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Towards regulation of Endocrine Disrupting chemicals (EDCs) in water resources using bioassays – A guide to developing a testing strategy

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ABSTRACT

Endocrine disrupting chemicals (EDCs) are found in every environmental medium and are chemically diverse. Their presence in water resources can negatively impact the health of both human and wildlife. Currently, there are no mandatory screening mandates or regulations for EDC levels in complex water samples globally. Bioassays, which allow quantifying *in vivo* or *in vitro* biological effects of chemicals are used commonly to assess acute toxicity in water. The existing OECD framework to identify single-compound EDCs offers a set of bioassays that are validated for the Estrogen-, Androgen-, and Thyroid hormones, and for Steroidogenesis pathways (EATS). In this review, we discussed bioassays that could be potentially used to screen EDCs in water resources, including *in vivo* and *in vitro* bioassays using invertebrates, fish, amphibians, and/or mammalians species. Strengths and weaknesses of samples preparation for complex water samples are discussed. We also review how to calculate the Effect-Based Trigger values, which could serve as thresholds to determine if a given water sample poses a risk based on existing quality standards. This work aims to assist governments and regulatory agencies in developing a testing strategy towards regulation of EDCs in water resources worldwide. The main recommendations include 1) opting for internationally validated cell reporter *in vitro* bioassays to reduce animal use & cost; 2) testing for cell viability (a critical parameter) when using *in vitro* bioassays; and 3) evaluating the recovery of the water sample preparation method selected. This review also highlights future research avenues for the EDC screening revolution (e.g., 3D tissue culture, transgenic animals, OMICs, and Adverse Outcome Pathways (AOPs)).

1. Introduction

Endocrine disrupting chemicals (EDCs) are exogenous compounds that “alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations” (WHO/IPCS, 2002). EDCs contaminate all components of the environment (atmosphere, hydrosphere, lithosphere, and biosphere; Metcalfe et al. this issue) Humans and wildlife are exposed to a wide variety of structurally diverse EDCs from many sources, including environmental matrices and food contaminants, therapeutic agents, chemicals from commercial and consumer products, personal care products, waste effluents and many other sources. As this is a major issue for health (e.g., reducing reproductive success on a global scale in multiple species (Marlatt et al. this issue), altered metabolism and

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cardiovascular function (Martyniuk et al. this issue)), a breadth of methodologies and approaches have been developed over the last decades by several international organizations (e.g., OECD) and governments (e.g., USA, EU, Japan) to screen and detect EDCs, especially in water resources. As of 2020, no country has yet to introduce mandatory EDC screening in water resource management (i.e., potable water, wastewater effluents, waterbodies). Nevertheless, some initiatives are currently underway to explore testing strategies for EDCs in wastewater in Canada, United States (US), and European Union (EU).

One of the main approaches used by the different EDC testing programs is based on bioassays to detect the adverse effects of EDCs on several aspects of the endocrine system. Bioassays can quantify the biological effects of a compound or a mixture either *in vivo* (whole organism) or *in vitro* (cells or receptors) and have been used globally to quantify non-endocrine related toxic effects for decades. Most specifically, tiered approaches including a combination of *in vitro* and short-term *in vivo* bioassays spanning mammals and non-mammals designed to capture estrogen (E), androgen (A), thyroid hormone (T), and steroidogenesis (S) pathways (EATS) have been proposed (Table 1). However, the EDC bioassays available and overall testing strategies were mainly designed to identify or to screen for potential EDCs on an individual chemical basis. As such, there is a lack of guidance for EDC testing methods to assess environmentally relevant mixtures that more accurately reflect human and wildlife exposure scenarios. For example, wastewater treatment plant (WWTP) effluent is a well-known source of EDCs for several wild fish species, and this finding was discovered via field-based studies demonstrating adverse effects related to the reproductive endocrine axis in fish living downstream of outfalls (Marshall Adams et al., 1992; Van Der Kraak et al., 1992; Jobling et al., 1995; Kovacs et al., 1995; Folmar et al., 1996; Cheek, 2006; Marlatt et al., this issue). This is not surprising since WWTP effluent contains a number of chemicals, such as pharmaceuticals and personal care products, steroid hormones, surfactants, industrial chemicals, and pesticides (reviewed in Luo et al., 2014). Mixtures comprised of such constituents could produce synergistic, antagonistic, or additive effects that would not be captured during single-compound tests by bioassays or by targeted analytical chemistry. Furthermore, due to the complexity of the number of potential interactions of multiple EDCs within a whole organism or a cell, the choice of *in vitro* bioassays over *in vivo* bioassays needs to include the most natural cellular milieu and careful extrapolation between animal models. These considerations are of paramount importance when selecting bioassays to estimate the risks of adverse effects of mixtures on biota.

This review aims to provide guidance to stakeholders, government agencies, and academics interested in developing an EDC bioassay-based testing strategy to assess complex water samples that contain a mixture of EDCs. Although we focus on bioassays targeting the EATS pathways, non-EATS endpoints are also relevant and are described further in Martyniuk et al. (this issue). The types of environmental samples included in this review are the following: wastewater (influent and/or effluent) from municipalities, hospital, and industry, surface water, agricultural runoff, drinking water, recycled water, and groundwater. We first review bioassays that are currently validated (or soon to be) for the identification of EDCs and then expand on how to adapt them for water testing when necessary. Second, we review the use of the effect-based trigger values (EBT) to enable the use of bioassays in regulations and its comparison to chemical analysis. We also discuss the most recent bioassay technologies which aim to improve the future of risk assessment evaluation of water. Lastly, we provide guidance on which bioassays to include in a testing strategy aimed at monitoring and regulating EDC levels in water resources, which can be adapted to various needs ranging from the protection of the human well-being to ecosystem health.

2. Currently used *in vivo* bioassays for EDC screening

The OECD Conceptual Framework (OECD, 2018) is made of five levels. Briefly, level 1 includes the use of existing data from bioassays or non-bioassay methods (i.e. *in silico*, physical/chemical properties); level 2 includes *in vitro* bioassays aimed at select endocrine mechanisms of action; level 3 consists of *in vivo* bioassays with data providing information on select endocrine mechanisms of action; while level 4 and 5 are *in vivo* assays providing data on adverse effects on endocrine-relevant endpoints, with level 5 consisting of bioassays over more extensive parts of the organisms life cycle compared to level 4. Based on the OECD guidelines (OECD, 2018), there may be sufficient weight of evidence to take a regulatory decision at level 3, which makes *in vivo* bioassays the current gold standard to confirm that a chemical is an EDC. For this exact purpose, various *in vivo* bioassays to screen water samples have been developed over the years in fish, amphibians, rodents and invertebrates, among others (Table 1, Table 2).

2.1. Invertebrate in vivo bioassays

Regulatory agencies are beginning to incorporate invertebrate *in vivo* bioassays into their guidance documents for evaluating EDCs (Ottinger et al., 2016; OECD, 2018). For example, the OECD has included a number of invertebrate *in vivo* assays in the conceptual framework outlined in their Revised Guidance Document 150 (OECD, 2018). For use in level 3 of their framework, the OECD is validating a short-term juvenile hormone activity screening assay with *Daphnia magna* (JHASA, OECD draft, Abe et al., 2015, Table 1, Fig. 1A), which will assess whether a chemical is a juvenile hormone agonist. Juvenile hormone (JH) has been studied the most in insects and has been found to have three isoforms, JH I, II, and III (Abe et al., 2015). Exposure to JH or JH analogues can cause daphnids to produce male offspring, which has consequences for the control of sex ratios to maximize reproductive fitness (Gerber et al., 2018). The effect of JH analogues on production of males can be assessed in the validated OECD *Daphnia magna* reproduction test (TG 211) but the draft OECD JHASA allows for an assessment of the effect of JH analogues in a third of the time (Olmstead and Leblanc, 2002, 2003). A number of validated and non-validated invertebrate tests have also been proposed for use in level 4 and 5 of the OECD framework (Table 2, OECD, 2018). US-EPA has also proposed including the estuarine mysid shrimp (*Americamysis bahia*) two-generation toxicity test (~60 d) or harpacticoid copepod (e.g., *Amphiascus tenuiremis*, *Nitocra spinipes*, *Acartia tonsa,* or *Tisbe battagliai*) development and reproduction test to the second tier of their Endocrine Disruption Screening Program (Verslycke et al., 2004; OECD, 2013b, 2013a; Ottinger et al., 2016).

Several studies have investigated endocrine disruption in a variety of invertebrate orders exposed to wastewater (Gross et al., 2001; Quinn et al., 2004; Schirling et al., 2005; Gust et al., 2010; Plahuta et al., 2017). However, the vast majority of studies have involved the phyla Mollusca and Arthropoda. For example, Gust et al. (2010) observed a significant decrease in reproduction and changes in the number of embryos with shells of New Zealand mudsnails (*Potamopyrgus antipodarum*) caged downstream of wastewater discharges. Plahuta et al. (2017) observed a significant decrease in molting frequency for the aquatic isopod *Asellus aquaticus* exposed to treated wastewater. Molting in arthropods is controlled by another group of hormones that have been well studied in insects, ecdysteroids (Lafont, 2000). Despite the recent addition of *in vivo* assays into regulatory frameworks for assessing endocrine disruption, there remains considerable research to be conducted to understand the basic endocrinology of invertebrates (Ford and Leblanc, 2020). The notable hurdle to understanding the basic endocrinology of invertebrates starts with the diversity of this group of organisms. Invertebrates represent all animal species except those in the sub-phylum Vertebrata. While important, a detailed understanding of the endocrinology of the different phyla is not necessary to determine whether chemicals interfere with the endocrine-mediated processes of growth,

Table 1
Bioassays used in existing framework/guidelines to characterize single compound EDCs: US-EDSP (EDSTAC, 1998), Japan MoE's (Japan-MOE, 2010, 2016), China's MoE (UN, 2017), OECD (OECD, 2018), European Union (Andersson et al., 2018).

Tier	US-EDSP	Japan MoE's EXTEND	China's MoE	OECD	European Union
Number of levels/ Tier	• 2 (+prioritization step)	• 2 (+prioritization step)	• 2 (+prioritization step)	• 5 (3 sufficient for decision)	• 3 minimum
Axis	• EATS	• EATSJhEc	• EATS	• EATSJhEc	• EATS
Tier 1/Level 2					
Characteristics	• *In vitro* and *in vivo* • Mammal and non-mammal vertebrates	• *In vitro* and *in vivo* • Non-mammal vertebrates and invertebrates	• *In vitro* • Mammals	• *In vitro* • Mammal and non-mammal vertebrates	• *In vitro* • Mammal and non-mammal vertebrates
Bioassays					
Estrogen (E)	• ER Binding, rat (890.1250) • ERTA, human (HeLa-9903, 890.1300) • Uterotrophic assay, Rat (890.1600)	• ERTA, medakas receptor α	• ERTA, human receptor	• ERTA, human receptor (HeLa-9903, VM7Luc4E2, ERα-CALUX®, TG 455) • ER Binding, human receptor (TG 493)	• ERTA (TG 455, 890.1300)
Androgen (A)	• AR Binding, rat (890.1150) • Hershberger assay, rat (890.1400)	• ARTA, medaka receptor β (method for antagonist u.d.) • Medaka anti-androgen detection assay (u.d.)		• ARTA (AR-EcoScreen, draft: AR-CALUX®, 22Rv1/MMTC-GR-KO, TG 458) • AR Binding Assay (890.1150, TG u.d.)	• ARTA (TG 458)
Steroidogenesis (S)	• H295R Steroidogenesis Assay, human (890.1550) • Aromatase assay, human (Recombinant, 890.1200)		• H295R Steroidogenesis Assay, human	• H295R Steroidogenesis Assay, human (TG 456) • Aromatase assay, human (890.1200, TG u.d.)	• H295R Steroidogenesis Assay, human (TG 456, 890.1550) • Aromatase assay, human (890.1200)
General reproduction (EAS)	• FSTRA, Fathead Minnow (890.1350) • Female (890.1450) and Male (890.1500) Rat Pubertal assay	• FSTRA (OECD TG229) or 21 day fish assay (OECD TG230), Medaka			
Thyroid (T)	• AMA, *X. laevis* (890.1100)	• TRTA, *X. tropicalis* receptor β (method for antagonist u.d.) • XETA (under consideration)			
Juvenile hormone		• JhRTA, Daphnid receptor (u.d.) • Daphnid juvenile hormone screening assay *in vivo* (u.d)			
Ecdysone		• EcRTA, Daphnid receptor • Daphnid ecdysone screening assay *in vivo* (u.d)			
Tier 2/Level 3					
Characteristics	• *In vivo* • Non-mammal vertebrates	• *In vivo* • Non-mammal vertebrates and invertebrates	• *In vivo* • Mammals	• *In vivo* • Vertebrates and invertebrates	• *In vivo* • Mammal and non-mammal vertebrates
Bioassays					
Reproduction	• Avian Two-Generation Toxicity Test in the Japanese Quail (890.2100) • MEOGRT (890.2200)	• MEOGRT (OECD TG 240)	• Hershberger assay, rat • Uterotrophic assay, rat • Female and Male Rat Pubertal assay • Two-generation *in vivo* testing	• FSTRA (TG 229) or 21 Day Fish Assay (TG 230), fathead minnow, medakas and zebrafish • EASZY assay (TG 250) • RADAR assay (u.d.) • REACTIV (u.d.) • AFSS (u.d.) • JMASA (u.d.) • Uterotrophic assay, rat (TG 440) • Hershberger assay, rat (TG 441)	• FSTRA (TG 229) • Uterotrophic assay, rat (TG 440) • Hershberger assay, rat (TG 441)
Thyroid	• LAGDA (890.2300)	• AMA (OECD TG 231) • LAGDA (OECD TG 241)	• Female and Male Rat Pubertal assay	• AMA (TG 231) • XETA (TG 248)	• AMA (TG 231)
Juvenile hormone and Ecdysone		• Daphnid Reproduction Test (OECD TG 211) • Daphnid Multi-generational test (u.d.)		• Short-Term Juvenile Hormone Activity Screening Assay Using *Daphnia magna* (u.d.)	

AFSS: Androgenised Female Stickleback Screen; AMA: Amphibian Metamorphosis Assay; EASZY: Detection of Substances Acting Through Estrogen Receptors Using Transgenic cyp19a1b-GFP Zebrafish Embryos; EcRTA: ecdysone RTA; FSTRA: Fish Short-Term Reproduction Assay; u.d.: under development; JhRTA: Juvenile hormone RTA; JMASA: Juvenile Medaka Anti-Androgen Screening Assay; LAGDA: Larval Amphibian Growth and Reproduction Assay; MEOGRT: Medaka Extended One Generation Reproduction Test; RADAR: Rapid Androgen Disruption Adverse Outcome Reporter; REACTIV: Rapid Estrogen Activity Tests *in vivo*; XETA: *Xenopus* Eleutheroembryonic Thyroid signaling Assay.

Table 2
Bioassays included in the OECD conceptual framework (OECD, 2018).

Level	Mammalian	Non-mammalian
4	TG 407: Repeated Dose 28-Day Oral Toxicity Study in Rodents TG 408: Repeated Dose 90-Day Oral Toxicity Study TG 451–3: Combined Chronic Toxicity/Carcinogenicity Studies TG 421: Reproduction/ Developmental Toxicity Screening Test TG 422: Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test TG 414: Prenatal Developmental Toxicity Study TG 426: Developmental Neurotoxicity Study TG 410: Repeated Dose Dermal Toxicity: 21/28-Day Study TG 411: Subchronic Dermal Toxicity: 90-Day Study TG 412 28-Day (Subacute) Inhalation Toxicity Study TG 413: Subchronic Inhalation Toxicity: 90-Day Study TG 409: Repeated dose 90-Day Oral Toxicity Study in Non-Rodents Pubertal Development and Thyroid Function Assay in Peripubertal Male (US EPA OPPTS 890.1500) and Female Rats (US EPA OPPTS 890.1450) (u.d.)	TG 242: *Potamopyrgus antipodarum* Reproduction Test (OECD, 2016c) TG 243: *Lymnaea stagnalis* Reproduction Test (OECD, 2016b) TG 218–219: Chironomid Toxicity Test (OECD, 2004b, 2004a) TG 211: *Daphnia* Reproduction Test (with Male Induction) (OECD, 2012a) TG 210: Fish Early Life Stage Toxicity Test TG 234: Fish Sexual Development Test (FSDT) TG 241: Larval Amphibian Growth and Development Assay (LAGDA) TG 206: Avian Reproduction Test New Guidance Document on Harpacticoid Copepod Development and Reproduction Test with *Amphiascus* (OECD GD 201) (draft u.d.) (OECD, 2013b)
5	TG 443: Extended One-Generation Reproductive Toxicity Study (EOGRTS) TG 416: Two-Generation Reproduction Toxicity Study	TG 233: Sediment Water Chironomid Life Cycle Toxicity Test (OECD, 2010) TG 240: Medaka Extended One-Generation Reproduction Test (MEOGRT) *Daphnia* Multigeneration Test for Assessment of EDCs (draft OECD TG) Mysid two generation test (draft test guideline) (OECD, 2013a) Fish Life cycle Toxicity Test (FLCTT) (US EPA OPPTS 850.1500) (u.d.) Zebrafish Extended One-Generation Reproduction Test (ZEOGRT) (draft OECD TG) (u.d.) Avian Two-Generation Toxicity Test in the Japanese Quail (ATGT) (US EPA OCSPP 890.2100/740-C-15-003) (u.d.)

reproduction, and behavior. However, to effectively assess endocrine-mediated effects on invertebrates as a group, species chosen for bioassays should represent the major invertebrate taxa in freshwater (Annelida, Crustacea, Insecta, Mollusca) and marine ecosystems (Annelida, Coelenterata, Crustacea, Echinodermata, and Mollusca). This represents an area that requires further research, particularly for marine taxa.

