathophaga stercoraria* L (*Scathophagidae*), *Musca autumnalis* De Geer (*Muscidae*))	4	Mortality Development Emergence (males and females)	
	OECD TG 233: Sediment–Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment	5	Mortality Development Emergence Time to emergence (males and females) Growth (weight) Reproduction Fertility **Sex ratio**	
Mollusca	OECD TG 242: *Potamopyrgus antipodarum* Reproduction Test	4	Mortality Reproduction (No. of embryos)	
	OECD TG 243: *Lymnaea stagnalis* Reproduction Test	4	Mortality Reproduction (No. of egg clutches)	
Nematoda	ISO 10872:2020: Determination of the toxic effect of sediment and soil samples on growth, fertility and reproduction *of Caenorhabditis elegans* (Nematoda)	5	Survival Growth Reproduction	

Endpoints in bold are more indicative of a potential ED mode of action

In relation to juvenile hormone and ecdysone modulation in chironomid, daphnid, and copepod tests, OECD [58] notes that there are no standardised in vitro screens for juvenile hormone or ecdysone (ant)agonists, although they cite relevant mechanistic assays reported by Cherbas et al. [61], Dinan et al. [62], Miyakawa and Iguchi [63], Smagghe et al. [64], and Swevers et al. [65]. Hartung et al. [66] and OECD [67] provide detailed guidance on good in vitro reporting standards which could be applied to these assays to ensure they are fit for purpose.

In conclusion, there are no validated tools to determine any invertebrate endocrine mode of action in vitro or in vivo. However, there are commonly used invertebrate toxicity tests that might capture adverse effects that could potentially result from an endocrine mode of action but would not identify the causal mechanisms. Therefore, the EU regulatory requirements for the identification of EDCs cannot currently be satisfied for invertebrates, either for invertebrates in general or for the specific invertebrates used in standard ecotoxicological studies.

Evidence for invertebrate endocrine disruption in the laboratory and field

In this section, we examine some of the in vivo laboratory and field evidence for endocrine disruption in invertebrates at OECD CF Levels 3 to 5. Later in this commentary, we discuss further development of in silico and in vitro approaches at CF Levels 1 and 2 which would complement and help prioritise these in vivo tests, so that they focus on the invertebrate endocrine pathways of greatest regulatory concern.

Research trends

Ford and LeBlanc [1] reviewed research progress on endocrine disruption in invertebrates and found that there were fewer research citations for invertebrate EDCs when compared with fish EDCs in every time period they examined. They concluded from a survey of 46 experts in the field of endocrine disruption that there had been only limited advances over the previous two decades because of misconceptions about the relevance of vertebrate hormones to invertebrate endocrine pathways, with a focus on EATS modalities [68]; lower public and regulatory interest in invertebrates when compared with vertebrates; lower funding for invertebrate endocrinology research; and a general lack of knowledge about invertebrate endocrinology which has hampered scientific understanding. In particular, several scientists who responded to the survey suggested a need for basic mechanistic endocrinology to allow full understanding of endocrine disruption and related population-level impacts in invertebrates.

We performed a further bibliographic assessment to assess whether there were any trends in invertebrate endocrine disruption research over the past decade. Derwent Innovation [69] was searched for published articles from 2010 to 2020 which included the terms INVERT* and ENDOCRIN* in either the title or the abstract. This provided a snapshot of relative research interest in different invertebrate taxa in relation to endocrinology. There were 1003 hits and these were reviewed manually to identify only primary research on potential invertebrate EDCs in either the laboratory or the field. One hundred and eighty-one published laboratory and field studies were identified in which invertebrates were either exposed in the laboratory or surveyed in the field, in studies with the explicitly stated purpose of detecting ED effects in invertebrates. In our analysis, mechanistic in vivo studies were defined as those at OECD CF Level 3, with apical studies defined as those at levels 4 and 5.

Almost 60% of the reported studies were in freshwater species, with 28% in saltwater species and 12% in terrestrial species. Tables 2, 3, and 4 summarise the invertebrate groups studied. This shows that although a wide range of invertebrates were used to assess potential endocrine disrupting effects, only the freshwater species *Daphnia magna* (Crustacea), *Chironomus riparius* (Insecta) and *Potamopyrgus antipodarum* (Mollusca), plus the saltwater species *Mytilus galloprovincialis* (Mollusca), were used extensively, with most other species studied only once or twice. Fourteen percent of freshwater studies were field- or semi-field (e.g. mesocosm) based, with the remainder being laboratory investigations, with mechanistic, apical, and mechanistic/apical studies split 45%, 22%, and 27%, respectively. Five percent of saltwater studies were field-based, with mechanistic, apical, and mechanistic/apical studies split 31%, 41%, and 29%, respectively. All terrestrial studies were laboratory-based, with mechanistic, apical, and mechanistic/apical studies split 13%, 61%, and 26%, respectively, and with the springtail *Folsomia candida* (Collembola), the fruitfly *Drosophila melanogaster* (Insecta), the woodlouse *Porcellio scaber* (Crustacea) and the worms *Eisenia fetida* and *Enchytraeus crypticus* (Annelida) studied most frequently.

There was a similar spread in the 126 separate substances examined for ED properties in invertebrates in papers published between 2010 and 2020. Most of these were industrial chemicals; pharmaceuticals; or agricultural/veterinary insecticides, herbicides, or fungicides. However, only 14 substances were tested in more than 5 studies (Table 5), most of which are known vertebrate EATS modulators and are likely to have been selected for this reason.

This bibliographic analysis suggests that most research interest in invertebrate endocrine disruption has been focused on standard laboratory-based freshwater model

species, especially *D. magna* and *C. riparius*, although a wide range of freshwater and, to a lesser extent, saltwater species have also been studied. Endocrine disruption in terrestrial invertebrates remains relatively understudied, although IGRs have received considerable attention [70]. Similarly, a wide range of potential EDCs (with a focus on vertebrate EATS modulators) have been tested across a wide range of different invertebrate species, although this makes it difficult to draw any conclusions about the utility of invertebrate models other than *D. magna* and *C. riparius* and, possibly, *P. antipodarum, L. stagnalis* and *M. galloprovincialis*.

Laboratory-based effects of EDCs on invertebrates

Reviews of laboratory evidence for invertebrate endocrine disruption are available for insects [24, 71], crustaceans [27, 72–74], molluscs [30, 40, 41, 75–78], echinoderms [37], cnidarians [39], and nematodes [79]. However, beyond effects of IGRs on insects and TBT on molluscs, very few studies have unambiguously identified endocrine disruption as the cause of adverse effects on invertebrate development, growth, or reproduction [80]. This is largely because of a lack of current methods to identify endocrine activity unambiguously in mechanistic tests with invertebrates.

