Acute toxicity, teratogenic and estrogenic effects of Bisphenol A and its alternative replacements Bisphenol S, Bisphenol F and Bisphenol AF in zebrafish embryo-larvae.

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ABSTRACT: Bisphenol A (BPA), a chemical incorporated into plastics and resins, has estrogenic activity and is associated with adverse health effects in humans and wildlife. Similarly-structured BPA analogues are widely used but far less is known about their potential toxicity or estrogenic activity *in vivo*. We undertook the first comprehensive analysis on the toxicity and teratogenic effects of the bisphenols BPA, BPS, BPF and BPAF in zebrafish embryo-larvae and an assessment on their estrogenic mechanisms in an estrogen-responsive transgenic fish Tg(ERE:Gal4ff)(UAS:GFP). The rank order for toxicity was BPAF>BPA>BPF>BPS. Developmental deformities for larval exposures included cardiac edema, spinal malformation and craniofacial deformities and there were distinct differences in the effects and potencies between the different bisphenol chemicals. These effects, however, occurred only at concentrations between 1.0 and 200 mg/L which exceed those in most environments. All bisphenol compounds induced estrogenic responses in Tg(ERE:Gal4ff)(UAS:GFP) zebrafish that were inhibited by co-exposure with ICI 182,780, demonstrating an estrogen receptor dependent mechanism. Target tissues included the heart, liver, somite muscle, fins and corpuscles of Stannius. The rank order for estrogenicity was BPAF>BPA=BPF>BPS. Bioconcentration factors were 4.5, 17.8, 5.3 and 0.067 for exposure concentrations of 1.0, 1.0, 0.10 and 50 mg/L for BPA, BPF, BPAF and BPS respectively. We thus show that these BPA alternatives induce similar toxic and estrogenic effects to BPA and that BPAF is more potent than BPA, further highlighting health concerns regarding the use of BPA alternatives.

INTRODUCTION

Endocrine disrupting chemicals (EDCs) possess structural similarities with endogenous hormones, and/or alter hormone biosynthesis, biodegradation or excretion, and exposure to them can alter biological homeostasis, in some cases at environmentally relevant exposure concentrations. Many groups of chemicals have been identified with endocrine disrupting properties and this, together with their widespread presence in the environment, has led to health concerns for both wildlife and humans. Disruption of sexual development in wildlife is a proven exposure consequence[1], and in fish exposure to environmental estrogens causes feminisation of males and alters sexual behaviour characteristics[2]. Estrogens, however, play much wider roles in many other developmental and homeostatic processes and therefore chemicals capable of disrupting normal estrogen signaling may have wider health effects. In humans, exposure to estrogenic chemicals has been associated with increased incidences of breast and testicular cancer, urogenital tract malformation, decrease in immune function, metabolic disease and heart disease (reviewed in Gore et al[3]).

Bisphenol A (BPA) is a chemical used in a variety of industrial materials, particularly polycarbonate plastics and epoxy resins. Due to increasing popularity of these durable, lightweight materials, the production of BPA has steadily increased, with production exceeding 2.4 million tonnes[4]. BPA has been described as slightly to moderately toxic to aquatic organisms[5] and has been identified also as an environmental estrogen. It is present in the urine of most humans[6, 7] and although BPA is relatively easily conjugated and excreted, there is an almost continuous exposure for both humans and wildlife.

In human epidemiological studies, BPA exposure (measured predominantly via urinary concentrations) has been linked to a variety of health symptoms including reduced sperm quality[8] and reduced fertilisation success[9], Polycystic Ovarian Syndrome[10], altered neural development [11,12], obesity[13], cardiovascular disease[14, 15], and type 2 diabetes[16]. However, it should be noted that

these reported effects are statistical associations only and the findings have been treated with some caution. Many of the associated health effects are based on a spot analysis of BPA and the studies have not necessarily considered historical exposures to BPA or exposure simultaneously to other chemicals. Controlled exposures to BPA, in rodents however, have shown effects similar to those identified in human epidemiological studies, and they include developmental defects in reproductive tissues, immune system effects, and neuro-developmental effects (reviewed in [17]). Some biological effects have also been reported in studies for environmentally relevant exposure doses and below the European Food Safety Authority currently recommended dose of 4 μg/kg body weight/day[18].

