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To cite this version:

Vincent Le Fol, Selim Aït-Aïssa, Manoj Sonavane, Jean-Marc Porcher, Patrick Balaguer, et al.. In vitro and in vivo estrogenic activity of BPA, BPF and BPS in zebrafish-specific assays. Ecotoxicology and Environmental Safety, 2017, 142, pp.150-156. 10.1016/j.ecoenv.2017.04.009 . hal-01604318

HAL Id: hal-01604318

https://hal.science/hal-01604318v1

Submitted on 26 May 2020

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Ecotoxicology and Environmental Safety 142 (2017) 150–156

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

In vitro and *in vivo* estrogenic activity of BPA, BPF and BPS in zebrafish-specific assays

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ARTICLE INFO

Keywords:
Zebrafish
Endocrine disruptors
Bisphenols
Estrogenic activity

ABSTRACT

Bisphenol A (BPA) is a widely used chemical that has been extensively studied as an endocrine-disrupting chemical (EDC). Other bisphenols sharing close structural features with BPA, are increasingly being used as alternatives, increasing the need to assess associated hazards to the endocrine system. In the present study, the estrogenic activity of BPA, bisphenol S (BPS) and bisphenol F (BPF) was assessed by using a combination of zebrafish-specific mechanism-based *in vitro* and *in vivo* assays. The three bisphenols were found to efficiently transactivate all zebrafish estrogen receptor (zfER) subtypes in zebrafish hepatic reporter cell lines (ZELH-zfERs). BPA was selective for zfERα while BPS and BPF were slightly more potent on zfERβ subtypes. We further documented the estrogenic effect *in vivo* by quantifying the expression of brain aromatase using a transgenic *cyp19a1b*-GFP zebrafish embryo assay. All three bisphenols induced GFP in a concentration-dependent manner. BPS only partially induced brain aromatase at the highest tested concentrations (> 30 μM) while BPA and BPF strongly induced GFP, in an ER-dependent manner, at 1–10 μM. Furthermore, we show that BPF strongly induced vitellogenin synthesis in adult male zebrafish. Overall, this study demonstrates the estrogenic activity of BPA, BPF and BPS in different cell- and tissue-contexts and at different stages of development. Differences between *in vitro* and *in vivo* responses are discussed in light of selective ER activation and the fate of the compounds in the models. This study confirms the relevance of combining cellular and whole-organism bioassays in a unique model species for the hazard assessment of candidate EDCs.

1. Introduction

Bisphenol A [BPA, 2,2,-bis(4-hydroxyphenol)propane] is one of the man-made chemicals with the highest volume of production, due to its wide use and great variety of applications (review by Michalowicz, 2014). An extensive amount of literature now demonstrates that BPA is an endocrine disrupting compound (EDC), leading to potential adverse health effects in human (review by Rochester, 2013) and wildlife (review by Oehlmann et al., 2009). Consequently, the use of BPA in food contact materials has been restricted or banned in several countries, and it is becoming evident that other bisphenols, intended to replace BPA for various industrial applications, are increasingly being used and detected in our environment. The question of the endocrinal effects of these substitutes is a current issue in human and environmental health studies.

BPS (2,2-bis [4-hydroxyphenol]sulfone) and BPF (2,2-bis [4-hydroxyphenol]methane) are among the main possible substitutes of BPA. BPS has been found in beverage and food cans (Vinas et al., 2010) and in thermal receipt papers (Becerra and Odermatt, 2012). It has been identified in more than 81% of urinary samples of the American population (Liao et al., 2012a). Recent studies have also reported significant environmental concentrations of bisphenols in aquatic ecosystems. BPF and BPA were reported to be predominant contaminants (Careghini et al., 2015; Liao et al., 2012b; Yamazaki et al., 2015), while increasing concentrations of BPS have been detected in sediment sections that represent the past decade, which is consistent with its

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http://dx.doi.org/10.1016/j.ecoenv.2017.04.009
Received 8 February 2017; Received in revised form 22 March 2017; Accepted 5 April 2017
Available online 11 April 2017
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recent introduction (Liao et al., 2012b).

Due to their structural similarity with BPA, it can be expected that BPS and BPF would share some of the effects of BPA. Up to now, most of the data related to the ED potency of BPS and BPF have been obtained in mammalian *in vitro* models. These data suggest that BPS and BPF are able to interfere with multiple nuclear receptors (Kitamura et al., 2005; Molina-Molina et al., 2013; Delfosse et al., 2012), to induce cell proliferation (Molina-Molina et al., 2013) or to alter testosterone secretion in fetal testis assay (Eladak et al., 2015). *In vivo* studies using mammalian and non-mammalian models are rarer but have shown that these compounds can impact the expression of hormone-regulated genes, producing adverse developmental and reproductive effects (Ji et al., 2013; Kinch et al., 2015; Naderi et al., 2014; Cano-Nicolau et al., 2016).