EFSA SC [81] and Munn and Goumenou [82] point out that although insect or crustacean reproduction lifecycle assays may show "downstream" (i.e. apical) effects, no "upstream" standardised mechanistic assays for invertebrate EDCs are currently available and that these apical tests on growth, development, and reproduction cannot provide a firm diagnosis of a specific endocrine activity linked to a given adverse effect. Limited understanding of invertebrate endocrinology means that read-across to untested groups from tests with other vertebrate or invertebrate taxa is uncertain, and the current focus on EATS modalities ignores important invertebrate endocrine modalities such as peptide hormone pathways.

Coady et al. [59] identify a significant data gap in understanding EDC hazards due to the lack of fundamental knowledge about endocrine pathways for many invertebrate species. They attribute at least some of the difficulty in addressing this to the large number of invertebrate species that exist, combined with the great diversity this group displays in the endocrine control of growth, development, and reproduction. They also identify this lack of understanding as leading to an unfortunate trend in the field, which is the assumption that indicators of endocrine activity in vertebrates (e.g. vitellogenin (VTG) induction by oestrogens in (male) fish) equally applies to invertebrates, when this is often not the case. For example, the transcriptomic response of the Vtg2 gene in *Daphnia magna* is not elevated in response to chemicals with known oestrogenic modes of action in vertebrates [83], and there is no valid evidence that vertebrate sex steroids have endocrine or reproductive roles in either molluscs [41, 84] or crustaceans [25, 85]. Other authors have also argued that measurement of VTG in invertebrates is inappropriate for several reasons, including evidence that vertebrate steroids can be absorbed from the environment and retained for very long periods, and key enzymes required for the biosynthesis of vertebrate steroids (e.g. aromatase) do not appear to be present in invertebrates (e.g. [86–88]). However, some researchers suggest that the presence of vertebrate steroids in invertebrates cannot be ignored because they can interact with multiple signalling components, leading to modulation of different physiological functions (e.g. [76, 89–92]). Measurement of VTG-like yolk proteins in invertebrates could potentially be relevant for ED identification in invertebrates when the endocrine control of reproduction has been elucidated. The problem with some previously reported analyses is that inappropriate methods have been used (e.g. use of alkali-labile phosphate as a surrogate for VTG-like proteins) as outlined by Morthorst et al. [84]. In addition, VTG-like protein changes have been linked to oestrogenic effects in mollusc species when the oestrogen receptor is inactive and does not bind oestrogens. In contrast to this controversy over invertebrate steroidal hormone signalling, there is considerable evidence for thyroid-like hormone signalling in several invertebrate phyla [93, 94].

In summary, the current lack of mechanistic laboratory methods to identify endocrine activity unambiguously in invertebrates hinders the application of the WHO-IPCS definition and established EU regulatory criteria for confirming a substance as an EDC.

Field effects of EDCs on invertebrates

Our bibliographic assessment suggests that recent field studies of potential invertebrate endocrine disruption are rare when compared to laboratory studies. Matthiessen et al. [15] also concluded that there was very little evidence that occurrences of invertebrate endocrine disruption from exposure to current-use chemicals are widespread in the field, with the evidence "essentially non-existent" for crustaceans and the causal evidence for molluscs "rather weak". This was for various reasons, including an overall lack of studies and a lack of exposure measurement in some studies that have been reported, potential confounding effects from other substances or stressors (e.g. parasites), and the assumption that invertebrate hormone systems are similar to those of vertebrates. They identified some limited evidence to suggest that bivalve molluscs may be feminised after exposure to presumably oestrogenic

Table 2 Freshwater invertebrate species investigated for ED-related mechanistic, apical, or mechanistic and apical effects in laboratory or field studies published between 2010 and 2020

Phylum	Species	Number of studies	Lab or Field	Mechanistic	Apical	Mechanistic/apical
Annelida	*Leech* spp	1	F		1	
	Lumbriculus variegatus	1	L		1	
Arachnida	*Arrenurus* spp.	1	F		1	
Cnidaria	*Hydra circumcincta*	1	L			1
	Hydra sp.	1	L		1	
Crustacea	*Amphipods*	1	F			1
	Astacus leptodactylus	2	L	2		
	Ceriodaphnia cornuta	1	L		1	
	Daphnia magna	18	L	1	7	10
	Diporeia spp.	1	L	1		
	Eudiaptomus gracilis	1	L			1
	Gammarus fossarum	4	L(3), F(1)	3	1	
	Gammarus locusta	1	L		1	
	Gammarus pseudolimnaeus	1	L	1		
	Gammarus pulex	1	L	1		
	Gammarus spp.	1	F		1	
	Hyalella azteca	3	L	2	1	
	Macrobrachium borellii	1	L	1		
	Macrobrachium potiuna	1	L	1		
	Macrobrachium rosenbergii	4	L	3		1
	Macrobrachium superbum	1	L		1	
	Mesocyclops luckarti	1	L			1
	Moina macrocopa	2	L		2	
	Monoporeia affinis	1	L		1	
	Procambarus clarkii	2	L	1		1
	Procambarus fallax	1	L	1		
Insecta	*Chironomus riparius*	20	L	12	1	7
	Chironomus sancticaroli	1	L		1	
	Hexagenia spp.	1	L		1	
	Hydropsyche sp	2	L/F	1		1
	Prodiamesa olivacea	1	L	1		
Mollusca	*Bithynia tentaculata*	1	L		1	
	Corbicula fluminea	2	L	1	1	
	Lampsilis fasciola	2	L		1	1
	Lampsilis siliquoidea	1	L		1	
	Lymnaea stagnalis	5	L		2	3
	Mollusc spp.	1	F			1
	Physa acuta	4	L	2	2	
	Physa pomilia	1	L		1	
	Planorbarius corneus	2	L, L/F		1	1
	Pomacea lineata	1	L		1	
	Potamopyrgus antipodarum	8	L(4), F(3), L/F		6	2
	Radix balthica	3	L(2), L/F		3	
	Unio tumidus	1	L	1		
	Viviparus	1	L/F		1	
Platyhelminthes	*Flatworm* spp.	1	L	1		
Rotifera	*Brachionus calyciflorus*	5	L	1	4	
Macroinvertebrates	Macroinvertebrates	3	F(2), L/F		2	1

Table 3 Saltwater invertebrate species investigated for ED-related mechanistic, apical, or mechanistic and apical effects in laboratory or field studies published between 2010 and 2020