2.2. Amphibian in vivo bioassays

Anurans, in particular, have been used to identify toxicity of aquatic contaminants for decades based on their susceptibility to waterborne contaminants via dermal, respiratory, and dietary routes of exposure, and more recently, have become the primary model organism to detect contaminants that disrupt the hypothalamic-pituitary-thyroid (HPT) endocrine axis. The OECD, US, Japan, and the EU have standardized *in vivo* amphibian bioassays within their EDC testing strategies (Table 1). Amphibians are useful models of thyroid function, because the amphibian metamorphic process is controlled by THs and mimics perinatal development in birds and mammals (Brown and Cai, 2007; Fini et al., 2012; Sachs and Buchholz, 2017; Mengeling et al., 2018). Specifically, the action of triiodothyronine (T3) in anuran metamorphosis is widely accepted as the initiator of frog metamorphosis and orchestrator of developmental processes, including tail and gill resorption, fore- and hindlimb growth, remodeling of the intestines, central nervous system, respiratory system, cranial cartilages and skin (Dodd and Dodd, 1976). Thus, abnormal changes in these developmental processes during metamorphosis or in *in vitro* cultures measuring sub-components of the HPT reflect a disturbance in the HPT axis.

Currently, three bioassays have been validated by the OECD (Table 1, Table 2) and have been recently compared in a review by Couderq et al. (2020). Each bioassay uses the African clawed frog, *Xenopus laevis*, a species that exhibits a fully aquatic lifecycle and is native to regions of Africa (IUCN, 2020). The first bioassay is the Amphibian metamorphosis assay (AMA; Conceptual framework Level 3, OECD TG 231; Fig. 1B). Of the various endpoints measured during AMA (see Fig. 1B), the thyroid gland histology is the most sensitive endpoint to detect chemicals inhibiting TH-mediated actions, while the determination of the developmental stage and hindlimb length are more sensitive to evaluate EDCs inducing TH-mediated actions (Pickford, 2010; Dang, 2019). The second bioassay is the Larval amphibian growth and development assay (LAGDA; Conceptual framework Level 4, OECD TG 241; Fig. 1C), which assesses and can validate HPT axis disruption using the same endpoints as in the AMA, but it also includes gonad histopathological observations and genotypic sex identification. The last standardized bioassay is the *Xenopus* eleutheroembryonic thyroid signaling assay (XETA; Conceptual framework Level 3, OECD TG 248; Fig. 1D). This transgenic *X. laevis* has been genetically modified to contain the Green Fluorescent Protein (GFP) under the control of a portion of the TH/bZIP, thus has the capacity to detect EDCs inducing or inhibiting TH-mediated GFP gene transcription (Furlow and Brown, 1999; Turque et al., 2005; Fini et al., 2007).

Although few studies using standardized amphibian bioassays have examined the impacts of wastewater effluents/complex mixtures of chemicals, the bioassay experimental designs to date appear to be capable of capturing changes *in vivo*. For example, Pablos et al. (2020) studied reclaimed water collected from a wastewater treatment plant that underwent tertiary treatment that still had measurable pharmaceuticals (carbamazepine) and personal care products (e.g. parabens) with the AMA. This study revealed that despite tertiary treatment, the reclaimed water caused stimulation of the thyroid gland indicated by accelerated development and altered thyroid gland histopathology (Pablos et al., 2020). Similarly, reports of the XETA detecting some thyroid disrupting activity in complex water samples (i.e., surface waters, Spirhanzlova et al., 2019; surface waters and treated wastewater extracts, Leusch et al., 2018b; untreated wastewater influents, Castillo et al., 2013), suggest XETA may be a sensitive and suitable assay to detect thyroid activity in environmental water samples as well.

Although the use of anuran models to detect disruption of other endocrine axes in standardized bioassays is not extensive with only one other standardized assay capable of examining impacts on the hypothalamic-pituitary gonad (HPG) axis, several lab-based studies report toxicant effects indicative of perturbation of the HPG axis using amphibian models (Abdalla et al., 2013; Hirakawa et al., 2013). Therefore, prior to the adoption of one or multiple amphibian bioassays into EDC testing regimes for monitoring complex environmental water samples, comparisons of the sensitivity and specificity of the aforementioned standardized assays for detecting HPT and/or HPG effects must be performed.

2.2. Fish in vivo bioassays

A significant advance in *in vivo* toxicity testing has been the standardization and validation of fish-based bioassays to screen chemicals in

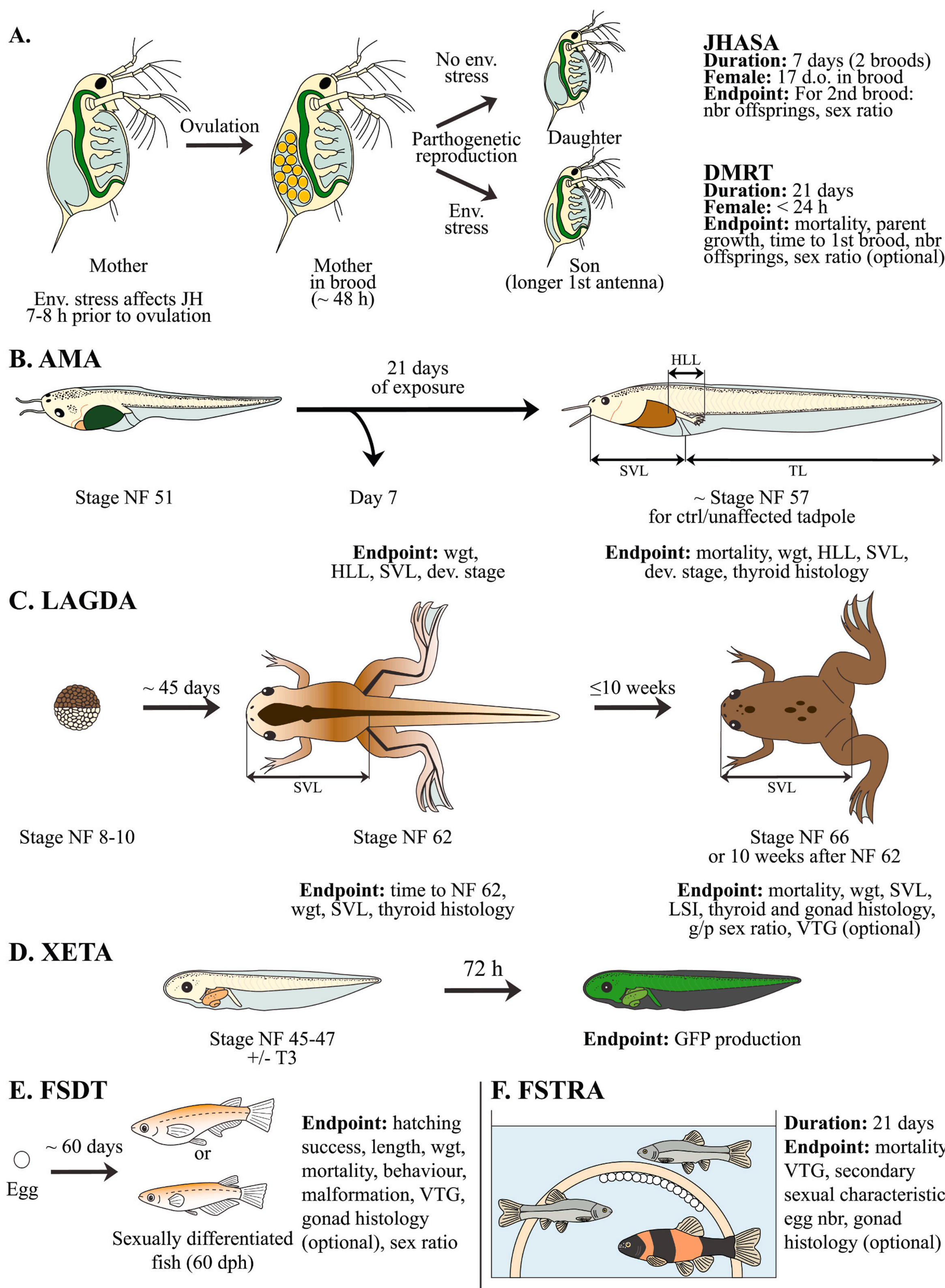

Fig. 1. Validated *in vivo* bioassays for non-mammalian species. (A) Comparison of *Daphnia magna* bioassay: Short-term Juvenile Hormone Activity Screening Assay using *Daphnia magna* (JHASA, OECD draft) and *Daphnia magna* reproduction test (DMRT, OECD TG 211); (B) Amphibian Metamorphosis Assay (AMA, OECD TG 231); (C) Larval amphibian growth and development assay (LAGDA, OECD TG 241); (D) *Xenopus* Eleutheroembryonic Thyroid signaling Assay (XETA, OECD TG 248); (E) Fish sexual development test (FSDT, OECD TG 234) using medaka here as an example, but it can also be conducted with other small-bodied fish; (F) Fish Short Term Reproduction Assay (FSTRA, OECD TG 229) using fathead minnow, but other small fish (e.g., medaka and zebrafish) can substitute. dev.: development; g/p sex ratio: genetic/phenotypic sex ratio; HLL: hind limb length; LSI: liver somatic index; nbr: number; SVL: snout to vent length; TL: tail length; VTG: vitellogenin level in plasma or liver; wgt: weight.

the late 2000s (approx. 2009–2012 for many of the established small fish OECD tests). Small-bodied fish, such as the fathead minnow (*Pimephales promelas*), zebrafish (*Danio rerio*), Chinese rare minnow (*Rhynchocypris oxycephalus*), Japanese medaka (*Oryzias latipes*), and mummichog (*Fundulus heteroclitus*), have significant advantages as animal models for EDC research over mammalian models. Some of these advantages include high fecundity, rapid embryonic development and generation turnover, and low cost of husbandry. These characteristics increase the experimental power of utilizing small-bodied fish in toxicity testing and there are several examples using small fish to assess estrogen receptor and androgen receptor agonists and antagonists (Martinović et al., 2008; Dang et al., 2011; Ankley and Jensen, 2014; Spirhanzlova et al., 2017), as well as those chemicals that disrupt steroidogenesis, for example through aromatase inhibition (Villeneuve et al., 2007; Muth-Köhne et al., 2016).

The OECD has harmonized fish tests designed to detect endocrine perturbations during sexual development and reproduction. The Fish Sexual Development Test (FSDT, OECD test guideline (TG) 234, OECD, 2011b, Fig. 1E) is designed to detect chemicals that act as (anti-) estrogens, (anti-) androgens, or those that impair steroidogenesis. The fathead minnow, a fish species that shows a wide distribution across North America, is the recommended OECD fish species of choice for the 21-day fish short-term reproduction assay (FSTRA, OECD TG 229, OECD, 2012b, Fig. 1F); however, other small-bodied fish can substitute (i.e., zebrafish and medaka). Comparable biological responses among the three species for aromatase inhibition have been noted in several studies (Celander et al., 2011). Following exposure to the test chemical at different concentrations in a flow-through system, both vitellogenin (VTG) and secondary sex characteristics (i.e., nuptial tubercles) are measured to determine the potential for EDC activity. VTG, the egg yolk protein precursor for female egg production, can be measured in plasma and is a useful biochemical endpoint to evaluate endocrine disruption for both males and females. The presence of VTG in males comes from exposure to estrogen-like compounds in the water. However, it is pointed out that there can be a very low, basal level of VTG in male plasma, as males also synthesize endogenous 17β-estradiol (E2). Reduced VTG concentration in females is an indicator of anti-estrogenic activity or of aromatase inhibition of the production of endogenous E2. The display of nuptial tubercles is also a useful biomarker. Males normally display this biomarker, while females do not. However, if females are exposed to androgens, they will also display nuptial tubercles. These two aforementioned biomarkers are among several responses that are indicative and sensitive to EDCs (Ankley and Johnson, 2004). Cumulative egg production and histopathology of the gonads are also useful indicators for estrogenic/androgenic agonism or antagonism (Ankley et al., 2003; Armstrong et al., 2016). Androgen-dependent biomarkers have also been identified in other species of fish such as spiggin production by male three-spined stickleback (*Gasterosteus aculeatus*) during breeding season (Katsiadaki et al., 2002) and the anal fin development in male mosquitofish (*Gambusia holbrooki* and *G. affinis*, Brockmeier et al., 2013).

Fish EDC studies have generated strong support to adverse outcome pathways (AOP) for estrogens (for example AOP #30), androgens (AOP #23), and aromatase inhibition (AOP #25). These AOPs are well described and constructed, and the molecular initiating events are clearly defined between chemical toxicant and receptor (or enzyme) and key events are predictable and measurable at the level of transcript and protein and whole organism (Martinović-Weigelt et al., 2017). For example, chemicals that act as androgens will significantly reduce VTG production from the liver of female fish, subsequently leading to underdeveloped ovaries and lower individual fecundity. Currently, one limitation for the EATS pathway is the lack of OECD guidelines for *in vivo* fish assays for thyroid hormone (TH) disruptors (Villeneuve, 2016, 2018); this hormone axis has been evaluated in predominantly amphibian-based assays, but there are several studies in fish that demonstrate the thyroid hormone axis is perturbed by different environmental contaminants (Lema et al., 2008; Chan and Chan, 2012; Pelayo et al., 2012; Dong et al., 2014; Thambirajah et al. this issue). Recently, efforts are underway to close this knowledge gap in fish (Knapen et al., 2020). In addition, undoubtedly new fish assays or additional measures within existing fish assays will be required to test for EDCs that occur via the non-EATS pathway - these include hormones that act as glucocorticoids, mineralocorticoids, neurohormones, and hormones associated with the regulation of feeding and lipid metabolism among others (for details, see Martyniuk et al. this issue). Additional work is also needed in the area of complex mixtures and effluent containing different chemicals with multiple MOAs, such as those which have mixtures of estrogens with anti-androgens and anti-estrogens, to discern the additive versus synergetic or antagonist effects in biological response. Laboratory studies with mixtures of (anti-) estrogens and (anti-) androgens show complex patterns of gene expression responses that suggest additional modes of action, for example from membrane receptors (Garcia-Reyero et al., 2009a, 2009b, 2018). While effluents from wastewater treatment plants are thought to be primarily estrogenic for wild fish captured downstream (Folmar et al., 1996, 2001a, 2001b; González et al., 2020); not all wastewater effluents are estrogenic and some in fact may be predominantly androgenic (Brockmeier et al., 2014). It is further possible that a complex effluent has activities that cancel each other out and to better understand the complexity, it is possible that fractionation and retesting are required. Another limitation is that the fish models used in the reproductive assays are semi-synchronous spawners, and fish that have alternative reproductive strategies, such as seasonal reproduction, are lacking in OECD validated protocols for toxicity testing.

2.4. Rodent in vivo bioassays

The use of rodents as an animal model for extrapolating to other mammalian wildlife and humans exposed to EDCs via water/food, air, soil, etc. either through oral, dermal or respiratory routes of exposure are prevalent in several standardized (Table 1, Table 2) and non-standardized tests (Marty et al., 2011). A wide range of *in vivo* standardized tests, including short term, chronic, and multi-generation tests, are available using rodent models for EDC testing *in vivo* (i.e. OECD level 3 to 5), with thorough coverage of detecting EDCs interfering with EATS-mediated pathways (or HPG and HPT endocrine axes). Currently, with the numerous life stages and exposure durations covered in the standardized *in vivo* rodent based tests particularly in the OECD conceptual framework (OECD, 2018), it is likely that these existing methods are capable of detecting and characterizing apical adverse effects due to EDC or non-endocrine etiologies in wastewater mixtures mainly by using oral or dermal exposure scenarios. Though, the addition of non-EATS biomarkers, such as associated with the HPA, somatotropic axis, retinoid signaling, peroxisome proliferators, vitamin D, gluco- and mineralo-corticoids would enhance modality detections in these bioassays (reviewed in Martyniuk et al. this issue). It is important to note however that the regulatory community is moving from *in vivo*-based bioassays to *in vitro*-based bioassays (Barton-Maclaren et al. this issue), and as such, the *in vivo* mammalian-based tests will not be further described here. For more information on mammalian testing, one should consult the OECD guideline (2018), which provides recommendations on the evaluation of chemicals for endocrine disruption using bioassays.