Among the molecular targets of BPA, its interaction with estrogen receptors (ER) is a well-established mechanism in different biological models. Furthermore, BPA and some congeners have been shown to exhibit differential binding affinities and activities depending on human ER subtypes, *i.e.* ERα and ERβ (Routledge et al., 2000; Delfosse et al., 2012; Molina-Molina et al., 2013). In contrast to humans, teleosts possess three ER subtypes, namely ERα, ERβ1 and ERβ2 (Menuet et al., 2002), which are differently expressed and regulated by estrogens. Because the measured estrogenic activity is known to be influenced by the species of origin, the cellular and the tissular context (Matthews et al., 2000) as well as the developmental stage of organisms, the effect of bisphenols on fish-specific models needs to be assessed.

In this study, we aimed at further investigating the capacity of BPA and its major substitutes to transactivate ERs in a model fish species, the zebrafish, a recognized model organism for investigating endocrine disruption (Segner, 2009). For this purpose, we used a set of *in vitro* reporter gene assays based on stable expression of subtypes of zebrafish ER (*i.e.* zfERα, zfERβ1, and zfERβ2) coupled to estrogen response element (ERE)-driven luciferase in a zebrafish liver cell line (ZFL) that was previously developed (Cosnefroy et al., 2012). Estrogenic activity of bisphenols was further evaluated by using an *in vivo* zebrafish embryo assay (EASZY assay) based on the *cyp19a1b-GFP* transgenic line expressing the Green Fluorescent Protein (GFP) under the control of the ER-regulated cyp19a1b gene in the brain (Brion et al., 2012). Finally, *in vivo* induction of vitellogenin synthesis (VTG) in male fish was examined to further assess the estrogenic activity in a fish liver context.

2. Materials and methods

2.1. Chemicals and cell culture reagents

2,2-Bis(4-hydroxyphenyl)propane (BPA, CAS # 80-05-7), 4,4′-sulfonyldiphenol (BPS, CAS # 80-09-1), bis(4-hydroxyphenyl)methane (BPF, CAS # 620-92-8), 17β-estradiol (E2, CAS #r 50-28-2), 17α-ethynylestradiol (EE2, CAS # 57–63-6) were purchased from Sigma-Aldrich (Quentin Fallavier, France). Leibovitz 15 culture medium (L15), 4-(2-hydroxyethyl) − 1-piperazineethanesulfonic acid (HEPES), F-12 nutrient mixture (Ham's F12) powder, Dulbecco's Modified Eagle Medium High Glucose (DMEM HG) powder, G418, hygromycin B, penicillin and streptomycin were purchased from Fischer Scientific (Illkirch, France). Fetal calf serum (FCS) and epidermal growth factor (EGF) were purchased from Sigma-Aldrich (Quentin Fallavier, France) and sodium bicarbonate and insulin from Dominique Dutscher (Brumath, France). ICI 182–780 (ICI) was purchased from Tocris (Bristol, UK) and flutamide (FLU) was obtained from Sigma-Aldrich (Quentin Fallavier, France).

2.2. Cell culture, chemical exposure and luciferase induction assay

ZELH-zfERs cell lines were previously established by a two-step stable transfection of the zebrafish hepatic cell line ZFL (ATCC CRL-2643) with the luciferase gene under the control of ERE (yielding ZELH cells) and each of the three zebrafish estrogen receptors yielding ZELH-zfERα, ZELH-zfERβ1 and ZELH-zfERβ2 cell lines (Cosnefroy et al., 2012; Sonavane et al., 2016). Cells were routinely cultured at 28 °C in a LDF-SVF culture medium, exactly as previously described (Cosnefroy et al., 2012). For chemical exposure, cells were cultured in phenol red free LDF with dextran-coated charcoal treated serum. The cells were seeded in 96-well plates at 25,000 cells per well for ZELH-zfERα and ZELH-zfERβ1, and at 75,000 cells per well for ZELH-zfERβ2, and were cultured for 24 h before exposure. BPA, BPS and BPF were freshly diluted in dimethyl sulfoxide (DMSO) to get a range of final concentrations from 3 nM to 10 μM in culture medium and a final concentration of solvent of 0.1% v/v. After 72 h of exposure, luciferase activity was assessed in living cells as previously described (Cosnefroy et al., 2012). Results were expressed as a percentage of maximal luciferase induced by E2 10 nM.

