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## The *Dicentrarchus labrax* estrogen screen test: A relevant tool to screen estrogen-like endocrine disrupting chemicals in the aquatic environment

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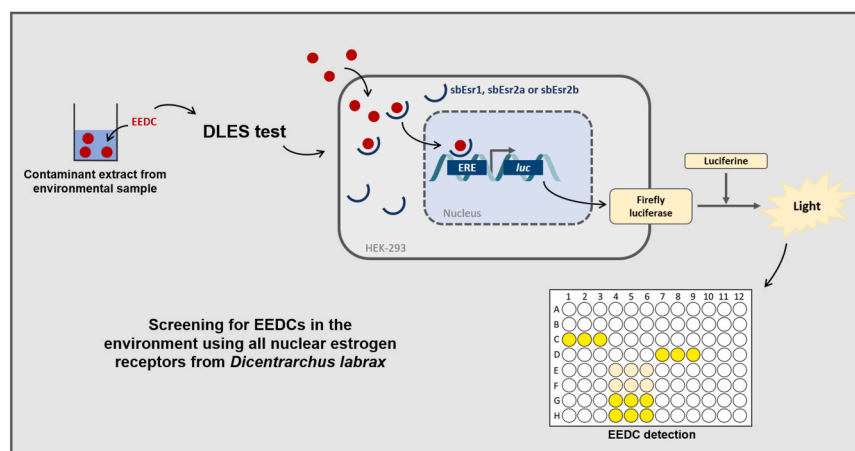
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### HIGHLIGHTS

- Improved screening of estrogenic compounds with all nuclear receptors from sea bass.
- High sensitivity and specificity of sbEsr2a and sbEsr2b different from hER $\alpha$ .
- Bioassay suitable to assess water quality regarding EEDC occurrence.
- An initial step in developing an AOP for the marine environment.
- Valuable for predicting adverse effects in teleost species.

### GRAPHICAL ABSTRACT



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### ABSTRACT

In response to the need for the diversification of regulatory bioassays to screen estrogen-like endocrine disrupting chemical (EEDC) in the environment, we propose the use of a reporter gene assay involving all nuclear estrogen receptors from *Dicentrarchus labrax* (i.e., sbEsr1, sbEsr2a, or sbEsr2b). Named DLES test (*D. labrax* estrogen screen), it aims at complementing existing standardized *in vitro* tests by implementing more estrogen receptors notably those that do not originate from humans.

Positive responses were obtained with all three estrogen receptors, and—consistently with observations from other species—variations in sensitivity to E2 were measured. Sensitivity and EC50 values could be classified as

Biomonitoring  
Sea bass

follows: sbEsr2b < sbEsr2a < sbEsr1. The pharmacological characterization with a human estrogen receptor antagonist (fulvestrant) successfully validated the specific involvement of each sbEsr and evidenced the capacity of the DLES test to highlight antagonist interactions. The DLES test was applied to WWTP contaminant extracts. A positive response was detected in the inflow sample in accordance with the YES test, but not in the outflow sample. Notwithstanding, the DLES test (sbEsr2b) exhibited greater sensitivity for the screening of those samples. This study demonstrates the need for more comprehensive testing including representatives of marine species for a better detection of EEDCs. The DLES test appears as a pertinent tool to predict adverse effects and to widen the scope of screening and hazard assessment of EEDCs in the environment.

## 1. Introduction

An endocrine disrupting chemical (EDC) can mimic or antagonize hormone action by binding to a hormone receptor or by interfering with several aspects of hormone production or action (La Merrill et al., 2020). Exposure to an EDC can alter physiological functions such as reproduction, immunity, growth, or metabolism, even at very low concentrations. At higher biological level, they can have a large-scale impact on the environment, for instance, by affecting the populations of aquatic organisms (Jobling and Tyler, 2003; Hayes et al., 2003; Rodríguez et al., 2007; Kidd et al., 2007). In humans, EDCs can cause diseases and disabilities (e.g., IQ loss and associated intellectual disability, autism, attention-deficit hyperactivity disorder, adult and childhood obesity, adult diabetes, cryptorchidism, male infertility, and mortality associated with reduced testosterone), leading to an estimated cost of \$209 billion per year within the European Union (Trasande et al., 2015) and \$340 billion in the USA (Attina et al., 2016). The ecological costs (i.e., impact on fisheries and livestock) associated with EDCs are likely to be substantially higher, but remain underestimated (Jaacks and Prasad, 2017).

Among EDCs, estrogen-like endocrine disrupting chemicals (EEDCs) are characterized by their estrogenic properties by which they can interfere with endogenous estrogen signaling (Shanle and Xu, 2011). Several studies reported the threats that EEDCs can pose to aquatic organisms and populations by targeting essential physiological functions (e.g., reproduction, immunity, growth) that are sensitive to estrogens (Moreira et al., 2021; Paula Alves da Silva et al., 2018; Rodríguez et al., 2007; Slaby et al., 2019; Zhou et al., 2010).

Today, EEDCs are ubiquitous, originating from various sources such as industrial activities, agriculture, and wastewater emissions (Casals-Casas and Desvergne, 2011; Kabir et al., 2015). Effluents from urban areas and hospitals are raising particular concern, as they represent a substantial source of pharmaceutical products, including synthetic estrogens (Pauwels and Verstraete, 2006). EEDCs are often found in high concentrations in the inflow and are still present in the outflow of wastewater treatment plants (WWTPs), which do not efficiently degrade and remove many of them, thus posing a risk to aquatic species and human health (López-Velázquez et al., 2021; Spataro et al., 2019; Tran et al., 2018; Zdarta et al., 2022). The efficiency of EEDC removal depends largely on the WWTP generation and on the properties of the molecules in question (Pauwels and Verstraete, 2006). As reviewed by Almazrouei et al. (2023), it can result in concentrations ranging from 0.3 to 670 ng.L<sup>-1</sup> in influents and 0.2–196.7 ng.L<sup>-1</sup> in effluents.

EEDCs can act at very low concentrations. Consequently, efficient detection of these compounds is essential to empower policy-makers and stakeholders to enact legislation aimed at reducing their emissions in the environment. In this context, *in vitro* bioassays offer high-throughput methods that complement chemical analysis, particularly when dealing with the detection of trace concentrations and contaminant mixtures and evaluate the risks of adverse effects, that can include emerging compounds and transformation products (Gou et al., 2016; Hettwer et al., 2018; Kunz et al., 2015; Leusch et al., 2014; Wernersson et al., 2015). Concerning EEDCs, available standardized *in vitro* solutions are valuable in assessing the estrogenic activities of various known substances (Durcik et al., 2022; Gramec Skledar et al., 2020), as well as in drinking water and environmental samples (Villanueva et al., 2021).

These assays can also be applied to evaluate the effectiveness of WWTPs (Itzel et al., 2020). As listed in the OECD (2024, 2018) guidelines, *in vitro* assays used to study EEDCs are:

- (i) The OECD TG 493, an estrogenic receptor binding assay conducted to detect compounds with an affinity for the human recombinant estrogen receptor alpha (hER $\alpha$ ). It is based on the competition between a test compound and a radiolabeled ligand for binding to the estrogen receptor.
- (ii) The OECD TG 455 (also referenced as ISO 19040-3), a stably transfected transactivation *in vitro* assay using luciferase reporter gene. It describes two methods involving the ER $\alpha$ -HeLa-9903 cell line and the VM1Luc-4E2 cell line which expressed estrogen receptors.
- (iii) The ISO 19040-1 & 2, the yeast estrogen screen (YES) conducted with either *Saccharomyces cerevisiae* (1) or *Arxula adenivorans* (2), both of which are transfected with hER $\alpha$ . This test uses a  $\beta$ -galactosidase reporter gene.