3. Commonly used *in vitro* bioassays for EDC screening

As demonstrated by the US-EPA's Endocrine Disruptor Screening Program (EDSP), the identification of EDCs with an approach that includes multiple *in vivo* bioassays (see Table 1) can be long and tedious. In 2009, the EDSP started evaluating a list of 52 chemicals in their Tier 1 of screening and published their first results in 2015 (EPA-EDSP, 2015). It could take even longer to obtain the results for 18 of those chemicals that were passed to the Tier 2 for further testing. This is concerning as it

is currently estimated that 40,000 to 60,000 industrial chemicals are being produced globally (ICCA and UNEP, 2019) and that at least 686 chemicals are potential EDCs based on an extensive literature review of only human and rodent studies (Karthikeyan et al., 2019). Testing all those chemicals with *in vivo* bioassays would require an unacceptable number of experimental animals given ethical, financial, and time limitations. Therefore, *in vitro* bioassays have become an essential first approach to the identification and evaluation of potential EDCs as demonstrated by the Toxicology in the 21st Century program (Tox21, National Research Council, 2007) and the Toxicity Forecaster (ToxCast, Dix et al., 2007), and are commonly used to evaluate the potential of endocrine activity of water samples in research. *In vitro* bioassays (cell-free and cell-based) typically evaluate one of the following mechanisms of action used by EDCs: (1) mimic/inhibit the binding of a hormone to its receptor or the transactivation of the receptor; (2) disrupt the synthesis or metabolism of the hormone or of its receptor; or (3) affect the transport of the hormone to its site of action.

3.1. Receptor transactivation assays

Various methods exist to evaluate a hormone binding to a hormone receptor, such as ligand binding assays, dimerization assays of nuclear receptors, and hormone-dependent proliferation assays (Soto et al., 1995; Judson et al., 2010, 2017; Knudsen et al., 2011). However, the most widely used method for measuring EDCs in environmental samples are the receptor transactivation assays (RTAs). In these bioassays, the binding of the ligand to the receptor is quantified by the transcription and translation of a protein under the control of a response element for a hormone of interest. The protein production is then quantified by an enzymatic reaction generating bioluminescence (luciferase), color (β-galactosidase), or fluorescence (β-lactamase) (Fig. 2A). The advantage of RTAs over other binding assays is that RTAs take into account both the receptor binding and the activation of transcription via the receptor. In contrast to proliferation assays, RTAs ensure specificity (ICCVAM, 2003). A variety of RTA models are listed in Table 3. RTAs range from recombinant yeast-cells expressing human receptors, to modified *X. laevis*, rat, fish and human cell lines. Considerations when selecting the most appropriate RTA model include: (1) the choice of species for the receptor, (2) the choice of cell line, and (3) the method sensitivity and accuracy.

As observed in Table 3, most methods utilize human receptors, except for the XL58-TRE-luc cells with the receptor of *X. laevis* (Sugiyama et al., 2005), the GH3-TRE-luc cells with the rat receptor (Freitas et al., 2011) and RTG-2-rtERα cells with the rainbow trout receptor (Ackermann et al., 2002). Other RTA assays using non-mammalian receptors exist and have primarily been developed by researchers in Japan (presented in Table 1). Commercially, there is the INDIGO Biosciences, Inc. that offers cell-luciferase kits for the human, rat and zebrafish receptors (https://indigobiosciences.com), but these assays are only starting to be used for the characterization of water samples (Castillo Meza et al., 2020). The choice of species for the receptor is important as it can modulate the sensitivity of the RTA. Nuclear receptors (NR) have evolved from the same ancestral gene through duplication events predating 400 million years ago and show high

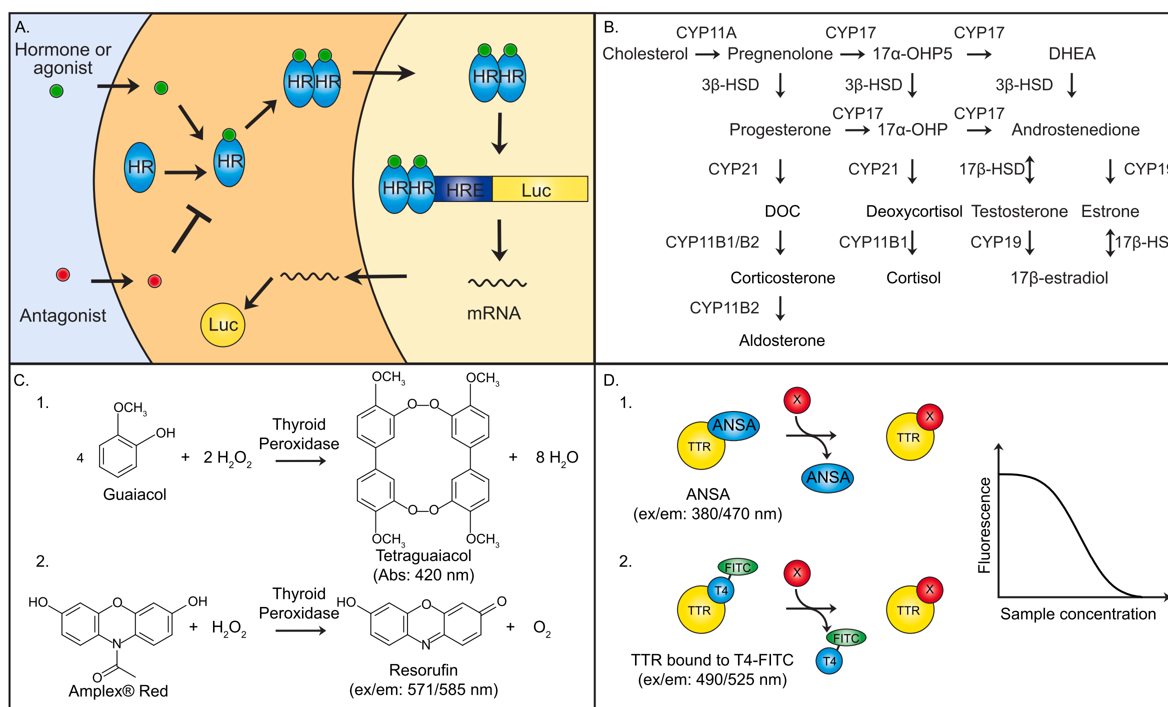

Fig. 2. *In vitro* bioassays. (A) (A) Receptor Transactivation Assay (RTA) using luciferase. In an RTA, the reference hormone or an agonist binds to the HR, which dimerizes. After dimerization, HR can translocate to the nucleus to activate the transcription of the luciferase (Luc) gene via the hormone response element (HRE). It is important to note that the species chosen for the receptor and the cells can vary and that the luciferase system can be replaced by one using the β-galactosidase or the β-lactamase; (B) the steroidogenesis pathways found in H295R cells, which are undifferentiated human fetal adrenal cells that can synthesize most steroids. The quantification of steroid production such as estrogen and testosterone can help determine if the steroidogenesis pathway is affected by compounds or mixtures (US-EPA, 2009a; OECD, 2011a; CYP: cytochrome P450, DHEA: dehydroepiandrosterone, DOC: 11-deoxycorticosterone, HSD: hydroxysteroid hydrogenase 17α-OHP5: 17α-OH Pregnenolone, 17α-OHP: 17α-OH Progesterone); (C) thyroid peroxidase (TPO) assay reaction using (C1) guaiacol, which generates the yellow colored tetraguaiacol (Chang and Doerge, 2000; Paul et al., 2013) and (C2) Amplex Red®, which generates the fluorescent molecule resorufin (Paul et al., 2014); (D) Transthyretin (TTR) displacement assay using (D1) ANSA (8-anilino-1-naphthalenesulfonic acid ammonium salt) and (D2) T4 tagged with FITC with X representing a xenobiotic which has affinity for TTR. In the ANSA method (Ferguson et al., 1975; Nilsson and Peterson, 1975 Montaño et al., 2012), the displacement of ANSA is quantified as ANSA is a ligand for TTR. For the FITC method (Ren and Guo, 2012; Ouyang et al., 2017), the displacement of T4 tagged with FITC is measured. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
In vitro methods to measure endocrine activity.

Endocrine axis	Method Name	Method	Limit of detection	Human EBT (Drinking and recycled water)	Ecological EBT (Surface water)	Reference
Estrogen	YES	Yeast strain with stably expressed human ER and ERE-lac-Z plasmid	<2 ng/L E2	12 ng/L EEQ [b]	0.2–0.4 ng/L EEQ [g]	Routledge and Sumpter (1996)
	ERα-CALUX® [a]	T47D or U2-OS cells stably transfected with pERE-TATA-Luc and pSG5-neo-hERα	T47D: 0.5 pM E2; U2OS: 0.8 pM E2	0.2 ng/L EEQ [b] 3.8 ng/L EEQ [e]	0.5 ng/L EEQ [c] 0.10 ng/L EEQ [d] 0.28 ng/L EEQ [f] 0.2–0.4 ng/L EEQ [g]	T47D: Legler et al. (1999); U2-OS: Sonneveld et al. (2005)
	ERβ-CALUX®	U2-OS cells stably transfected with pERE-TATA-Luc and pSG5-neo-hERβ	n.d.			Sonneveld et al. (2005)
	T47D-KBluc	T47D cells (ATCC #HTB133) stably transfected with pGL2.TATA.Inr.luc. neo and express naturally ERα, ERβ and GR	0.1–1 pM E2 (no clear calculation)			Wilson et al. (2004)
	MELN	MCF-7 cells stably transfected ERE-βGlob-Luc-SVNeo plasmid and express naturally ERα	1–10 pM E2 (no clear calculation)		0.37 ng/L EEQ [d]	Balaguer et al. (1999)
	MVLN	MCF-7 cells transfected stably with pVit-tk-Luc and express naturally ERα	n.d.		0.1–0.3 ng/L EEQ [g]	Demirpence et al. (1993)
	ERα-GeneBLAzer	HEK293T cells stably transfected with recombinant hERα and GeneBLAzer β-lactamase (BLA)	EC50 E2: < 1 nM	1.8 ng/L EEQ [b]	0.34 ng/L EEQ [d] 0.24 ng/L EEQ [g]	Invitrogen, Huang et al., 2011
	RTG-2-rtERα	RTG-2 cells (rainbow trout (*Oncorhynchus mykiss*) gonad cell) stably transfected with pCI-neo-rtERα and pERE-TK-luc	EC50 E2: 0.33 nM			Ackermann et al. (2002)
	Hela-9903[a]	HeLa cells stably transfected with ERE-MT-TATA-luc and hERα	PC10 and 50 E2: <10 nM	0.6 ng/L EEQ [b]	1.0 ng/L EEQ [d] 0.18 ng/L EEQ [f]	US-EPA, 2009c; OECD, 2016a
	E-SCREEN	Proliferation of MCF-7 cells	n.d.	0.9 ng/L EEQ [b]	0.1–0.3 ng/L EEQ [g]	Soto et al. (1995)
Androgen	YAS	Yeast strain with stably expressed human AR and the gene of the β-galactosidase under an ARE	EC50 DHT: 3.5 nM			Gaido et al. (1997)
	AR-CALUX® [a]	U2-OS cells stably transfected with HRE-TATA-Luc and pSG5-neo-hAR	3.6 pM DHT	11 ng/L DHT EQ [e]	For anti-AR: 25 μg/L Flutamide EQ [c] 14 μg/L Flutamide EQ [d]	Sonneveld et al. (2005)
	AR-LUX	T47D cells stably transfected with pPB-ARE2-tataluc expressing naturally AR	46 pM DHT			Blankvoort et al. (2001)
	MDA-kb2	MDA-MB-453 cells (ATCC #HTB131) stably transfected with pMMTV.luc. neo and express naturally AR and GR	0.1 nM DHT		For anti-AR: 3.5 μg/L Flutamide EQ [d]	Wilson et al. (2002)
	AR-GeneBLAzer	HEK293T cells stably transfected with recombinant hAR and GeneBLAzer β-lactamase (BLA)	EC50 17α-OH progesterone: 2 nM	14 ng/L Testosterone EQ [b]	For anti-AR: 3.3 μg/L Flutamide EQ [d]	Invitrogen, Huang et al., 2011
Thyroid	TRβ-CALUX ®	U2-OS cells stably transfected with pGL3-2xTRE-Luc and pSG5-neo-hTRβ	13.2 pM T3			Collet et al. (2019)
	TTR-TRβ-CALUX ®	Quantification of T4 bound to TTR with TRβ-CALUX ®	n.d.			Collet et al. (2019)
	TRβ-Geneblazer	HEK293T cells stably transfected with recombinant hTR TRβ-Geneblazer and GeneBLAzer β-lactamase (BLA)	EC50 T3: <1 nM			Huang et al. (2011)
	GH3.TRE-Luc	GH3 cells (rat) expressing stably pGL4CP-SV40-2xtaDR4 which has a 2xTRE and a luciferase reporter, and express naturally TRβ and TRβ	n.d. (~1–10 pM T3)		For anti-TR:0.60 μg/L Bisphenol A EQ [d]	Freitas et al. (2011)
	TPO inhibition assay	Quantification of H_2O_2 by guaiacol oxidation or Amplex UltraRed in rat thyroid microsome	n.d.			Paul et al. (2014)
	TTR displacement assays with ANSA	Evaluation of the displacement of bound ANSA on TTR by fluorescence	IC50 T4: 26 nM			Montaño et al. (2012)
	TTR and TBG displacement assay with FITC	Evaluation of displacement of T4 tagged with fluorescein on TTR or TBG	TTR: IC50 T4: 26 nM; TBG: IC50 T4 : 85 nM		0.49 μg/L Thyroxine EQ [d]	Ren and Guo (2012)
	hTR-GRIP1	Yeast transformed with pGBT9-hTRβ and pGAD424-GRIP1/FL	10 pM T3 and T4 (based on range, limit of detection not calculated)			Li et al. (2008)
	XL58-TRE-luc	XL58 cells transduced with LV-TRE-Luc and expressing naturally TRβ	10 pM T3			Sugiyama et al. (2005)
Steroidogenesis	H295R[a]	Quantification of steroids after exposure of H295R cells which are undifferentiated human fetal adrenal cells				US-EPA, 2009a; OECD, 2011a
	Aromatase					US-EPA (2009b)

(*continued on next page*)

Table 3 (*continued*)

Endocrine axis	Method Name	Method	Limit of detection	Human EBT (Drinking and recycled water)	Ecological EBT (Surface water)	Reference
		Quantification of ^{3}H-androstenedione in estrone by the aromatase from microsome				
Progestins	PR-CALUX ®	U2-OS cells stably transfected with HRE-TATA-Luc and pSG5-neo-hPR	1.3 pM Org 2058	724 ng/L Levonorgestrel EQ [e]	For anti-PR: 1967 ng/L Endosulfan EQ [d]	Sonneveld et al. (2005)
Gluco-corticoid	GR- CALUX ®	U2-OS cells stably transfected with HRE-TATA-Luc and pSG5-neo-hGR	0.2 pM Dexamethasone	150 ng/L Dexamethasone EQ [b] 21 ng/L Dexamethasone EQ [e]	100 ng/L Dexamethasone EQ [c]	Sonneveld et al. (2005)
	GR-GeneBLAzer	HEK293T cells stably transfected with recombinant hGR and GeneBLAzer β-lactamase (BLA)	EC50 Dexamethasone: 20 nM			Invitrogen, Huang et al., 2011

CALUX: Chemically Activated Luciferase eXpression, TPO: Thyroid peroxidase, TTR: TH transporter transthyretin.
References for EBT.
[a] Validated by OECD.
[b] Escher et al. (2015).
[c] van der Oost et al. (2017).
[d] Escher et al. (2018a).
[e] Brand et al. (2013).
[f] Brion et al. (2019).
[g] Jarošová et al. (2014a).

conservation from 250 million years onward across vertebrates (Bertrand et al., 2004). Duplications of this ancestral NR gene resulted in protein receptors for THs, a diverse array of steroid hormones, the pregnane- and retinoid-X receptor (PXR and RXR) and the ecdysone receptor (Bertrand et al., 2004; Mitsis et al., 2020), as well as ER isoforms like ERα and ERβ, the two ERβ isoforms in teleost fish and orphan ER-related receptors in different species (Thornton, 2001; Bertrand et al., 2004; Zhang et al., 2012; Asnake et al., 2019). In general, each of the NRs maintained a common structure with a ligand-binding domain (LBD) to bind to the hormone and a DNA-binding domain to activate transcription. While the percentage of sequence identity for a given receptor can vary from 60 to 70% between human and fish (Thornton, 2001; Sabo-Attwood et al., 2004; Asnake et al., 2019), the amino acids constituting the ligand-binding pocket are highly conserved (Zhang et al., 2012; Asnake et al., 2019; Mitsis et al., 2020). Some amino acids and the pocket size can vary across species as shown for ER isoforms of human and fish (Asnake et al., 2019), which can give more flexibility to the pocket to better accommodate ligand binding and increase affinity for the receptor. This variation in affinity can lead to notable difference in responses to ligand such as the complete absence of response to bisphenol A (BPA) from the mouse PXR in contrast to the human one (Barrett, 2012). A ligand-binding study of ERα across five vertebrate species for 34 compounds further illustrates differences in affinity (Matthews et al., 2000). Species specific variability among the ERs and AR in human and species of fish has also been established (Wilson et al., 2007; Blum et al., 2008; Rider et al., 2009; Miyagawa et al., 2014; Pinto et al., 2014; Asnake et al., 2019). The significance of the differences in ligand-receptor binding and affinity in terms of *in vitro* screening methodology is that water samples containing EDCs may pass undetected if the selected receptor binding assay is less sensitive to a compound in the mixture due to lower affinity. Conversely, the detection of an EDC may occur if the receptor binding assay utilized ERs from a more sensitive species. Studies demonstrate that the determination of whether chemicals are estrogenic or androgenic based upon species specific assays is relatively consistent among taxa (Wilson et al., 2007; Blum et al., 2008; Rider et al., 2009; Miyagawa et al., 2014; Pinto et al., 2014; Asnake et al., 2019). Nevertheless, the choice of species will influence the sensitivity of the method of detection to some extent.