2.3. Zebrafish embryos, chemical exposure and in vivo imaging

Newly fertilized *cyp19a1b*-GFP transgenic zebrafish (*Danio rario*, from AB strain) eggs were collected from the breeding stock of adult zebrafish, which were maintained under controlled photoperiod (14/10 h light/dark cycle) in charcoal filtrated water at 28 ± 1 °C and fed with SDS-400 (Special Diet Services, Dietex, Argenteuil, France) twice a day and live brine shrimp (Artemia spp.; Ocean Nutrition). Chemical exposures were performed from 0 to 4 days post-fertilization (dpf) in an incubator at 28 °C under semi-static conditions with a total renewal of the medium each day. Each condition consisted of a minimum of 10 embryos in 100 ml of water contaminated by the diluted chemical in DMSO to get a final solvent concentration of 0.01% v/v. Zebrafish embryos were exposed to a range of concentrations of BPA, BPS or BPF. For each chemical, zebrafish embryos were co-exposed with either ICI (1 μM) or FLU (1 μM). Ethinylestradiol (EE2) 0.05 nM was used as a positive control. At the end of exposure, transgenic zebrafish larvae were collected for fluorescence measurement by image analysis. Each larva was observed and photographed in dorsal view under a fluorescence microscope combined with an AxioCam Mrm camera (Zeiss GmbH, Göttingen, Germany). All photographs were taken with the same parameters and analyzed using Axiovision Imaging Software, as previously described (Brion et al., 2012). Fluorescence quantification was based on the measurement of the integrated density of the region of interest for each picture using ImageJ software as previously described (Brion et al., 2012). Results were expressed as mean fold of induction above control.

2.4. BPF exposure and plasmatic vitellogenin measurement in adult zebrafish

Six-month-old male adult zebrafish (*Danio rerio*, AB strain) were exposed to BPF (0.1, 1 and 10 μM) or E2 (10 nM), using DMSO as the vehicle (concentration in water of 0.006%, v/v). For every condition, eight zebrafish were exposed for 7 days in 4 l of water with a total water renewal every 24 h. At the end of exposure, fish were euthanized. Blood (5 μl) was collected *via* a micropipette tip through a ventral incision between the abdomen and the head and stored at −80 °C. Vitellogenin concentrations were measured using a competitive zebrafish vitellogenin enzyme linked immune-sorbent assay (zf-Vtg ELISA) as previously described (Brion et al., 2002). Results were expressed as a mean with 95% confidence interval, in ng per ml of plasmatic vitellogenin and analyzed using the Mann-Whitney test. Animal handling and experimentations were in accordance with the EU Directive 2010/63/EU for animal experiments.

2.5. Data analysis

For cell exposure, each chemical was tested in triplicate in at least two independent experiments. Concentration–response curves (CRC) were modeled by using the Regtox Microsoft Excel™ macro (version 7.0.7, available at http://eric.vindimian.9online.fr/), which uses the four-parameter Hill equation model. From CRCs, either EC50 (the concentration of a test chemical which induces 50% of its maximal effect) or PC50 (the concentration of a test chemical inducing 50% of the maximal positive control response) was determined. Whenever an incomplete CRC was obtained, *i.e.* a CRC not reaching a plateau, the maximal effect parameter was fixed to 100% activation. When this was the case, the PC50 of the tested compound was determined. In all other cases, *i.e.* a full CRC with a plateau at an intermediate (partial agonist pattern) or maximal level (full agonist pattern), the maximal effect was not fixed in the model and the EC50 was determined. For *in vivo* assays, GFP induction by BPA, BPS and BPF was expressed as mean ± SEM and values were compared to DMSO control using one way ANOVA followed by a Dunnett post-hoc test. Dose-response of GFP induction in transgenic larva was modeled by using the Regtox EV7.0.7 Microsoft Excel™ macro to derive *in vivo* PC50. The effect of co-exposure of bisphenols with ICI or flutamide was analyzed using the Mann-Withney test.

3. Results and discussion

3.1. In vitro estrogenic activity of BPA, BPS and BPF in ZELH-zfERs cells

In this study, EC50 values of E2 in ZELH-zfERα, -zfERβ1 and -zfERβ2 cell lines were highly concordant with the values reported in our previous studies (Cosnefroy et al., 2012; Sonavane et al., 2016), thus confirming the reproducibility of our established cell systems, and allowing the examination of the activity of BPA, BPF and BPS in these cell lines. All three bisphenols were found to be active in the three cell lines, but with different transactivation efficiencies and potencies according to the zfER subtype (Fig. 1, Table 1). BPA showed a selective activity towards zfERα (EC50 1 μM) as compared to zfERβ1 (EC50 17 μM) and zfERβ2 (EC20 18 μM). Such zfERα selectivity was not observed for BPS and BPF, which were slightly more potent towards zfERβs than zfERα (Fig. 1, Table 1). Similar conclusions were noted when assessing the relative estrogenic potencies (REP), with BPA having a higher REP on zfERα while BPF and BPS REPs did not markedly differ between cell lines (Table 1).

In terms of EC50, the values reported in the zebrafish-specific ZELH cell lines were generally in the same range (1–20 μM) as those reported in other *in vitro* models (Molina-Molina et al., 2013; Pinto et al., 2014; Tohyama et al., 2015). The main issue may concern ER subtype selectivity and agonist profiles of the studied chemicals, which may reflect a cell-context specific pattern response.