The aquatic environment is a major route for the disposal of industrial and domestic chemicals, including bisphenols, and health impacts of BPA exposure are documented for a range of aquatic animal species. The majority of studies into the effects of bisphenol exposure, have focused on fish[19, 20] and effects predominantly relate to sexual development and function, and include the induction of the egg precursor protein vitellogenin, intersex (the presence of female oocytes in male gonads), inhibitory effects on sperm maturation and numbers, oocyte atresia, alterations in sex steroid level and modified behaviour. Despite the possible wide range of health effects associated with exposure to BPA proposed in mammals and fish, studies have been restricted largely to effects on sexual development/reproduction. Effects of BPA exposure on development in fish for the most part, have been reported only for concentrations far exceeding those found in most natural environments[21, 22].

In response to the significant data sets published supporting adverse health effects associated with BPA exposure, several authorities have taken steps to reduce human exposure. As an example, use of BPA in food contact materials has now been banned in Japan and Canada and in 2011 the European Union prohibited the manufacture and import of baby bottles containing BPA. Public awareness about BPA has increased demand for BPA-free products and the manufacture

and use of alternative compounds which possess the same basic structure as BPA (the most commonly used being Bisphenol AF -BPAF, Bisphenol F -BPF and Bisphenol S -BPS). The similarities in structure of the alterative bisphenols to BPA make them ideal as replacements for use in polymers, however these structural similarities also give cause for concern that they may also have estrogenic activity and induce similar associated health effects as BPA[23].

BPF, BPS and BPAF are detected in food and beverage products (in the USA) at concentrations generally below 1 ng/g[24]. BPS has also been detected in thermal receipt papers, currency bills and other paper products, with the highest concentration of 22 mg/g in thermal paper[25]. Bisphenols also occur in house dust with BPA, BPF and BPS accounting for >98% of the total bisphenol content. River and sediment samples also contain BPF and BPS, occasionally at concentrations similar to BPA[26, 27]. BPAF is generally found in rivers and sediments at lower concentrations than for BPA, BPF and BPS[28].

The toxicity of bisphenol analogues has received little attention when compared to BPA. Studies assessing BPF, BPS and BPAF for estrogenic activity in *in vitro* reporter systems[23, 29, 30] have reported varying potencies. Overall, however, findings appear to indicate BPF has a similar estrogenic potency, and BPAF greater potency, than BPA. Some *in vitro* studies have reported BPS being weakly active as a ligand for the estrogen receptor, but others indicate an equal potency to BPA[31, 32]. BPA and BPS have been reported to induce DNA damage *in vitro* in HepG2 cells at 0.1 μM, whereas BPF and BPAF showed no such effects at 10 μM[33]. The mechanism(s) of action for this effect has not been established.

Few studies have assessed the endocrine disrupting potential of these alternatives to BPA *in vivo*. In mammals, BPS, BPF and BPAF have been shown to be estrogenic in the uterotrophic assay[34, 35]. Other effects of BPAF observed in mammals include reductions in cholesterol, testosterone and white blood cell counts, increases in serum thyroxin values, and a disruption of the estrous cycle[36, 37]. In zebrafish, exposure to 0.5 μg BPS/L has been shown to impact

negatively on reproductive endpoints and gonadosomatic index[38]. An increase in concentrations of plasma 17β-estradiol (E2) in males and females (at concentrations of 0.5 and 50 μg/L BPS, respectively) and reduced testosterone concentrations in males (at 50 μg/L BPS) have also been reported for aqueous exposures[38]. Two recently published studies have also shown that BPS, BPAF and BPF induced estrogenic responses in the brain of a *cyp19a1b*-GFP transgenic zebrafish[39, 40]. These findings highlight that bisphenols marketed as safer alternatives to BPA, may similarly induce widespread and varied health effects.