Phylum	Species	Number of studies	Lab or Field	Mechanistic	Apical	Mechanistic/apical
Annelida	*Galeolaria caespitosa*	1	L			1
	Nereis succinea	1	L		1	
	Platynereis dumerilii	1	L	1		
Crustacea	*Acartia tonsa*	1	L		1	
	Amphiascus tenuiremis	1	L	1		
	Artemia salina	1	L		1	
	Callinectes sapidus	1	L			1
	Carcinus maenas	2	L/F	2		
	Clibanarius vittatus	1	L		1	
	Diaphanosoma celebensis	1	L			1
	Echinogammarus marinus	1	L			1
	Eurytemora affinis	2	L	2		
	Homarus gammarus	1	F		1	
	Paracyclopina nana	2	L			1
	Tigriopus japonicus	1	L			1
	Tisbe battagliai	1	L			1
Mollusca	*Chlamys farreri*	2	2	1		1
	Crassostrea angulata	1	L			1
	Crassostrea gigas	3	L	2		1
	Crepidula onyx	1	L		1	
	Haliotis diversicolor supertexta	1	L			1
	Heleobia australis	1	L		1	
	Mytilus edulis	4	L(3), F	2		2
	Mytilus galloprovincialis	10	L	9	1	
	Mytilus spp.	1	L			1
	Nucella lapillus	1	L	1		
	Plicopurpura pansa	1	L			1
	Ruditapes decussatus	1	L	1		
	Ruditapes philippinarum	2	L	1	1	
	Scrobicularia plana	1	L	1		
Tunicata	*Ciona intestinalis*	4	L	1	2	1
	Phallusia mammillata	1	L			
Macroinvertebrates	Macroinvertebrate spp.	1	F		1	

sewage effluent or other sources. However, they concluded that, with the exception of organotins and molluscs, no studies have shown population-level impacts on invertebrates in the field.

In contrast, Cuvillier-Hot and Lenoir [16] suggest that there *is* evidence of field ED effects in invertebrates, citing studies by Amiard and Amiard-Triquet [95] and Jin et al. [96]. However, the latter study only investigated effects in fish and Amiard and Amiard-Triquet [95] draw extensively on Bergman et al. [97] in their review of invertebrate field effects, so theirs is not a primary source. In fact Bergman et al. [97] concluded that little is known about the manifestation of endocrine effects on the reproductive system of either male or female invertebrates; field-based evidence of endocrine-mediated reproductive disorders in invertebrate males is scarce and solely concerns aquatic crustaceans and molluscs; chemical-related sex ratio imbalances associated with TBT, DDT, and municipal effluent exposure have been reported for wild molluscs; and little information is available on endocrine neoplasias in invertebrate species, with even less information linking any incidence of invertebrate neoplasia with contaminant exposure. Organotin effects on molluscs therefore remains the single conclusive example of ED effects on aquatic invertebrate populations in the field,

Table 4 Terrestrial invertebrate species investigated for ED-related mechanistic, apical, or mechanistic and apical effects in studies published (all laboratory) between 2010 and 2020

Phylum	Species	Number of studies	Mechanistic	Apical	Mechanistic/apical
Annelida	*Eisenia fetida*	2	1		1
	Enchytraeus crypticus	2		2	
Crustacea	*Porcellio scaber*	3	1	2	
Hexapoda (Collembola)	*Folsomia candida*	4	1	3	
Insecta	*Bombyx mori*	1			1
	Drosophila melanogaster	4		2	2
	Eurborellia annulipes	1		1	
	Lasius niger	1		1	
	Spodoptera exigua	1		1	
	Spodoptera littoralis	1			1
	Tenebrio molitor	1		1	
Nematoda	*Caenorhabditis elegans*	2		1	1

Table 5 Substances tested five or more times in studies on invertebrate ED published between 2010 and 2020

Substance	Substance type	Number of studies
Bisphenol A [BPA]	Industrial chemical	32
Tributyltin [TBT]	Biocide	15
17 alpha-ethinylestradiol [EE2]	Pharmaceutical	13
17 beta-estradiol [E2]	Pharmaceutical	13
Vinclozolin	Insecticide/herbicide/fungicide	9
Nonylphenol	Industrial chemical	8
Fluoxetine	Pharmaceutical	8
Benzophenone-3 [BP3]	UV filter	8
Di(2-ethylhexyl) phthalate [DEHP]	Industrial chemical	7
Chlordecone	Insecticide/herbicide/fungicide	6
Cadmium	Metal/metalloid	6
WWTP effluent	Mixture	6
4-Methylbenzylidene camphor [4MBC]	UV filter	6
Triclosan	Biocide	5

with both mechanistic and apical supporting studies from the laboratory, although the precise mechanism of this EDC still remains unclear [14, 15, 98].

In terrestrial systems, Cuvillier-Hot and Lenoir [16] implicate substances such as IGRs in potential adverse endocrine effects on terrestrial invertebrates such as honeybees, wild bees, moths, parasitic wasps, and beetles. However, as with aquatic invertebrates, the evidence is weak that such effects occur in natural field populations of non-target arthropods [99]. The lack of evidence for any widespread ED effects on invertebrate wildlife populations might suggest that the hazards are negligible, although it is unclear whether this is a case of "absence of evidence" or "evidence of absence" [15]. It is therefore appropriate to ask a question posed more widely by Bergkamp [100]: are we searching for "phantom risks" or is there plausible field evidence for endocrine-mediated effects on invertebrates from exposure to current-use chemicals?

In their survey of experts, Ford and LeBlanc [1] identified field investigations to answer this question as the first of four research needs relevant to invertebrate endocrine disruption assessment:

- Field investigations: the evaluation of invertebrate field populations with sensitivity to adverse demographic effects;
- Biological target discovery: evolutionary studies to identify common potential invertebrate EDC targets and any unique targets for particular phyla, and the development of biomarkers for specific interactions between EDCs and invertebrate molecular targets;
- AOP construction for plausible ED effects on invertebrate populations; and
- Laboratory corroboration of field observations to investigate adverse outcomes at environmentally relevant concentrations, although this is less relevant under a European hazard-based approach.

Developing a framework for invertebrate EDC identification

The preceding overview suggests that there are two main obstacles to developing a coherent and scientifically defensible framework for invertebrate EDC identification, comparable to the vertebrate OECD CF:

1. Limited scientific understanding of invertebrate endocrinology, especially for non-arthropods. This problem has been well known for at least two decades [7] and is unlikely to be resolved soon [1]. Therefore, regulatory authorities can currently only make reliable decisions about EDCs based on vertebrate data.
2. A lack of mechanistic assays to identify endocrine modes of action in invertebrates [72]. This creates difficulties in attributing adverse effects on individuals or populations to a specific endocrine mode of action and therefore also makes it difficult to satisfy the WHO-IPCS definition of an EDC and to comply with EC [46] criteria for identifying EDCs.

Despite these obstacles, a defensible framework can be developed now and subsequently updated and improved as knowledge increases. This framework requires the following features:

1. Clear definition of invertebrate protection goals at the population level (which may differ between species in a similar way to protection goals for vertebrates);
2. Identification of assays which measure Molecular Initiating Events, Key Events, and Key Event Relationships along invertebrate-relevant AOPs [101] and which are sufficient to link adverse outcomes plausibly to a substance with an invertebrate-relevant endocrine pathway, and
3. Identification of representative invertebrate model test species and assay measurement endpoints to support population protection goals for invertebrate-relevant endocrine pathways.

