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Environment International 130 (2019) 104896

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Monitoring estrogenic activities of waste and surface waters using a novel *in vivo* zebrafish embryonic (EASZY) assay: Comparison with *in vitro* cell-based assays and determination of effect-based trigger values

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ARTICLE INFO

Handling Editor: Adrian Covaci

Keywords:
Zebrafish
Estrogen monitoring
In vitro and *in vivo* bioassays
Correlation
Effect-based trigger value

ABSTRACT

This study reports the use of the recently developed EASZY assay that uses transgenic cyp19a1b-GFP zebrafish (*Danio rerio*) embryos to assess *in vivo* estrogenic activity of 33 surface (SW) and waste water (WW) samples collected across Europe that were previously well-characterized for estrogen hormones and *in vitro* estrogenic activity. We showed that 18 out of the 33 SW and WW samples induced estrogenic responses in the EASZY assay leading to a significant and concentration-dependent up-regulation of the ER-regulated *cyp19a1b* gene expression in the developing brain. The *in vivo* 17β-estradiol-equivalents (EEQs) were highly correlated with, both, the chemical analytical risk quotient (RQ) based on steroidal estrogen concentrations and EEQs reported from five different *in vitro* reporter gene assays. Regression analyses between the *vitro* and *in vivo* effect concentrations allowed us to determine an optimal cut-off value for each *in vitro* assay, above which *in vivo* responses were observed. These *in vitro* assay-specific effect-based trigger values (EBTs), ranging from 0.28 to 0.58 ng EEQ/L define the sensitivity and specificity of the individual *in vitro* assays for predicting a risk associated with substances acting through the same mode of action in water samples. Altogether, this study demonstrates the toxicological relevance of *in vitro*-based assessment of estrogenic activity and recommends the use of such *in vitro/in vivo* comparative approach to refine and validate EBTs for mechanism-based bioassays.

1. Introduction

During the last decades, numerous studies have reported the contamination of aquatic environments by endocrine disrupting chemicals (EDCs) resulting in adverse health effects on sensitive aquatic species including fish (Sumpter, 2005; Tyler et al., 1998). Among EDCs, much attention has been paid to substances acting as agonists of the estrogen receptor (ER), notably natural and synthetic steroidal estrogens such as 17β-estradiol (E2), estrone (E1), and 17α-ethinyl estradiol (EE2), as they are widely released from waste water effluents into aquatic ecosystems at low but active concentrations on the reproductive fitness of aquatic species (Brion et al., 2004; Kidd et al., 2007; Kidd et al., 2014; Nash et al., 2004).

Monitoring of environmental estrogens has thus become of increasing relevance to assess the quality of water bodies (Kase et al., 2018). In that respect, the use of *in vitro* reporter gene assays to monitor estrogenic activity has proven relevant as they enable an integrative and quantitative assessment of ER-active contaminants in terms of 17β-estradiol equivalents (EEQs), considering complex environmental mixtures of both, known and unknown, compounds (Mehinto et al., 2015; Snyder et al., 2001; Vethaak et al., 2005). The high specificity and sensitivity of most established *in vitro* ER cell-based assays allow an EEQ

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https://doi.org/10.1016/j.envint.2019.06.006
Received 10 January 2019; Received in revised form 6 May 2019; Accepted 3 June 2019

quantification for a wide concentration range of water contamination with ER-agonists, including water with very low levels of estrogenic chemicals. Therefore, these types of bioassays are relevant for the monitoring of estrogenic activities in water bodies under different pressure levels (Kunz et al., 2015; Kunz et al., 2017). However, the cell-specific transactivation of ER measured *in vitro* may not necessarily reflect the estrogenic activity observed *in vivo* in fish as many factors can influence the response of a given biological model such as inter-species differences for ER and toxicokinetics (adsorption, distribution, metabolization and excretion of chemical) resulting in differences between cell-based assay and responses in organisms (Le Fol et al., 2017; Van den Belt et al., 2004). This has raised the question of the relevance of *in vitro* measurement of estrogenic activity to predict risk for aquatic species and ecosystems (Hotchkiss et al., 2008; Van den Belt et al., 2004).

The determination of *in vitro* threshold values is an important element for the use of cell-based ER transactivation assays for the monitoring of the estrogenic potential in surface waters in order to discriminate between good and poor quality of water bodies. Effect-based trigger values (EBT) can be used as such threshold values. Different methodologies based on either environmental quality standards (EQS) of priority estrogenic chemicals (Escher et al., 2018; Kunz et al., 2015; van der Oost et al., 2017) or measured values in environmental waters (Jarosova et al., 2014) have been proposed to derive EBT values for *in vitro* estrogenic activity, all proposed EBT values falling in the low ng/L range, *i.e.*, an EEQ ranging from 0.1 to 1 ng E2/L (Escher et al., 2018; Jarosova et al., 2014; Kase et al., 2018; Kunz et al., 2017; van der Oost et al., 2017). However, a harmonization of EBT for *in vitro* effect-based methods is still missing (Kase et al., 2018). Furthermore, although the methodologies to derive EBT values based on PNEC (predicted no-effect concentration) or EQS consider *in vivo* responses in aquatic organisms, the links between *in vitro* estrogenic activity and *in vivo* effects in fish to define EBTs have been less investigated experimentally. Notwithstanding, qualitative and quantitative linkage between human or fish cell-based ER transactivation assays with estrogenic effect in aquatic organisms have been addressed previously using model estrogenic compounds and/or environmental samples (Cavallin et al., 2014; Henneberg et al., 2014; Ihara et al., 2015; Mehinto et al., 2018; Sonavane et al., 2016; Sonavane et al., 2018; Van den Belt et al., 2004). To some extent, these studies showed the relevance of using *in vitro* assays to predict endocrine disruption in exposed-fish. Good correlations were found between *in vitro* ER transactivation and estrogenic responses in medaka but no specific EBT was derived from this study (Ihara et al., 2015). In some studies, the absence of *in vivo* estrogenic responses for samples with low estrogenicity *in vitro* was correctly predicted (Mehinto et al., 2018) while in others estrogenic activity in fish was confirmed for the most active samples *in vitro* (Henneberg et al., 2014; Sonavane et al., 2016). However, these studies were based on a limited number of active samples encompassing a broad range of estrogenic activity *in vivo* precluding a quantitative and robust assessment of the thresholds. Moreover, *in vitro* and *in vivo* comparison often relies on single cell-based assay without considering the variability between *in vitro* models (Kunz et al., 2017) that are commonly used for screening estrogenic activity.