Here we used the zebrafish, including an estrogen-responsive transgenic fish Tg(ERE:Gal4ff)(UAS:GFP)[41], to compare the toxicity, sub-lethal effects, and relative potency and target tissues for estrogenic effects of BPS, BPF and BPAF to BPA.

MATERIALS AND METHODS

Fish Source, Culture and Husbandry. Adult zebrafish for the provision of embryos were kept in a custom-built zebrafish aquaria facility at the University of Exeter. Effects of bisphenols on embryo development and toxicity were studied in wild-type WIK strain (originally from the Max Planck Institute, Tubingen, Germany) and the estrogenic potency and tissue targets for estrogenic responses were analysed in the transgenic zebrafish Tg(ERE:Gal4ff)(UAS:GFP)[41]. Fish were allowed to breed naturally and eggs were collected via collection chambers approximately 1 h post-fertilisation (hpf). Eggs were sorted to remove any unfertilised embryos prior to use.

Chemical Exposures. Stock solutions of Bisphenol A (BPA, CAS 80-05-7), Bisphenol F (BPF, 620-92-8), Bisphenol S (BPS, 80-09-1) and Bisphenol AF (BPAF, 1478-61-1) (≥ 97% purity) Sigma-Aldrich Company Ltd. were prepared in ethanol in glass bottles. Stocks were further diluted in ethanol to the required concentrations prior to dilution in embryo test water. All tests were performed in reconstituted purified water in accordance with ISO guideline 7346-3. Chemicals in ethanol stock were dissolved in test water to give a final ethanol concentration of

0.01%. Solvent controls contained the equivalent ethanol concentration in ISO water. To confirm that the responses observed in Tg(ERE:Gal4ff)(UAS:GFP) exposed to the different bisphenol chemicals were mediated by an ER-dependent pathway, embryos were co-exposed with estrogen receptor antagonist ICI 182,780 (CAS, 129453-61-8) Tocris Bioscience, Bristol, UK. ICI was dissolved in ethanol and exposed to embryos at a concentration of 607 µg/L (1 µM).

Each experimental group consisted of 20 embryos exposed in 100 mL of water and was run in triplicate. Experiments were conducted in temperature controlled laboratories (28 ± 1 °C) under semi-static conditions. Exposures to determine toxic effects and morphological abnormalities were conducted from 0 hpf to 96 hpf in accordance with OECD guidelines for Fish Embryo Acute Toxicity Test (Test No. 236). Larvae were assessed on a regular basis (3, 24, 48, 72 and 96 hpf) and mortality, hatching rate and abnormalities recorded. Mortality was determined based on no visible heartbeat. Morphological abnormalities were observed and photographed using an Olympus SZX16 microscope equipped with an Olympus XC10 camera. Exposures to determine estrogenic response by GFP induction assessments were conducted from 0 hpf to 120 hpf. At the end of this exposure period 120 hpf old larvae were processed for fluorescent imaging analysis.

Image Analysis of Tg(ERE:Gal4ff)(UAS:GFP) Zebrafish. Tg(ERE:Gal4ff)(UAS:GFP) larvae were anesthetised with 0.4% tricane, mounted in 3% methylcellulose in ISO water and placed onto a glass-bottom 35 mm dish. All larvae were observed in lateral view and images were obtained using a Zeiss Axio Observer.Z1 equipped with an AxioCam Mrm camera (Zeiss, Cambridge, UK). All photographs were taken using the same parameters using a X10 objective. Exposure times adopted were dependent on the region photographed due to differing levels of fluorescence intensity in different target organs, (50 ms for head region, 20 ms for mid body region and 400 ms for tail section). Exposure time was kept consistent for specific regions across the fish body. Photographs were processed using the Axiovision Imaging software and fluorescence quantification was calculated using the ImageJ software (http://rsb.info.nih.gov/ij/).