We address each of these features in the subsections below.

Invertebrate protection goals

Both European regulation [46] and public opinion [102] identify invertebrate *populations* as the focus of interest when developing regulatory protection goals for invertebrate wildlife. However, the enormous diversity of invertebrates when compared to vertebrates means that criteria must be agreed when selecting which species populations to prioritise for research into potential ED because it is not practically possible to test every invertebrate phylum. The ecosystem services approach is one framework that could be used to prioritise invertebrates of importance to humans (e.g. pollinators). Non-target invertebrate wildlife populations provide a wide variety of ecosystem services including food (for consumption by humans and other wildlife), pollination, genetic resources (biodiversity), education and inspiration, aesthetic values, pest and disease regulation (e.g. spiders feeding on insect pests), seed and propagule dispersal, and recreation and ecotourism (e.g. butterfly-watching and shellfish collection) [71, 103]. The European Commission also identifies societal and ecosystem benefits as a key driver for research on potential EDCs [44]. The EFSA Scientific Committee [104] uses the concept of ecosystem services to derive specific protection goals (SPGs) for service-providing units (SPUs). An SPU can be any ecological entity that provides an ecosystem service (provisioning, regulating, cultural, or supporting services) to humans. EFSA SC [104] states that the following need to be defined before setting an SPG: the ecological entity (e.g. individual, population, functional group, or ecosystem), the attribute of that entity (e.g. behaviour, growth, abundance, biomass, or ecosystem processes), the magnitude of effects (i.e. negligible, small, medium, or large), the temporal scale of effect for the attribute (e.g. duration and frequency), and the spatial scales (e.g. in-field and off-field patches of landscapes). If the ecological entity to protect is the population of a particular species, as stated in Regulation (EU) 2018/605 on EDCs [21], then EFSA SC [81, 104] suggests that in most cases the attribute to be protected will be population dynamics (recruitment, size, and stability) in terms of abundance (e.g. numbers of individuals and their fitness) or biomass. For example, Table 6 shows definitions of SPGs for invertebrates potentially exposed to an insecticide [105].

The proposed "horizontal approach" by the European Commission [21] to identify EDCs that cause population-relevant effects might therefore involve the following if it is based, as stated, on the EU's current approach to plant protection products and biocides:

1. Identification of key invertebrate SPUs within an ecosystem services framework to ensure that all major groups are covered; and
2. Prevention of changes in the population abundance and biomass of these species which take them out of their range of natural variability (this might also include prevention of changes in species diversity).

Definition of invertebrate SPUs and SPGs can draw upon an expanding literature on the ecosystem services provided by both aquatic and terrestrial invertebrates, including insects [106], terrestrial and freshwater invertebrates [107], marine and estuarine invertebrates [108, 109], bivalve aquaculture [110], and non-cultured shellfish [111]. In the absence of any additional ecological or toxicological information on the functional importance or vulnerability of particular invertebrate phyla, we propose that the selection of appropriate invertebrate SPUs may be based on relative species richness and phylogenetic relationships, as well as information on any unique invertebrate endocrine pathways. This would ensure that the most important invertebrate groups in relation to abundance and biomass are considered, phylogenetic similarities and dissimilarities between groups are taken into account, and toxicity testing is kept to a reasonable minimum.

We recognise that an ecosystem services approach is an explicitly anthropocentric and contested framework [112]. However, the conceptual domain of invertebrate endocrine disruption must be bounded somehow, even if only imperfectly. This can then be subject to regular review and the boundaries can, if necessary, be redrawn in the light of new knowledge. If such boundaries are not set then regulatory authorities are faced with an apparently limitless and therefore impossible task: to protect an ill-defined set of "all invertebrates", including currently unknown or understudied species and endocrine pathways, against exposure to currently unknown EDCs.

Adverse Outcome Pathways for invertebrate EDC identification

There is a developing consensus in the (eco)toxicological and regulatory communities that different outputs from in silico predictions, in vitro and in vivo assays, and population modelling may usefully be considered within an AOP framework. The AOP concept is a robust way to organise information on potential EDCs and help support regulatory decision-making [101, 113–118]. The concept is chemically "agnostic" (i.e. not specific to an individual substance) and can be used to describe the actions of a group of chemicals [119, 120]. It can therefore be used to reflect the definition of an EDC: requiring an endocrine mechanism (i.e. a Molecular Initiating Event [MIE]), causally linked (via key events [KEs] and key event relationships [KERs]) to a population-relevant adverse outcome (AO), although the KER that links an individual outcome to a population-relevant AO is usually derived "by extension" [101], see also [121]. An AOP may describe a sequence of KEs from MIE to AO either linearly or, in most cases and more realistically, through network effects if KEs are shared amongst AOPs [122, 123].

A cascade of effects through an AOP from an MIE to an AO requires sufficient chemical potency and exposure for a KE to activate the next step in the chain [121]. Consequently, an AO may not manifest if a non-responding KE interrupts the process. Criteria for determining the biological plausibility of an AOP, for both vertebrate and invertebrate endocrine disruption, must therefore include, as a minimum [113, 124]:

1. Biological plausibility: Is there a mechanistic (i.e. structural or functional) relationship between upstream and downstream KEs which is consistent with established biological knowledge?
2. Essentiality: Are downstream KEs or the AO prevented if an upstream KE is blocked?
3. Empirical evidence:

a. Does the empirical evidence support the inference that a change in an upstream KE leads to an appropriate change in a downstream KE?
b. Does each upstream KE occur at lower doses and earlier time points than the associated downstream KE and is the incidence of the upstream KE greater than that for the downstream KE?
c. Are there inconsistencies in empirical support across taxa, species, and stressors that do not align with an expected pattern for the hypothesised AOP?

There is now considerable guidance on best practice for constructing AOPs and defining their constituent MIEs, KEs, KERs, and AOs [58, 117, 124–126]. Development of fully quantitative AOPs (qAOPs) is the "holy grail" [119, 120, 127], but even a semi-quantitative AOP is likely to be useful for regulatory purposes [120]. This is because quantitative, invertebrate-relevant, in vitro mechanistic assays can be anchored to one end of the pathway, and a quantitative invertebrate population-relevant AO anchored to the other end, with

Table 6 Example definition of Specific Protection Goals for invertebrates potentially exposed to an insecticide [105]

Ecosystem services key driver	Problem	Focal species	Entity	Attribute	Spatial scale	Temporal scale	Model type	Model output
Soil invertebrates	Effects of application scenarios on populations	*Eisenia fetida*, *Folsomia candida*	Population	Abundance & biomass	In-crop	1 year	Spatially explicit Individual-Based Model (IBM)	Time to recovery
Terrestrial invertebrates	Recovery of populations	Linyphyiid spiders, carabid beetle	Population	Abundance	In-field/off-field	A few years	Spatially explicit IBM	Time to recovery
Aquatic invertebrates	Effects of time variable exposure on populations	*Gammarus*, *Chaoborus*, *Daphnia*	Population	Abundance	Edge of field water body	1 to a few years	Toxicokinetic–Toxicodynamic and IBM	Magnitude and duration of effect

intermediate KEs inferred. (Semi)quantification overcomes the criticism that qualitative AOPs do not demonstrate exceedance of a toxic threshold and therefore do not demonstrate the plausibility and essentiality of each KE [118].