Another aspect to consider while choosing an RTA is the type of cell model. The comparison of estrogenic RTAs by Kunz et al. (2017) highlighted the impact of the cell-line on measured activity of the test chemical. For five different RTAs using the human receptor (YES, ERα-CALUX ®, MELN, ERα-GeneBlazer, and T47D-KBluc), the sensitivity for estrogens and estrogenic chemicals (estrone (E1), E2, 17α-ethinylestradiol (EE2), and BPA) varied across cell lines. For example, the E2 equivalence factor (EEF; express in ng/mL) for E1 varied from 0.02 for ERα-CALUX ® and MELN, to 0.26–0.27 for YES and ERα-GeneBlazer, and 0.53 for T47D-KBluc. For EE2, the variability was even more drastic with EEF varying from 0.9 to 6.1 across cell-lines. Comparable variability in EC50 values were obtained by comparing T47D-KBluc cells, the YES and the E-SCREEN (Alvarez et al., 2013). Both studies (Alvarez et al., 2013; Kunz et al., 2017) warns that the high potency of T47D-KBluc cells for E1 could lead to the overestimation of estrogenic activity of a sample rich in E1. However, the reverse could be said about ERα-CALUX ® and MELN, which could underestimate the activity of such samples. A more extensive characterization of the sensitivity of each cell line for well-known estrogenic compounds would help understanding how the evaluation of estrogenic activity in mixtures depends on cell-lines. Moreover, the use of yeast cells is no longer recommended by both the OECD (2018) and the US-EPA (EDSTAC, 1998) for testing EDCs even if the methods are reproducible (Kunz et al., 2017) and available for multiple receptors (Routledge and Sumpter, 1996; Zhen et al., 2009; Allinson et al., 2011; Bovee et al., 2011; Li et al., 2016). There are several reasons for this. First, yeast RTAs have less sensitivity (higher detection limit) than human cell's RTAs (Leusch et al., 2010, 2017; Van Zijl et al., 2017). In addition, the transport through the yeast cell wall can be less efficient than in vertebrate cells (ICCVAM, 2003), which could affect the detection of substances that cannot pass through this barrier. Moreover, yeast cells exhibit differences in metabolism (Odum et al., 1997; Petit et al., 1997; Beresford et al., 2000; ICCVAM, 2003), which can affect the activation of some compounds. One such example is the pesticide methoxychlor, for which the metabolites possess estrogenic activity (Shelby et al., 1996; Beresford et al., 2000). Furthermore, yeast RTAs do not always discriminate between agonist and antagonist substances (Fang et al., 2000; ICCVAM, 2003), which could falsify the detected activity of a sample. Finally, the viability of yeast cells can be impaired by fungicides, those which are known to be EDCs (Petit et al., 1997; Hotchkiss et al., 2003; Skolness et al., 2013; Melvin et al., 2018). Hence, yeast RTAs can be less reliable to identify the activity of compounds and thereby, the activity of complex mixtures such as environmental water samples. Their use should be avoided, but the low cost and low infrastructure requirement associated

with the yeast RTAs make them still an attractive option for some laboratories.

The reproducibility and sensitivity of the bioassay is also an important factor to take into account when choosing an RTA. Kunz et al. (2017) evaluated the intra- and inter-day variability of five ER-RTAs (i.e., YES, ER-CALUX®, T47D-KBluc, MELN, and ER-GeneBlazer) for well-known estrogenic compounds. ER-CALUX® had overall the lowest coefficient of variation (~13%) which never reached more than 30% of variation for all the performed tests in contrast to other bioassays. Mehinto et al. (2015) did an interlaboratory comparison of the GeneBLAzer suite of RTAs with recycled water samples. The coefficient of variation was the lowest for GR-GeneBLAzer (<30%), while ER-GeneBLAzer had the highest variation (>30%). Such studies suggest more work needs to be done to reduce the variation across laboratories, as well as careful standardization of such methods. Other studies have also compared the sensitivity of ER-RTAs. One study compared five estrogenic assays (e.g., YES, ER-CALUX®, T47D-KBluc, MELN, and E-SCREEN) on 16 environmental samples (Leusch et al., 2010). The limit of quantification (LOQ) was similar among bioassays varying from 0.1 to 0.27 ng/L of EEQ except for the YES assay which had a LOQ of 4.25 ng/L. Another study compared ER-CALUX®, ER-GeneBlazer, MELN, hERα-HeLa-9903 and planar-YES on 33 environmental samples (Könemann et al., 2018). The LOQ throughout the samples varied from 0.002 to 0.2 ng/L with no significant differences between methods. A comparison of three ER (ERα-Geneblazer, BG1Luc4E2 and E-SCREEN) and two (anti-)AR (AR-GeneBlazer and MDA-kb2) on three environmental samples showed the detected signal can vary by 1–3 orders of magnitude (König et al., 2017). In another study, an inter-laboratory comparison was made for 12 *in vitro* bioassays for ER, seven for AR, five for GR, two for PR and four for TR using 10 water samples (Escher et al., 2014). The detection of endocrine activity was reproducible among bioassays for ER, GR and (anti-)AR bioassays with 1–2 orders of magnitude between signals, while no activity was detected for PR (<10% of activation), TR and AR. Moreover, the estrogenic equivalency factors (EEF) for known compounds varied over 1–2 orders of magnitude between assays, but also between studies using the same assay (reviewed in Jarošová et al., 2014a). Similar observations were made by the re-calculation of the EC10 of standards of various agonist and antagonist bioassays (11 for AR, six for PR, five for GR, five for TR and eight for ER) (reviewed in Leusch et al., 2017). While all these studies reveal that there is variability across bioassays (1–3 orders of magnitude), they also demonstrate that most bioassays are sensitive enough to detect an activity in any given water sample and that those data are generally reproducible across methods. However, in contrast to ERs, there is a lack of comparison of bioassays for other receptors. As such, it is suggested that efforts be placed into the comparison of RTAs for AR, TR, GR, PR, RXR, and PPAR as activities for these receptor pathways have been detected in water samples (Ishihara et al., 2009; Jugan et al., 2009; Li et al., 2011; Metcalfe et al., 2013; Schriks et al., 2013; Bain et al., 2014; Escher et al., 2014; Leusch et al., 2014b; Roberts et al., 2015; Conley et al., 2017; König et al., 2017; Kassotis et al., 2018; Müller et al., 2018).

To interpret the hormone-like effects of mixtures of chemicals in water, we should also investigate further the effect concentrations of single bioactive compounds. What is striking about hormone RTAs is that there are typically very few, high-potency agonists. Only 6.4% of 7465 chemicals were active in the Tox21 collection of the ERα-GeneBLAzer assay (Escher et al., 2020b), but most had low to medium potency, nowhere near reaching the potency of the natural hormones and EE2. The specificity ratio (Escher et al., 2020b) describes how much more potent a chemical is in reporter gene activation, i.e., its specific mode of action, here activation of the ER, in comparison to the quantitative Structure Activity Relationship (QSAR) for baseline cytotoxicity of the same cell line (Escher et al., 2019). A specificity ratio (SR) of 1 relates to non-specific toxicity (baseline toxicity), $1 < SR \leq 10$ is moderately specific and $SR > 10$ indicates a specific effect (Escher et al., 2020b). The specificity ratio of the high-potency ERα-agonists (E2, estriol (E3), EE2, and 17α-estradiol) ranges from 10^6 to 10^7 (Hashmi et al., 2018; Escher and Neale, 2021), while the specificity ratios of the Tox21 data collection ranged from 0.1 to $10^{4.7}$ only, with the majority smaller than 1000. The specificity ratios of estrogenic agonists are in fact bimodal log-normally distributed (Fig. 3A) with a difference of almost six orders of magnitude between the distributions 50th percentiles (Fig. 3B). Thus, in mixtures as they occur in water samples, the high-potency agonist often dominates despite their low concentrations as it will be discussed in more details in section 5.

3.2. *Analyzing hormone synthesis and transport*

While the analysis of EDC activity with RTAs is highly informative, one limitation is that they measure only how a chemical binds and transactivates a hormone-receptor and does not measure other impacts on the endocrine system. Some EDCs can also impact hormone synthesis as in the case of steroidogenesis which can be impaired by contaminants found in environmental samples (water: Gracia et al., 2008; Maletz et al., 2013; sediment: Kim et al., 2014). The most widely used steroidogenesis bioassay uses the H295R cell line (US-EPA, 2009a; OECD, 2011a) for which a high-throughput protocol was recently developed (Karmaus et al., 2016; Haggard et al., 2018). The H295R cells are undifferentiated human fetal adrenal cells, which are able to synthesize a wide variety of steroids (Fig. 2B). After the exposure of the H295R cells to EDCs, E2 and testosterone levels are usually quantified in the media using LC-MS or ELISA (US-EPA, 2009a; OECD, 2011a), but an extensive list of other steroids can also be quantified (Karmaus et al., 2016; Haggard et al., 2018). Another approach to assess steroidogenesis is to measure each of the specific enzymatic steps, for example the activity of aromatase (US-EPA, 2009b); however, such bioassays may require the use of radioactivity and are focused on single enzymatic conversions. Measuring the effects of EDCs on steroidogenesis is paramount in water samples as demonstrated in a case study on hospital wastewater (Maletz et al., 2013). In this study, ozonation treatment removed estrogenic activity from the water, but it did not mitigate the steroidogenic activity and it could even increase the estrogen production by aromatase, which suggests endocrine active metabolites were created through the process (Maletz et al., 2013). Such results raise the importance to assess the activity of mixtures in different endocrine pathways.

In addition to the reproductive hormones, endocrine disruption through EATs-mediated pathways can occur during the synthesis of THs (Boas et al., 2006; Thambirajah et al. this issue). One of the key steps of TH synthesis known to be inhibited by EDCs is the addition of iodide to tyrosines of the thyroglobulin molecule by the thyroid peroxidase (TPO). TPO can be inhibited by anti-thyroid drugs (e.g., 6-propylthiouracil, methimazole; Sugawara et al., 1999), isoflavones in soybeans (Divi et al., 1997), and pesticides, such as mancozeb (Kackar et al., 1997). The inhibition of TPO is usually measured in rat or porcine thyroid microsomes through its peroxidase activity via the oxidation of guaiacol by colorimetric measurements (Chang and Doerge, 2000; Paul et al., 2013, Fig. 2C1). A high-throughput method using Amplex UltraRed is also used to detect the peroxidase activity of the TPO (Paul et al., 2014, Fig. 2C2). Other EDCs can alter THs through different mechanisms. Both PBDEs and PCBs can bind strongly to transthyretin (TTR) and the thyroxine-binding globulin (TBG) resulting in the displacement of THs impairing their blood transport (Meerts, 2000; Montaño et al., 2012; Ren and Guo, 2012). Various free-cell methods exist to quantify the displacement of T4 on TTR or TBG (radioligand-based: Lans et al., 1993; Hill et al., 2017; plasmon resonance-based: Marchesini et al., 2006; Marchesini et al., 2008); however, the most sensitive and scalable methods are fluorescence-based illustrated in Fig. 2D (ANSA: Ferguson et al., 1975; Nilsson and Peterson, 1975; Montaño et al., 2012; T4-FITC: Ren and Guo, 2012; Ouyang et al., 2017). Both methods were recently compared by Leusch et al. (2018b) for pure compounds and various types of water samples. The comparison revealed that the T4-FITC based assay has a lower detection limit and generates less auto-fluorescence

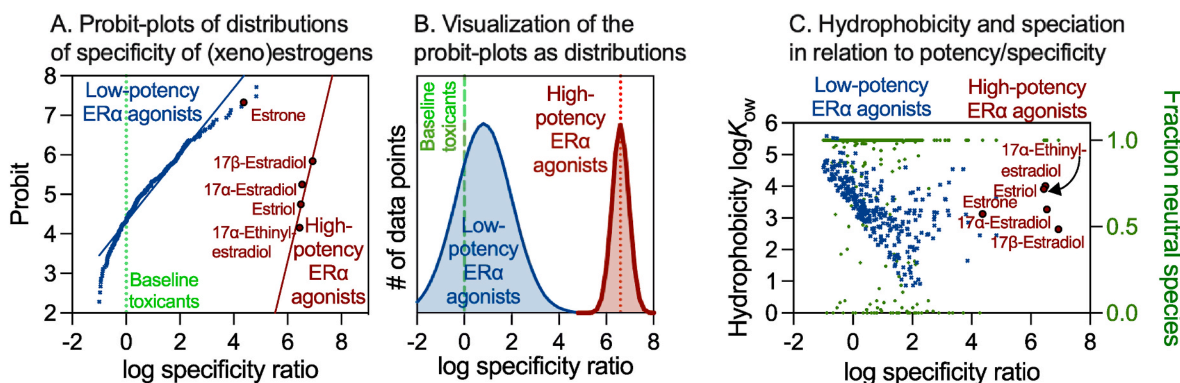

Fig. 3. (A) Probit plots of the specificity ratios (measure of enhanced effect (activation of the ER) over predicted baseline toxicity (cytotoxicity); specificity ratio = IC_{10}(baseline cytotoxicity QSAR)/EC_{10}(activation)) for low-potency ERα-agonists in the ERα-GeneBLAzer assay (blue crosses, data from Tox21, listed in Table S5 of (Escher et al., 2020b)) in comparison to high-potency ERα-agonists (red dot, data from Hashmi et al., 2018. Figure adapted from Escher and Neale, 2021). (B) Visualization of the probit plots as distributions of specificity ratios (using regression parameters from A.) C. Relationship between hydrophobicity expressed as $\log K_{ow}$ and specificity (left y-axis) as well as fraction neutral species (green, e.g. right y-axis) is depicted for high- (red) and low-potency (blue) ERα-agonists in the ERα-GeneBLAzer assay (data from A). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

which would make it more suitable for the analysis of environmental samples. It is also important to note that even though the thyroid axis is arguably one of the more widely studied hormone axis in terms of endocrine disruption, no *in vitro* bioassay has been yet validated by the OECD (OECD, 2018). A group of experts assembled by the OECD (2014) determined that the TPO inhibition assay and TTR/TBG displacement assay were the most relevant to assess toxicity of the thyroid axis, as well as the most "ready to validate" methods for OECD. However, validation of such bioassays was still not done when the revised guidelines were published (OECD, 2018). Other bioassays should also be developed for other mechanisms of thyroid disruption such as iodine uptake by the thyroid gland, deiodination and the binding to thyroid receptors (OECD, 2014, 2018).

3.3. Cell viability

As with any *in vitro* bioassays, it is mandatory to assess the viability of cells. Ignoring cytotoxicity and cell viability can lead to false positive and false negative responses (over-estimating/under-estimating). Analysis of the vast dataset of Tox21 showed that cells near death can activate multiple nonspecific pathways known as the "cytotoxicity burst"(Judson et al., 2016; Escher et al., 2020a). Hence, cell-based data investigating hormone activity could be biased by cell death. For example, one could conclude that some compounds do not act as EDCs as no luminescence is detected, but in reality, cells are non-viable or metabolically inactive. This is of relevance for assays run in antagonistic mode because cytotoxicity is difficult to differentiate from antagonism at low cytotoxicity levels. Of the 62 articles reviewed herein (Table 4, Table 5), only 27 mentioned assessing cell viability, and just 12 of them reported their criteria of bioassay's data acceptability for cell viability. It is recommended that any concentration generating over 10% cytotoxicity should be excluded of any further analysis (Escher et al., 2018b). It is also important to note that like the previously discussed *in vitro* bioassays, cytotoxicity and cell viability assays are also subject to variability. While a comparison of cytotoxicity/viability assays has not been done to our knowledge for water samples, different measures for cytotoxicity, cell viability, metabolic activity, and cell confluence have been compared to each other in various toxicological studies (Putnam et al., 2002; Fotakis and Timbrell, 2006; Pohjala et al., 2007; Bopp and Lettieri, 2008; Zwolak, 2014; Kleijn et al., 2016; Masson-Meyers et al., 2016; Braun et al., 2018; Escher et al., 2019). Based on those studies, the sensitivity of the assays appears to vary depending on cell-line and toxicant. Hence, any assay could under/overestimate cytotoxicity depending on the situation. Thus, the use of multiple assays (at least two ideally) are required to prevent mischaracterizing the toxicity of the sample.

4. Preparation of water sample for bioassay

While *in vivo* bioassays can be conducted directly with the water sample (Thorpe et al., 2009; Werner et al., 2010; Altmann et al., 2012; Castillo et al., 2013; Cavallin et al., 2016; Bakos et al., 2019; Pablos et al., 2020), for some animals, especially embryos, contaminated water can be lethal and therefore, prevent the study of endocrine endpoints. In the case of *in vitro* bioassays, cells require a specific environment provided by the cell culture media (nutrients, salt, pH) to ensure their viability and growth (Waymouth, 1970; Arora, 2013). Hence, some animals and cell cultures cannot be directly exposed to contaminated water samples without affecting their viability. To perform such endocrine bioassays, the water samples need to be prepared adequately. Currently, two main methods are used for water sample preparation. The first type consists in preparing the culture media directly with the water sample to be tested (herein referred to as the Media Method), which has only been used for *in vitro* bioassays to our knowledge. The second and most popular method uses Solid Phase Extraction (SPE) columns (e.g., C18, HLB) to concentrate the water contaminants (herein referred to as the SPE Method). Other methods to prepare samples exist. For example, liquid-liquid extraction can be used (for details see van der Linden et al., 2008; Maletz et al., 2015), but is time consuming and require significant amounts of solvent. Passive sampling can also be used (Alvarez et al., 2013; Creusot et al., 2014; Spirhanzlova et al., 2019; Elkayar et al., 2022) and enables integration of contamination over time for surface water or in air. However, this method will not be discussed in this review.