However, these tests primarily focus on human estrogen receptors. Given the diversity of estrogen receptors in vertebrates, one might question whether these *in vitro* assays can provide specific information on the effects of EEDCs elicited in marine organisms, namely in fish that suffer lifelong exposures to pollutants and are typical sentinel species for chemical pollution (Yost et al., 2014).

*Dicentrarchus labrax* is a marine fish that spends sensitive larval and juvenile life stages in estuaries (Ramos et al., 2012), which are often highly contaminated ecosystems. It is a species of significant economic importance in fishing and aquaculture with its genome well-annotated (Mazón et al., 2015; Tine et al., 2014). *D. labrax* possesses three nuclear estrogen receptors known as sbEsr1, sbEsr2a, and sbEsr2b (also designated as sb-ER $\alpha$ , sb-ER $\beta$ 1, and sb-ER $\beta$ 2, respectively), and two membrane estrogen receptors isolated and characterized (expression, tissue distribution/localization and regulation, transactivation properties and disruption) (Halm et al., 2004; Pinto et al., 2018; Zapater et al., 2024). This difference likely derives from a gene duplication event in the teleost lineage, leading to the emergence of duplicate genes that code for sbEsr2a and sbEsr2b (Hawkins et al., 2000; Katsu et al., 2008).

The roles of sbEsr1, sbEsr2a, and sbEsr2b are not yet fully understood; however, they are likely to be involved in various physiological functions beyond reproduction. For instance, Moreira et al. (2021) provided strong evidence of their involvement in the regulation of immune system development. Zapater et al. (2019) provided information as to the distribution of these receptors in various tissues and organs, but also as a function of the ovarian developmental cycle of *D. labrax*. They showed that sbEsr1 was predominantly expressed in the pituitary gland, but that it could also be found in the gonads, brain, and head kidney of males and females alike. Interestingly, sbEsr2a, which was principally expressed in the pituitary gland, gonads, and kidneys, showed higher expression level in males, whereas sbEsr2b exhibited strong expression levels in the pituitary gland, gonads, liver, intestines, and kidneys, with higher levels observed in the gonads of females and in the kidneys of males. Furthermore, during ovarian development, the expression of sbEsr1 and sbEsr2a in ovaries increased, with a peak at the maturation/ovulation stage, whereas sbEsr2b was mainly expressed at early

vitellogenesis and the maturation/ovulation stages. In the liver, the expression pattern during ovarian development was similar to that of sbEsr1 in ovaries, but not for sbEsr2a, which remained constant throughout the cycle, and sbEsr2b with the disappearance of the peak at the maturation/ovulation stage.

Despite a growing understanding of the diversity of estrogen receptors in fish as well as their tissue distribution, regulation and functional characteristics, normalized *in vitro* assays designed to detect EEDCs still predominantly focus on hER $\alpha$  (OECD, 2018). The extensive diversity of EEDCs potentially implies variability in responses across species and a wide range of effects on physiological functions. To cover this species dependent variability, it would be advantageous to take into account other estrogen receptors instead of solely hER $\alpha$ . This can help to inform extrapolations from human to fish estrogen receptors, as their respective selectivity can differ (Cosnefroy et al., 2012; Pinto et al., 2014; Sonavane et al., 2016; Yost et al., 2014). Thus, it appears pertinent to widen the scope of EEDC testing and to integrate more species and receptors in bioassay design, in order to provide more comprehensive information that can help to predict adverse outcomes in different apical endpoints and to provide information of their putative mode of action. In this work, we propose a test to screen EEDCs in the environment based on a reporter gene assay incorporating the three nuclear estrogen receptors from *D. labrax* (Muriach et al., 2008; Pinto et al., 2019b; Quesada-García et al., 2012; Zapater et al., 2019). Quesada-García et al. (2012) previously used a similar test to assess with pertinency the estrogenic activity of fish diet and to screen EEDCs in environmental samples, such as a WWTP effluent. However, their test focused on sbEsr1 only. Hence, our study is an extension to their work, proposing a reporter gene assay encompassing sbEsr1 as well as sbEsr2a and sbEsr2b as a tool to more comprehensively detect the presence of EEDCs—*D. labrax* estrogen screen (DLES) test. We compared the sensitivity and responsiveness of each nuclear estrogen receptor and apply our test to contaminant extracts from the inflow and outflow of a WWTP. To assess the reliability of DLES test, the results were confronted with those obtained from the normalized ISO 19040-1 test (YES test).

## 2. Materials and method

### 2.1. Reagents and substances

Chloridric acid (HCl, 37%), dichloromethane (DCM), methanol (MeOH), dimethyl sulfoxide (DMSO), Gibco™ Dulbecco's Modified Eagle Medium (DMEM, with high glucose, sodium pyruvate, GlutaMAX™ and phenol red), Gibco™ DMEM Nutrient Mixture F-12 (DMEM/F-12, no phenol red), Gibco™ penicillin-streptomycin (10,000 U.mL<sup>-1</sup>), Gibco™ Opti-MEM™ I Reduced Serum Medium (no phenol red), Invitrogen™ Lipofectamine™ 3000 Transfection Reagent, Thermo Scientific™ 17- $\beta$  estradiol (E2, purity: 98%), and fulvestrant ICI 182,780 were all purchased from Fisher Scientific (Waltham, USA). Ultrapure water was obtained from Merck Millipore (Burlington, USA). Charcoal stripped FBS (South America origin) were obtained from Dutscher (Bernolsheim, France). Steady-Glo® luciferase assay system was procured by Promega (Madison, USA). Fetal bovine serum (FBS, non-USA origin) and thiazolyl Blue Tetrazolium Bromide (MTT) were purchased from DMLabo (Bretteville-sur-Odon, France).

### 2.2. Extraction of contaminants from WWTP influent and effluent

#### 2.2.1. Sample collection and treatments

In April 2022, inflow and outflow samples (2 L) were provided by the operators of the WWTP located in Le Havre (France). This WWTP is operating wastewater for approximately 300,000 inhabitants and has the capacity to process up to 80,000 m<sup>3</sup> d<sup>-1</sup> of both stormwater and wastewater. As a control, a blank sample consisting of ultrapure water was subjected to the same contaminant extraction protocols as employed for the WWTP samples. On the day of collection, a volume of

1.1 L from each sample was filtered through pyrolyzed 2.7- $\mu$ m glass fiber filters, pH-adjusted to 2 using 37% HCl, and stored at 4 °C in the dark.

#### 2.2.2. Solid phase extraction

The extraction protocol was based on methods outlined in Dufour (2017) and Fuster (2017). Briefly, the procedure consisted of extracting contaminants with 3 cc Oasis® HLB cartridges, each containing 60 mg of the sorbent phase (Hydrophilic Lipophilic Balance, Waters Corporation, Milford, USA) installed on a solid-phase extraction vacuum manifold. The sorbent phases were successively conditioned with DCM (2  $\times$  3 mL), MeOH (2  $\times$  3 mL), and ultrapure water (2  $\times$  3 mL, pH 2) before loading each sample onto the Oasis HLB cartridge. Then, the sorbent phases were completely dried. This step was checked by gravimetric control. The molecules adsorbed onto the sorbent phase were eluted successively using 3 mL of MeOH, 3 mL of MeOH:DCM (1:1), and 3 mL of DCM. The eluates were collected in glass test tubes and stored in the dark at -20 °C until the solvent evaporation phase. Throughout these procedures, the maximum percolation speed was maintained at 15 mL min<sup>-1</sup>. Ultimately, the eluates were completely evaporated under a gentle nitrogen stream, and the resulting extracts were immediately resuspended in DMSO to achieve a concentration rate of 20,000 $\times$ . These stock solutions were stored in amber glass vials (Low Adsorption QsrtVial™ 0.3 mL, Supelco Inc., Bellefonte, USA) at -20 °C in the dark.