Hecker [128] noted that only a limited number of "mature" AOPs are available, especially for microorganisms, invertebrates, and plants, because most work has focused on vertebrates [129]. Currently (December 2021), there are three AOP Wiki descriptions of specific relevance to invertebrate endocrine disruption:

- An AOP for juvenile hormone receptor agonism leading to male offspring induction and associated population decline, with taxonomic applicability to *D. magna* and *D. pulex* (and potentially other crustaceans and insects) (https://aopwiki.org/aops/201).
- Ecdysone receptor (EcR) agonism leading to incomplete ecdysis-associated mortality, with taxonomic applicability to *D. magna* (and potentially other crustaceans and insects) (https://aopwiki.org/aops/4).
- 5-Hydroxytryptamine transporter (5-HTT) inhibition leading to population increase, with taxonomic application to molluscs (https://aopwiki.org/aops/195).

Song et al. [130] provide a detailed AOP for ecdysone receptor agonism leading to lethal moulting disruption in arthropods, which illustrates the utility of the approach (Table 7). This AOP should be applicable to both steroidal (e.g. ecdysone) and non-steroidal (e.g. tebufenozide) EcR agonists. The AOP includes empirical data from insects (Diptera, Lepidoptera, and Coleoptera) and crustaceans, although the authors note that crustacean-based evidence for certain elements of the pathway is sparse. However, they point out that both the EcR and the role of ecdysis triggering hormone (Eth), in stimulating muscle contraction behaviour required for ecdysis, are considered well conserved across arthropods. They therefore conclude that "based on evaluation of known sequence conservation and phylogenetic relationships, it is expected that this AOP may be applied broadly to most arthropods, although differences in the exact nature of quantitative relationships between some of the KEs may vary among taxa."

As Fay et al. [131] point out in case studies for ecdysone receptor agonism and 5-HTT inhibition, these AOPs are based on substantial prior knowledge of invertebrate endocrinology and MIEs. This highlights the significant resource investment that would be required to implement such approaches even for a limited number of pathways and relevant surrogate species with "adequate" taxonomic coverage. However, the AOP framework also encompasses useful approaches for identifying previously unknown MIEs, as addressed below.

Identification of Molecular Initiating Events at OECD CF levels 1 and 2

An MIE is the initial interaction between an exogenous molecule and a biomolecule or biosystem that can be causally linked to an outcome via a pathway [132]. Identification of relevant MIEs is not just an important technical prerequisite when developing an AOP. In the case of invertebrate endocrine disruption, it is probably *the* key requirement when one considers the current lack of knowledge about invertebrate endocrinology and the lack of tools to assess chemical interactions with invertebrate endocrine activity.

MIEs possibly relevant for invertebrate endocrine disruption may be identified in four main ways:

1. Prior knowledge of invertebrate endocrine pathways. However, as we have seen, this knowledge is patchy, limited to a few taxa (e.g. honeybees, silk moths, shrimps, and mussels), and considers only a small number of pathways.
2. Regulatory authorities could request that additional studies might be performed if concerns about potential ED effects are triggered, either by findings in core guideline (toxicology and ecotoxicology) studies (e.g. at OECD CF Levels 3, 4, or 5) or if there is a concern triggered by a substance's mode of action and its potential to cause an MIE [133]. However, in a hazard-based framework there is little point in performing an invertebrate test to determine whether a substance is an EDC if it has already been classified as such from vertebrate tests, and a means must also be found to confirm an *endocrine* mode of action if in vivo adverse effects on apical endpoints are found in non-target invertebrates. For example, 17α-ethinylestradiol is a known vertebrate EDC with population-level effects in fish [134], so it is unnecessary to test this substance with invertebrates to determine whether it is an EDC specifically for hazard classification purposes. In contrast, a substance that has *not* been classified as a vertebrate EDC can only be classified as an invertebrate EDC if observed adverse effects are linked causally to an endocrine mode of action in these organisms.
3. By using chemical structural alerts to prioritise substances with structures known to disrupt vertebrate pathways which appear to be conserved in invertebrates [135–139], or with structures known to disrupt only invertebrate pathways (e.g. [140]). However, once again this approach would be redundant for hazard classification if a substance is already known to be a vertebrate EDC, and it does not solve the problem of potential effects on currently unknown invertebrate endocrine pathways.
4. By using high-throughput "'omics" datasets (e.g. transcriptomics, metabolomics, lipidomics, and proteomics [141–145]) to explore changes in genetic, metabolic, lipid, or protein structures after exposure to a chemical at any CF Level. Data from these assays can then be used in "reverse engineering", "right-to-left", or "top-down" AOP development to identify MIEs [146–152].

It is the last of these that holds the greatest promise for providing reassurance that potential EDCs which specifically interact within non-target invertebrate endocrine pathways will be identified and adequately regulated. This highly scientifically complex, organisationally complicated and financially expensive approach might be something akin to the IMI PREMIER project on pharmaceuticals in the environment (https://imi-premier.eu/)—involving the scientific expertise, organisational capabilities and (crucially) funding potential of a large consortium of relevant stakeholders.

There is a pressing need for research to support development of additional invertebrate-specific EDC screening tests and a first step is to characterise at the molecular and functional level the many nuclear receptors present in invertebrates [59]. For example, Oliveira et al. [153] list 36 nuclear receptor families and their physiological ligands which are known to occur in arthropods. These authors and others (e.g. [154, 155]) recommend high-throughput screening tools and other rapid and relatively inexpensive alternatives to in vivo vertebrate testing. Castro and Santos [156] have also called for comprehensive analysis and functional characterisation of nuclear receptors across invertebrate lineages so that the extent of receptor conservation can be determined and relevant in vitro assays developed for cost-effective high-throughput testing. Drug discovery already uses invertebrate models such as *Caenorhabditis elegans* and *Drosophila melanogaster* to identify bioactive compounds and to understand their mechanism of action [157]. Kaur et al. [158] provide a recent systematic review of computational techniques and tools for 'omics data analysis which identifies promising techniques that might be used to identify MIEs.