Currently there is no standard method for water sample preparation, despite this being a critical step that can impact the sensitivity and accuracy of the bioassays. This section will describe the advantages and disadvantages of each method, as well as the various protocols that exist along with their pitfalls. To evaluate the existing methods, a literature search was performed in PubMed in 2020–2021 for the commonly used bioassays in the literature (Table 3), except for the YES/YAS assay which will not be considered further as described in the RTA section. Every article related to the evaluation of EDCs in any type of water matrix was considered, as well as other relevant papers found when searching for the other sections of this present review. In total, the methodology of 61 published articles was evaluated. We acknowledge that some articles may have been missed and the dataset may be incomplete; nevertheless, we are confident we collected the majority of the methods most commonly used in the field.

Table 4
Methods of preparation of media with effluent.

In vitro assay	Type of sample	Media preparation	Final sample concentration	Reference
ER-CALUX (T47D cells)	WW	Sterilization with 0.2 μm filter, dilution of sample with PBS at needed concentration for the bioassay, addition of diluted sample to test media for 1:5 dilution	>1/5	Avberšek et al. (2013)
MELN	WW	Sterilization with 0.2 μm filter, dilution 1:1 media 2X: sterilized sample, addition of diluted sample to the well in a 1:1 ratio (sample final concentration 1:3)	1/3	Balaguer et al. (1999)
T47D-KBluc	PPM-WW	Dilution 1:4 sample: media 1X, adjustment of pH as required by the media preparation, sterilization with 0.22 μm filter	1/4	Davis et al. (2013)
MDA-kb2	RW creating ARO	Preparation of media with water sample in small volume (10 mL), supplementation of DCC-FBS, sterilization with 0.22 μm filter	1	Horn et al. (2020)
VM7Luc4E2 (TG457)	WW	Sterilization with 0.2 μm filter, dilution of concentrated media 10X to 1X with sterilized sample	0.72	Niss et al. (2018)
T47D-KBluc, MDA-kb2 (not for Wehmas et al., 2011)	WW, PPM-WW, ARO	Preparation of media with water sample in small volume (20–40 mL), adjustment of pH as required by the media preparation, sterilization with 0.22 μm filter	1	Wehmas et al. (2011); Cavallin et al. (2014); Cavallin et al. (2016)

ARO: agricultural runoff; PPM-WW: pulp and paper mill; RW: rainwater; WW: wastewater (alone refers to municipal WWTP).

4.1. Media method

As stated earlier, the Media Method consists of preparing the cell culture media directly with the water samples and was used by only 8 articles out of 61 (Table 4). There are two different approaches to apply the Media Method. The first approach is to prepare the cell media with the raw water samples (Wehmas et al., 2011; Cavallin et al., 2014, 2016; Horn et al., 2020), while the second approach is to dilute a media (prepared with pure water) with the water samples. For the former approach, the initial media condition used can either be at a normal concentration (Avberšek et al., 2013; Davis et al., 2013) or concentrated (powder media diluted in smaller water volume; Balaguer et al., 1999; Niss et al., 2018). Depending on the chosen methodology, the starting concentration of water samples for the *in vitro* bioassays can vary between 1/5 and 1 of the original sample's concentration. While most protocols (Table 4) used the same numbers of steps overall, the utilization of the concentrated media approach could be more advantageous for high throughput settings to standardize the media preparation. Overall, the Media Method modifies the sample as little as possible, while being cost and time effective at the same time. However, the Media Method will usually have a higher limit of detection as the samples are diluted rather than being concentrated. Moreover, it is difficult to have a suitable control considering that the composition of salt and dissolved organic carbon (DOC) vary from one sample to another. Due to those disadvantages, the Media Method is less commonly used.

4.2. Solid phase extraction (SPE) method

For EDC testing, the Solid Phase Extraction (SPE Method) allows the concentration of hydrophobic to neutral contaminants present in water samples, as well as allowing the removal of salts, inorganics, metals and most of the DOC which may cause interferences with bioassays. Moreover, the SPE allows a lower limit of detection for quantification of EDCs and provides a unique method of preparation for multiple bioassays. This method is currently the most popular method (55/61 articles) to prepare water samples for bioassays. In this method, the water sample is (1) passed on a pre-conditioned column; (2) the column is washed and dried; (3) the contaminants are eluted; (4) the eluate can be concentrated even more by evaporation of the solvents; and (5) the concentrated eluate is reconstituted into a small volume with a solvent of choice. While those basic steps are found in every article using the SPE method, various parameters vary among all studies, such as the initial volume of sample, the pre-treatment of the sample, the sorbent used, the choice of solvent, and the temperature of evaporation. All these differences are noted in Table 5 and will be detailed further in this section.

4.2.1. Sample pre-treatment

The initial volume of samples used varies from 0.1 to 43 L, but most articles used a volume comprised between 0.25 and 2 L (Table 5). There are no clear guidelines to determine the optimal volume required to maximize the sensitivity of the bioassay being conducted, but the volume used must take into consideration the amount of sorbent present in the column, the level of contaminants in the sample, the target analytes and the background contaminants. Simon et al. (2019) showed that the sample volume had no influence on recovery between 0.5 and 2 L of wastewater effluent and 1–4 L of surface water spiked with a mixture of four estrogenic compounds (E1, E2, EE2 and BPA) using 300 mg LiChrolut EN/RP-18 sorbent. Samples with low levels of contaminants (e.g., effluent from WWTP, surface water, and drinking water) typically require higher volumes of water for analysis compared to samples that are highly contaminated (e.g., influent from WWTP) to be able to detect any endocrine disrupting activity. In studies assessing the endocrine disrupting activity of various types of water samples (Murk et al., 2002; Furuichi et al., 2006; Muller et al., 2008; Jugan et al., 2009; Maletz et al., 2013; Neale et al., 2017; Leusch et al., 2018b), the volume of water needed was usually doubled for any water source considered cleaner than the contaminated sample.

After collecting the water, the sample should be kept on ice until arrival to the laboratory and some pre-treatment steps should be performed immediately. Often, SPE samples are acidified with HCl or H_2SO_4 (Table 5). The main reason for sample acidification is to prevent bacterial growth, which could degrade contaminants over time. For this same purpose, some studies add methanol, formaldehyde, or copper sulfate (Chen et al., 2004; Schilirò et al., 2004, 2009, 2012; Li et al., 2011; Conley et al., 2017; Kibambe et al., 2020; Spina et al., 2020). However, acidification could impact the recovery of some chemicals. For example, sample acidification can decrease the recovery of some contaminants, such as progestins and mifespristone (Šauer et al., 2018). In contrast, Stalter et al. (2016) showed that acidification could improve the recovery of disinfection by-products for various SPE sorbents as evaluated by chemistry and cytotoxicity assays. Since EDCs are comprised by various classes of compounds, acidification could have

Table 5
Condition of SPE column to extract.

Cartridge	Acidification and other preparation	Filtration or centrifugation	Conditioning of Column[a]	Type of sample	Volume of sample	Step before elution	Solvent of elution[a]	Step after elution	Final volume and solvent	Bioassay for endocrine endpoint	Reference
Autoprep® EDS-1 (500 mg)	pH 3 with AcOH	1 µm glass fiber	10 mL MeOH, 10 mL upH_2O	ARO	IW: 0.1 L, EW: 0.4 L	Dried by centrifugation at 1000 rpm for 10 min	10 mL 5 mM TMA in MeOH	Evaporation under N_2-stream at 30 °C	500 µL MeOH	MVLN	Furuichi et al. (2006)
Bond Elut ENV (1 g, 6 cc)	pH 2.5 with 1 M H_2SO_4, 10 mL MeOH and conductivity adjusted to 8500 µS with 0.5% NaCl	No	6 mL Ace, 10 mL MeOH, 6 mL pH 2 dH_2O	WW, SW, IWW	2 L, except for Spina et al., (2020) which uses 0.2 L	Rinsed with 6 mL pH 2 dH_2O, dried under vacuum	2 x 2.5 mL Ace	Evaporation under N_2-stream to 1 mL, 100 µL removed and replaced by DMSO, evaporated to 100 µL	100 µL DMSO, filter-sterilized (0.22 µm)	E-screen (not included in Schilirò et al., 2004), MELN	Schilirò et al. (2004); Schilirò et al. (2009); Schilirò et al. (2012); Spina et al. (2020)
C18 (Atlantic, disk)	pH 3 with 1 M HCl compared to not acidified	5 and 1 µm atlantic fast flow glass fiber	ACN, dH_2O	WW	1 L	Rinsed with H_2O, air dried for 15 min	10 mL ACN	Evaporation under N_2-stream	2 x 20 µL DMSO	(anti-)AR- and (anti-)PR- CALUX (U2OS)	Šauer et al. (2018)
C18 (disk, 6 cc)	No	1 µm filter	MeOH, upH_2O	PPM-WW	1 L	N.M.	4 x 1 mL MeOH	Evaporation under N_2-stream to 100 µL	200 µL 1:1 MeOH: upH_2O (5,000X)	T47D-KBluc	Wehmas et al. (2011)
C18 (Empore, 47 mm disks, 3 M)	6 mg $CuSO_4·5H_2O$ in the field and 24 mg Na_2EDTA and 5 ng BPA-d16	No	N.M.	SW	0.6 L	N.M.	9 mL 90:10 MeOH: Ace	Evaporation to dryness of 1.5 mL	50 µL 100% EtOH	BLYES, T47D-Kbluc, MDA-kb2, CV-1 with hGR and MMTV-Luc	Conley et al. (2017)
C18 (Empore, FF, 47 mm disk)	No	Glass fiber	N.M.	WW, SW	0.3–1 L	Dried completely for 1 h at 37 °C	2 x 4 mL Hex, 2 x 4 mL 3:1 Hex: DCM, 2 x 4 mL DCM, 2 x 4 mL 1:1 DCM: MeOH, 2 x 4 mL MeOH	Evaporation under N_2-stream	DMSO (1,000X)	Competitive binding assay for TTR and TR from *X. laevis*, *X. laevis* XL58-TRE-luc cells and *X. laevis* embryo exposure (only for Murata and Yamauchi, 2008)	Murata and Yamauchi (2008); Ishihara et al. (2009)
C18 (Baker, 200 mg)	No	Sea sand	N.M.	RW	9 L	N.M.	Ace	Evaporation under N_2-stream at 30 °C, dissolved in 25 µL 1-propanol, evaporated again	50 µL DMSO	ER-CALUX (T47D)	Hamers et al. (2003)
C18 (Varian Mega Bond Elut, 1 g)	pH 2.5–3	N.M.	N.M	EW	1 L	Dried O/N by lyophilization	2 x 5 mL MeOH	N.M.	50 µL DMSO	E-SCREEN	Henneberg et al. (2014)
Two subsequent C18 (500 mg, 6 cc)	No	No	Hex, DCM, Ace, MeOH, upH_2O	DW	2 L x 5 (different cartridges)	N.M.	10 mL Hex, 10 mL 4:1 Hex: DCM, 10 mL 1:1 DCM: MeOH	Combination of eluates, evaporation to near dryness, dissolved in 500 µL DCM, evaporated again	200 µL DMSO	MDA-kb2	Hu et al. (2013)
Two subsequent C18 (500 mg, 6 cc)	No	No	Hex, DCM, Ace, MeOH, upH_2O	SW	2 L	Dried with N_2-stream	10 mL Hex, 10 mL 4:1 Hex: DCM, 10 mL 1:1 DCM: MeOH	Evaporation with rotatory evaporator, evaporation under N_2-stream to near dryness	200 µL DMSO	MDA-kb2, CV-1 with pUAS-tkluc, pGal4-L-TR (TRTA assay)	Shi et al. (2016)
	No			WW	6–43 L						

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Table 5 (*continued*)

Cartridge	Acidification and other preparation	Filtration or centrifugation	Conditioning of Column[a]	Type of sample	Volume of sample	Step before elution	Solvent of elution[a]	Step after elution	Final volume and solvent	Bioassay for endocrine endpoint	Reference
Chromabond HR-X (10 g) between two glass filter plates		Sartopure GF, Midicap filters	200 mL EtOAc, 200 mL MeOH, 100 mL dH_2O			Dried with N2-stream O/N	0.1 L EtOAc, 0.1 L MeOH, 0.1 L 1% FA in MeOH, 0.1 L 2% 7 N NH_3 in MeOH	pH neutralized to 7, filtered through filter paper (GF/F), evaporated with rotatory evaporator to dryness	MeOH (5,000X)	AR and ER-CALUX (U2OS), REACTIV (medaka-gfp for ER), XETA	Välitalo et al. (2017)
Isolute ENV+ (200 mg)	pH 2 with 4 M HCl	Glass fiber (GF/C) washed with acidified H_2O, freeze-dried and extracted with acetone	N.M.	Landfill leachate	0.25 L and 1 L	N.M.	Ace	Evaporation under N_2-stream to near dryness	1 mL EtOH, and filter sterilized (0.2 μm)	MVLN	Coors et al. (2003)
Multilayer: envicarb (200 mg), 1:1:1.5 Strata X–CW: Strata X-AW: Isolute Env+ (350 mg), Oasis HLB (200 mg)	pH 6.5–6.7 before filtration	0.7 μm glass fiber	5 mL MeOH, 10 mL upH_2O	WW	SW and IW: 1.5 L, EW: 0.5 L	Dried and inverted for elution	6 mL 50:50 EtOAc: MeOH with 0.5% NH_3, 3 mL 50:50 EtOAc: MeOH with 1.7% FA and 2 mL MeOH	Evaporation at 40 °C	N.M.	MELN, MDA-kb2, cyp19a1b -GFP zebrafish	Neale et al. (2017)
Lichrolut®-EN (100 mg)	pH 3 with HCl, 5 mL MeOH	Glass-fiber filter	Hexane, Ace, MeOH, H_2O (pH 3)	WW	1 L	Dried with N2 for 1 h	4 x 1 mL Ace	Evaporation under N_2-stream	100 μL DMSO	RTG-2-rtERα	Ackermann et al. (2002)
LiChrolut EN/RP18 (100 mg EN and 200 mg RP-C18, 6 cc)	pH 3 with HCl	Glass fiber	2 x 1 mL hexane, 2 x 1 mL Ace, 3 x 2 mL MeOH, 3 x H_2O (pH 3)	EW, SW	EW: 0.5 or 2 L; SW: 1 or 4 L	Rinsed with 10 mL H_2O (pH 3), dried with N_2-stream for 1 h	4 x 1 mL Ace, 1 mL MeOH	Reduced under N_2-stream to 500 μL	Filled to 1 mL or 4 mL for larger volumes with EtOH (EW: 500X, SW: 1000X)	ERα-CALUX	Simon et al. (2019)
Oasis HLB (200 mg, 5 cc)	No	0.7 μm glass-fiber	5 mL MeOH, 4 mL upH_2O	ARO	1 L x 5 (different cartridge)	Rinse with 1 mL upH_2O, vacuum dried (20 min)	2 × 4 mL MeOH	Evaporation under N_2-stream at 45 °C to 0.5–1 mL, replicates combined, concentration, addition of 10 μL upH_2O and evaporation to 10 μL	Brought to 500 μL with EtOH	T47D-Kbluc, E-screen, YES	Alvarez et al. (2013)
Oasis HLB (500 mg, 6 cc)	No	No	3 mL MeOH, 3 mL dH_2O	WW	0.5 L	N.M.	10 mL MeOH, 10 mL ACN	Evaporation under N_2-stream	100 μL DMSO	ERα-, GR-, anti-AR- and anti-PR- CALUX (U2OS)	Alygizakis et al. (2019)
Oasis HLB (60 mg, 3 cc)	pH 2.5 with HCl	Glass fiber (GF/C) and 0.45 μm nylon fiber	3 mL EtOAc, 3 mL MeOH, 3 mL pH 2.5 upH_2O	WW, HWW	0.2 L	Air dried and stored at −20 °C	3 mL EtOAc	Evaporation under N2-stream to 0.5 mL, clean with ISOLUTE cartridge (500 mg) with EtOAc, evaporation under N_2-stream until dry	EtOAc (volume unclear)	ER-CALUX (T47D)	Avberšek et al. (2011); Avberšek et al. (2013)
Oasis HLB (6 cc)	Addition of 0.5% H_2SO_4	0.7 μm glass fiber	5 mL MeOH, 5 mL upH_2O	WW	0.5 L	Washed with H_2O with 10% MeOH	2 x 3 mL MeOH, 2 x 3 mL DCM	Evaporation under N_2-stream to dryness, reconstituted in 2 mL MeOH. 1 mL of the	50 μL DMSO	(anti-)ERα-, (anti-)AR-, PR-, GR-, PPARγ2-CALUX (U2OS)	Bain et al. (2014)

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Table 5 (*continued*)