In this context, our main objectives were 1) to determine whether an estrogenic activity measured *in vitro* triggers as well an *in vivo* response in a biological fish model, and 2) to test - based on this *in vitro/ in vivo* comparison - whether there exists an *in vitro* threshold value with a predictive power for the occurrence of effects *in vivo*. For that purpose, the *in vivo* estrogenic activities of 16 surface water (SW) and 17 wastewater (WW) samples were analyzed on a small-scale whole-organism assay that uses transgenic zebrafish cyp19a1b-GFP (Green Fluorescent Protein) embryos (Brion et al., 2012; Tong et al., 2009). The samples were collected across Europe and analyzed in the frame of a previous Science-Policy Interface/Chemical Monitoring of Emerging Pollutants project. These samples have been previously well-characterized for estrogenic activities using five *in vitro* effect-based methods (ERα-CALUX, MELN, ER-GeneBlazer hERα-Hela9903, pYES) and for estrogen hormones using three analytical methods based on LC-MS/MS for E1, E2 and EE2 (Kase et al., 2018; Konemann et al., 2018). Herein they were further analyzed for their *in vivo* estrogenic activity using the EASZY assay (Detection of **E**ndocrine **A**ctive **S**ubstance, acting through estrogen receptors, using transgenic cyp19a1b-GFP **Z**ebrafish embr**Y**os), which allows the sensitive detection and quantification of environmental estrogens at an early developmental stage (0 to 4 days post fertilization) by quantifying the induction of the ER-regulated *cyp19a1b* gene (Menuet et al., 2005) in the developing brain by means of *in vivo* fluorescence imaging (Brion et al., 2012). The EASZY assay has been shown to sensitively respond to a diversity of ER-active compounds that belong to different chemical classes (Brion et al., 2012; Cano-Nicolau et al., 2016; Le Fol et al., 2017; Neale et al., 2017) and to be a useful *in vivo* tool for evaluating binary and multi-component mixtures (Brion et al., 2012; Hinfray et al., 2016; Hinfray et al., 2018; Petersen et al., 2013) or complex environmental matrices (Fetter et al., 2014; Sonavane et al., 2016; Sonavane et al., 2018). Before analyzing the thirty-three environmental samples, a first step was to quantify the *in vivo* estrogenic potency of steroidal hormones in the EASZY assay using E1, E2 and EE2 standards and to assess the ability of the assay to quantify EE2 in spiked-water samples.

2. Material and methods

2.1. Chemicals

17β-estradiol (E2), 17α-ethinylestradiol (EE2) and estrone (E1) were obtained from Sigma-Aldrich (St-Quentin Fallavier, France). Stock solutions of chemicals were prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and stored at −20 °C. Fresh dilutions of test chemicals were prepared before each experiment.

2.2. Water samples

All extracts of surface water (SW) and waste water (WW) tested in the present study were identical to the ones tested in the previous *in vitro* study (Konemann et al., 2018). A total number of 16 SW and 17 WW samples were collected at selected sampling sites located in seven European countries. The samples were extracted using solid phase extraction (SPE) yielding 1000-fold concentrated organic extracts (Konemann et al., 2018). For performing the EASZY assay, 1 mL of each extract (equivalent to 1 L of native sample) was further concentrated and resuspended in 0.1 mL of dimethylsulfoxide (DMSO), which was further diluted 1000-fold in the exposure medium. This means that the highest relative enrichment factor (REF) tested was 10.

In addition, surface water samples collected from the Netherlands were spiked with EE2 at two concentrations, 0.6 ng/L and 6.0 ng/L, and analyzed in the EASZY assay to demonstrate the ability of the assay to quantify estrogenic activity of water samples. Ultrapure water (1 L) was run in parallel to spiked water samples and used as extraction blank. All the protocols used for spiked water preparation, field sampling, and waste and surface water samples extraction were previously detailed in Konemann et al. (2018).

2.3. Zebrafish exposure to single steroidal estrogens and to environmental samples: the EASZY assay

Newly fertilized transgenic cyp19a1b-GFP zebrafish eggs (up to 4 h post-fertilization) were exposed to the test substance or the environmental samples for 96 h under semi-static conditions with a complete renewal of the medium every 24 h. According to the EU Directive 2010/63/EU on the protection of animals used for scientific purposes, the EASZY assay does not fall into the regulatory frameworks dealing with animal experimentation (Strahle et al., 2012) and is considered as an

alternative method for animal experiments.

Each experimental group consisted of 20 embryos exposed in a crystallizing dish covered by a glass lid. For single test chemicals, the total volume of water was 25 mL and the final test concentration of DMSO was 0.01% (V/V). For environmental samples, the volume of water was set to 10 mL and the organic extract was diluted 1000-fold meaning that the final test concentration of DMSO was 0.1% (V/V). No effect on either the development of zebrafish or the GFP signal was observed in both DMSO control groups (0.01% and 0.1% (V/V)) compared to the water control group.

Zebrafish embryos were exposed to serial dilutions of single steroidal estrogen or extracts of water samples, using a dilution factor of 3 between serial concentrations. Typically, the organic extracts were tested at six different relative enrichment factors (REF), *i.e.* 10, 3, 1, 0.3, 0.1 and 0.03. For each experiment, a concentration-response curve of the reference substance (E2) was run in parallel to allow the quantification of the estrogenic activity present in the sample as ng E2-equivalent (EEQ) per liter. For water samples spiked with EE2, a concentration-response curve of EE2 was used to quantify the estrogenic activity as ng EE2-equivalent (EE2-EQ) per liter.

Embryos were kept in an incubator at 28 °C. At the end of the exposure period, 4-day post fertilization (dpf) old zebrafish were processed for fluorescence measurement by *in vivo* imaging using wide-field fluorescence microscopy according to Brion et al., 2012. Living *cyp19a1b*-GFP embryos were observed in dorsal view and the head was photographed using a Zeiss AxioImager.Z1 fluorescence microscope equipped with a AxioCam Mrm camera (Zeiss Gmbh, Göttingen, Germany) using the X10 objective, with a 134 ms exposure time at maximal intensity. Photographs were analyzed using the Zen software. The fluorescence signal was quantified using a specific ImageJ (Rasband, 1997-2018) macro developed for the EASZY assay. The Fluorescence image AnalysiS Tool (FAST) macro is freely available at https://imagej.net/FAST/. For each picture, the integrated density was measured. It corresponds to the sum of the gray-values of all pixels, above 290 defined as background value, within the region of interest.