A key requirement in developing AOPs is to build a community of biologists and modellers because both high throughput, mechanistic in vitro and in vivo assays, and predictive computational modelling are necessary to define MIEs and early KEs [159]. For example, Hodges et al. [160] discuss how the use of genome-wide RNA profiling and non-targeted metabolomics can be used to analyse networks of genes and metabolites showing reproducible correlations across multiple samples and test conditions. Machine learning techniques can relate the different 'omics data types in a way that is more powerful than reliance on shared sequence similarity to infer functional homology. In another example, Perkins et al. [148] describe use of a network inference approach to pathway discovery.

LaLone et al. [161] suggest that if the molecular target of a chemical is unknown then in vitro data (e.g. from USEPA ToxCast [162–165]) might be used to identify potential protein molecular targets, or it may be possible to assign tentative molecular targets based on information from structurally similar chemicals that have been tested. Hodges et al. [160] note that while there is a wealth of results from receptor binding assays (e.g. from ToxCast), these have not yet been systematically reviewed to determine how many are relevant and valid

Table 7 Adverse Outcome Pathway of ecdysone receptor agonism leading to incomplete ecdysis-associated mortality [130]

KE	Event description	Level of organisation	Upstream to downstream KE relationship	Support for essentiality	Biological plausibility	Empirical support	Overall WoE	Quantitative understanding	Detection method	Target for detection
MIE	EcR, activation	Macromolecular	D	S	S	S	S	W	In vitro ECR binding assay; transcriptional analysis	EcR transfected mammalian cells; mRNA from cell, tissue, and whole organism
KE-1	*E75b* gene, induction	Molecular/cellular	D	S	S	S	S	W	Transcriptional analysis	mRNA from cell, tissue, and whole organism
KE-2	*Ftz-f1* gene, suppression	Molecular/cellular	D	M	S	M	M	W	Transcriptional analysis	mRNA from cell, tissue, and whole organism
KE-3	Release of circulating Eth, reduction	Tissue/organ	I	S	S	M	M	W	Enzyme immunoassay; immunohistochemical staining	Haemolymph; isolated endocrine tissue
KE-4	Release of circulating Ccap, reduction	Tissue/organ	D	S	S	M	M	W	Enzyme immunoassay; immunohistochemical staining	Haemolymph; isolated endocrine tissue
KE-5	Ecdysis motoneuron bursts, reduction	Tissue/organ	D	S	S	M	M	W	Electrophysiological recording	Isolated CNS, abdominal ganglion
KE-6	Excitatory postsynaptic potential, reduction	Tissue/organ	D	M	S	M	M	W	Electrophysiological recording; FM1-43 fluorescent labelling	Skeletal muscles
KE-7	Abdominal muscle contraction, reduction	Tissue/organ	D	M	S	M	M	W	Electrophysiological recording; behavioural (air/water swallowing) assays	Skeletal muscles; whole organism
KE-8	Incomplete ecdysis, induction	Individual	D	S	S	S	S	S	Light microscope, histopathology	Cuticle; whole organism
AO	Mortality, increased	Individual	D	S	S	S	S	St	Survival test	Whole organism

MIE Molecular Initiating Event, *KE* key event, *AO* Adverse Outcome, *EcR* ecdysone receptor, *E75b* nuclear receptor E75B, *Ftz-f1* Fushi tarazu factor-1, *Eth* ecdysis triggering hormone, *Ccap* crustacean cardioactive peptide *D* direct, *I* indirect, *S* strong, *M* moderate, *W* weak

for invertebrates. Madden et al. [166] also note that in vitro tools complement in silico tools by verifying the domain of applicability of structural alerts identified in silico and corroborating proposed mechanisms, and Schroeder et al. [167] show how they can be used to trace mixture toxicity pathways and effects within an AOP framework.

The number of screening tests required to cover each important invertebrate-specific endocrine pathway need not be large but, depending on the number of pathways required to be investigated, could multiply rapidly. However, Judson et al. [168] demonstrated for vertebrates that adequate predictive power could be obtained from using a subset of only four out of 16 USEPA screening tests for oestrogen agonism. A similar approach can be used to identify a minimum set of in vitro assays for reliable determination of juvenile hormone receptor agonism, ecdysone receptor agonism, 5-HTT inhibition, and any other identified invertebrate endocrine pathways of concern.

Mihaich et al. [169] highlight the wide range of different species that need to be protected, which presents a challenge because the molecular targets and associated toxicity pathways for EDCs can differ among species. As a result, there has been a focus on developing computational approaches to compare target molecules of MIEs or KEs among taxonomic groups to enable initial predictions to be made about adverse outcomes. An example tool is the USEPA's Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS) which aligns the sequence of the functional molecule representing an MIE, such as a receptor or enzyme which has been shown to trigger an adverse effect [139, 161, 170–172]. There is a strong correlation between SeqAPASS susceptibility predictions for vertebrate and invertebrate aquatic species and empirical toxicity data, so this and other molecular target sequence tools can identify taxa affected by common endocrine MIEs. LaLone et al. [172] conclude that high-throughput screening targets of regulatory relevance are likely to be broadly applicable across most vertebrate taxa and some targets may be applicable to certain invertebrates. Subsequent in vitro and in vivo studies can then provide further empirical evidence to determine whether a substance is an EDC. This creates positive feedback, particularly between in silico predictions and high-throughput in vitro tests for these predictions. SeqAPASS uses the National Center for Biotechnology Information protein database, which includes protein sequences for thousands of vertebrates, invertebrates, plants, bacteria, and viruses. Houck et al. [173] show the predictive potential of SeqAPASS across vertebrate taxa, and LaLone et al. [172] show how SeqAPASS can be used to identify high-throughput mammalian ToxCast screens for steroidogenic and thyroid targets that may also be relevant for invertebrate taxa. SeqAPASS analyses of enzymes involved in steroidogenesis suggest that results from the human cell-based High Throughput-H295R assay may be broadly extrapolated to other vertebrates, but not invertebrates. Level 1 and 2 evaluations of human THRα and THRβ and their respective ligand binding domains showed that these receptors are well conserved across vertebrates, with the exception of Ceratodontimorpha (lungfish). Conservation of THRβ but not THRα is also found for several invertebrate taxa, including Polychaeta (sandworms), Gastropoda (snails), Lingulata (lampshells), Bivalvia, Enteropneusta (acorn worms), Asteroidea (starfish), Branchiostomidae (lancelet), and Ascidiacea (sea squirts, tunicates). Similarly, Iodothyronine Deiodinase 1 (DIO1) and DIO3 are also found in invertebrate species, but DIO2 is not. Further work is required to understand the functional role of these proteins in invertebrates and to determine whether tools such as SeqAPASS are useful for non-vertebrates. If so, such tools could be augmented further by integrating information on chemical toxicodynamic and toxicokinetic properties so that species differences in absorption, distribution, metabolism, and excretion are also taken into account [128]. This approach is not currently immediately applicable to invertebrates because the necessary in silico approaches and in vitro assays are still missing, and knowledge of chemical toxicodynamics and toxicokinetics in invertebrate taxa of interest for endocrine disruption assessment is very fragmented.