### 2.3. Reporter gene assay using *Dicentrarchus labrax* nuclear estrogen receptors

#### 2.3.1. Plasmid production

The plasmids used in this work were three pcDNA3 based plasmids containing the complete coding sequences of *sbesr1* (Genbank Acc. n. AJ505009.1) as described in Muriach et al. (2008), *sbesr2a* (GenBank Acc. n. KY968340.1), and *sbesr2b* (Genbank Acc. n. KY968341.1) as described Zapater et al. (2019), the pERE-TK-LUC plasmid, which expresses the firefly luciferase gene under the control of several estrogen response element (ERE) binding sites (Le Page et al., 2006), and the pEGFP-N3 plasmid (Clontech, Mountain View, USA), constitutively expressing the green fluorescent protein gene. Plasmid amplification and production was done by standard techniques (Green and Sambrook, 2012). High-copy plasmid purification was carried out using Nucleo-Bond® Xtra Midi Endotoxin-free (Macherey-Nagel, Düren, Germany), following the manufacturer's recommendations. Plasmid concentrations were determined using the NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Scientific, Waltham, USA). Finally, the plasmids were aliquoted and stored at -20 °C until use.

#### 2.3.2. Transient transfection

The experimental methodology was based on and slightly modified from Muriach et al. (2008) and Zapater et al. (2019). Human embryonic kidney 293 cells (HEK-293, ATCC reference CRL-1573, devoid of estrogen receptors) were grown at 37 °C and 5% CO<sub>2</sub> in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin until reaching 80–90% confluence. Cells were resuspended in DMEM containing 10% FBS and seeded in 24-well plates (Cellstar®, Greiner Bio-One, Kremsmünster, Austria) at a concentration of 125,000 cells per wells (600  $\mu$ L). After 24 h of incubation at 37 °C and 5% CO<sub>2</sub>, a DNA-lipid complex was prepared using Lipofectamine™ 3000 reagent, following the manufacturer's recommendations, with a total of 0.75  $\mu$ g of DNA used for each well (details are given in supplementary Table A1.). The cell culture medium was replaced by 200  $\mu$ L of DMEM/F-12 supplemented with 10% charcoal-stripped FBS. Subsequently, 50  $\mu$ L of the DNA-lipid complex were added to each well. The cells were incubated at 37 °C and 5% CO<sub>2</sub> for an additional 24 h.

The success of the transfection process was confirmed if more than 50% of the cells expressed the green fluorescent protein, observed using a fluorescent microscope. Then, cells were resuspended in 600  $\mu$ L of DMEM per well containing 10% FBS and 1% penicillin/streptomycin.

They were collected, pooled in polystyrene centrifuge tubes, and homogenized according to the receptor type. At last, 100  $\mu\text{L}$  of cells were plated in 96-well white polystyrene microplates with clear bottom (Corning®, Somerville, United States) and incubated at 37 °C and 5%  $\text{CO}_2$  for 24 h before initiating contaminant exposures.

### 2.3.3. Exposures

Exposure solutions were freshly prepared before each experiment. DMSO was used as a solvent to obtain a stock solution of E2 (0.01 M), fulvestrant (0.01 M), and WWTP extracts (20,000 $\times$ ). E2 (10-fold dilutions from 0.1 pM to 100 nM), fulvestrant (10 and 100 nM), and WWTP extract (0.001 $\times$ , 0.01 $\times$ , and 0.1 $\times$ ) concentrations to be tested were prepared by serial dilutions in DMEM/F-12 (10% FBS charcoal stripped + 1% penicillin/streptomycin). All these exposure conditions contained a final proportion of 0.001% DMSO. All experiments included a negative control containing 0.001% DMSO and a negative control without DMSO. Transfected cells were exposed to a final volume of 100  $\mu\text{L}$  for 24 h, at 37 °C and 5% of  $\text{CO}_2$ .

### 2.3.4. Luciferase assay

The activity of the firefly luciferase in cells was determined using the Steady-Glo® luciferase assay system (Promega) according to the manufacturer's recommendations. In each well containing transfected cells exposed to the different mixtures explained above, a volume of 100  $\mu\text{L}$  of reagent was added to the 100  $\mu\text{L}$  of exposure mixture. The plate was incubated for 5 min at room temperature. Enzymatic activity was then assessed by quantifying the luminescence produced in each well using the Tecan Infinite® M200 microplate reader (Tecan, Männedorf, Switzerland).

### 2.4. Cytotoxicity assay

As a higher number of viable cells would surely lead to a higher production of luciferase and therefore more bioluminescence, any cytotoxicity may lead to misinterpretations of the outcome of the DLES assay. Thus, the assessment of the compound cytotoxicity is an essential step to allow for a correct interpretation of the reporter gene assay.

To assess the transfected cell viability, MTT tests were conducted. The cells, which were transfected with pcDNA3-sbEsr2a, were incubated for 3 h, at 37 °C and 5%  $\text{CO}_2$  after adding 10  $\mu\text{L}$  of MTT at the concentration of 5 mg  $\text{mL}^{-1}$  to each well. Then, cells were lysed, and thereafter the purple formazan crystals were dissolved by adding 150  $\mu\text{L}$  of DMSO. Absorbance values for each well were measured using a Tecan Infinite® M200 microplate reader at a measurement wavelength of 570 nm and a reference wavelength of 630 nm.

### 2.5. YES and anti-YES tests

The YES test was performed according to the methodology of Routledge and Sumpter (1996) modified by Denier et al. (2008). This procedure followed ISO 19040-1 standard. Different concentrations of E2 (0, 0.01, 0.03, 0.1, 0.32, 1, 3.2, and 10 nM), and WWTP samples (0 $\times$ , 0.0001 $\times$ , 0.0003 $\times$ , 0.001 $\times$ , 0.003 $\times$ , 0.01 $\times$ , 0.03 $\times$ , 0.1 $\times$ , 0.32 $\times$ , 1 $\times$ , 3.2 $\times$ , and 10 $\times$ ) with or without E2 (0.5 nM) were tested. After 48 h incubation at 30 °C, the enzyme activity was quantified by measuring the absorbance.

### 2.6. Limit of detection determination and statistical analysis

All analyses and graphical illustrations were performed on RStudio (build 576, R v. 4.1.2, R Core Team, 2021; RStudio Team, 2022). In all experiments, triplicate measurements were averaged and normalized according to the negative control (without DMSO for MTT and DLES tests, and with DMSO for the YES test). The results were expressed as the mean of experiments  $\pm$  standard error of the mean.

The limits of detection (LOD) for E2 in the DLES test on the one hand and the YES test on the other hand were determined according to a statistically robust method adapted to bioassays, as recommended by Holstein et al. (2015). In brief, the limit of blank ( $L_c$ ) was determined using Eq. (1):

$$L_c = \mu_{\text{blank}} + t(1 - \alpha, n - 1)\sigma_{\text{blank}} \quad (\text{Eq. 1})$$

where  $\mu_{\text{blank}}$  represents the averaged response for  $n$  negative control replicates,  $\sigma_{\text{blank}}$  is the standard deviation (SD) of the negative control replicates, and  $t(1 - \alpha, n - 1)$  the  $1 - \alpha$  percentile of the Student-distribution ( $\alpha = 0.05$ ) given  $n - 1$  degrees of freedom (DF). Then, the SD for the test concentrations was calculated following Eq. (2):

$$\sigma_{\text{test}} = \sqrt{\frac{\sum_{i=1}^m \sigma_i^2}{m}} \quad (\text{Eq. 2})$$

where  $\sigma_i$  is the SD of the response for  $n$  replicates of the  $i$ th test conditions and  $m$  is the number of test conditions. Eqs. (1) and (2) enable to calculate the limit of detection in the response domain ( $L_D$ ) as presented in Eq. (3):

$$L_D = L_c + t(1 - \beta, m(n - 1))\sigma_{\text{test}} \quad (\text{Eq. 3})$$

where  $t(1 - \beta, m(n - 1))$  is the  $1 - \beta$  percentile ( $\beta = 0.05$ ) of the Student distribution with a DF equal to  $m(n - 1)$ .