Few recent studies have reported the *in vitro* activation of zfERs by BPA. All were based on reporter gene assays in human cell lines. In line with our data, Pinto et al. (2014) showed marked selectivity of BPA towards zfERα *vs.* zfERβs in stably transfected HeLa cells (HELN). Also, using GAL4-LDB fusion proteins transiently transfected in human HEK293 cells, Tohyama et al. (2015) reported an almost equipotent transactivation of zfERα (ESR1 LBD) and zfERβ2 (ESR2a LBD) by BPA, while its REP was found to be 3- to 100-fold higher on zfERα, compared to zfERβ2 and zfERβ1 respectively. Several BPA analogues, including BPF, were reported to induce zfERα but not zfERβs transactivation in human U251 glial cells in transient transfection experiments, while BPS was not found to be active, whatever the receptor (Cano-Nicolau et al., 2016). However, the authors reported single concentration (1 μM) data, which renders the quantitative comparison between chemicals and receptors difficult. Interestingly, in HELN reporter cells expressing human ERs, Molina-Molina et al. (2013) reported a higher activity of BPA on hERα while BPF was more active on hERβ, which, to some extent, parallels our findings on zfERs. Altogether, these data as well as the results of the current study support the view that bisphenols can act as selective ER modulators in mammals and in fish. Slight variations between studies may reflect the influence of different cell contexts related to the tissue and/or species of origin (Le Fol et al., 2015).

In zebrafish ZELH-zfER cells, bisphenols were found to behave as partial or weak agonists (Fig. 1). To some extent, similar patterns have been reported for BPA on zfERs in other cell systems (Pinto et al., 2014; Tohyama et al., 2015). This is in agreement with the fact that BPA is a weak estrogenic compound that exhibits a distinct mechanism of action than E2 on human ERs (Gould et al., 1998; Routledge et al., 2000). This was further documented by crystallographic analyses describing a discrete binding mode of BPA congeners to human ERs leading to weak agonism (by bisphenol AF) or antagonism (by bisphenol C) (Delfosse et al., 2012). Our data suggest that a similar mechanism could be involved in zebrafish systems and could contribute to differential transactivation efficacy in a zfER subtype-dependent manner. Such ER selectivity of bisphenols may lead to differential estrogenic response *in vivo* depending on the target tissue.

3.2. In vivo estrogenic activity of BPA, BPS and BPF

In vivo estrogenic activity of a large range of concentrations of bisphenols was assessed by quantifying the fluorescence signal in the developing brain of 4-dpf old transgenic *cyp19a1b*-GFP zebrafish (Fig. 2). BPA exposure led to significant induction of the fluorescence intensity from 5 μM ($p < 0.001$; Fig. 2). The maximal fold-induction of GFP for BPA 10 μM was similar to that measured in zebrafish exposed to EE2 0.05 nM (Brion et al., 2012). Interestingly, in zebrafish exposed to low BPA concentrations (0.01–0.06 μM), a weak but not statistically significant 2-fold increase of GFP was noted. Therefore, this observation did not confirm the effects of low concentrations of BPA on the brain aromatase in zebrafish reported previously (Kinch et al., 2015).

In BPF-exposed zebrafish embryos, a concentration-dependent induction of GFP was observed with a significant effect from 1 μM (Fig. 2). At higher concentrations, the GFP signal reached a plateau at 5 μM (maintained up to 20 μM) with a maximum fold of induction similar to BPA 10 μM and EE2 0.05 nM. The EC50 of BPF was 1.2 μM suggesting its higher estrogenic activity *in vivo* as compared to BPA (EC50 5.7 μM). BPS was the less efficient molecule as it induced GFP by 4-fold only at 30 and 60 μM. Our data thus support the inducing effect of these bisphenols on *cyp19a1b* transcript levels in the hypothalamus of zebrafish embryos (Cano-Nicolau et al., 2016) and further provide a quantification of their estrogenic activity in this model.

In the presence of the ER antagonist, ICI 182–780 (ICI), BPA and BPF-induced aromatase expression was partially suppressed, demonstrating that functional ERs were involved in the effect mediated by these compounds (Fig. 3). However, ICI 1 μM was unable to block the effect of BPS. Previous studies have shown that (xeno-)estrogens can strongly stimulate cyp19a1b expression in zebrafish larvae (Brion et al., 2012; Menuet et al., 2005). It has been clearly demonstrated that the estrogen-dependent regulation of brain aromatase gene expression involves functional ERs and the binding of liganded-ER on ERE and ½ ERE located in the promoter region of the cyp19a1b gene (Menuet et al., 2005). Our study confirms the involvement of these elements in BPA and BPF observed effects.