2.4. Data analysis

2.4.1. Quantification of relative estrogenic potency (REP) of steroidal hormones and estrogenic activity of water samples (EEQ)

The statistical analysis was performed on fold-induction data relative to negative (solvent) control. A Kruskal-Wallis test was performed for the analysis of the variance first, followed by a Dunn's post-hoc test. If the response was significantly increased compared to the negative control, the sample was defined as an active sample. Statistical analyses were performed using GraphPad Prism 5.00 (GraphPad Software, San Diego California USA). Concentration-response curves were modelled using log-transformed data. The Regtox 7.0.6 Microsoft Excel TM macro (http://www.normalesup.org/~vindimian/fr_index.html) uses the Hill equation model and allows calculation of EC_{50}-values. For a given chemical, the EC_{50} was defined as the concentration inducing 50% of its maximal effect. Relative estrogenic potencies (REP) were determined as the ratio of the EC_{50}-value for the reference compound E2 to the EC_{50}-value of the test chemical. If a concentration-response relationship of a compound did not reach the upper plateau, the modeling was performed using fixed parameters for slope and maximum taken from the E2-reference curve.

For active environmental samples in the EASZY assay, the estrogenic activity is expressed as ng E2-equivalent (EEQ) per liter. The EEQ was calculated as the ratio of the EC_{20} of E2 (in ng/L) to the EC_{20} of a given sample, which was expressed as a relative enrichment factor (REF) that considers both the concentration factor during the water extraction step and the dilution factor applied to the extract when performing the bioassay. For water samples spiked with EE2, the same approach was performed to quantify the estrogenic activity with EE2 as reference substance and the data were expressed as ng EE2-EQ per liter.

2.4.2. Linear regression analysis and logistic regression

The linear regression between log EEQ *in vivo* and log EEQ *in vitro* was performed using GraphPad Prism 5.00 (GraphPad Software, San Diego California USA). A *t*-test of Pearson's correlation coefficient was performed to analyze the statistical significance of the correlation.

Logistic regression was used to investigate the relation between the *in vitro* EEQs and the activity of the samples after exposure of transgenic embryos zebrafish, coded as a categorical variable with only two possible outcomes, activity or inactivity. The active samples were defined as described previously. Logistic regression was performed using function glm in R 3.3.1 (Team, 2016). The optimal cut-off with the maximal sensitivity (or true positive rate) and specificity (or true negative rate) was selected using package ROCR (Sing et al., 2005).

2.4.3. Sensitivity and specificity analysis

The sensitivity and specificity for the various combinations of *in vitro* assays with *in vivo* responses were expressed in percent and calculated according to Eqs. (1) and (2) respectively (Kase et al., 2018).

$$Y_{sensitivity}(\%) = \frac{t_p}{t_p + f_n} \bullet 100 \quad (1)$$

$$Y_{specificity}(\%) = \frac{t_n}{t_n + f_p} \bullet 100 \quad (2)$$

with t_n true negative, *i.e. in vitro* estrogenic activity below the EBT with no *in vivo* estrogenic response; t_p true positive, *i.e. in vitro* estrogenic activity above the EBT with *in vivo* estrogenic response *in vivo*; f_n false negative, *i.e. in vitro* estrogenic activity below the EBT but *in vivo* response and f_p false positive, *i.e. in vitro* estrogenic activity above the EBT but no estrogenic response *in vivo*.

Similarly, a sensitivity and specificity analysis for the EASZY assay regarding known mixture risk quotient, based on three high end chemical analytical data sets (Kase et al., 2018), was performed with t_n true negative, *i.e.* no risk indicated by chemical analysis and *in vivo* EASZY; t_p true positive, *i.e.* risk indicated by chemical analysis and *in vivo* EASZY; f_n false negative, *i.e.* risk indicated by chemical analysis but not by *in vivo and* f_p false positive, *i.e.* no risk indicated by chemical analysis but by *in vivo*.

3. Results and discussion

3.1. EASZY sensitively quantifies the estrogenic activity of steroidal estrogens

Exposure of transgenic zebrafish to synthetic (EE2) and natural (E1, E2) steroidal estrogens led to a strong and significant induction of GFP fluorescence in the developing brain of zebrafish (Fig. S1). The EC_{20} and EC_{50} values (Table 1) derived from modeling full concentration-response curves of the GFP-signal (Fig. S2) revealed pronounced differences between these substances in the EASZY assay: EE2 is by far the most active steroidal estrogen with an EC_{50} in the sub-nM range (EC_{50} = 0.007 nM).

This result confirms the high responsiveness of the *cyp19a1b* gene and the EASZY assay to synthetic estrogens such as EE2, hexestrol and diethylstilbestrol (Brion et al., 2012; Vosges et al., 2010). The responsiveness of the EASZY assay for EE2 is very similar to the one observed in several cell based ER transactivation assays based on the comparison

Table 1
Effective concentrations (EC) expressed in nM (and ng/L) of the major steroidal estrogens in the *in vivo* EASZY assay.

Substance	EC_{20} nM (ng/L)	EC_{50} nM (ng/L)
17β-estradiol (E2)	0.26 (59)	0.62 (168)
Estrone (E1)	0.43 (116)	0.97 (254)
17α-ethinylestradiol (EE2)	0.003 (0.89)	0.007 (2.01)

Table 2
In vivo and *in vitro* EC_{50} values expressed in nM (and ng/L), and relative estrogenic potency (REP) for the steroidal estrogens in the *in vivo* EASZY assay and *in vitro* cell-based assays. The REP for the different *in vitro* effect-based methods were from (Konemann et al., 2018). The REP for E2 was set to 1 in each assay. For the pYES, no EC50 data were derived and only the limit of quantification for E1, E2 and EE2 were reported in Table S1 as well as REP.