Cartridge	Acidification and other preparation	Filtration or centrifugation	Conditioning of Column[a]	Type of sample	Volume of sample	Step before elution	Solvent of elution[a]	Step after elution	Final volume and solvent	Bioassay for endocrine endpoint	Reference
								sample is evaporated to dryness			
Oasis HLB (500 mg, 6 cc)	a: pH 7.5; b: pH 1–2 with H_2SO_4	No	N.M.	DW	0.25 L	N.M.	2 x 5 mL EtOAc	Evaporation	40 µL DMSO (1,000X)	ER-CALUX (U2-OS)	Bourgin et al. (2013a); Bourgin et al. (2013b)
Oasis HLB (6 cc)	pH 2 with HCl	No	5 mL 75:25 EtOAc: Ace mixture, 5 mL MeOH, 5 mL pH 2 upH_2O	Landfill leachate	0.4 L	Dried 10 min	8 mL 75:25 EtOAc: Ace	Evaporation under N_2-stream at 65 °C to dryness	150 µL DMSO	BG1Luc4E2 (ERα positive cells, pGudLuc7ERE)	Giudice and Young (2011)
Oasis HLB (500 mg, 6 cc)	No	0.7 µm glass fiber (GF/F)	10 mL Ace-hexane (1:1), 10 mL MeOH, 10 mL 0.05 mM HCl	IWW	0.5–1 L	Dried 2 h under N2-stream	10 mL MeOH, 10 mL Ace:Hex (1:1)	Evaporation under N_2-stream to dryness	1 mL MeOH	T47D-KBluc	He et al. (2020)
Oasis HLB (500 mg, 6 cc)	No	2 µm glass fiber	6 mL MeOH, 8 mL dH_2O	WW	0.5 L	Dried for 10 min	6 mL MeOH	Evaporation under N_2-stream	MeOH (1,200X)	MVLN	Jarošová et al. (2014b)
Oasis HLB (500 mg)	No	1 µm glass fiber	10 mL MeOH, 10 mL upH_2O	DW, WW	SW: 1 L, EW: 0.5 L, IW: 0.25 L	Dried under vacuum for 5 min	10 mL MeOH	Evaporation with rotatory evaporator at 40 °C	DMSO	MELN and PC-DR-LUC (TRα1)	Jugan et al. (2009)
Oasis HLB (500 mg, 12 cc, up to 12 cartridges per sample)	pH 3 with HCl and 1% formaldehyde	0.7 µm glass fiber (GC/F)	5 mL dH_2O, 5 mL MeOH, 5 mL dH_2O	WW	0.25 L	Dried under vacuum for 20 min	6 mL MeOH	Evaporation under N_2-stream	1 mL EtOH	T47D-Kbluc	Kibambe et al. (2020)
Oasis HLB	pH 2	centrifuge at 4 °C, 2000g for 12 min to remove large particles and AP20 filter (Millipore)	N.M.	WW, SW, GW	N.M.	Dried under vacuum for 1–2 h and stored at 4 °C until elution (max 10 days)	5 mL MeOH, 5 mL 1:1 Ace: Hex	Evaporation under N_2-stream to dryness	500 µL ethanol split in aliquot of 40 µL and dried	YES, ER-CALUX (T47D), MELN, T47D-KBluc and E-SCREEN	Leusch et al. (2010)
Oasis HLB (20 cc)	No	AP20 glass fiber	10 mL MeOH, 10 mL upH_2O	WW, RecW	N.M.	Dried for 2 h	2 x 10 mL MeOH	Evaporation under N_2-stream to near dryness	500 µL MeOH	E-SCREEN and AR-CALUX (U2OS)	Leusch et al. (2014a)
Oasis HLB (500 mg)	2 mL MeOH/L of sample	0.45 µm glass fiber	N.M.	WW	4 L	Dried under vacuum for 5 min	2 x 5 mL of 7:3 Hex: DCM, 2 x 5 mL MtBE, 2 x 5 mL 9:1 DCM: MeOH, 5 mL MeOH	Filtration by anhydrous sodium sulfate and evaporated with rotatory evaporator at 40 °C to 2 mL and to dryness under N_2-stream	400 µL DMSO	β-Galactosidase assay of TR in yeast strain containing hTR-GRIP1	Li et al. (2011)
Oasis HLB (1 g, 20 cc)	0.0001% sodium thiosulfate, 5 mM HCl (final concentration)	Glass fiber	10 mL MeOH, 20 mL 5 mM HCl	WW, DW, RecW	0.5–4 L (varied by type of water)	Dried under vacuum for 2–3 h	10 mL MeOH, 10 mL Ace:Hex (1:1)	Evaporation under N_2-stream	500 µL	E-SCREEN	Macova et al. (2011)
Oasis HLB (6 cc)	No	No	N.M.	HWW	EW: 2 L for 3 cartridges, IW: 0.5 L	Dried with N_2-stream	MeOH	Evaporation under N_2-stream	EtOH (33,000X)	LYES, ER-CALUX (T47D), H295R	Maletz et al. (2013)
Oasis HLB (6 cc)	pH 3 with HCl	1.6 µm glass fiber	4 mL Hex, 4 mL Ace, 4 mL	SW, GW, artificial pound	1 L	Dried with N_2-stream	3 x 4 mL Ace, 4 mL MeOH	Evaporation under N_2-stream	1 mL DMSO	LYES, ER-CALUX (T47D), H295R	Maletz et al. (2015)

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Table 5 (*continued*)

Cartridge	Acidification and other preparation	Filtration or centrifugation	Conditioning of Column[a]	Type of sample	Volume of sample	Step before elution	Solvent of elution[a]	Step after elution	Final volume and solvent	Bioassay for endocrine endpoint	Reference
			MeOH, 4 mL pH 3 dH_2O								
Oasis HLB (200 mg)	pH 3–5 with 10% HCl	0.7 µm glass fiber (GF/F)	N.M.	WW	IW: 1 L; EW: 2 L	N.M.	Conjugated E2: 5 mM TMA in 60:40 MeOH: H_2O, free estrogens: MeOH	No	Conjugated E2: 5 mM TMA in 60:40 MeOH: H_2O, free estrogens: MeOH	MELN	Muller et al. (2008)
Oasis HLB (500 mg)	No	No	10 mL MeOH, 10 mL EtOAc	WW, SW	SW: 2 L	Dried by vacuum	10 mL MeOH, 10 mL EtOAc	Filtration with 0.2 µm polyethersulfone filter, evaporation under N_2-stream to dryness at 40 °C	MeOH (1,000X)	ERα-, GR-, AR-, (anti-)PR-, PPARγ-GeneBLAzer	Müller et al. (2018)
Oasis HLB (1 g, 6 cc)	Acidified with H_2SO_4	Glass fiber filter	MeOH and upH_2O	WW, SW	0.5 L	Washed with upH_2O with 2% MeOH, dried under vacuum for 30 min	MeOH and DCM	Evaporation under N_2-stream at 37 °C to dryness	50 µL DMSO	(anti-)ERα-, (anti-)AR-, (anti-)PR-, GR-, PPARγ2-CALUX (U2OS)	Roberts et al. (2015)
Oasis HLB (200 mg)	No	No	2 x EtOAc, 1 x MeOH, 2 x H_2O	SW	1 L	Dried	3 x 2.5 mL EtOAc	Evaporation under N_2-stream at 56 °C to 3 µL	50 µL DMSO	GR-CALUX	Schriks et al. (2013)
Oasis HLB (500 mg, 6 cc)	No	0.22 µm glass fiber	10 mL DCM, 10 mL MeOH, 10 mL upH_2O	DW	24 L	N.M.	10 mL MeOH, 10 mL upH_2O	Evaporation under N_2-stream to 500 µL	1.5 mL DMSO	ERα- and AR-CALUX (U2OS)	Shi et al. (2018)
Oasis HLB (12 mg)	No	0.45 µm glass fiber	N.M.	WW	0.05 or 1 L	N.M.	10 mL Ace, 10 mL DCM	Evaporation under N_2-stream	1 mL DMSO (1000X)	MDA-kb2, YES	Sun et al. (2017)
Oasis HLB (200 mg)	pH 3 with 32% HCl	No	5 mL 10% MeOH in MtBE, 3 mL MeOH, 3 mL dH_2O	DW	1 L	Washed with 3 mL 5% MeOH in dH_2O and dried	6 mL 10% MeOH in MtBE	Evaporation under N_2-stream at 37 °C to dryness	1 mL EtOH (1,000X for bioassays)	T47D-Kbluc, YES	Van Zijl et al. (2017)
Oasis HLB followed by silica cartridge	No	0.45 µm glass fiber	HLB: 6 mL EtOAc, 6 mL ACN, 12 mL dH_2O; Silica: 4 mL H_2O saturated with EtOAc, 4 mL 1:9 EtOAc: Hex	SW	20 L	HLB: Rinsed with 10 mL dH_2O and dried with N2-stream; Silica: 3 mL 1:9 EtOAc: Hex	HLB: 15 mL EtOAc, 6 mL 1:1 EtOAc: ACN; Silica: 3 mL 62:38 EtOAc: Hex	HLB: Evaporation to dryness and redissolved in 2 mL 1:9 EtOAc: Hex; Silica: Evaporation to dryness	250 µL DMSO	MVLN	Song and Wang (2016)
Oasis HLB (500 mg, 6 cc), Supelclean coconut charcoal (Sigma-Aldrich, 2 g, 6 cc), in series	pH 3 with HCl, sodium thiosulphate (1 g/L)	1.6 µm glass fiber (GF/A)	10 mL Ace: Hex (1:1), 10 mL MeOH, 10 mL 5 mM HCl	SW, EW, RecW, DW, WW	1 L x 8 or 4 depending on water sample	Dried under vacuum	10 mL MeOH, 10 mL Ace:Hex	Combination of 8 or 4 samples, evaporation under N_2-stream	1 mL MeOH	103 bioassays with 39 targeting EDC pathways (ER, AR, GR, PR, TR and steroidogenesis)	Escher et al. (2014)
Oasis HLB (6 cc), Supelclean coconut charcoal (Sigma-	No	No	5 mL MeOH, 5 mL upH_2O	RW, RecW, WW,	2 L	Dried	2 x 5 mL MeOH	Evaporation under N_2-stream to dryness	1 mL MeOH (2,000X)	(anti-)ERα-, (anti-)AR-, GR-, PR-, TRβ-CALUX (U2OS)	Leusch et al. (2014b)

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Table 5 (*continued*)

Cartridge	Acidification and other preparation	Filtration or centrifugation	Conditioning of Column[a]	Type of sample	Volume of sample	Step before elution	Solvent of elution[a]	Step after elution	Final volume and solvent	Bioassay for endocrine endpoint	Reference
Aldrich), in series											
Oasis HLB (6 cc), Supelclean coconut charcoal (Sigma-Aldrich), in series	Sodium azide (1 g/L), if chlorine, sodium thiosulfate (50 mg/L)	No	N.M.	RecW	4 x 1 L (1 cartridge per L)	Rinsed with upH_2O, dried by vacuum for 2 h	10 mL MeOH, 10 mL Ace:Hex (1:1)	Combination of the 4 eluates, evaporation under N_2-stream	4 mL MeOH	AR-, ERα-, GR-, PR-GeneBLAzer	Mehinto et al. (2015)
Oasis MCX (150 mg, 6 cc)	pH 2.3 with 30% HCl	0.45 µm cellulose nitrate	8 mL ACN, 8 mL MeOH, 8 mL pH 2.3 H_2O	SW	1 L	Washed with pH 2.3 H_2O, dried with N2-stream	2.5 mL ACN, 2 x 2.5 mL 5% NH_4OH in ACN	Evaporation under N_2-stream at 58 °C to 50–100 µL, 50 µL DMSO added, evaporation under N_2-stream at 65 °C for 10 min	50 µL DMSO	ERα-, AR-, GR-, TRβ- and PR-CALUX (U2OS)	Kolkman et al. (2013)
Oasis SDB (500 mg)	No	Glass fiber	6 mL MeOH, 8 mL dH_2O	EW	1 L	Dried	6 mL MeOH	Evaporation under N_2-stream	MeOH (1,200X)	Hela-9903, MDA-kb2	Henneberg et al. (2014)
SDB-XC disk (6 for surface water and 1 for wastewater)	No	1.2 and 0.45 µm glass fiber	N.M.	SW, WW	SW: 9 L, WW: 1–2 L	N.M.	3 x 5 mL MeOH	Evaporation at 60 °C	DMSO or EtOH	YES, ER-CALUX (T47D) and ER-binding assay	Murk et al. (2002)
Sep-Pak RP18	pH 2.5 with H_2SO_4 + 5 mL MeOH and adjusted conductivity for 5 g/L NaCl	1 µm glass fiber and 0.45 µm cellulose acetate membrane	6 mL Ace, 10 mL MeOH, 6 mL pH 2 dH_2O	WW of fossil fuel production	1 L	N.M.	3 x 2 mL Ace	Evaporation under N_2-stream to 0.5 mL, 0.5 mL DMSO added, evaporation under N_2-stream to 0.5 mL	500 µL DMSO (2,000X) and filter sterilized (0.2 µm)	MVLN	Chen et al. (2004)
Strata X (200 mg)	pH 3 with AcOH and 200 mg Na_2EDTA	0.7 µm glass fiber (GF/F) washed with 0.5 mL MeOH	2 x 2 mL MeOH and 3 x 2 mL dH_2O	Coastal SW and WW	1 L	Dried	3 x 2 mL MeOH	Evaporation under N_2-stream at 30 °C to 1 mL	1 mL MeOH	H295R	Gracia et al. (2008)
Strata X (200 mg)	pH 2	No	MeOH, pH 2 upH_2O	SW, WW, DW	SW, DW: and upH_2O: 1 L per cartridge for 20 L, WW: 0.5 L per cartridge for 10 L	Dried	MeOH, ACN, Ace	Evaporation under N_2-stream to dryness	2 mL MeOH (10,000X for SW, 5,000X for WW)	TPO inhibition assay, TTR displacement assay, TRβ-CALUX (U2OS), TRβ-Geneblazer and GH3.TR-Luc and XETA	Leusch et al. (2018b)
Supelclean™ LC-18 (500 mg)	No	centrifugation at 13,500×*g* for 30 min at 4 °C, filtered with 2.0 µm and 1.2 µm glass fiber filters	5 mL upH_2O, 5 mL 5% MeOH in upH_2O	ARO	0.5 L	Rinsed with 1 mL upH_2O, dried with vacuum	2 x 4 mL MeOH	Addition of 10 µL upH_2O, evaporation under N_2-stream at 40 °C	1 mL EtOH	T47D-KBluc, YES	Yost et al. (2014)
XAD-2/8 (Sigma)	pH 3 with 4 M H_2SO_4	0.45 µm glass fiber	MeOH, ACN, DCM	SW	5 L	Dried	MeOH, ACN, DCM	Evaporation under N_2-stream	1 mL DMSO	MDA-kb2, YES	Sun et al. (2016)

Ace: acetone; ACN: acetonitrile; ARO: agricultural runoff; DCM: dichloromethane; dH_2O: deionised or distilled water; DW: drinking water; EW: effluent water; FA: formic acid, GW: groundwater; Hex: hexane; HWW: hospital WW; IW: influent water; IWW: industrial WW; LYES: Lyticase YES; MeOH: methanol; MtBE: methyl tert-butyl ether; N.M.: not mentioned; O/N: overnight; PPM-WW: pulp and paper mill; RW: rainwater; RecW: recycled water; SW: surface water; TMA: trimethylamine; upH_2O: milliQ or ultrapure water; WW: wastewater (alone refers to municipal WWTP).

**To simplify the table, some parts of the methods that were not varying as much across articles were not mention and are described briefly here. The collection of water samples was usually made in amber glass bottles. Not all the article mentioned the flow rate of the SPE, but when mentioned the flow rate varied around 5–15 mL/min. Moreover, the concentrated sample is typically stored at −20 °C prior to the bioassays. Additionally, the order of the step is performed in the order of the column of the table except if mention otherwise.

[a] The different volumes indicated are done subsequently.

various effects on the recovery of the SPE and should be studied further.

In addition to acidification, the water samples are also often treated to remove large particulates, which can obstruct the SPE column. Those particulates can be removed by filtration with 0.22–5 μm pore's filters (usually glass-fiber), or alternatively, by centrifugation (Table 5). After such pre-treatments, it is recommended to get the sample on the SPE cartridge quickly. After this step, it will be possible to dry the cartridge for storage.

4.2.2. Recovery

The majority of known EDCs exhibit neutral to medium hydrophobicity. In fact, of the almost 500 chemicals that were active in the ERα-GeneBLAzer assay, the ones with the highest potency expressed as specificity ratio SR were typically of moderate hydrophobicity expressed as octanol-water partition constant $2 < \log K_{ow} < 5$ and were fully neutral at pH 7 (Fig. 3C). In contrast, low-potency ER agonists have high diversity of K_{ow} (Fig. 3C, left y-axis). Many low-potency ER agonists are also charged (Fig. 3C, right y-axis), but these will play a minor role for the mixture effects in water samples. Neutral compounds with a hydrophobicity range $2 < \log K_{ow} < 5$ have typically a good recovery on most SPE materials that are based on C18 or organic copolymers (HLB, HRX, ENV+). Indeed, the most popular sorbent selected for EDCs in the reviewed articles was the Oasis HLB (26/55 articles), followed by the C18 (11/55 articles) and the Bond Elut ENV + of Agilent (4/55 articles). Other types of columns are also used and are reported in Table 5. Some articles used MCX and XAD sorbents, which allow having a higher range of hydrophobicity, but can reduce the recovery of non-ionised or cationic chemicals.