159 For each picture intensity was measured as the mean grey value of all the pixels within a region
160 of interest, and the region of interest was kept consistent between individuals. Background was
161 subtracted using the ImageJ rolling ball algorithm which removes any spatial variations of the
162 background intensities as described in Sternberg (1983)[42].

163 **Determination of Chemical Concentration in Exposure Water.** Measured concentrations of
164 test chemicals were determined in triplicate from each exposure tank. Up to 100 mL of tank water
165 was collected to which 2% methanol and 0.1% of acid acetic were added. The water samples
166 were extracted through an Oasis HLB (6 mL, 200 mg) cartridge (Waters, Manchester, UK),
167 previously conditioned with 5 mL of methanol and 5 mL ultrapure water at a flow rate of 5-10
168 mL/min. Prior to the Solid Phase Extractions (SPE), two internal standard (BPA-d8 and 2,2'-
169 BPF) were added. The amount of internal standard (IS) added was calculated so that the ratio of
170 compound/IS was 1/1. The cartridge was washed with 5 mL of distilled water, dried under
171 vacuum, and elution was performed with 5 mL methanol. Extracts were dried, reconstituted in
172 water/acetonitrile (7/3v/v) and passed through 0.22 μm centrifuge filters before performing LC-
173 MS. Recovery test of the SPE protocol performed at a low and high concentration for each
174 compound (n=4 for each concentration) gave values ranging from 83 ± 2 to 108 ± 9%. See
175 Supporting Information for full details of LC-MS analysis "Water Chemistry LC-MS
176 Quantification".

177 **Determination of Internal Chemical Concentration.** For a series of selected exposure
178 concentrations, 1.0 mg/L BPA, 0.10 mg/L BPAF, 1.0 mg/L BPF and 50 mg/L BPS, analyses
179 were run to measure internal whole body concentrations. The concentrations for these analyses
180 were selected based on the production of a strong GFP signal in several tissues in the ERE-TG
181 zebrafish approximately comparable in intensity across treatments.

182 Fish were exposed for 120 hpf for these uptake assessments. Five zebrafish larvae, previously
183 anaesthetised with tricaine (0.5 g/L, non-recoverable) in 300 μL of test solution, were transferred

to a 96 well MultiScreen$_{HTS}$ BV Filter Plate (Merck Millipore, Ireland). The test solution was removed under vacuum and larvae were washed with culture water (containing tricaine) to eliminate residual test solution and transferred in 300 µL of pure water to a 96-well plate (Porvair Sciences, UK). Then 300 µL HPLC- grade acetonitrile was added and samples were homogenised for 3 minutes to achieve extraction of analytes. LCMS grade water (900 µL) was added to each well and after mixing the plate was centrifuged at 4000 rpm for 30 min. Supernatant solutions from each well were transferred to a 96-well plate and removed for LC-MS analysis. See Supporting Information for full details of LC-MS analysis "Internal Chemical Concentration LC-MS Quantification".

Data analysis. Concentration response curves were modelled using a generalised linear model (GLM) in R (http://www.r-project.org/) and allows for calculation of LC/EC50. For a given chemical, LC50 and EC50 were defined as the concentration inducing 50 % mortality or of the maximal effect, respectively. Abnormality occurrence is expressed as mean percentage ± standard error of the mean (SEM). Fluorescence data is expressed as mean fold induction above the solvent control ± standard error of the mean (SEM). Statistical analyses of fluorescence data were performed in IBM SPSS Statistics 23. Statistical significance is indicated at the $p < 0.05$(*) or <0.01(**) level and calculated using an ANOVA and Games-Howell post hoc test.