Data were fit into a four-parameter logistic (4PL) model (Eq. (4)) using the R package "dr4pl" (An et al., 2019; Landis et al., 2021):

$$\text{Response} = d + \left( \frac{a - d}{1 - \left( \frac{\log_{10}(C + 0.0002)}{c} \right)^b} \right) \quad (\text{Eq. 4})$$

where  $a$ ,  $b$ ,  $c$ , and  $d$  were the 4PL parameters corresponding to the lower limit, the slope, the inflection point (EC50), and the upper limit, respectively, and  $C$  the concentration. The logistic method was applied to obtain initial parameter estimates, and the robust estimation method utilized the sum of the squares loss function. EC10 was also determined by using the 4PL curve.

At last, the  $L_D$  value was translated into the LOD according to the 4PL equation inversion shown in Eq. (5):

$$\log_{10}(\text{LOD} + 0.0002) = c \left[ \left( \frac{a - d}{L_D - d} \right) - 1 \right]^{1/b} \quad (\text{Eq. 5})$$

Since the assumption of homoscedasticity and normal distribution of the data were not met, statistical differences between groups in fulvestrant and WWTP experimentations were assessed using the Kruskal-Wallis test and Conover's all-pairs test with the  $p$ -value adjustment method of Benjamini and Hochberg (1995), employing the R packages "stats" (R Core Team, 2022) and "PMCMRplus" (Pohlert, 2022).

## 3. Results and discussion

### 3.1. DLES test sensitivity and applicability

The LOD is a critical parameter of a screening bioassay as it directly concerns the method sensitivity. To assess the quality and the efficiency of the DLES test, it underwent testing with a range of concentrations of the natural ligand E2. The LOD values were determined using the robust method outlined by Holstein et al. (2015). This method considers not only the variability of the response observed within the control, but also the variability of responses within test conditions. Table 1 provides the LOD values for E2. They can be arranged in order of decreasing sensitivity: sbEsr2b < sbEsr2a < hER $\alpha$  < sbEsr1. Except for sbEsr1, LOD can be considered as very low, indicating high sensitivity. Determination of LODs was conducted using a stringent method compared to the

**Table 1**  
Four-parameter logistic curve details and extrapolations.

|                       | DLES test           |                 |                  | YES test          |
|-----------------------|---------------------|-----------------|------------------|-------------------|
|                       | sbEsr1              | sbEsr2a         | sbEsr2b          | hER $\alpha$      |
| <b>4PL model</b>      |                     |                 |                  |                   |
| Lower limit (%)       | 94.52               | 104.67          | 100.6            | 65.53             |
| 95% CI                | [71.56, 117.49]     | [93.68, 115.67] | [74.64, 126.55]  | [-413.6, 618.08]  |
| Slope                 | 2.22                | 2.5             | 1.53             | 1.22              |
| 95% CI                | [-9.8, 14.24]       | [-3.19, 8.19]   | [-0.28, 3.35]    | [0.3, 2.85]       |
| log(EC50 + 2) (nM)    | 0.99                | -1.01           | -1.19            | -0.08             |
| 95% CI                | [0.85, 1.13]        | [-1.13, -0.89]  | [-1.59, -0.79]   | [-0.4, 0.19]      |
| Upper limit (%)       | 426.46              | 246.33          | 294.57           | 2248.77           |
| 95% CI                | [351.46, 501.46]    | [233.7, 258.97] | [268.08, 321.06] | [1623.1, 2656.74] |
| <b>Extrapolations</b> |                     |                 |                  |                   |
| LOD (nM)              | >11.69 <sup>a</sup> | 0.18            | 0.08             | 0.94              |
| EC10 (nM)             | >3.66 <sup>a</sup>  | 0.04            | 0.02             | 0.19              |
| EC50 (nM)             | >9.84 <sup>a</sup>  | 0.1             | 0.06             | 0.84              |

4PL model: Four-parameter logistic model, 95% CI: 95% confidence interval, LOD: Limit of detection, EC50: 50% maximal effective concentration, EC10: 10% maximal effective response concentrations, E2 efficacy: Maximal response, <sup>a</sup> Data extrapolated despite the absence of a clear upper limit on the 4PL model.

commonly used “mean value of the control condition + 3SD” (Holstein et al., 2015). As evidence, with this usual approach, the LOD would be estimated as 4.38 nM for sbEsr1, 0.005 nM for sbEsr2a, and 0.0005 nM for sbEsr2b. For all sbEsrs, 10 nM of E2 strongly induced luciferase activity, as already observed in previous works (Muriach et al., 2008; Pinto et al., 2019b; Zapater et al., 2019). Thus, this E2 concentration was selected as a positive control for further experiments.

Differences in the nuclear estrogen receptor responses were described in other fish species (Chakraborty et al., 2011; Hawkins and Thomas, 2004; Katsu et al., 2007; Le Page et al., 2006; Menuet et al., 2002; Serra et al., 2019, 2020). They may be attributed to variations in the estrogen receptor transactivation, ligand affinity, or coactivator recruitment (Hawkins and Thomas, 2004; Menuet et al., 2002). Herein, after E2 exposure, variations in the EC50 and EC10 values between nuclear estrogen receptors or methods (i.e., DLES and YES tests) were observed (Table 1). Both can be arranged as follows: sbEsr2b < sbEsr2a < hER $\alpha$  < sbEsr1. Equations and graphical representations of logistic curves for each receptor (used to determine EC50 and E10 values) are shown in Fig. 1, and the 95% confidence intervals of 4PL curve parameters are provided in Table 1. These results are consistent with the findings of Zapater et al. (2019), demonstrating that E2 was more potent to induce a response with sbEsr2b than with sbEsr2a. However, EC50 values calculated in their study were higher for both estrogen receptors (255.6 nM for sbESR2a and 7.25 nM for sbEsr2b) which may be explained by slight differences in the experimental methodology. Concerning sbEsr1, in the present work, EC50 and EC10 were derived from experimental data that mainly focused on low concentrations. Therefore, the concentration range may not have been broad enough to reach upper level on the 4PL model. Consequently, these data must be handled with caution. In comparison, Quesada-García et al. (2012) applied higher E2 concentrations ranging from 1 nM to 1 mM and reported an EC50 of 32 nM with sbEsr1. Similarly, Muriach et al. (2008), who also employed higher E2 concentrations (0.1 nM–10  $\mu$ M), observed a significant induction of luciferase activity at a minimal concentration of 1 nM with sbEsr1, while we observed such effect at 10 nM. At last, Table A.2. provides effect concentrations obtained with other bioassays based on estrogen receptor activation of with E2 (Modified from Escher et al., 2018). It appears that the sensitivity of the DLES test is comparable to, or better than other assays, except for the MELN and the