Substance	Assay		EC_{50} nM (ng/L)	REP
17β-estradiol (E2)	EASZY	*in vivo*	0.62 (168)	1
	ERα-CALUX	*in vitro*	0.008 (2.26)	1
	MELN	″	0.015 (4.19)	1
	ER-GeneBLAzer	″	0.102 (27.81)	1
	Hela-9903	″	0.024 (6.56)	1
Estrone (E1)	EASZY	*in vivo*	0.97 (254)	0.64
	ERα-CALUX	*in vitro*	0.287 (77.6)	0.01
	MELN	″	0.053 (14.3)	0.29
	ER-GeneBLAzer	″	1.32 (354)	0.08
	Hela-9903	″	1.37 (371)	0.02
17α-ethinylestradiol (EE2)	EASZY	*in vivo*	0.007 (2.01)	96.10
	ERα-CALUX	*in vitro*	0.006 (1.96)	1.30
	MELN	″	0.018 (5.34)	0.79
	ER-GeneBLAzer	″	0.056 (16.67)	1.67
	Hela-9903	″	0.016 (4.71)	1.18

of the EC50 values (Table 2) (Cosnefroy et al., 2012; Gutendorf and Westendorf, 2001). In comparison, the natural hormones E2 and E1 were less active *in vivo* on brain *cyp19a1b* gene expression than EE2. The responsiveness of the EASZY assay to E2 markedly differed from *in vitro* models as the EC_{50} values derived from cell-based assays were 6- to 75-fold lower than in transgenic model (Table 2). For E1, comparing EASZY and *in vitro* cell models revealed a higher sensitivity than ER-GenBLAzer and Hela-9903 while a lower sensitivity as compared to ERα-CALUX and MELN cell lines (Table 2).

Such differences between the estrogenic activity of the two natural steroidal hormones (E1, E2) and EE2 have been previously reported *in vivo* based either on a ER-responsive transgenic zebrafish model (Legler et al., 2002), endogenous hepatic ER-regulated gene expression in different fish species (Caldwell et al., 2012; Thorpe et al., 2003; Van den Belt et al., 2004) (Table S2). Based on its REP, EE2 is about 100 times more potent than E2 *in vivo* in the EASZY assay which agrees with the relative estrogenic potencies reported in other fish studies using *in vivo* reporter gene assay or induction of testis-ova in juvenile medaka (Legler et al., 2002; Metcalfe et al., 2001). However lower REPs for EE2 were reported based on vitellogenin induction, and varied from 12 to 30 depending on the fish species and the life stage of exposure (Caldwell et al., 2012). The rationale behind a higher REP for EE2 based on *cyp19a1b* induction compared to vitellogenin may rely on the cell-context (*i.e.* glial *versus* hepatic cell context). In glial cells, a positive auto-regulation loop for the *cyp19a1b* gene contributes to an enhanced gene response signal and thus an increased sensitivity to potent ER ligand such as EE2.

Marked differences were also noticed for E1. This compound is 10 to 100 times less active than E2 in *in vitro* assays (except for MELN and pYES that present a higher sensitivity for E1), while it is only 1.5 times less active compared to E2 *in vivo* on the induction of the brain aromatase (Table 2). This lower REP for E1 agrees with the data reported in different fish species based on vitellogenin concentrations (Table S2), while in other studies E2 and E1 were found to be equipotent (Legler et al., 2002; Metcalfe et al., 2001).

Altogether, these data illustrate the high sensitivity of the EASZY assay to steroidal estrogens especially to EE2 and to some extent to E1 as compared to *in vitro* cell-based assays. Furthermore, the quantification of the estrogenic activity measured in the short-term embryonic EASZY assay agrees with the estrogenic response measured in fish after prolonged exposure to steroidal hormones notably for EE2 (Table S2), albeit more sensitive responses were reported for E2 and E1 after chronic exposure of rainbow trout, an estrogen-sensitive fish species (Thorpe et al., 2003). The *in vivo* data also highlighted marked differences of relative potencies between EE2 and the natural steroidal hormones, E2 and E1. The lower *in vivo* responsiveness of natural steroidal estrogens compared to EE2 is likely to be explained by a metabolization of E2 and E1 *in vivo,* that lacks or is less prominent in the respective *in vitro* assays. Also, the tissue distribution, bioaccumulation potential of steroidal estrogens *in vivo* are important factors which the *in vitro* assays do not account for. This finding underlines the relevance of assays based on whole-organisms for the assessment of estrogenicity in environmental samples.

3.2. EASZY accurately quantifies the estrogenic activity of surface water spiked with EE2

The performance of the EASZY assay was first evaluated by quantifying the estrogenic activity of water samples spiked with EE2 at 0.6 ng/L (“low”) and 6.0 ng/L (“high”) as nominal concentrations. No estrogenic activity was detected in the blank sample whereas the two spiked water samples were active in the EASZY assay (Fig. S3). The comparison of measured EE2 concentrations and the determined *in vivo* EE2-EQ showed a high agreement between these two methods (Table 3) demonstrating the analytical performance of the EASZY assay to quantify the estrogenic activity of water samples.

A similar analytical performance was reported for the cell-based assays with average recoveries of 112% and 93% in terms of *in vitro* EEQ compared to measured EE2 concentrations for the low and the high spike-levels in the water samples, respectively (Konemann et al., 2018).

3.3. In vivo quantification of estrogenic activity of waste and surface waters using EASZY

Each SW or WW sample was evaluated for its capacity to induce GFP-expression in the transgenic *cyp19a1b*-GFP zebrafish embryos. The results showed that 18 out of 33 samples significantly induced GFP compared to the solvent control (Fig. 1).

Waste water (WW) samples induced the GFP-expression more frequently and to a higher extent than surface water (SW) samples. Twelve out of 17 tested WW samples and 6 out of 16 SW samples were assessed estrogenic in the EASZY assay. (Figs. S4 and S5). For WW samples, marked GFP induction was measured with maximal induction signal similar or even higher than in positive control zebrafish exposed to E2 (Fig. S4). Furthermore, concentration-dependent inductions of GFP were often reported for lower REF as compared to SW samples. For the most active WW sample (# S23), the lowest REF leading to a significant

Table 3
Measured chemical concentrations of 17α-ethinylestradiol (EE2) and EE2 equivalent concentrations (EE2-EQ) of spiked water samples with high or low EE2 concentrations are compared with the nominal EE2 concentrations. Measured chemical concentrations were from Supplementary data of (Konemann et al., 2018).