RESULTS

Bisphenol Exposure Concentrations and Uptake into Embryos. Chemical analysis of working solutions confirmed bisphenol test concentrations at the start of the exposures. All bisphenols were determined to be within 10% of their nominal concentrations with the exception for exposure to 0.001 and 0.01 mg/L BPAF and 10 mg/L BPS treatments where concentration was measured to be 80, 68 and 78% of nominal respectively (Table S2). Controls did not contain bisphenols at the respective limits of detection (40 ng/L for BPA; 100 ng/L, BPF; 10 ng/L, BPAF

209 and 1.5 ng/L, BPS). Measured internal chemical uptake in the larvae for the different bisphenols
210 are summarised in Table 1. Uptake of BPA was determined to be 0.31 ± 0.01 and 4.5 ± 0.4
211 ng/larvae for fish exposures to 0.1 and 1.0 mg/L respectively, for 1000 µ/L BPF, 18 ± 1
212 ng/larvae, for 100 µg/L BPAF, 0.53 ±0.00 ng/larvae and for 50 mg/L BPS, 3.34 ± 0.15 ng/larvae
213 respectively. Larval volume was estimated to be 1 µL and based on these analyses, the BCF for
214 BPA, BPF, BPAF and BPS were 4.5 ± 0.4, 17.8 ± 1.3, 5.3 ± 0.04 and 0.067 ± 0.003, respectively.
215 **Relative Toxicity of Bisphenols on Zebrafish Embryo Development.** Acute toxicities of the
216 bisphenol chemicals were observed to differ. The 96 hpf LC50 values for the different bisphenols
217 are shown in Table 2 (full data in Figure S1) giving a rank order of BPAF>BPA>BPF>BPS from
218 the most toxic to the least toxic. In controls mortality rates were between 0 and 10%.
219 Embryos in control groups developed normally with a hatch rate between 85 - 100% by 72 hpf.
220 All bisphenols were shown to delay the time of hatching with EC50 (72 hpf) values shown in
221 Table 2 (and Figure S2). Thus, the same rank order for bisphenols occurred for hatching delay as
222 for mortality.
223 The incidence and type of deformity differed for different exposure concentrations and
224 bisphenol type. Common malformations observed are shown in Figure 1 with full details in Table
225 3. Teratogenic effects observed with exposure to BPA included cardiac edema and craniofacial
226 abnormality, with significant effects on these endpoints observed at or above 5.0 and 10.0 mg/L
227 respectively. Ten percent of fish were also observed to have cranial haemorrhage at an exposure
228 of 12.5 mg/L BPA. BPF induced a range of morphological defects including cardiac edema at ≥
229 10 mg/L and craniofacial abnormality, spinal malformation, cranial haemorrhage and yolk sac
230 deformity at ≥ 20 mg/L. There was also a marked decline in pigmentation in zebrafish exposed to
231 10 mg/L BPF. The most potent bisphenol for developmental effects was BPAF, causing cardiac
232 edema at concentrations of 1.0 mg/L. BPS caused developmental effects only at very high
233 exposure concentrations; cardiac edema, craniofacial abnormalities and pronounced spinal

deformity were induced above 200 mg/L. No deformities were observed in any of the control groups.

Estrogenic Effects of Bisphenol Chemicals Measured in ERE-TG Zebrafish Larvae. Transgenic zebrafish without chemical exposure had some detectable GFP expression in the otic vesicle (Figure 2). This fluorescence was not found to be inducible by any of the bisphenol chemicals, with a similar level of occurrence and similar intensity across all treatments. No inducible GFP expression was detected in other tissues of unexposed larvae, however very low levels of autofluorescence were present in some tissues such as the yolk sac. This did not affect quantitation of the estrogenic responses and was accounted for in background quantitation and subtraction for the individual tissues.

For BPA exposure, GFP expression was first detected for exposure to 0.1 mg/L BPA where a significant increase (3.2- fold GFP induction above controls) was observed in the pericardial region (Figure 2). This was confirmed as the heart by periodic contractile movement of the expression domains. No significant GFP expression was observed in any other tissues at this exposure concentration. At the highest exposure concentration for BPA (1.0 mg/L) a strong GFP signal was observed in the heart (23.6-fold GFP induction), liver (60.4-fold GFP induction) and tail (11.2-fold GFP induction).