In a recent study, Kinch et al. (2015) found that co-exposure to BPA and flutamide (6 μM) reduced cyp19a1b mRNA expression when these compounds were administered from 8 to 48 hpf, hence suggesting that BPA can induce transcription of AroB (cyp19a1b) *via* androgen receptors (ARs) specifically during this developmental period. In our study, co-exposure of zebrafish embryos to bisphenols and flutamide (1 μM) from 0 to 96 hpf failed to down-regulate bisphenol-induced GFP intensity (Fig. 3). It has been established that the up-regulation of the brain aromatase gene by androgens such as testosterone or dihydrotestosterone (Brion et al., 2012; Mouriec et al., 2009) does not involve a

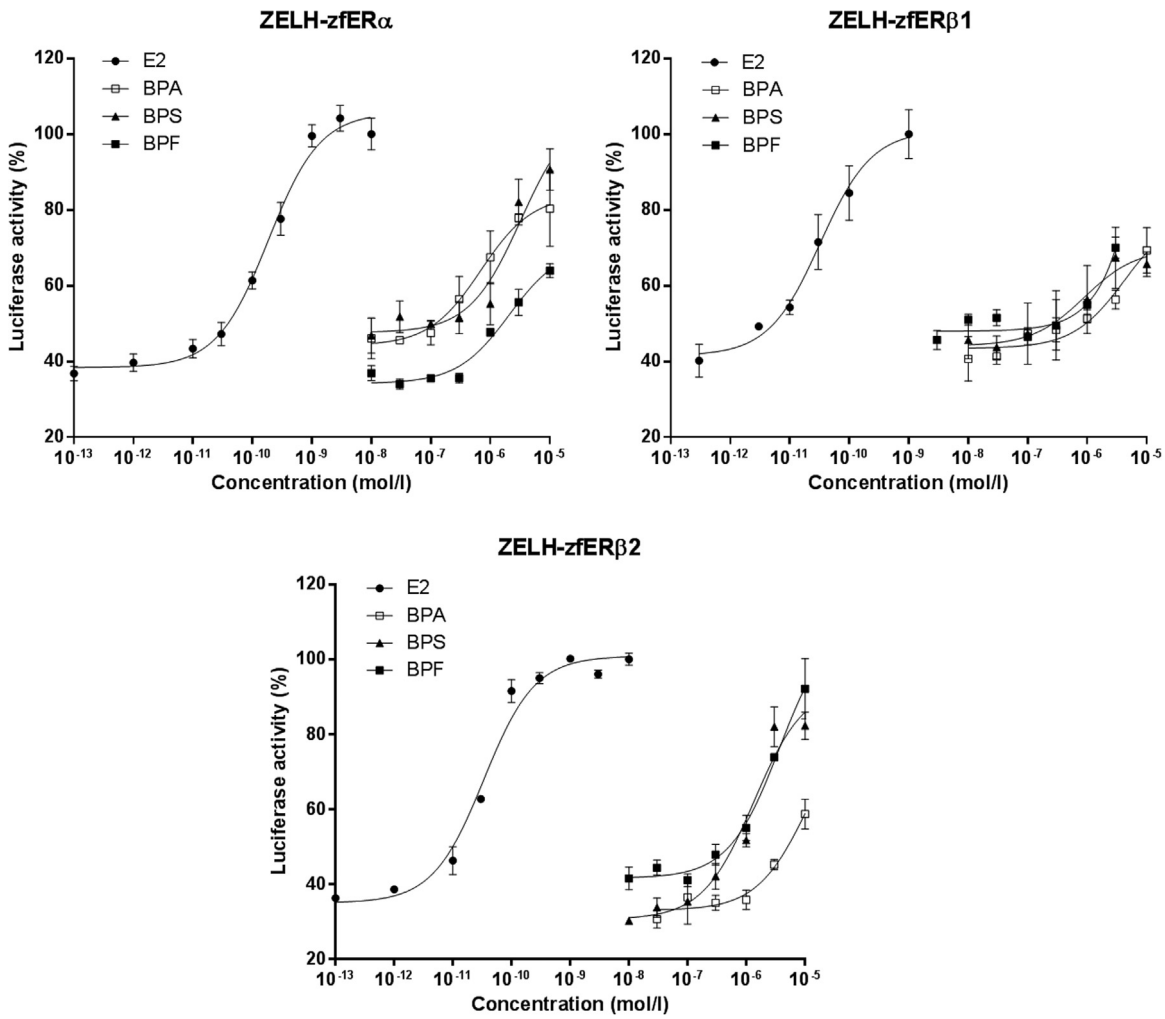

Fig. 1. Concentration-dependent induction of luciferase by BPA, BPS and BPF in (A) ZELH-zfERα, (B) ZELH-zfERβ1, and (C) ZELH-zfERβ2 cells exposed for 72 h. Values are expressed as percentage of luciferase induction by E2 10 n M (mean +/- SD, n = 3).

Table 1
Effective concentrations (EC50 in nM) and relative estrogenic potency (REP) of BPA, BPF and BPS in ZELH-zfERs zebrafish cell lines.