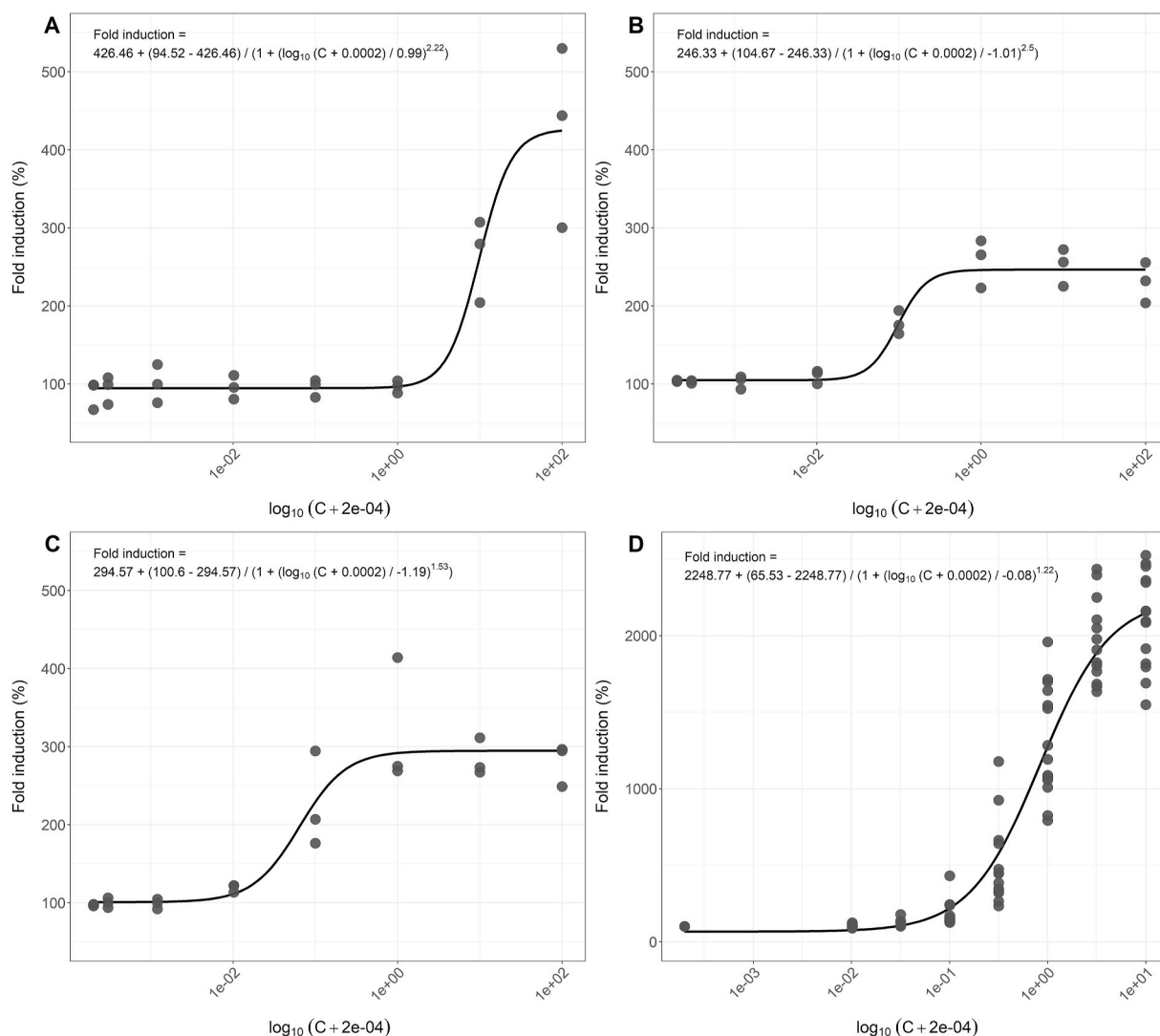
ER-CALUX assays (EC<sub>10</sub> = 0.002 and 0.0007 nM, respectively). Similarly, the EC<sub>50</sub> obtained with bisphenol A (EC<sub>50</sub> = 1360.82 nM, Figure A3) using sbEsr1 fell within the same range as those obtained with ER $\alpha$  from *Danio rerio* (EC<sub>50</sub> = 807 nM, Le Fol et al., 2017), but were higher than those obtained using U2OS ER $\alpha$  CALUX bioassay (EC<sub>50</sub> = 270 nM, Wang et al., 2014). No EC<sub>50</sub> could be estimated for sbEsr2a within the concentration range tested, while EC<sub>50</sub> is provided for sbEsr2b (3152.9 nM); however, cautions should be taken as the upper limit was not reached at all.

Fulvestrant is known to be an antagonist to hER $\alpha$  (Long and Nephew, 2006) or *D. rerio* nuclear estrogen receptors (Notch and Mayer, 2011; Sonavane et al., 2016). Therefore, it was used to assess the ability of the DLES test to deal with contaminant mixtures and their potential interactions (e.g., antagonism, synergism). As expected, no effect for fulvestrant alone was observed with any of the *D. labrax* nuclear estrogen receptors (Fig. 2). When applying 10 nM of E2 together with 100 nM of fulvestrant, a significant inhibition of the luciferase activity compared to the positive control was observed with all three *D. labrax* estrogen receptors (sbEsr1 *p*-value <0.001, sbEsr2a *p*-value = 0.009, sbEsr2b *p*-value = 0.009). Therefore, fulvestrant can be considered as an anti-estrogenic compound for *D. labrax*. This also demonstrated that the DLES test is suitable to assess antagonist effects of estrogen-like endocrine disrupting chemicals.

Estrogen receptor-selective activity has been observed depending on substances and species, and those differences among estrogen receptors can even lead to either agonism or antagonism for a given compound (Escande et al., 2006; Sun et al., 1999; Xin et al., 2010). Herein, we observed that *D. labrax* estrogen receptors do not display the same response to E2 (Fig. 1) or BPA (Figure A3), and that the receptor showing the highest responsiveness can vary. These observations support the idea that different receptors may have evolved to exert distinct physiological roles in an organism. This functional differentiation is further supported by their differential distribution among organs or tissues, as well as between developmental stages as demonstrated in *D. labrax* (Halm et al., 2004; Moreira et al., 2021; Zapater et al., 2019), and in other fish species (Filby and Tyler, 2005; Hawkins et al., 2005; Katsu et al., 2007; Menuet et al., 2002; Sabo-Attwood et al., 2004).

The scientific community has not been unaware of this problem. Consequently, complementary functional bioassays based on fish estrogen receptors have been developed and used. However, they were primarily designed with estrogen receptors from only three species: *D. rerio* (Asnake et al., 2019; Bardet et al., 2002; Cosnefroy et al., 2012; Hinfray et al., 2018; Le Fol et al., 2015, 2017; Le Page et al., 2006; Menuet et al., 2002; Miyagawa et al., 2014; Notch and Mayer, 2011; Pinto et al., 2014, 2019a,b; Sassi-Messai et al., 2009; Serra et al., 2019, 2020; Sonavane et al., 2016; Tohyama et al., 2015), *Oncorhynchus mykiss* (Ackermann et al., 2002; Cosnefroy et al., 2009; Kunz and Fent, 2006; Le Guevel and Pakdel, 2001; Molina-Molina et al., 2008; Petit et al., 1997), and *Oryzias latipes* (Chakraborty et al., 2011; Ihara et al., 2014; Miyagawa et al., 2014; Tohyama et al., 2015; Yost et al., 2014).