BPAF, BPF and BPS demonstrated similar patterns of GFP expression to BPA, though concentrations necessary for comparative induction varied (Figure 2). BPF had the most similar estrogenic potency to BPA, inducing an increase in fluorescence in the heart region at both 0.1 and 1.0 mg/L, with GFP inductions of 3.4-fold and 30-fold, respectively. The heart was again the only tissue expressing GFP at 0.1 mg/L BPF. At 1.0 mg/L BPF, significant GFP induction was observed in the liver and posterior tail region (124-fold and 13- fold, above controls, respectively). BPAF was the most potent estrogen of all bisphenol chemicals tested. GFP was induced in the heart (2.4-fold increase) at 0.01 mg/L BPAF increasing to a 22-fold induction in

GFP expression at 0.1 mg/L. At 0.1 mg/L, BPAF also induced GFP induction in the liver and tail region (59-fold and 7.3- fold increases above controls, respectively). BPS was relatively weak as an estrogen in ERE-TG larvae; but concentrations of 20 mg/L and 50 mg/L induced 2.7-fold and 10.8-fold inductions in GFP in the heart respectively.

Estrogen Receptor Inhibitor suppression of GFP expression in ERE-TG zebrafish. ICI 182,780 is a high affinity nonselective estrogen receptor antagonist, devoid of any partial agonism. Co-exposure ICI 182,780 in tandem with various bisphenols was used to determine if estrogen-induced GFP expression in the ERE-TG larvae was dependent on classic estrogen receptors (ERs). Exposure to ICI 182,780 completely removed the induction of GFP expression in all tissues by all bisphenols tested (Figure 3).

DISCUSSION

We have provided a comprehensive assessment on BPA and its commonly used alternatives, BPF, BPAF and BPS to illustrate that all of these bisphenols can be toxic in zebrafish embryo-larvae causing lethality, albeit for very high exposure concentrations (Table 2). Sub-lethal effects observed included pericardial edema, craniofacial abnormality, pigment reduction, spinal malformation and yolk sac deformity (Figure 1, Table 3). These effects occurred for lower exposure concentrations and there were distinct differences in the effects and potencies between the different bisphenol chemicals. The concentrations of the bisphenols required to induce toxic and developmental effects were, however, several orders of magnitude higher than concentrations commonly measured in the environment[2, 26, 27]. The highest levels of BPA reported in environmental waters are typically below 1 µg/L although concentrations up to 21 µg/L[20] have been reported. For BPF, BPAF and BPS, concentrations in environmental samples, when detected, are generally below 1 ng/L but concentrations up to 19 ng/L for BPS, 246 ng/L for BPAF and 123 ng/L for BPF[26, 28] have been recorded. The current trend for replacing BPA with

its structurally similar alternatives, however, will inevitably lead to increased concentrations of these chemicals in environmental and biological samples in the near future[28].

The 72-h EC50 values for hatching rate (5.7 mg/L) and 96-h LC50 (12 mg/L) for BPA in our study in zebrafish are similar to those determined in previous studies[21, 22]. Despite their similar structure, the BPA analogues had different potencies for effects on hatching rate and mortality with BPAF approximately 6-7-fold more potent than BPA. In contrast, BPS was approximately 30- and 20-fold less potent than BPA for hatching rate and mortality, respectively.

Similar morphological abnormalities, including cardiac edema, spinal malformation and craniofacial abnormalities, induced by the different bisphenols are consistent with those reported in the literature for BPA and may suggest similar modes of toxicity, albeit with variable potency[21, 43]. However, lesions were also observed that were more associated with the different bisphenol analogues. For example, lack of pigmentation for exposure to BPF at concentrations of 10 mg/L and above (Figure 1). This may be due to an effect on thyroid signaling as there is evidence that BPA can bind directly to, and block, the TR[44, 45]. Pigmentation, however, also involves cross-talk between estrogen and thyroid signaling, complicating possible effect mechanisms[44]. Some pigment loss was observed in BPA exposed fish but the effect was much more pronounced for BPF exposures. Another phenotype that was more distinctive to an individual bisphenol analogue was intracranial haemorrhage for exposure to BPF (Figure 1, Table 3). This may be due to a weakening of local vasculature which can arise through disruptions to thyroid and estrogen signaling[21]. Although BPS was the least toxic of the bisphenols tested, this analogue induced tail abnormalities not seen for other bisphenols and also induced the highest degree of pronounced curvature of the spine (Figure 1, Table 3), again suggesting differences in the mechanisms of toxicity.