Chemicals		ZELH-zfERα	ZELH-zfERβ1	ZELH-zfERβ2
E2	EC50	0.15	0.03	0.06
BPA	EC50	807	17154	18918*
	REP	1.9×10^{-4}	1.7×10^{-6}	3.2×10^{-6}
BPF	EC50	5795	5337	2418
	REP	2.6×10^{-5}	5.6×10^{-6}	2.5×10^{-5}
BPS	EC50	4058	1016	2468
	REP	3.7×10^{-5}	3.0×10^{-5}	2.4×10^{-5}

Each chemical was tested in triplicate in at least two independent experiments. Data are mean of values of EC50 (n = 3) except those marked by an asterisk (*) which are PC50; REPs were determined as the ratio of E2 EC50 to that of bisphenol.

direct effect of AR on the regulation of *cyp19a1b* expression, but rather ER activation (Mouriec et al., 2009). Overall, our data support an up-regulation of the brain aromatase by BPA and BPF mediated by ER, at the developmental stages studied.

The reason why ICI 1 μM did not counteract, even partially, the effects of high concentrations of BPS is not known. Treatment of newly fertilized embryos with ICI alone during 24 h, followed by a co-treatment with BPS and ICI, also failed to block the action of BPS on GFP (data not shown). This result is supported by *in vitro* data obtained in a reconstituted glial cell model in which BPS was unable to induce *cyp19a1b*-luciferase activity, independently of the estrogen receptor sub-type, while both BPA and BPF were active (Cano-Nicolau et al., 2016). Thus, our results suggest that BPS acts differently on brain aromatase as compared to BPA and BPF. One hypothesis is the involvement of ER-independent signaling pathway(s). It is known that BPA can directly activate estrogen related receptor γ (ERRγ) and can induce development abnormalities in zebrafish embryos (Tohmé et al., 2014). However, to what extent BPS can bind to and activate zfERRγ to induce brain aromatase in zebrafish remains to be determined.

Overall, we were able to demonstrate that the three bisphenols induced a differential estrogenic response in the brain of embryos, characterized by different activation profiles of the *cyp19a1b* gene. BPA and BPF seem to act as full agonists and BPS as a partial agonist *in vivo*. As mentioned above, our *in vitro* data together with other reports (Delfosse et al., 2012) suggest that some bisphenols would act as selective ER modulators (SERM), which may account for the cell- and tissue-specific differences in ER activation by bisphenols observed *in vitro* (Delfosse et al., 2012) and *in vivo* in BPA-exposed zebrafish embryos (Gorelick et al., 2011). Based on our study, the strong estrogenic activity of BPA and BPF in radial glial cells of zebrafish could reflect such SERM property.

3.3. *In vivo estrogenic activity of BPF on vitellogenin synthesis in adult male fish*

In adult as well as in juvenile fish, the ability of BPA and BPS to

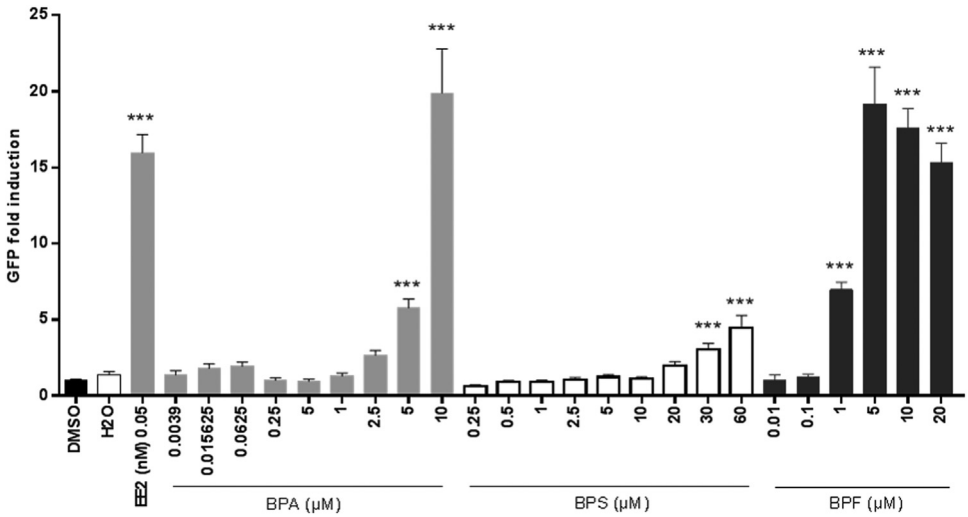

Fig. 2. Concentration-dependent induction of GFP by BPA, BPS and BPF in *cyp19a1b-GFP* transgenic larva after 96 h of exposure. Values are expressed as mean +/- SEM (n = 10–20). One way ANOVA was used for DMSO comparison (***: $p < 0.001$).