The choice of volatile polar organic solvents used for elution (acetone, ethylacetate, methanol) will not impact as much the recovery because exhaustive extraction is already assured by a surplus of solvent. However, the choice of solvent still varies significantly across articles. Of the 55 articles listed in Table 5, 12 articles used MeOH alone, 8 articles used acetone, 5 articles used ethyl acetate and 1 used acetonitrile. However, over half of the studies (29/55 articles) used various solvents to elute the contaminants on the column by either combining solvents or by sequentially eluting with different solvents.

To evaluate the performance of the SPE, the recovery of selected compounds should ideally be evaluated. However, only 13 studies out of 55 assessed the recovery of their methods by chemical analysis. Other articles mentioned measuring the recovery but were excluded because they cited other articles, or they did not specify the recovery per chemical. This lack of assessment of recovery for SPE in a bioanalytical context has also been noted by Neale et al. (2018). It is recommended to assess recovery by analytical methods, such as liquid chromatography coupled with mass spectrometry (LC-MS), but also by bioassay recovery with natural or spiked water samples (Neale et al., 2018; National Water Research Institute, 2020). For commonly tested estrogenic compounds (e.g., E1, E2, EE2, BPA, and nonylphenol), the recovery varied between 64 and 167% for Oasis HLB (Jugan et al., 2009; Avberšek et al., 2011; Van Zijl et al., 2017; Kibambe et al., 2020). Moreover, Shi et al. (2018) obtained recoveries with Oasis HLB ranging from 71.3 to 117.6% for 97 compounds (estrogenic compounds, PCBs, pesticides, phthalate, and PAHs). Furthermore, Alygizakis et al. (2019) assessed the recovery of 280 compounds (pharmaceutical, steroid, drug of abuse, artificial sweetener, pesticide, plasticizer, surfactant, flame retardant and more) using Oasis HLB. The majority of tested chemicals had a recovery between 60 and 120%. Only 25 compounds had a recovery below 60% and 5 compounds had a recovery over 120%. Overall, those high recovery rates shown by Oasis HLB for a large class of compounds demonstrate its usefulness for the extraction of EDCs for bioassay analysis. Similarly, high recoveries can be obtained by C18 columns. When using 2 consecutive C18, the recovery of 59 compounds (e.g., estrogenic compounds, PCBs, pesticides, and phthalates) varied from 82 to 113% (Hu et al., 2013) and was comprised between 82 and 111% specifically for the phthalate family (Hu et al., 2013; Shi et al., 2016). Moreover, Yost et al. (2014) had over 70% recovery for the majority of 37 compounds (mostly steroids), except for progesterone (57%), norgestimate (58%), and estriol-3-glucuronide (30%) when using the Supelclean™ LC-18 column. High recoveries can also be achieved by other types of columns. Simon et al. (2019) used the LiChrolut EN/RP18 and had a recovery over 80% for 11 herbicides and 4 estrogenic compounds (E1, E2, EE2 and BPA) except for metribuzin for which the recovery went as low as 38% because of its high binding affinity for the sorbent. Kolkman et al. (2013) obtained recoveries ranging from 1% (2-napthylamine and triglyme) to 99% (testosterone) for 39 compounds for an average of 61% with the Oasis MCX ® column. Ion exchange material is not required for the mainly neutral EDCs, but it does not do any harm given the good recoveries. By using a multilayer SPE (envicarb, strata X–CW, Strata X-AW, Isolute Env+ and Oasis HLB), Huntscha et al. (2012) obtained a recovery over 80% for 88 tested compounds (pesticides, pharmaceuticals) spiked in various water matrices. However, the use of many different SPE materials plus the use of multiple solvents can generate activity in blanks due to the amounts and diversity of materials the sample gets in touch with. Sample preparation should be as parsimonious with materials as possible.

No matter the chosen approach, the recovery of at least one reference compound should be done to ensure the quality of the method. When analysing recovery by analytical chemistry, one should aim at recoveries of 80–120% for individual target chemicals. For bioassays, the recovery should be within a factor of two (50–200%), given that concentration-response curves are on a log-scale and a factor of 2 in recovery will only yield a 0.3 increment on a log scale, which is within the reproducibility of the assays.

4.2.3. Evaporation and storage

After elution, solvents are generally evaporated to concentrate further the sample (Table 5). Evaporation is usually performed with a nitrogen stream (44/55 articles) or sometimes with a rotatory evaporator (4/55). Another aspect of evaporation is that the sample can be heated. Out of the 55 articles reviewed, only 16 specified the temperature of the sample during evaporation. In those articles, the selected temperature varied from 30 to 65 °C (Table 5). While the impact of temperature used during evaporation has not been fully studied on endocrine activity, temperature was shown to affect chemical recovery of a battery of pharmaceuticals and illicit drugs extracted by SPE and analyzed by LC-MS (Baker and Kasprzyk-Hordern, 2011). The authors showed that the optimal temperature of evaporation was 20 °C, but this temperature increases dramatically the time of sample preparation. Thus, the authors suggest using a temperature lower than 40 °C to have a good balance between sample recovery and time of preparation. A way to avoid loss of recovery linked to temperature can be to use a SpeedVac® and to freeze the sample prior to evaporation (Zhang et al., 2015). Hence, while the evaporation step of the method has less impact on the recovered chemicals than the choice of sorbent, some precautions still need to be taken to avoid decreasing recovery.

After elution and evaporation, samples are reconstituted typically in another solvent of choice (mostly DMSO, EtOH, or MeOH) for the bioassays and/or for storage. Avberšek et al. (2011) have suggested EtOAc should be used preferentially for resuspension when conducting bioassays as it appears to have the least effect on cell viability for the ER-CALUX cells, followed by MeOH compared to DMSO, EtOH and acetone. The reason for this is that more volatile solvents escape to the headspace during incubation of the cells at 37 °C. Another way to avoid the cytotoxic effect of the solvent could be to aliquot the sample in a solvent, evaporate the solvent prior to each bioassay (except for DMSO) and reconstitute the sample in the assay medium. For example, this method is routinely used for water samples in the Escher laboratory (Müller et al., 2018; Nivala et al., 2018; Neale et al., 2020a, 2020b). Apart from cell viability, another aspect to consider is the conservation of the sample over time, which can be critical if the sample cannot be analyzed immediately. For this, DMSO is generally recommended. A

study showed the steady activity of extracted water samples stored in DMSO for 6 weeks at 4 °C and at −20 °C, and a decrease of 27% when stored at room temperature (Murk et al., 2002). In contrast, samples preserved in EtOH evaporated over time and were degraded following a period of six weeks (Murk et al., 2002). Also, the volume of reconstitution should also be considered carefully, as it can affect the dissolution of compounds and thus, the observed signal as described by Murk et al. (2002). In addition, the samples should be weighed before storage and prior to running the bioassays to account for evaporation of the solvent. To prevent evaporation, the use of crimped vials is recommended.

5. Effect-based trigger values

With the use of SPE, we can enrich samples sufficiently to a point which elicits an effect. But which effect is acceptable? Given the utility of RTAs for detection of even low levels of hormone active chemicals through their bioactivity, these bioassays have been applied frequently for assessment of treatment efficacy of wastewater treatment plants (Purdom et al., 1994; Desbrow et al., 1998; Prasse et al., 2015; Neale et al., 2020b), advanced water treatment plants (Leusch et al., 2014a; Leusch and Snyder, 2015; Volker et al., 2019), and drinking water treatment plants (Neale et al., 2020a), but also to assess the quality of surface water (Murk et al., 2002; Vermeirssen et al., 2006; Leusch et al., 2018a; Serra et al., 2020). However, the high sensitivity of the hormonal RTAs also means that mere detection of an effect does not necessarily mean the water body poses a risk to biota. Therefore, safe effect levels, so called effect-based trigger values (EBT) have been defined for water for human consumption as well as for surface waters by numerous different approaches, most of which relies on reading across from drinking water guidelines and environmental quality standards.

For environmental protection and surface waters, the environmental quality standards (EQS) or other surface water guideline values can provide the point of departure for the derivation of the EBTs (Escher et al., 2018a). For drinking water, the point of departure is the ADI (acceptable daily intake) of a potent reference compound translated into water concentration assuming a consumption of 2 L per day and a lifetime of 70 years. The resulting concentration is similar to a drinking water guideline value, which can also be used as a point of departure for the derivation of EBTs (Escher et al., 2015).

The acceptable concentration of the reference chemical is then translated to an equivalent concentration for the bioassay of choice. For example, Kunz et al. (2015) derived a generic EBT-EEQ of 0.4 ng/L by directly reading across from the EU proposed environmental quality standard for E2. Such an approach does not account for mixture effects and potency differences between different active chemicals in a given bioassay. Hence different versions of EBT derivations differ by which and how many single chemicals' effect data were considered for the derivation of EBTs and how mixtures are dealt with. Escher et al. (2018a) used the relative effect potency of the potent estrogenic chemicals and their typical relative abundance in surface water to weigh their contributions in a mixture EBT. Table 3 provides an overview about EBTs for various RTAs for hormonal effects. By far, the most abundant EBT values are for estrogenicity. The EBTs for one effect endpoint (e.g. estrogenicity) vary depending on the RTA used due to differences in inherent sensitivity of the bioassays (Escher et al., 2018a). Regardless of the derivation method, the EBT for a given bioassay and endpoint showed sufficient consistency and robustness for practical applicability in research studies but may have to be more refined when it comes to regulatory application.

6. Prediction of endocrine activity using the concentrations of detected chemicals

The mixture effects of water samples indicative of binding to hormone receptors are typically driven by the natural hormones and by hormonal therapy drugs with a small contribution from other xenohormones. In the case of estrogenic activity, E2, E3, and EE2 are highly potent and dominate the mixture effects as discussed previously. The estrogenic mixture effects in wastewater expressed as E2 equivalent concentration (EEQ_{chem}) can be predicted by summing up the product of the relative effect potencies and detected concentrations of E1, E2, and EE2. The EEQ_{chem} agreed well with the E2 equivalent concentration (EEQ_{bio}) measured in wastewater and surface water using numerous estrogenicity bioassays (Könemann et al., 2018). Part of the experimental EEQ_{bio} could not be explained by E1, E2, and EE2 at low concentrations because in some samples E1, E2, and EE2 fell below the detection limit (Könemann et al., 2018), but if their concentration was assumed to be half the limit of quantification (LOQ/2), the EEQ_{chem} matched well with the EEQ_{bio} without the need to include any further chemicals. A very similar picture was obtained for several estrogenicity assays, namely MELN, HeLa-9903, ER-GeneBLAzer, and pYES (Könemann et al., 2018). When using the quantification of E1, E2, EE2 and E3, the correlation between EEQ_{chem} and EEQ_{bio} was often high (>0.9 of correlation coefficient, Avberšek et al., 2011; Avberšek et al., 2013; Conley et al., 2017), but was lower when more chemicals of lower potency were included in the prediction (correlation coefficient of 0.625–0.834; Yost et al., 2014; Conley et al., 2017; Shi et al., 2018). As expected, the estrogenic activity is not always well predicted by the concentration of xenoestrogenic compounds explaining as low as 0.4% of the activity (Bain et al., 2014; Leusch et al., 2014a; Mehinto et al., 2015; Neale et al., 2017). This lack of prediction could be due to a poor quantification of the more potent natural estrogenic chemicals E1, E2 and E3, which are at a low concentration and can require a targeted analysis. Another aspect that could affect the prediction is the presence of specific chemicals at a site of study. For example, He et al. (2020) obtained a correlation coefficient of 0.805 between EEQ_{chem} and EEQ_{bio} by only looking at the concentration of the nonylphenol and its biodegradation products in the wastewater surrounding a textile industry. Similarly, Hu et al. (2013) could predict the anti-androgenic activity of drinking water with the concentration of only 6 phthalates. For androgenic activity, steroid content correlates well with the activity (Leusch et al., 2014a; Conley et al., 2017). However, explanation of GR activity by known chemicals (e.g., dexamethasone, hydroxycortisone) was lower than 1% (Mehinto et al., 2015; Conley et al., 2017), which demonstrated that unknown chemicals generated the effect.

It will soon be possible to identify those unknown chemicals with the help of high-resolution effect-directed analysis (EDA) methods. During an EDA, the sample is first fractionated using chromatographic separation and its fractions are subsequently analyzed with both LC-MS and bioassays. The data generated through the EDA allows building "bioassay chromatograms" to identify the active compounds in complex samples. To evaluate the relevance of using EDA in prioritizing contaminants of emerging concerns, such as EDCs, a European-wide demonstration program was conducted (Tousova et al., 2017). This demonstration program was able to detect known ER, AR, and GR agonists in surface water samples collected across Europe. Moreover, the study identified 21 prioritizing compounds, including two xenoestrogens (nonylphenol and nonylphenoxyacetic acid). With EDA, Houtman et al. (2020) were able to detect known substances such as the potent ER agonists α/β E2 and EE2, and several GR agonists, as well as identifying for the first time the anti-androgenic compound tebuconazole in wastewater treatment plant effluent. This kind of approach could help identify new EDCs for receptors such as AR, GR and PR for which the nature of environmental contaminants activating them is often unknown.

7. The future of EDC testing via bioassays?

While there are no current official guidelines for the use of *in vivo* and/or *in vitro* bioassays for detecting EDCs in water resources (e.g., complex effluents), the bioassays described in the previous sections were validated internationally for identifying single EDCs and are also being

used in research to analyze environmental samples. Nevertheless, novel approaches are being developed to improve the detection of EDCs using bioassays ranging from 3D tissue culture to simulate more accurately tissue physiology, to transgenic fish which simplifies the experiment and decreases exposure time, and to use complementary OMICs analysis to integrate different endocrine pathways at the same time.

7.1. Transgenic models for in vivo EDC testing

Transgenic animal models have played an integral role in evaluating the effects of EDCs *in vivo*. Recently, the XETA model has been validated by the OECD (OECD TG 248; Fig. 1D) which opens the path to other transgenic models for other species. Over the past decade, there has been a plethora of fish models developed using state of the art gene editing approaches to screen wastewater with estrogenic activity. These transgenic models have primarily been developed for zebrafish strains (Schreurs et al., 2002; Damdimopoulou and Treuter, 2011; Gorelick and Halpern, 2011; Brion et al., 2012; Lee et al., 2012), but other small-bodied fish have been used such as the Japanese medaka (Kurauchi et al., 2005; Spirhanzlova et al., 2016; Abdel-moneim et al., 2018; Spirhanzlova et al., 2020). The basic idea of these *in vivo* assays has been to insert reporter genes (i.e., green fluorescent protein) into the genome of fish which are subsequently driven by hormone response elements (HRE) or larger hormone-responsive promoters. These *in vivo* biosensors can then be used to screen wastewater effluents or complex mixtures for hormonally active agents.

Perhaps not surprisingly, some of the earliest transgenic models were developed for detecting estrogenic effluent. Legler et al. (2000) first developed the pEREtata-Luc transgenic model incorporating luciferase into zebrafish in the form of a supercoiled DNA-construct driven by an estrogen response element (ERE). Later on, Zeng et al. (2005) developed a transgenic medaka with a GFP under an estrogen-inducible promoter derived from VTG 1. Moreover, a zebrafish transgenic cell lines has been made using the red fluorescent protein mCherry with the telegenic promoter Tg(vtg1:mCherry) (Bakos et al., 2019; Csenki et al., 2020). Other estrogen biosensors have utilized pigment free zebrafish termed the 'Casper' phenotype, which has the advantage of quantifying whole-body fluorescence responses to estrogens in a high throughput manner without the confounder of skin pigment (Green et al., 2016).

In addition to models that utilize ERE and partial promoters for estrogen-responsive transcripts, such as VTG, advances have also been made in developing transgenic fish lines for steroidogenic enzymes, in particular aromatase which converts terminal androgens into estrogens (e.g., cyp19a1a-eGFP zebrafish line; Hinfray et al., 2018; De Oliveira et al., 2020). One transgenic model, the tg(cyp19a1b-GFP) zebrafish line, was originally developed to study aromatase expression in radial glia of fish (Tong et al., 2009), and was subsequently used to test chemicals for estrogenicity (Petersen et al., 2013) and responsiveness to aromatizable androgens, non-aromatizable androgens, and synthetic progestogens alone and in mixtures (Brion et al., 2012). This year, this assay deemed EASZY assay was approved by the OECD (Conceptual framework Level 3, OECD TG 250) as a 96-h test for embryos treated with chemicals and investigated with a fluorescence imaging microscope. Similarly, an OECD test guideline draft (OECD, 2020) is being written on the choriogenin h-gfp (chgh-gfp) medaka line (Spirhanzlova et al., 2016), known as the Rapid Estrogen Activity Tests *in vivo* (REACTIV). This transgenic line allows to either quantify ER activation by GFP quantification, or it can evaluate indirectly the aromatase activity by supplementing testosterone in the assay. The OECD is also currently reviewing RADAR (Rapid Fluorescent Detection of (anti)androgens with spiggin-gfp medaka; Sébillot et al., 2014) for the evaluation of androgenic compounds.