BPA has been shown to induce estrogen-related effects in both fish and mammals including at environmentally relevant concentrations[2, 19, 20]. In the ERE-TG zebrafish we found the different

309 bisphenol analogues induced similar target tissue response patterns to that seen for BPA and
310 included the heart, liver, tail muscle somites and corpuscles of Stannius (Figure 2). For all
311 bisphenols tested the most responsive tissue was the heart, though at higher concentrations the
312 greatest response level occurred in the liver. In the heart, the bisphenol A analogues affected the
313 atrioventricular valves and the bulbus arteriosus, as reported previously for BPA[41, 46]. Responses
314 in the liver are consistent with reports of BPA, BPAF and BPS inducing the hepatic synthesis of
315 vitellogenin in fish[20, 47, 48]. Responses in the muscle somites are consistent with the role of
316 estrogen in muscle growth and for the corpuscles of Stannius in calcium handling[49]. As
317 mentioned above, the brain too has been shown to be responsive to BPA and it analogues BPAF
318 and BPF in a *cyp19a1b*-GFP transgenic zebrafish[39].
319 BPAF was the most potent estrogen in the ERE-TG zebrafish inducing a response in the heart
320 at 0.01 mg/L and other tissues at 0.1 mg/L compared with threshold concentrations of between
321 0.1 mg/L and 1.0 mg/L for BPA and BPF. BPS was between 50- and 500-fold less potent as an
322 estrogen than the other bisphenols. The potency order for the different bisphenols reflects that
323 seen for their toxicity, but may operate mutually exclusive mechanisms.
324 Several studies have shown that BPAF is around 10 times more potent than BPA as estrogen in
325 *in vitro* cell systems[23, 29]. The reported estrogenic activities of BPF and BPS compared to BPA,
326 however, are much more variable[23, 30, 50]. Data from *in vitro* studies can be difficult to interpret as
327 the metabolic capabilities for most cell-based assay systems can vary according to tissue or
328 species type[40]. In addition little is known about whether required co-factors for receptors are
329 expressed in those cells, and they do not take into account the potential for bioconcentration, all
330 of which can have a significant bearing on the biological effect of an EDC[51]. Few *in vivo* studies
331 have investigated BPA analogues but BPS has been reported to affect egg production, plasma
332 steroid concentration and hatching and survival rates in zebrafish from 0.01 mg/L[38, 47].

BPF is a common replacement for BPA, but here we show that both chemicals share a similar level of toxicity and *in vivo* estrogenic potency, with other possible off-target effects not seen for BPA. BPAF is not yet used as widely as BPF or BPS in BPA-free materials but given its estrogenic and toxicity potencies we would argue that it is not an appropriate alternative to BPA. BPS, the most commonly used monomer in thermal paper and BPA-free replacement products[25] has been reported to share a similar potency to BPA based on *in vitro* studies. Our data for zebrafish would suggest that this is not the case *in vivo*. There is nevertheless some remaining concern over BPS because of its reported resistance to degradation and persistence in the environment[52].