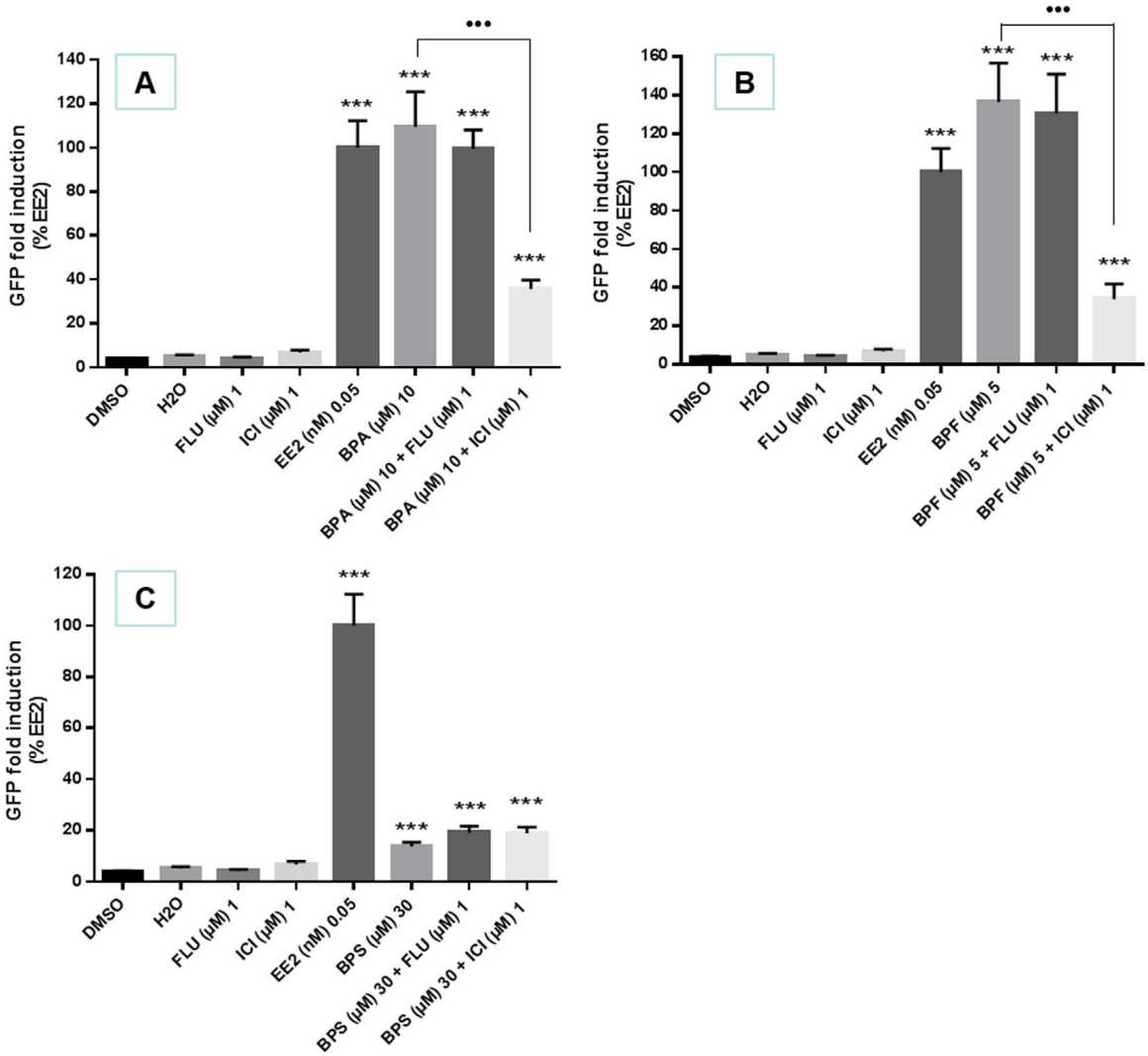

Fig. 3. Effect of ICI-182,780 (ICI) and flutamide (FLU) on GFP induction by (A) BPA, (B) BPS and (C) BPF in *cyp19a1b-GFP* transgenic larva after 96 h of exposure. Values are expressed as mean +/- SEM (n = 10–20). One way ANOVA followed by a Dunnett post-hoc test was used for statistical comparison with DMSO exposed larva (***: $p < 0.001$). Mann-Whitney test was used for co-exposure conditions (***: $p < 0.001$).

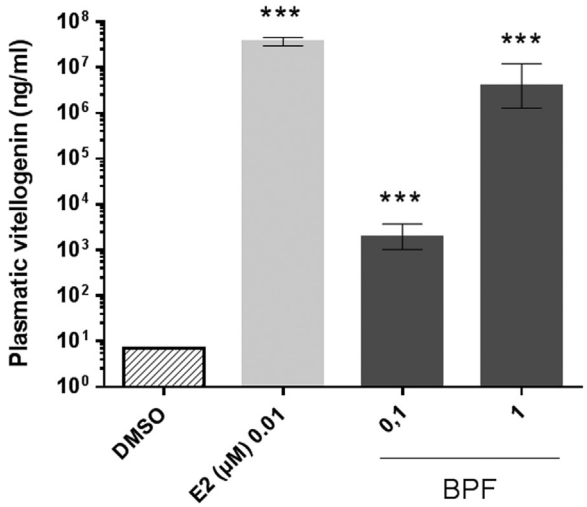

Fig. 4. Plasmatic vitellogenin concentration in adult male zebrafish after a 7-days exposure to BPF (µM). Eight fish were exposed for each condition and blood Vtg was measured in each animal (***: $p < 0.001$, Mann-Whitney test). Data are expressed as geometric mean ± 95% confidence interval.

induce ER-regulated hepatic vitellogenin synthesis has already been reported in various fish species (Brian et al., 2005; Tabata, 2004) including the zebrafish (Ji et al., 2013; Naderi et al., 2014; Song et al., 2014; Van den Belt et al., 2003).