CRISPR/Cas-based gene editing is poised to become a widely used technology for developing transgenic fish models to study EDCs. Most recently, CRISPR/Cas9 has been utilized to develop gene "knock-in models" to study EDCs, specifically a zebrafish transgenic line which expresses GFP in response to estrogenic chemicals, such as bisphenol A (Abdelmoneim et al., 2020). Moving out of the laboratory into the field, the use of such transgenic assays has been demonstrated with environmental samples collected directly from river systems and other bodies of water (Gorelick et al., 2014; Sonavane et al., 2016), further supporting the use of transgenic fish models as environmental monitoring tools for estrogenic effluent and water.

7.2. Use of co-culture and 3D tissue culture for in vitro EDC testing

In vitro bioassays are often single cell-type reporter assays or cells in (primary) monoculture. Although such *in vitro* bioassays have considerably improved our understanding of chemical mechanisms of toxicity, they determine single endpoints in single cell types, which poorly reflect the reality at the tissue level or intact organism that have complex cell-cell interaction between present cell types. One of the driving research

Table 6
AOPs endorsed by OECD in the AOPWiki.

AOP number	MIE	Organism	AO
3	Binding of inhibitor, NADH-ubiquinone oxidoreductase (complex I)	Human/rat	Parkinsonian motor deficits
6	Antagonist binding to PPARα leading to body-weight loss	Human/rat// mouse/ fathead minnow/ quail	Decreased Body Weight
10	Binding to the picrotoxin site of ionotropic GABA receptors leading to epileptic seizures in adult brain	Human/rat/ mouse/quail/ zebrafish	Epileptic seizure
12	Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development leads to neurodegeneration with impairment in learning and memory in aging	Human/rat/ mouse/ monkey/ zebrafish	Neurodegeneration, impairment of learning and memory
13	Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities	Human/ mouse/ monkey/rat	Impairment of learning and memory
21	Aryl hydrocarbon receptor activation leading to early life stage mortality, via increased COX-2	Zebrafish/ medaka/ chicken	Early life stage mortality
23	Androgen receptor agonism	Fathead minnow	Ovary development
25	Aromatase Inhibition	Medaka/ zebrafish/ fathead minnow	Ovary development
42	Thyroperoxidase Inhibition	Human/rat/ mouse/ chicken/ zebrafish/ fathead minnow	Development impairment
48	Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	Human/rat/ mouse	Impairment of learning and memory

goals in the study of EDCs is to better reproduce *in vivo* interactions by developing co-culture and 3D cell models that incorporate physiologically relevant intercellular communications (e.g., Yancu et al., 2020), with a focus on steroidogenesis, an important, but poorly studied target for EDCs. We point out that there are efforts to develop such tools to study thyroid hormones; however, in the interest of brevity, we only provided examples only focused on steroidogenesis. Several *in vitro* cellular co-culture models with enhanced physiological and toxicological relevance to the effects of EDCs on steroidogenesis have been developed. These include co-culture models of the feto-placental unit and human breast tumor micro-environment. These co-cultures successfully model the essential steroidogenic interactions between the placenta and the fetal adrenal gland during pregnancy (Thibeault et al., 2014), or between mammary epithelial tumor cells and the surrounding adipose stromal cells in the case of hormone dependent breast cancer (Yancu et al., 2020). These models have elucidated a number of novel mechanisms of endocrine disruption by pesticides (Caron-Beaudoin et al., 2017), antidepressant drugs (Hudon Thibeault et al., 2017), and natural compounds (Yancu and Sanderson, 2019; Yancu et al., 2019, 2020) at the level of hormone production, expression of steroidogenic enzymes key to the biosynthesis of progestins, androgens and estrogens, and particularly the regulation of aromatase (cytochrome P450 19; CYP19) by alternate tissue-specific promoters (Caron-Beaudoin et al., 2016, 2018; Yancu et al., 2019, 2020). Current efforts focus on the development of piscine and avian co-culture models that reproduce the HPG axis for the study of potential disruption by EDCs with consideration for emerging and persistent pesticides.

Although cellular co-culture models that allow different cell types to interact in a manner close to intercellular communication *in vivo*, they still consist of two types of cells in monolayers, either mixed together in multi-well cell culture plates or in multi-well plates with one cell-type at the bottom of the wells and the other attached to inserts. To better mimic the architecture and physiological structure of the *vivo* tissues in humans, numerous 3D culture models have been developed. 3D cell cultures ideally form a structure that is similar to the way cells are organized in an *in vivo* tissue. Such structures, referred to as organotypic, organoid or spheroid, better reflect *in vivo* cell-cell interactions and cell signaling behavior, gene expression profiles and transport dynamics closer to that exhibited *in vivo* (Alépée, 2014). 3D cell cultures usually require a matrix or scaffold to help the cells organize into relevant *in vivo*-like structures (Rimann and Graf-Hausner, 2012). In the area of endocrine disruption, the application of 3D cell culture and 3D co-culture models is less common in part to various reasons. One reason is the lack of standardization of 3D culture. Projects of the European Cluster to Improve Identification of Endocrine Disruptors (EURION; https://eurion-cluster.eu/) are aiming to develop and standardize 3D culture. Another reason delaying the adoption of 3D cell culture is the problem of extrapolation between species. For example, van den Brand et al. (2019) compared the human rat 3D co-culture of endometrial cell-types and found that in response to progesterone the gene expression of ERα increased in the human model but decreased in the rat model. Estrogens and progesterone did not affect the aryl hydrocarbon (AhR) gene expression in the human model but decreased it in the rat model. Progesterone had no effects on the induction of AhR-mediated CYP1A1 expression by the AhR ligands in the human model, whereas it reduced the inducibility of CYP1A1 in the rat model compared to no hormonal exposures (van den Brand et al., 2019). These observations indicate that extrapolation between 3D models is not completely reliable, which complicates further *in vivo* extrapolation. Clearly 3D culture and co-culture models are underrepresented in the mechanistic study of

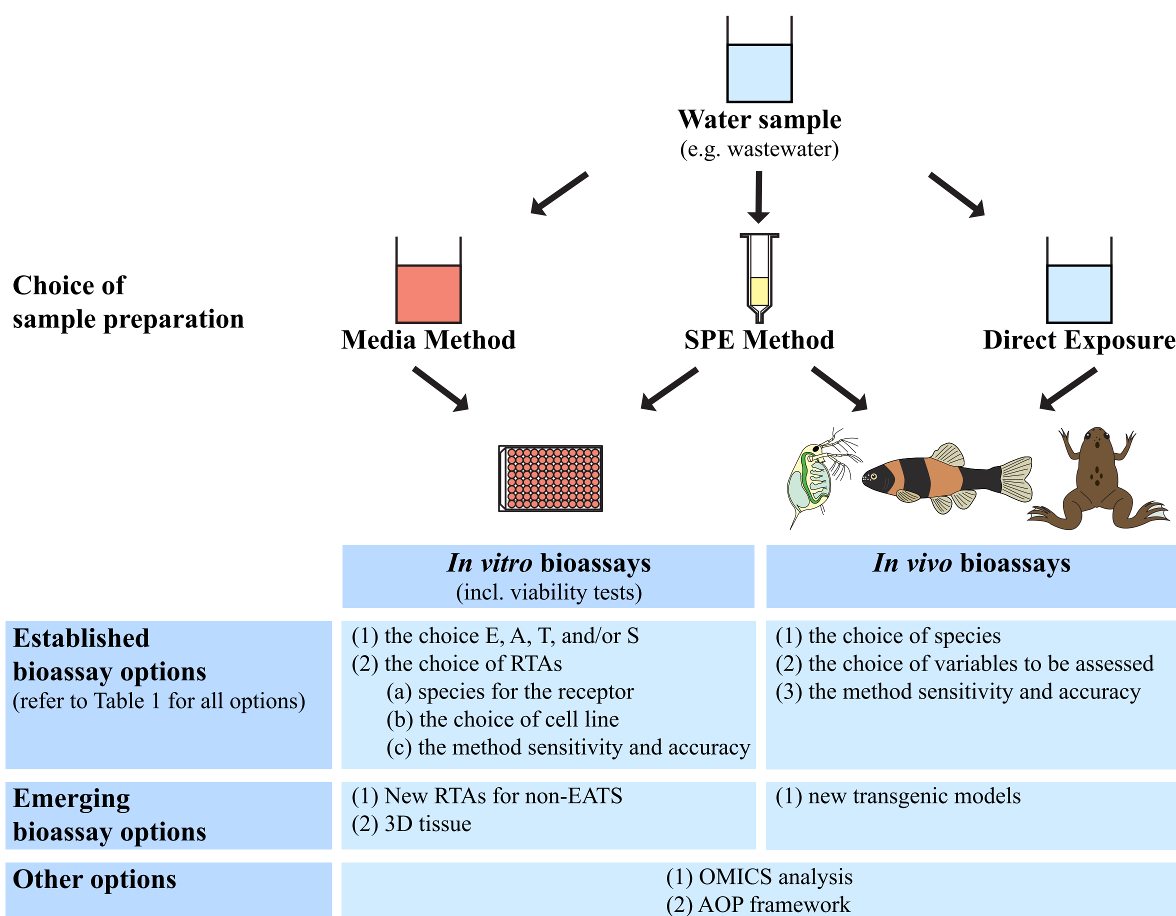

Fig. 4. Summary of the options available to develop a testing strategy based on bioassay.

EDCs making the development of such models a major priority and area of growth in the field of endocrine toxicology.

7.3. OMICS and adverse outcome pathways (AOPs)

One limitation to some bioassays is they only target one type of mechanism of action of EDCs, for example, ER-RTA will only screen for EDCs that act through the ER. Therefore, the use of complementary OMICs analysis can aid integrating the different endocrine pathways at the same time. Indeed, the development of simple, robust and high throughput assays to assess endocrine disruption is greatly facilitating our ability to detect changes at the transcriptomic level (messenger RNA content, mRNA) to reveal early biomarkers of EDCs exposures (Corton et al., 2019; Soufan et al., 2019). In this regard, screening of EDCs can now be performed using targeted multiplex qPCR analysis assessing the expression of genes involved in EATS and non-EATS endocrine axes, simultaneously, and this, in cell lines, embryos, or whole animals. Needless to say, these approaches have not been limited to aquatic species and OMIC-based studies have been leveraged in mammalian species, including humans to discern EATS and non-EATS mechanisms of action and links to diseases. Moreover, with the rapid development of toxicogenomic tools data integration at different molecular levels (epigenetic, transcriptomic, proteomic, metabolomic, lipidomics), new mechanism-based biomarkers can be developed for Adverse Outcome Pathway (AOP) validation. In this regard, a multi-omics data integration approach, recently used in several species including for example zebrafish, mice and rats, have allowed researcher to better understand the modes of action of EDCs and identify biomarkers of exposure or effect (Mesnage et al., 2017; Ortiz-Villanueva et al., 2017; Shu et al., 2019; Tarazona et al., 2019; Mustieles et al., 2020). While it is clear that many different pathways can lead to effects in development, growth, reproduction and susceptibility to disease, each pathway is initiated to begin with by the interaction of a contaminant with a molecular target and that initial interaction sets in motion a series of key events at the cellular, tissue, organ and individual level that result in the adverse outcome (Ankley et al., 2010). In the case of aromatase inhibition as a molecular initiation event where, testosterone is not converted to E2, a hormone that is necessary for reproduction, it is clear that all subsequent key events from activation of the ER to the production of VTG, and the spawning of eggs will be inhibited downstream. AOPs are compound agnostic tools, so rather than focusing on specific chemicals, the AOP concept focuses on the pathways.

While the first AOPs were by necessity linear trajectories, they have now matured into more complex networks that better describe biological systems, and molecular pathways that interact with each other. Some of the points of interactions can be considered as hubs, serving to direct several pathways which join together at these points to result in the same adverse outcome. It is now relevant to map networks of AOPs (Knapen et al., 2015) to get a complete and accurate vision of how EDCs may perturb a biological system. As an example, Knapen et al. (2015) illustrates in an AOP framework how the thyroid hormone axis can be disrupted during fish development. Here three different AOPs (#42, #156, and #157) are established for the same apical outcome, reduced swimming performance. In AOP #42, which involves the juvenile stage of fish, thyroid peroxidase inhibition results in decreased serum concentrations of T4, followed by a reduction of T3 (which is also impacted by inhibition of deiodinase, as in the embryonic stage). For AOPs #156 and #157, the inhibition of deiodinase impacts the production of T3, resulting in the reduction of inflation of the posterior swim bladder. Integrating these AOPs together makes it clear that the ultimate adverse effect would be observable at the population level (Knapen et al., 2015).

The AOPwiki (https://aopwiki.org) is the official repository for AOPs. AOPs submitted in this platform are peer reviewed and finally endorsed by organizations such as the OECD. Remarkably, there are only sixteen AOPs that have received full OECD endorsement to date and of these only eight relate to endocrine disruption (Table 6). The EDC-related AOPs include three related to AhR-mediated activities (# 21, #131, and #150), one related to AR-mediated activity (#23), one related to aromatase inhibition (# 25), two related to the thyroid hormone axis (#42 and #54) and one related to action through peroxisome proliferator activated receptor (PPAR) (#6), which is not normally included in the EATS group. Of the eight non endocrine related AOPs, three relate to glutamate receptors (#14, #15, #19), one to mitochondrial activity in the brain (#3), one relates to a chloride channel in the brain (#10), and the other three relate to irreversible modifications of proteins or DNA (#15, #38, and #40). There are at least 13 additional AOPs that are under review, several that are open for comment or adoption and many more that are in development.

Numerous authors encourage the use of AOPs to organize the knowledge gathered in studies, for example to assess contaminants that affect reproduction, the thyroid axis, or that can be used for read-across to other vertebrates (Johansson et al., 2020; Knapen et al., 2020; McArdle et al., 2020). Future studies should investigate how to incorporate the AOP output into EBT calculations.

8. Final recommendations to developing an EDC testing strategy now

As described throughout this review, there are various options of bioassays that allow quantifying specific effects related to EATS pathways in various water matrixes (Fig. 4). To monitor EDCs in water resources (including in complex effluents), *in vitro* bioassays 1) provide cost effective methods, 2) reduce the use of animals and 3) generate numerous data that can be integrated into AOPs, which are becoming increasing used to conduct chemical risk assessments (Barton-Maclaren et al. this issue). In addition, by developing appropriate EBTs, *in vitro* bioassays can provide an adequate tool to monitor the presence of EDCs and could eventually help with future EDC regulations.

For example, EBTs for estrogenicity in water resources are making their way slowly into regulation of the state of California. The Water Quality Control Policy for Recycled Water of now recommends a trigger level of 3.5 ng/L EEQ using an ERα-RTA (State Water Ressources Control Board, 2019). This is one the first government to advice on EBT values for EDCs. Likewise, the *Ministère de l'Environnement et de la Lutte contre les Changements Climatiques du Québec* (MELCC) has initiated the development of a screening strategy using a mix of bioassays in order to monitor EDCs in wastewater effluents. The strategy used in Quebec consists of a two-Tier approach. The first Tier aims to target the EAS pathways using the ER-RTA, AR-RTA, and H295R steroidogenesis assays. If a given sample exhibits any positive activity exceeding the calculated EBTs, the sample would be further tested in a modified version of the fish reproduction assay using fathead minnow (Tier 2) to validate the effects observed in Tier 1. This approach is being currently validated by the Langlois laboratory. Moreover, the Ontario Ministry of the Environment, Conservation and Parks (Canada) in collaboration with the Yargeau laboratory has also been investigating the application of bioanalytical tools for the rapid assessment of residual biological activity of wastewater using *in vitro* cell based assays (Petosa et al., Submitted). These examples highlight the increasing number of countries considering regulatory screening of EDCs in their effluents.

No matter the initiative, here is a list of recommendations when establishing a battery of *in vitro* bioassays:

- Select *in vitro* bioassays for multiple endpoints of the EATS pathways, and if possible include non-EATS pathways;
- Conduct two cytotoxicity assays – assessing two different pathways/ mechanisms of cell death;
- Include various animal species if possible;
- Prioritize internationally standardized and/or commercial assays that come with quality control, e.g., GeneBlazer (ThermoFisher), Indigo, and CALUX. Of note, Indigo and GeneBlazer do not require a signed license;

- Define EBT for each endocrine endpoint comprised in the bioassay battery, which will act as a threshold for risk assessment.

There is also a current need for standardization and validation of methods. The following points should be considered:

- Determine quality controls and internal standard for QA/QC for the bioassay and sample preparation of any type of water – much like those developed by NIST;
- Conduct inter/intra laboratory validation to verify the methodology;
- Continue developing new methodologies (e.g., 3D tissue culture, OMICS) to be included in the international and governmental frameworks.

Author contributions

Julie Robitaille: conceptualization, data curation, writing (original draft, review & editing), visualization, supervision, project administration, Nancy D. Denslow: writing (original draft, review & editing), Beate I. Escher: writing (original draft, review & editing), Hajime Kurita: writing (original draft, review & editing), Vicki Marlatt: writing (original draft, review & editing), Christopher J. Martyniuk: writing (original draft, review & editing), Laia Navarro-Martin: writing (review & editing), Ryan Prosser: writing (original draft, review & editing), Thomas Sanderson: writing (original draft, review & editing), Viviane Yargeau: writing (review & editing) and Valerie S. Langlois: conceptualization, supervision, project administration, writing (original draft, review & editing), funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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