Some studies involving estrogen receptors from other freshwater species were proposed (Katsu et al., 2008; Miyagawa et al., 2014; Tohyama et al., 2015). To the best of our knowledge, few studies have focused on marine species. They involved *Micropogonias undulatus* (fusion protein, Kitano et al., 2006), *Paralichthys olivaceus* (Hawkins and Thomas, 2004), *Sparus auratus* (Passos et al., 2009; Pinto et al., 2006), and *D. labrax* (Muriach et al., 2008; Pinto et al., 2019b; Quesada-García et al., 2012; Zapater et al., 2019). The present work originates from the latter. As showed in their gene/cDNA sequences and reflected in their phylogenetic trees analysis, there are significant differences, notably among the amino acid sequences of the different types of estrogen receptors in fish (Nelson and Habibi, 2013; Zapater et al., 2019). Marine species form a distinct cluster separated from other freshwater species, notably *D. rerio*, *O. mykiss*, and *O. latipes*. The phylogenetic distances suggest potential differences in estrogen receptor functioning, a hypothesis supported by experimental studies on ligand-ER interactions



**Fig. 1.** Four-parameter dose-response curves and associated equation for E2 as a function of sbEsr1 (A,  $n = 3$ ), sbEsr2a (B,  $n = 3$ ), and sbEsr2b (C,  $n = 3$ ) from the DLES test, and hER $\alpha$  (D,  $n = 14$ ) from the YES test.

among various fish species (Asnake et al., 2019; Miyagawa et al., 2014). Thus, there is a need for a regulatory screening test involving multiple estrogen receptors from marine fish to diversify the detection of EEDCs. This would enhance our understanding of the impact of these contaminants in an estuarine and coastal context, notably by evaluating potential adverse outcomes through various signaling pathways in marine fish and providing insights into their modes of action. Validated screening methods in environmentally relevant species would improve the basis for decision making in environmental risk assessment (Grignard et al., 2022) by complementing established bioassays that solely rely on human estrogen receptors.

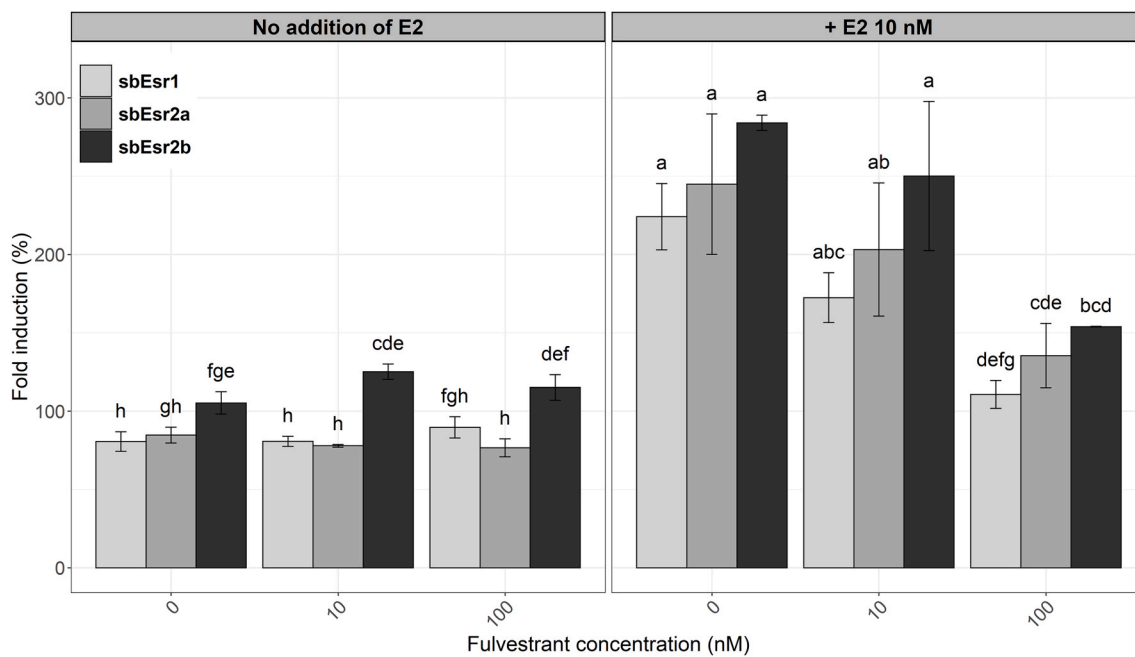
### 3.2. DLES test application to wastewater

Averaged viability of cells exposed to WWTP extracts ranged between  $85.14 \pm 3.56\%$  (UP 0.1X + 10 nM E2) and  $109.95 \pm 0.49\%$  (UP 0.1X). No significant difference between conditions was detected ( $p$ -value = 0.467), which indicates the absence of significant cytotoxicity (Fig. 3). It can thus be excluded that the reporter gene assay was biased by cytotoxicity. In the same way, the blank control did not induce any significant variation in the cell viability (Figure A1.).

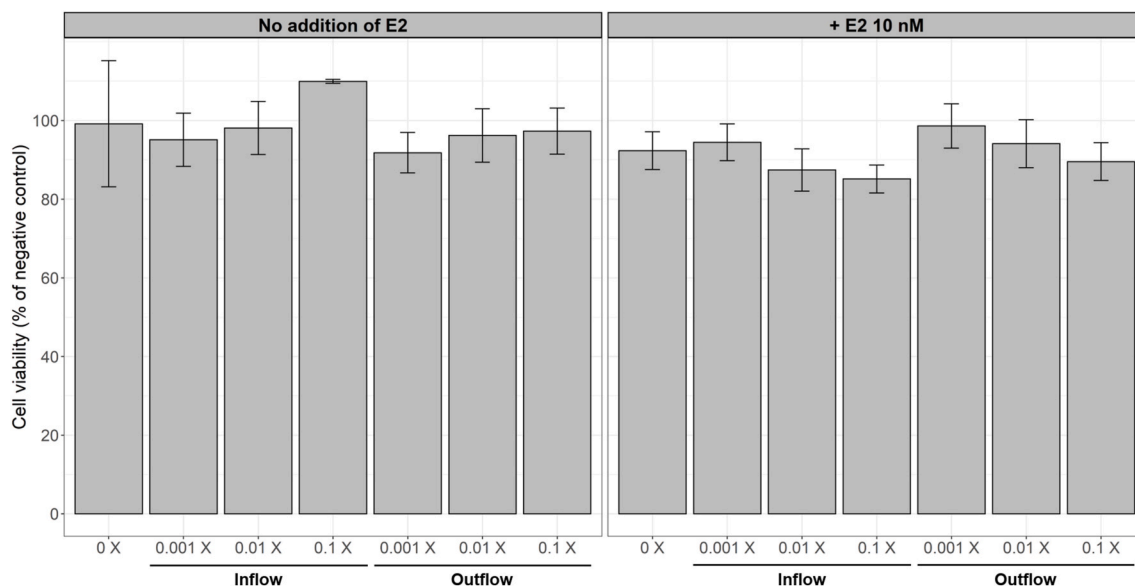
Exposure to 0.1X of contaminant extracts from WWTP inflow led to a significant induction of the luciferase activity compared to the negative

control ( $p$ -value = 0.043) in sbEsr2b transfected cells (Fig. 4). Such a significant increase was not observed in any other condition without addition of E2, regardless of the nuclear estrogen receptor or the tested sample from either influent or effluent of the WWTP ( $p$ -value >0.05, Figs. 4 and 5). This result indicated the occurrence of one or more potential EEDCs in the inflow sample. As mentioned earlier, a variation in the observed sensitivity between the different sbEsrS was demonstrated, with the sbEsr2b being particularly sensitive, thus supporting the importance of screening with different estrogen receptors in order to obtain a comprehensive evaluation of estrogenic activities.

Our results were confronted with the data obtained by the normalized OECD YES test. Both were consistent, allowing us to draw the same conclusions, but in a complementary way. First, the YES test highlighted an agonist effect on hER $\alpha$  with the inflow contaminant extract confirming the efficacy of the DLES test to assess the occurrence of EEDCs in water. Moreover, no effect was detected on sbEsr1 and its analog form hER $\alpha$  at equivalent concentrations ( $\leq 0.1$ X, Figs. 4 and 6). In fact, exposures to WWTP inflow extract induced a significant effect with the YES test only at 1X, 3.2X, and 10X ( $p$ -value <0.001, Fig. 6). It was technically not feasible to apply these conditions with the DLES test because the concentrations of the contaminant extract stock solutions (in DMSO) were too low to obtain them in final proportion of DMSO at 0.001%. A 10 times higher concentration of the inflow contaminant



**Fig. 2.** Fold induction of the luciferase activity measured with the DLES test using sbEsr1 (light grey), sbEsr2a (dark grey), and sbEsr2b (black) after the exposure to fulvestrant with (left) or without (right) the addition of E2 at 10 nM. All conditions contained 0.001% DMSO. Results are expressed as means ( $\pm$ SEM) normalized relative to the negative control without DMSO. Different letters indicate significant differences ( $n = 2$ , Kruskal Wallis test and Conover's all pairs test, no significant difference).