	Nominal spike concentration (ng/L)	Analytically determined spike concentration (ng/L)	EASZY EE2-EQ (ng/L)	Ratio of *in vivo* EE2-EQ to measured EE2 concentration (%)
EE2 low	0.6	0.770	0.720	93.5%
EE2 high	6.0	6.341	6.380	100.6%

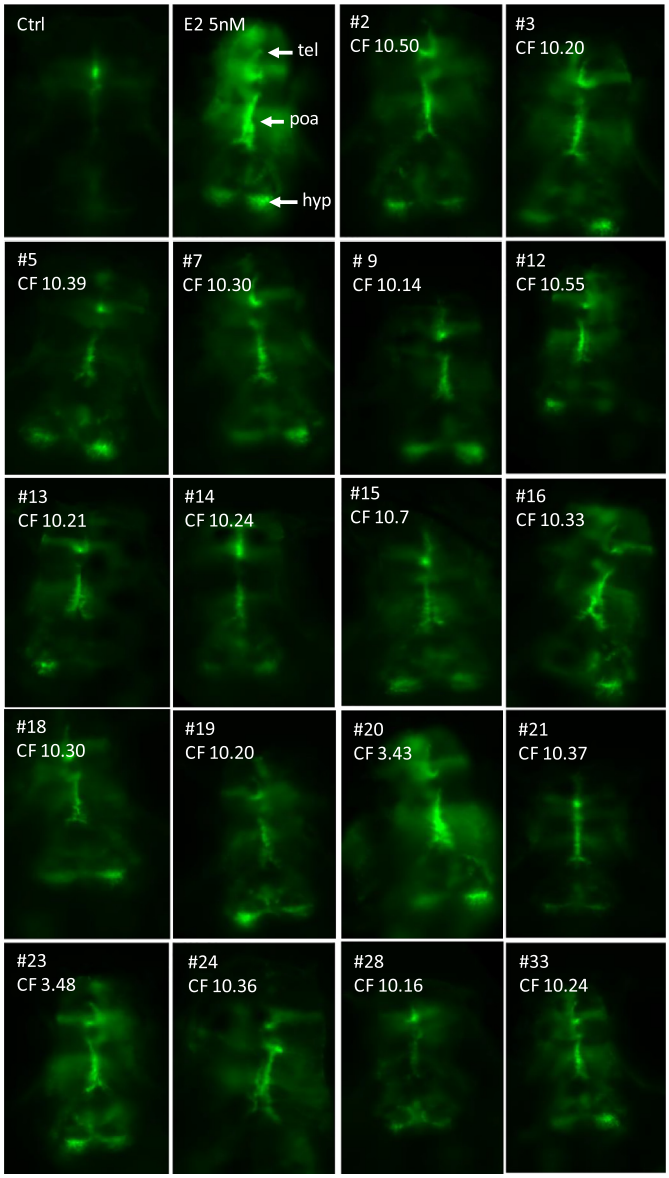

Fig. 1. *In vivo* imaging of the brain of 4-dpf old live transgenic cyp19a1b-GFP zebrafish embryos exposed to waste water (WW) and surface water (SW) sample extracts that were all able to induce a significant response above control in the EASZY assay. Fluorescent signal (green color) reveals induced GFP expression in the developing brain. Dorsal views (anterior to the top) of the telencephalon (tel), preoptic area (poa), and the caudal hypothalamus (hyp). For each water samples, the concentration factor (*CF*) of tested extract is indicated. CTRL: solvent control, E2 = 17β-estradiol, # = sample number. Scale bar = 100 μM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

induction was 0.39 (Dunn's multiple comparison test, $p < 0.05$) and led to a full concentration-response curve. For SW samples, the measured activities were in general less pronounced (Fig. S5) with statistically significant GFP inductions most often observed at the highest REF tested, *i.e.* REF ~10 (Dunn's multiple comparison test, with $p < 0.05$ or < 0.01). An exception was noted for sample # S3 for which significant inductions were reported at both REF = 3.43 and REF = 10.21 (Dunn's multiple comparison test, $p < 0.01$).

As a result, the *in vivo* EEQs derived from these concentration-response relationships covered a broad range of estrogenic activity from the less active (15.9 EEQ ng/L) to the most active sample (673 EEQ ng/L) (Fig. 2).

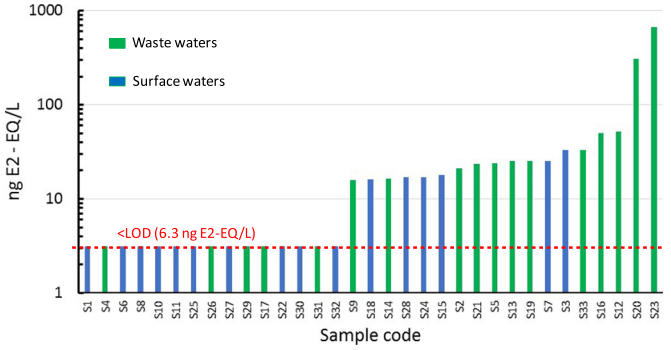

Fig. 2. *In vivo* measured 17β-estradiol equivalents (EEQ) expressed in ng/L for all the waste and surface water samples. The value of the limit of detection (LOD, red dashed line) of the EASZY assay was assigned for all inactive samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

It is noteworthy that as compared to *in vitro* EEQs, the estrogenic activities quantified *in vivo* were higher. Such difference may be related to a lower EC50 of E2 in *in vitro* assays, which raises the question of the reference substance for estrogen-equivalent calculation. For instance, since the EC50 values of EE2 are similar in *in vitro* and *in vivo* assays, the use of EE2 as a reference substance would have led to quantitative data with comparable order of magnitude (*i.e.*, from 0.1 ng/L to 10 ng/L). Nevertheless, this data show the ability of the EASZY assay to detect ER-agonists in complex mixtures and to quantify EEQs, as previously reported for other environmental matrices and samples (Sonavane et al., 2016; Sonavane et al., 2018). Complementary to *in vitro* ER transactivation assays, the responses observed with the EASZY assay inform on the capacity of environmental contaminants to induce the ER-signaling in the developing brain of fish. The findings demonstrate that environmental estrogenic contaminants can reach internal organs and in particular the brain to target radial glial cells and impact the expression of the brain aromatase that has been associated with altered neurogenesis and behavioral changes in zebrafish (Diotel et al., 2010; Kinch et al., 2015).

3.4. Correlation between in vitro and in vivo estrogenic activity: determination of effect-based trigger (EBT) values for in vitro estrogenic activities

When comparing *in vivo* and *in vitro* estrogenic profiles, all analyzed samples were found to be active *in vitro* (Konemann et al., 2018) while only 54% were active *in vivo* (Fig. 2). Nevertheless, correlation analysis between *in vivo*- and *in vitro*-derived EEQs revealed that *in vivo* and *in vitro* estrogenic activities were significantly correlated for all tested *in vitro* assays (Table 4).