Coady et al. [174] provide an example from vertebrate toxicology which shows how regulatory pressure can stimulate work on MIE identification and the development of appropriate high-throughput assays. They describe how the USEPA identified 15 potential MIEs for thyroid-based AOPs, including those related to thyroid hormone synthesis, transport, nuclear receptor binding, and effects in peripheral tissues [123, 175]. The USEPA then ranked these MIEs based on their relevance to the thyroid pathway, their toxicological potential, and the current status of high-throughput bioassay development. Four MIEs from this thyroid AOP network (the sodium iodide symporter, thyroperoxidase, iodothyronine deiodinase, and hepatic nuclear receptors involved in thyroid metabolism) were ranked highest for bioassay development. Similar regulatory pressure to identify invertebrate-specific endocrine MIEs would most likely stimulate and accelerate similar research and development activity.

Invertebrate model species at OECD CF Levels 3, 4, and 5

Bioinformatic reverse engineering, from high-throughput in vitro 'omics assays, is proposed above as the most

efficient and effective way to determine MIEs with a potential ED mode of action. However, can we ever hope to provide a reasonably comprehensive framework that will identify EDCs across all invertebrate taxa without substantially expanding the range of invertebrate model species used in vivo?

Chapman [2] reviewed the number of species in each invertebrate phylum and identified the 12 with the greatest estimated number of species, in order of richness, as Insecta, Arachnida, Nematoda, Mollusca, Crustacea, Myriapoda, Platyhelminthes, non-insect Hexapoda, Annelida, Porifera, Echinodermata, and Cnidaria. Integration of knowledge about the relative number of species within different invertebrate phyla, their conservation status, and their phylogenetic relationships [176–178] suggests that a reasonably comprehensive testing strategy for invertebrate EDCs could be based on representative models from the following phyla:

1. Arthropoda (Insecta, Arachnida, Crustacea, or Myriapoda);
2. Mollusca;
3. Annelida; and
4. Cnidaria

Of these four phyla, only Cnidaria are not currently included in international test guidelines for testing chemicals with invertebrates, although protocols for suitable test species are available [179–181]. A case for testing based primarily on species richness and numerical dominance might also be made for inclusion in this list of Nematoda, with use of *C. elegans* as a representative model species [79] and for which an ISO test standard exists. However, there is no indication from either terrestrial or aquatic field studies that reliably suggests EDC-related population effects in any invertebrate phyla other than molluscs, although this may be due to a lack of relevant studies. The added value of annelid, cnidarian, and nematode models is therefore debatable.

There does not appear to be a compelling case to expand the battery of invertebrate in vivo tests for endocrine disruption unless further research reveals unique endocrine pathways sensitive to EDCs in invertebrates other than arthropods and molluscs.

Invertebrate population modelling

Adverse population effects are the AOs most commonly identified as a requirement by regulatory authorities in AOPs for non-endangered wildlife species [116], although the regulatory approach for endangered vertebrate species often focuses more on the protection of individuals and a similar approach may also be relevant for endangered invertebrate species. Devillers and Devillers [182] review models for projecting the population consequences of effects on juvenile hormone pathways in non-target species, including invertebrates. They describe simple equation-based models (e.g. [183]) and slightly more complex matrix models [184–186] that have been used to project the effects of methoprene exposure on aquatic crustacean populations. They also compare the utility of compartment models [187] versus individual-based models (IBMs, sometimes known as agent-based models [ABMs], [188]) for projecting the effects of insecticide exposure on honeybees. They conclude that IBMs provide more realistic and robust results than other methods because they account for the continuous development and interaction of individuals throughout their lifetimes and within their population in ways that can be related to environmental parameters.

In contrast to a species-specific model, a biological traits-based approach [189–191] could be used to develop a generic invertebrate model which includes realistic worst case sensitivity traits for adverse population effects. Invertebrate traits that have been considered include voltinism; asexual/sexual reproduction; maximum lifespan; lifecycle duration; lifecycles per year; maximum body size; feeding type and habit; oxygen source and respiration type; mobility; dispersal mechanisms; and current, salinity, temperature, and pH preferences [192]. For example, Rubach et al. [192] found that self-fertility/asexuality versus sexual reproduction, plus temperature preference, were the traits most associated with sensitivity to organophosphate insecticides in aquatic macroinvertebrates. van den Berg et al. [191] also found that in aquatic macroinvertebrates, carbamate toxicity was positively associated with pH preference and negatively associated with lifecycle duration and numbers of lifecycles per year. An analysis of invertebrate traits most associated with sensitivity to known EDCs would provide parameters for construction of population models either for focal species with these traits or for "generic" invertebrate species with biologically compatible sets of these traits. Model projections can then be used to assess whether any effects observed in endocrine disruption-relevant invertebrate toxicity tests will translate into population-level effects for the most demographically sensitive focal or generic species. ECETOC [60] also supports a traits-based approach when extrapolating AOPs across species and argues that aspects other than taxonomic relatedness should be considered, such as reproductive strategies (e.g. uni- versus multi-voltinism and r- versus K-strategists), which can compensate for stress at the population level. There are multiple modelling and comparative studies showing that some traits that are sensitive to toxicants, such as reproduction in certain species, can have a very low impact on population

growth (e.g. [193, 194]) although this will depend on the life history strategy of each species.

EFSA SC [195] suggests that population models can be used for setting a critical effect level (i.e. a benchmark response). They envisage that models of focal species could be used to determine endpoints corresponding to cut-off values set by ecosystem service specific protection goals. These models can be used for calculating critical effect levels for certain types of effect. Forbes et al. [196] also show how mechanistic dynamic energy budget models can be used to link organism-level responses measured in standard toxicity tests to protection goals relevant to ecosystem services. EFSA SC [104] specifies that population resilience depends on the ecological context and is related to the degree to which induced fluctuations in the population density are buffered by density-dependent feedback mechanisms and competition with other species. For example, small effects on fecundity in density-regulated systems (e.g. a slightly reduced number of eggs for insects that produce many more eggs than develop into adults) will not translate adversely to the population level if egg quality remains unaffected. Although implicit in the use of organism-level toxicity data in effects assessments, it is invalid to assume that responses at the organism level are directly proportional to responses at the population level [197]. This is why qualitative population inferences, based only on individual organism effects observed in toxicity tests, should be quantitatively examined with population models, preferably also including interspecific interactions. If this is not performed then such qualitative inferences remain speculative and may lead to false conclusions.