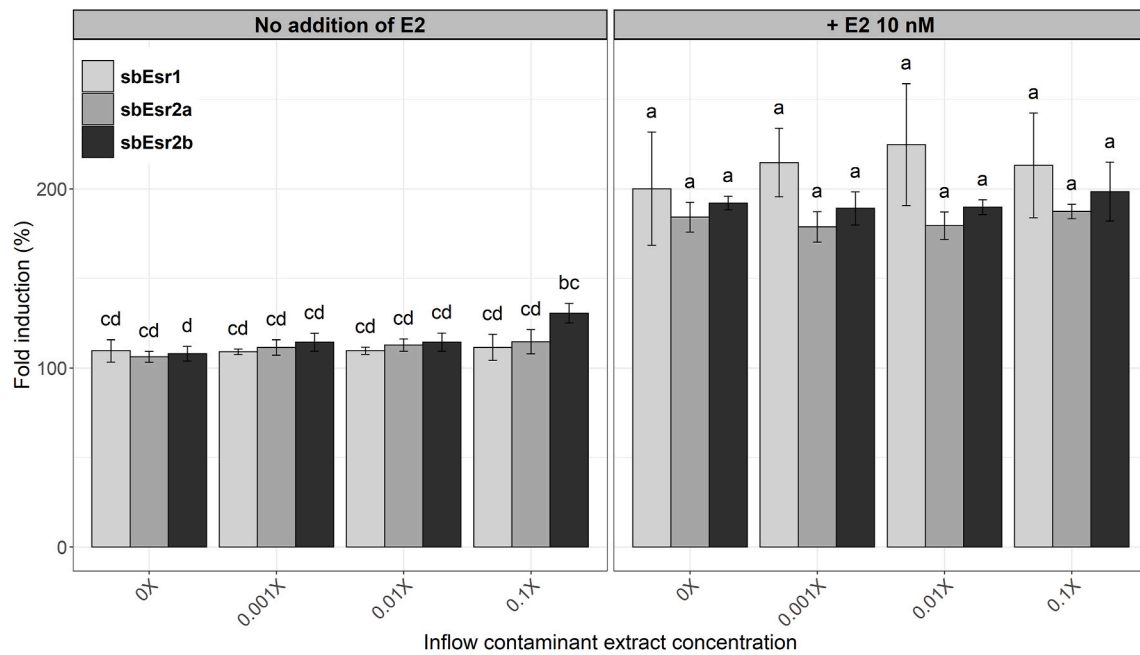
**Fig. 3.** Cell viability assessed with the MTT test on cell transfected with sbEsr2a after the exposure to the contaminant extract from the inflow and outflow of a WWTP with (left) or without (right) the addition of E2 at 10 nM. All conditions contained 0.001% DMSO. Results are expressed as means ( $\pm$ SEM) normalized relative to the negative control without DMSO. No significant differences were detected ( $n = 2$ , Kruskal Wallis test and Conover's all pairs test).

extract was necessary to induce a significant positive response in the YES test, and thus to detect the presence of EEDC(s). The absence of a response with sbEsr1 may be due to the tested concentration in combination with the lower sensitivity of the sbEsr1.

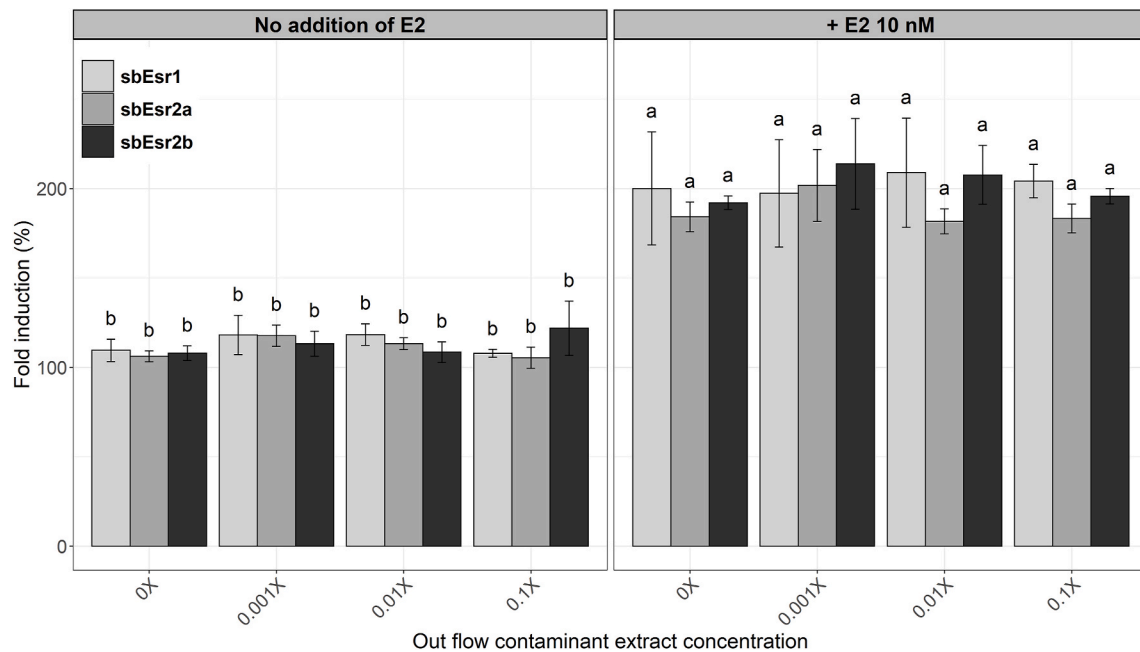
The data suggest an interaction (cumulative) between the contaminant extract from the WWTP inflow and E2 detected with the YES test, which, however, never became statistically significant with the DLES. When cells were exposed to E2 (0.5 nM) together with the wastewater extracts, the  $\beta$ -galactosidase activity increased significantly only at 10 $\times$  ( $p$ -value = 0.032) of contaminant extract from the WWTP inflow

(Fig. 6). Regarding the outflow contaminant extract, the DLES test and the YES test did not detect any effect at the range of concentrations tested (Figs. 5 and 7). However, a non-significant inhibition of the  $\beta$ -galactosidase activity may be noticed in conditions containing 10 $\times$  of contaminant extract from the WWTP outflow ( $p$ -value = 0.746), which could be related, as for the DLES test, to the presence of a substance(s) able to inhibit hER $\alpha$ , or to disturb the yeast viability at this strong concentration (10 $\times$ ) in addition of E2 (Fig. 7).





**Fig. 4.** Fold induction of the luciferase activity measured with the DLES test using sbEsr1 (light grey), sbEsr2a (dark grey), or sbEsr2b (black) after the exposure to the contaminant extract from the inflow of a WWTP with (left) or without (right) the addition of E2 at 10 nM. All conditions contained 0.001% DMSO. Results are expressed as means ( $\pm$ SEM) normalized relative to the negative control without DMSO. Different letters indicate significant differences ( $n = 3-4$ , Kruskal Wallis test and Conover's all pairs test,  $p$ -value  $< 0.05$ ).