In the present study, we further documented the *in vivo* estrogenic activity of bisphenols and provided new data on BPF by measuring the induction of the circulating concentrations of vitellogenin in males after 7 days of exposure (Fig. 4). We demonstrated that BPS significantly induced VTG at 0.1 µM and 1 µM ($p < 0.001$) while the highest concentration tested (10 µM) led to a high mortality rate, an effect not yet been observed in the embryo assay. Comparison of LOECs obtained in the EASZY assay and LOECs collected from the *in vivo* fish VTG assays revealed that the estrogenic potency of BPA was similar in both assays. Indeed, three week-exposure to BPA induced a significant vitellogenin induction in male zebrafish at LOECs 0.5 mg/L (Song et al., 2014) and 1 mg/L (Van den Belt et al., 2003) while the lowest BPA concentration inducing GFP in the EASZY assay was 1.4 mg/L (this study). Conversely, BPF and BPS were much more potent in the VTG assay as compared to the EASZY assay. For instance, developmental exposure from embryos to adults to BPS resulted in a significant induction of VTG in males at concentrations ranging from 0.1 mg/L (Naderi et al., 2014) while, in our study, 4-day exposure from 0 to 96 hpf resulted in significant brain aromatase induction at 7.5 mg/L of BPS. Furthermore, 7-day exposure of adult male zebrafish to 0.02 mg/L of BPF resulted in VTG induction (Fig. 4). In the EASZY assay, significant induction of GFP occurred from 0.2 mg/L in transgenic cyp19a1b-GFP embryos (Fig. 2). Our results show that BPF is the most effective compound in inducing ER-signaling *in vivo*. This highlights the hazard of this BPA substitute and its potential risk for aquatic species, due to the fact that the active concentrations of BPF are environmentally relevant as regards to the contamination of surface waters (Yamazaki et al., 2015).

3.4. Relevance of in vitro and in vivo zebrafish-specific mechanism-based assays

In our study, we observed that BPA, BPF and BPS were all effective in activating the three zfERs subtypes and in inducing the expression of the aromatase gene in the developing brain. We further extended the study to BPF, which revealed its capacity to induce brain aromatase expression in embryos, as well as vitellogenin synthesis in adults.

Observed differences in the estrogenic responses to these bisphenols may depend on different pharmacokinetic parameters between ZELH-zfERs cells lines, zebrafish embryo and male fish. It was previously demonstrated that phase I and phase II drug-metabolizing enzymes are functional in zebrafish larvae (Brox et al., 2016; Alderton et al., 2010; Le Fol et al., 2017; Kurogi et al., 2013). In a recent study, the metabolism of BPS was evaluated in zebrafish embryos and adults and revealed a relatively low bioavailability and an efficient metabolism of BPS into conjugated metabolites (Le Fol et al., 2017), which may explain its lower *in vivo* activity which was not predicted solely on the basis of *in vitro* data.

Importantly, our study is in line with the view that bisphenols act as SERMs, which may account for the cell- and tissue-specific responses (Routledge et al., 2000; Delfosse et al., 2012, Gorelick et al., 2011). As a consequence, combining different models and endpoints with appropriate design appears particularly important in hazard assessment. In this context, an integrated approach based on *in vitro* and *in vivo* zebrafish-specific mechanism-based assays seems relevant in the context of the environmental hazard assessment of EDCs and their substitutes. Yet, the comparison of the estrogenic activity of the tested substances, based on the different models, revealed marked differences in terms of effective concentrations leading to a significant estrogenic effect. This highlights once again the necessity of interpreting the molecular mechanism observed at the cellular level with enough caution in order to achieve a comparison of *in vitro* data with data obtained in whole-organism models, and, ultimately, to conduct further studies so as to efficiently and quantitatively predict *in vivo* effects of EDCs based on *in vitro* data.

Acknowledgements

We wish to thank Emmanuelle Maillot-Maréchal, Benjamin Piccini and Olivier Palluel for their invaluable technical help with the cell and zebrafish experiments. This study was supported by the French Ministry of Ecology (P181-DRC50) and by doctoral fellowships to Vincent Le Fol (INERIS) and to Manoj Sonavane (FP7-PEOPLE-2011-ITN EDA-EMERGE project under the grant agreement number 290100).

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