Knowledge gaps

An expert group convened by the European Commission (EC 2018b) identified the following priority knowledge gaps in relation to invertebrate endocrine disruption assessment:

- Invertebrate endocrinology/physiology (highest priority);
- Mechanistic understanding for invertebrates (particularly molluscs);
- Echinoderm developmental research; and
- Retinoic X Receptor (RXR) research in invertebrates, specifically molluscs, as an example of the most vulnerable species.

The group recommended that no further mollusc guideline development for endocrine disruption endpoints should take place until further research has adequately described mollusc physiology, endocrinology, and metabolic pathways. They also suggested that the six reporter assays for trans-activation of retinoic acid receptors in the ToxCast battery could be developed and validated for screening [59, 175]. This is because RXR and Retinoic Acid Receptors (RAR) are well conserved and would therefore be relevant across many different taxa potentially exposed to retinoids [198]. Further suggestions were that the role of RXR in invertebrates should be investigated in molluscs (it can be cloned for several mollusc species Vogeler et al. [199]); and there should be development of in vitro receptor assays for juvenile hormone and ecdysteroids. This would link adverse outcomes to these pathways and provide additional mechanistic data to support endpoints for male production in the *Daphnia* reproduction test and in a short-term juvenile hormone activity screening assay currently under development (SJHASA—see Table 1). Invertebrate hormone analysis within existing apical invertebrate tests was also suggested as potentially useful (e.g. ecdysis triggering hormone levels or ecdysterone levels measured in arthropods).

Specific test development recommendations from this expert group were:

- Growth and development
- Validation by OECD of in vitro assays for RXR and RAR (OECD CF 2)
- Validation by OECD of in vitro Peroxisome Proliferator-Activated Receptor (α,β/δ,γ) transactivation assays (OECD CF 2)
- In vitro daphnid juvenile hormone and ecdysone agonist assay development (OECD CF 2)
- In vivo assay development for ecdysis triggering hormone levels (OECD CF 3)
- In vivo assay development for ecdysterone levels in arthropods (OECD CF 3)
- Reproduction
  In vivo spawning assay development in echinoderms (OECD CF 3)

Bopp et al. [200] documented a survey of experts who also identified endocrine mechanistic screening tests for invertebrates as a priority research need.

Our assessment of the current state of knowledge largely supports these conclusions. However, our main initial focus would be on the development of 'omics data that can be reverse engineered through use of bioinformatics techniques to identify a comprehensive set of invertebrate-specific, endocrine-related MIEs, which represent the most important invertebrate taxonomic groups.

Conclusions

For more than 20 years, the knowledge gap in relation to invertebrate biodiversity and endocrinology has been a common theme in the scientific and technical literature. In this commentary we have tried to focus more on what we *do* know and whether this knowledge is sufficient to construct a robust regulatory framework for identifying invertebrate EDCs.

Regulatory authorities agree on the WHO-IPCS [22] definition of an endocrine disruptor, which forms the basis of current EU regulation [21]. The common protection goal for invertebrate wildlife is at the population level, which translates into no individual adverse effects that are relevant for population dynamics, abundance, or biomass. There is very limited evidence for endocrine-mediated effects of current-use chemicals on non-target invertebrate populations in the field, with effects on arthropods or molluscs demonstrated or inferred in only a very small number of studies. However, the low number of relevant field studies means that it remains unclear whether this is evidence of absence or just an absence of evidence.

Arthropods and molluscs comprise almost 80% of the estimated total number of living invertebrate species and are also amongst the most important in providing ecosystem services, so it is a logical starting point to focus regulatory attention on these phyla. Several major invertebrate-relevant endocrine pathways are reasonably well understood, particularly for insects and crustaceans (and therefore probably for most arthropods) and also for molluscs. For insects these pathways involve peptide hormones, ecdysteroids, and juvenile hormones; for crustaceans these pathways also involve peptide hormones and ecdysteroids, plus methyl farnesoate; and for molluscs these pathways are mainly based on peptide hormones. In silico and in vitro mechanistic assays are available or under development for some of these endocrine pathways, and high-throughput 'omics approaches combined with bioinformatics could be used to reverse engineer AOPs to identify additional invertebrate ED MIEs for currently unknown pathways. Once endocrine MIEs are identified, in silico tools such as SeqAPASS are available to assess the likely susceptibility of different invertebrate taxa based on receptor homology. However, much of the information required to build reliable AOPs which are meaningful for regulatory use is still unavailable. There are some promising initiatives, but the scientific community remains far from being able to cover the diversity of signalling pathways within the major invertebrate taxa, not to mention those of lesser scientific interest.

Data on AOP KEs further downstream can be obtained from invertebrate tests performed according to internationally validated test guidelines available for insects, crustaceans, and molluscs with apical endpoints potentially relevant at the population level. Population models, including those based on sensitive invertebrate traits, can then be built to determine whether apical effects found in invertebrate tests are likely to cause adverse population-level effects in a similar way to that recommended for non-target vertebrates by Crane et al. [53]. An AOP for each known invertebrate endocrine pathway can therefore be anchored at both ends (mechanistic and population adverse outcome), although some intermediate KEs and KERs may at first be poorly understood. This approach is tractable but would require significant resource investment for development and implementation. The timescale and scientific and organisational complexity of such a financially expensive initiative should not be underestimated.

Abbreviations

5-HTT: 5-Hydroxytryptamine transporter; AO: Adverse outcome; AOP: Adverse Outcome Pathway; CF: Conceptual framework; CHH: Crustacean hyperglycaemic hormone; DIO: Iodothyronine deiodinase; EATS: Estrogen, androgen, thyroid, and steroidogenesis; EC: European Commission; ECETOC: European Centre for Ecotoxicology and Toxicology of Chemicals; ECHA: European Chemicals Agency; EcR: Ecdysone receptor; ED: Endocrine disruptor; EDC: Endocrine Disrupting Chemical; EFSA: European Food Safety Authority; Eth: Ecdysis triggering hormone; EU: European Union; IGR: Insect growth regulator; IPCS: International Programme on Chemical Safety; KE: Key event; KER: Key event relationship; MIE: Molecular Initiating Event; OECD: Organisation for Economic Cooperation and Development; qAOP: Quantitative Adverse Outcome Pathway; RAR: Retinoic acid receptor; RXR: Retinoic X receptor; SC: Scientific Committee; SeqAPASS: Sequence Alignment to Predict Across Species Susceptibility; SJHASA: Short-term juvenile hormone activity screening assay; SPG: Specific protection goal; SPU: Service-providing unit; TBT: Tributyl tin; THR: Thyroid hormone receptor; USEPA: United States Environmental Protection Agency; VTG: Vitellogenin; WHO: World Health Organization.

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Authors' contributions

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Availability of data and materials

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