Fig. 3 shows the relationship between *in vivo* EEQs derived from EASZY and *in vitro* EEQs derived from the GeneBLAzer and MELN assays.

Correlation graphs between EEQs from EASZY and those derived from HeLa9903, pYes and ERα-CALUX assays can be found in Fig. S6.

Based on this graphical representation, a cut-off value allowing a

Table 4
Pearson correlation coefficient between log EEQs derived from *in vivo* (EASZY) and *in vitro* assays for SW, WW and SW + WW samples. All the correlations were significant with p value < 0.0001 except for SW sample EASZY *vs* HeLa with p value = 0.0003. *n* = number of samples analyzed.

Sample	ERα-CALUX	HeLa	MELN	ER-GeneBLAzer	pYES
SW (*n* = 16)	0.88	0.79	0.86	0.88	0.91
WW (*n* = 17)	0.96	0.93	0.89	0.94	0.83
SW + WW (*n* = 33)	0.92	0.90	0.88	0.93	0.83

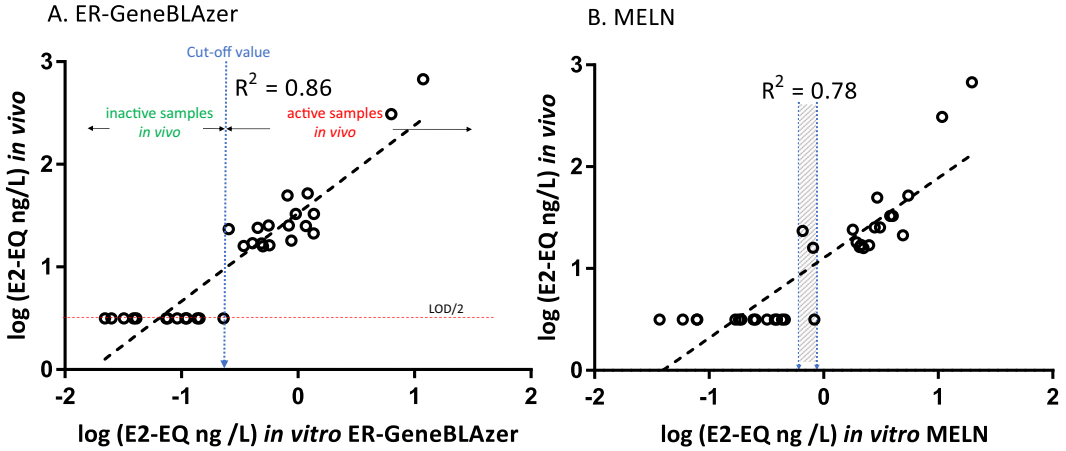

Fig. 3. Correlation between *in vivo* EEQs derived from the EASZY assay and *in vitro* EEQs measured with the ER-GenebLAzer assay (A) and MELN (B). The blue arrow (panel A) indicates the putative *in vitro* EBT value determined graphically for the ER-GenebLAzer assay. The gray area (panel B) indicates the area in which an *in vitro* EBT value could be determined for MELN. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

clear discrimination between non-estrogenic and estrogenic samples *in vivo* could be clearly defined in case of the ER-GeneBLAzer. A similar finding resulted from the comparison between *in vitro* EEQs measured by ERα-CALUX and the EASZY assay *in vitro* (Fig. S6). However, for the MELN, HeLa9903 and pYES assays, such a graphical determination of *in vitro* cut-off values discriminating inactive and active samples was less obvious using this data set although the existence of a threshold is strongly suggested (Fig. 3B and Fig. S6).

To determine an optimal cut-off value with highest discriminative power for each bioassay, the relationships between *in vivo* and *in vitro* EEQs were further analyzed using a logistic regression model (by coding EASZY data as either "inactive" or "active" as described above). The generation of sensitivity and specificity curves as a function of the cut-off value allowed defining discriminative cut-off values for each assay, *i.e.* a value for which both, the sensitivity and the specificity were maximal. An example of such analysis for the GeneBLAzer and MELN assays is presented in the Fig. 4 (see also the Fig. S7 for the other *in vitro* assays).

Based on the comparison of *in vitro* EEQs and *in vivo* estrogenic activities, experimentally derived cut-off values were determined and suggested as assay-specific EBT values. These assay-specific EBTs allowed to identify true active (sensitivity) and true negative (specificity) samples with high probabilities (Table 5).

It is noteworthy that the assay-specific EBTs values defined herein are all within the same range of previously published EBTs for *in vitro* estrogenic activity, ranging from 0.1 to 1.01 EEQ/L (Escher et al., 2018; Kase et al., 2018; Kunz et al., 2017; Jarosova et al., 2014; van der Oost et al., 2017), thereby reinforcing the relevance of these proposed approaches. Notwithstanding the slight differences noticed between all the proposed EBTs, the EBTs derived from the *in vitro* and *in vivo* comparison allowed to substantially increase the sensitivity and the specificity of the *in vitro* assays and thus reducing the risk of false negative and false positive assessments (Table S3).

3.5. Do the in vivo responses inform on the risks posed by environmental estrogens to aquatic ecosystems?

While it appears relevant to link the measured effect in *in vitro* effect-based methods with relevant *in vivo* responses to define EBTs values, the question of the relevance of the *in vivo* bioassay response with respect to the risk for aquatic ecosystems has to be discussed (Mehinto et al., 2018). Based on E1, E2 and EE2 concentrations, a cumulative risk quotient (RQ) representing the combined risk for the mixture of the three steroidal estrogens was calculated in all SW and WW samples investigated (Kase et al., 2018). An RQ > 1 indicated an unacceptable risk for aquatic species while RQ < 1 indicated an acceptable risk for aquatic species. Interestingly, a strong association was found between the calculated RQ and *in vivo* estrogenic responses (Fig. 5).

Among the 15 inactive samples *in vivo*, 13 samples presented no risk (RQ < 1) and for 14 out of 18 active samples the risk was evaluated as being unacceptable (RQ > 1). This means that the *in vivo* response reliably informs on the risk of mixtures of steroidal estrogens for aquatic species with a high sensitivity (87.5%) and specificity (75.5%) (see Table S4).