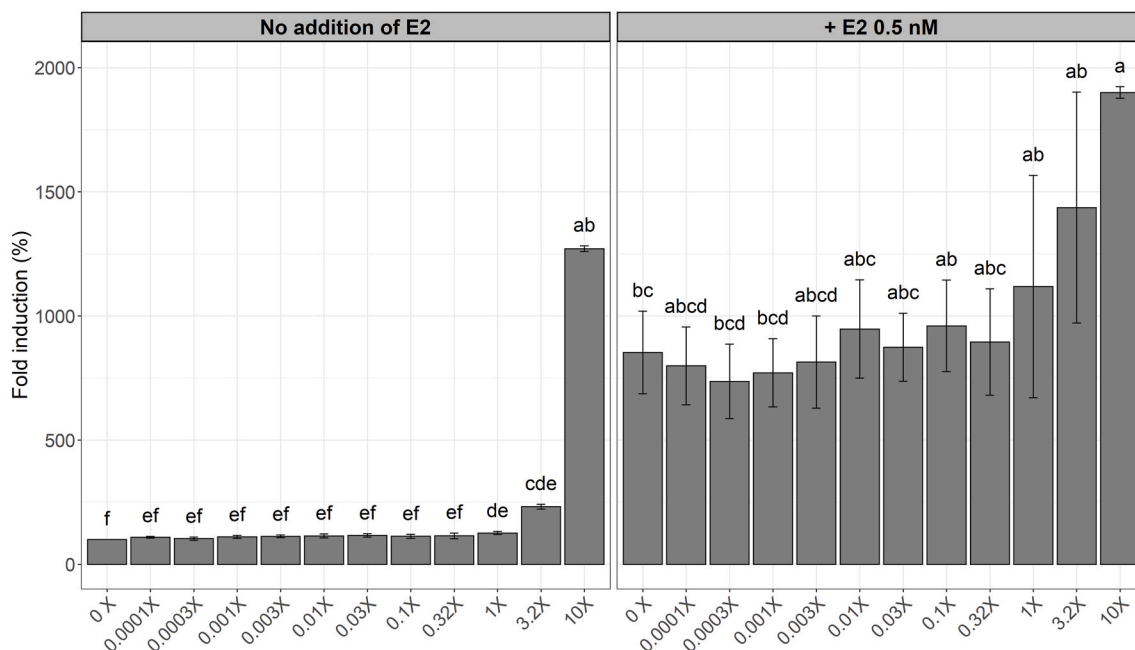


**Fig. 5.** Fold induction of the luciferase activity measured with the DLES test using sbEsr1 (light grey), sbEsr2a (dark grey), or sbEsr2b (black) after the exposure to the contaminant extract from the outflow of a WWTP with (left) or without (right) the addition of E2 at 10 nM. All conditions contained 0.001% DMSO. Results are expressed as means ( $\pm$ SEM) normalized relative to the negative control without DMSO. Different letters indicate significant differences ( $n = 3-4$ , Kruskal Wallis test and Conover's all pairs test,  $p$ -value  $< 0.05$ ).

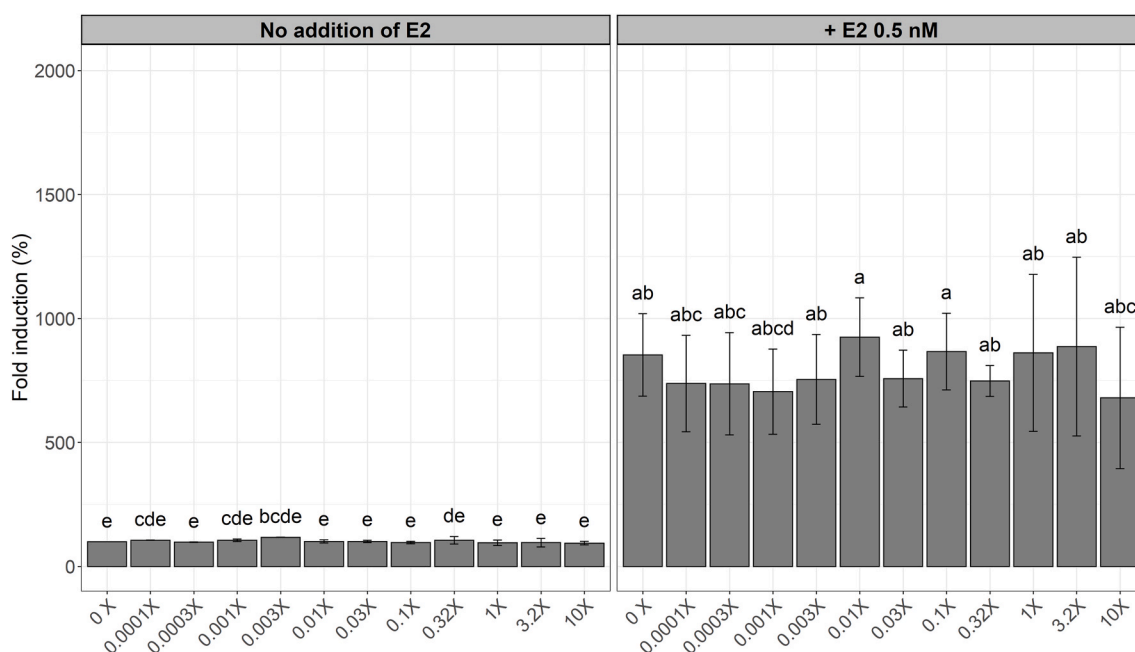
#### 4. Conclusions

The large variety of EEDCs and differences in estrogen receptors of non-target species, i.e., fish of marine and freshwater environments, suggest that the established bioassays used to screen surface waters for estrogenic activity should be complemented by a wider range of estrogen screening to better detect EEDCs and to understand their impact on

organisms. In this study, the gene reporter assay was targeting several estrogen receptors from a marine fish species, unlike similar *in vitro* tests mainly using freshwater species. In particular, the third receptor from *D. labrax* (i.e., sbEsr2b) proved to be highly sensitive and capable of specifically detecting estrogenic activity that did not induce the other receptors. The DLES test, therefore, has the potential to complement existing bioassays for chemical hazard assessment, but also provide



**Fig. 6.** Fold induction of the  $\beta$ -galactosidase activity measured with the YES test using hER $\alpha$  after the exposure to the contaminant extract from the inflow of a WWTP with (left) or without (right) the addition of E2 at 0.5 nM. Results are expressed as means ( $\pm$ SEM) normalized relative to the negative control condition. Different letters indicate significant differences ( $n = 2-4$ , Kruskal Wallis test and Conover's all pairs test,  $p$ -value  $< 0.05$ ).



**Fig. 7.** Fold induction of the  $\beta$ -galactosidase activity measured with the YES test using hER $\alpha$  after the exposure to the contaminant extract from the outflow of a WWTP with (left) or without (right) the addition of E2 at 0.5 nM. Results are expressed as means ( $\pm$ SEM) normalized relative to the negative control condition. Different letters indicate significant differences ( $n = 2-4$ , Kruskal Wallis test and Conover's all pairs test,  $p$ -value  $< 0.05$ ).

information to evaluate the potential adverse outcomes for marine species (namely fish) and to give information of their mode of action. However, more EEDCs need to be tested and confirmation by water chemistry would be beneficial.

#### CRediT authorship contribution statement

**Sylvain Slaby:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Auréli**

**Duflot:** Writing – review & editing, Validation, Investigation. **Cinta Zapater:** Writing – review & editing, Methodology. **Ana Gómez:** Writing – review & editing, Resources, Methodology. **Jérôme Couteau:** Validation, Resources, Investigation. **Géraldine Maillet:** Validation, Investigation. **Thomas Knigge:** Writing – review & editing, Conceptualization. **Patrícia I.S. Pinto:** Writing – review & editing, Resources, Conceptualization. **Tiphaine Monsinjon:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Conceptualization.

## 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.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2024.142601>.

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