There were, however, some exceptions as elevated risk based on chemical analyses were noticed for two samples for which no *in vivo* responses were measured (*i.e.* # 27 RQ = 2.27 and # 17 RQ = 3.49). Such differences between chemically determined risk and *in vitro* effect-based method had already been noticed as these two samples were characterized by low *in vitro* estrogenic activities in all the cell-based assays (with the exception of the pYES for sample 17 with an EEQ = 0.690 ng/L) (Kase et al., 2018). Therefore, the *in vivo* responses reported herein support the responses measured *in vitro*. The samples #17 and #27 showed elevated levels for EE2 but comparably low levels of the other two estrogenic compounds analyzed by LC/MS, *i.e.* E1 and E2 (Konemann et al., 2018). The fact that the RQ is based on the suggested EQS-levels explains this finding. The EQS is defined to be protective for the whole aquatic ecosystem which means that at EQS-level effects *in vivo* should not occur. Since the used model organism may not be the most sensitive fish species and the hazardous concentration thresholds for 5% of species (HC5) of 70 pg/L EE2 determined by a Species Sensitivity Distribution (SSD) approach was modified by an assessment factor of 2, it may happen that a sample shows a RQ > 1 but is not assessed as estrogenic by an *in vivo* assay. However, in most samples this was not the case and risks indicated based on the chemical analysis were captured by means of the biological assay. On the other hand, few other samples (samples #5, #14, #19 and #21) induced estrogenic effects in the developing fish brain whereas the risk quotient was below the value of one (RQ < 1) indicating no risk based on the chemical analysis of the three steroids. *In vitro*, moderate to high estrogenic activities were quantified in most cellular assays for these samples thereby indicating elevated risks (Kase et al., 2018; Konemann et al., 2018) that were further confirmed by the *in vivo* assay in the present study. Since the induction of the brain aromatase is not an adverse effect *per se* it is not justified to define a risk for the aquatic environment in complete analogy to the risk assessment based on EQS-exceedance. However, the results clearly demonstrate that ER-agonists were present in these samples that were not captured by chemical analysis. It means that these compounds were bioavailable and that this or these compounds were distributed in the whole organism resulting in

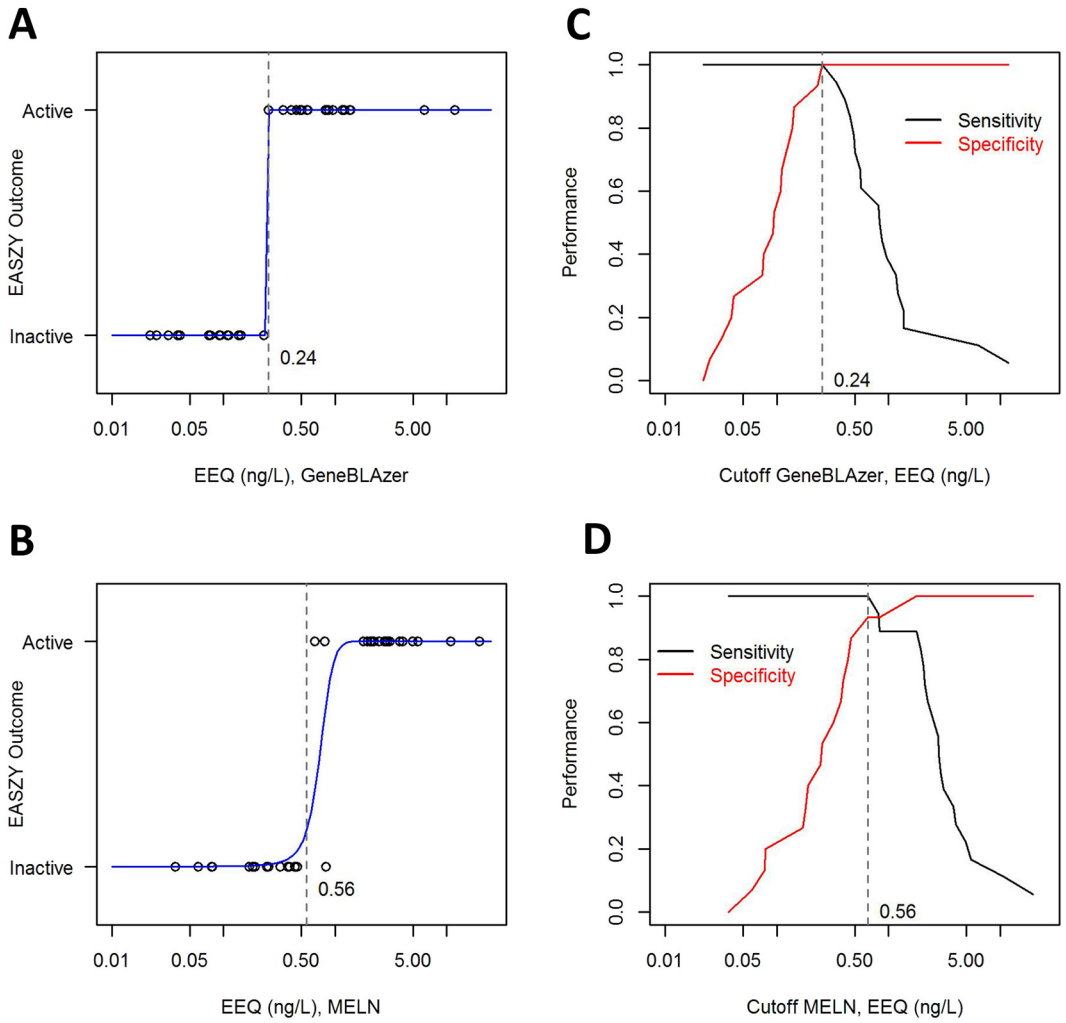

Fig. 4. Logistic regression curves between *in vitro* EEQs measured in ER-GeneBLAzer (A) and MELN (B) assay. and *in vivo* responses found in EASZY. A sensitivity-specificity analysis as a function of cut-off value probabilities was performed for GeneBLAzer (C) and MELN (B) to determine optimal cut-off value leading to maximal sensitivity and specificity (see also Fig. S7 for the other *in vitro* assays).

Table 5
Sensitivity and specificity of *in vitro* estrogenic assays based on assay-specific effect-based trigger values (EBTs) defined in regard to *in vivo* effects measured in zebrafish embryos.

Assay	EBT ng/L	Sensitivity (%)	Specificity (%)
ERα-CALUX	0.28	100	100
MELN	0.56	100	93
ER-GeneBLAzer	0.24	100	100
Hela-9903	0.18	94	93
pYES	0.50	83	93
Mean ± SD	0.35 ± 0.15	95 ± 6.6	96 ± 3.4

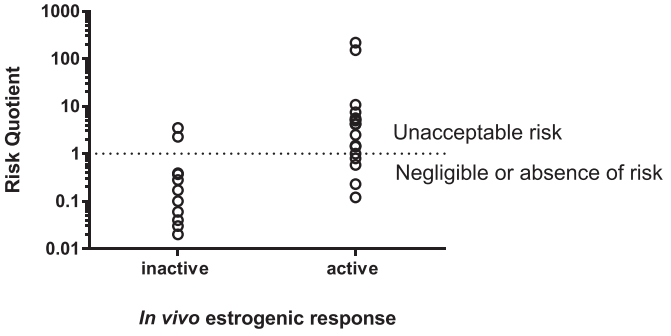

Fig. 5. Relation between the *in vivo* responses measured in EASZY, categorized as inactive or active, and the cumulative risk quotients (RQ) for WW and SW samples. RQ is based on chemically measured concentrations of E1, E2 and EE2 in each sample and used for chemical status assessment. All the RQ data were from Kase et al. (2018). A strong association between RQ and *in vivo* estrogenic responses was found (Chi2 test, p = 0.0002).

brain tissue concentrations that were sufficient to trigger a biological response on the molecular level *in vivo*. This finding underlines the strength of this kind of *in vivo* bioassay, namely, the integral and unbiased detection of bioactive compounds in the environment.

Altogether, these findings illustrate the relevance of the measured *in vivo* estrogenic responses regarding a chemical analysis risk assessment and the ability of effect-based methods (*in vitro* and *in vivo*) to capture mixture effects of (known) steroidal estrogens but also of other (unknown) substances acting by the activation of the ER (Altenburger et al., 2018; Kunz et al., 2015; Neale et al., 2015). Finally, the finding that *in vivo* responses inform on the risk for aquatic species further supports the relevance of the suggested *in vitro* EBT values derived from the *in vitro / in vivo* correlation for estrogenic activities in the present study. This increases the discriminating power when interpreting *in vitro* estrogenic activities to assess the quality of the water bodies thereby lowering the probability of false positive and false negative assessments (Table 6 and Table S5).

Table 6
Sensitivity and specificity of *in vitro* assays as regard to the chemically cumulative risk assessment of steroidal estrogens (Risk Quotient). For *in vitro* assays, the proposed EBTs were derived from the correlations between *in vitro* and *in vivo* estrogenic activities measured in SW and WW samples.

Assay	EBT (ng/L)	Risk quotient	
		Sensitivity (%)	Specificity (%)
ERα-CALUX	0.28	87.5	76.5
MELN	0.56	87.5	70.6
ER-GeneBLAzer	0.24	87.5	76.5
Hela-9903	0.18	93.8	82.4
pYES	0.50	87.5	88.2

It has to be pointed out that the suggested EBT-values have to be further validated with independent data sets but the proposed sensitivity and specificity analysis allows the assessment of EBT-values in a straightforward manner.

4. Conclusions and outlooks

This study reports the *in vivo* estrogenic activities of surface and waste water samples based on their capacities to induce the ER-regulated *cyp19a1b* gene expression in the developing brain of zebrafish, thereby demonstrating that environmental ER ligands were bioavailable, distributed within the organism to target radial glial cells and induce brain aromatase expression. While the short- and long-term adverse outcome of brain aromatase disruption needs to be further explored (Diotel et al., 2013; Kinch et al., 2015; Vosges et al., 2012; Vosges et al., 2010), this early molecular event might indicate a negative impact of substances capable of affecting the ER-signaling pathway in an intact organism.

Interestingly, the *in vivo* estrogenic activities were correlated with *in vitro* responses and to EQS mixture risk assessment. A sensitivity and specificity analysis allowed us to determine *in vitro* cut-off values with high discriminative power for each *in vitro* assay that were suggested as assay-specific effect-based trigger values (EBTs) above which *in vivo* responses were observed. This result further adds toxicological relevance to *in vitro* effect-based methods as predictive tools for observed responses at the organism level. It is also remarkable that, based on such *in vitro vs in vivo* correlations, the suggested EBT values were close to existing EBT-proposals. The refinement of EBTs based on the *in vitro* and *in vivo* comparison improves the sensitivity and specificity of five *in vitro* assays for predicting the risks associated with substances acting through the same mode of action. Thanks to this approach is was possible to achieve a high specificity and sensitivity gain for EQS mixture effects and biological relevance of 5 different *in vitro* EBM in mean of > 95%. This further supports the relevance of *in vitro* tools as specific and reliable tools for an efficient environmental monitoring of substances acting as estrogens.

To our knowledge, no other mode of action of endocrine disrupting chemicals has so far been able to provide a level of information comparable with the one gained with the present study on 33 realistic samples of seven EU member states investigated with three high resolution HPLC MS/MS, five *in vitro* ER transactivation assays (Kase et al., 2018; Konemann et al., 2018) and the *in vivo* EASZY assay (this study). Neverthless, it would be advisable to extend it to other case studies (*e.g.*, WFD monitoring sites) to validate the proposed EBTs. Furthermore, the *in vivo* responses were measured on a specific zebrafish assay (having its own advantages and limits) hence questioning if similar EBTs would have defined using other fish models and endpoints of estrogenic activity. However, the data provided are sufficiently robust to illustrate a proof of concept to define or refine EBTs. Hence, similar experimental approaches can be recommended for other mode of action, notably those that are mediated through nuclear receptors. Such approach would however require robust and reliable *in vivo* screening biological models which are currently lacking for most of NR-mediated effects.

Acknowledgments

This study was supported by AQUAREF, the NORMAN network for emerging pollutants, the French Agency for Biodiversity (AFB), the P181-DRC50 of the French Ministry of Ecology and the project SOLUTIONS (European Union Seventh Framework Programme FP7-ENV-2013-two-stage Collaborative project under grant agreement number 603437). SB was supported by the German Federal Ministry of Environment. The authors would like to express their thanks to Cléo Tebby (Models for Ecotoxicology and Toxicology, INERIS) for her help on Logistic regression analysis using R. and to Beate I. Escher of Helmholtz Center for Environmental Research (UFZ, Leipzig) for helpful discussion and her review of an earlier version of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.06.006.

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