The mechanisms of action of BPA have been relatively well studied. BPA appears to be mechanistically pleiotophic. The best established mechanism is its ability to bind to ERs and modify gene expression, albeit at effective concentrations several orders of magnitude lower than that of 17β-estradiol[53, 54]. Some effects may be exerted through rapid nongenomic pathways independent of classic ER signaling[55, 56]. BPA also has the ability to bind strongly to the estrogen related receptor ERRγ while E2 is inactive on that pathway[57, 58]. These ERRs can bind to EREs in ER target genes, inducing translational responses. It is therefore theoretically possible that fluorescence responses observed in ERE-TG fish resulted partly from ERR-induced promotion. Co-exposure of ERE-TG zebrafish larvae to the ER antagonist ICI 182 780 abolished the GFP expression observed in all tissues for all chemicals tested (Figure 3) strongly supporting the hypothesis that when activation of genes is induced via the ERE promoter, in these tissues, for BPA, BPF, BPAF and BPS this activation is mediated through the classical ERs. For BPS this differs from the findings of Le Fol et al 2017, who demonstrated that in the brain of *cyp19a1b*-GFP transgenic zebrafish BPA and BPF-induced aromatase was reduced in ICI co-exposed fish but not for those exposed to BPS[40]. It is difficult to reconcile these differences across the different experimental models. The brain of *cyp19a1b*-GFP transgenic zebrafish is much more restrictive

358 for studies on estrogens in that only the brain fluoresces in response to estrogens in accordance
359 with the location of the *cyp19a1b* gene, thus with the exception of the brain, we cannot compare
360 responses to BPS across comparable responding tissues in the different transgenic models. It may
361 be the case that tissue specific factors differentially affect the interactions of the different
362 bisphenols with the ERs in those tissues. It is also possible that BPS interacts with ERRγ to
363 induce the observed fluorescence, though this was not confirmed. It should be recognised that the
364 ERE-TG model only indicates presence of activity through ERE based gene activation, so cannot
365 give evidence for nongenomic effects. Also rapid signaling effects interact with more traditional
366 nuclear hormonal receptor pathways[59]. Whether these bisphenol analogues have effects on
367 estrogen signaling, either directly or indirectly, through androgen and thyroid signaling
368 pathways[23, 50, 60, 61] also warrants investigation.
369 It is possible that the reason, or part thereof, for differences observed between the estrogenic
370 potency of the bisphenols is due to preferential binding to a particular estrogen receptor subtype
371 (esr1, esr2a and/or esr2b). One *in vitro* study has indicated that BPA was most selective for esr1
372 (reported as ERα) while BPS and BPF did not appear to have strong binding preference for any
373 one estrogen receptor subtype[40]. In our study we found the heart was the primary tissue target for
374 all bisphenols, followed by the liver, and given that in 5 days post fertilization zebrafish
375 (determined via the use of whole mount *in situ* hybridization) esr1 transcripts predominate in the
376 heart, esr2a (reported as esr2b) in the liver, and esr2b (reported as esr2a) is not expressed[46], the
377 preferential binding of the different bisphenols seen *in vitro* for the different ER subtypes would
378 not appear to be a key factor in determining differences in bisphenol activity we observed *in vivo.*
379 Potency differences between the bisphenols may also relate to differences in their
380 bioavailability. Albeit a limited analysis, we show uptake differed between some of the bisphenol
381 analogues (Table 1). There was an approximate 4-fold higher uptake of BPF in exposed fish
382 compared with uptake of BPA (both with 1000 µg/L exposure). In contrast, the uptake of BPAF

383 resulted in a similar BCF to BPA (5.3 and 4.5 respectively). The uptake amount of BPS was
384 similar to that for BPA, however, exposure concentrations were much higher for BPS, leading to
385 a much lower BCF of 0.067. The similarities in the BCF of BPAF and BPA would suggest their
386 marked differences in comparative estrogenic potency (10-fold higher for BPAF) predominantly
387 relates to their comparative interactions with the ER(s). However, the differences in the
388 bioavailability of BPF and BPS compared to BPA, also appears to indicate that uptake may play a
389 key role in the response of zebrafish to these chemicals. These analyses are based on whole body
390 burdens only and do not consider uptake into the different body tissues which could also affect
391 their comparative potency. A previous study investigating the bioconcentration of BPS in 96 hpf
392 zebrafish, reported a BCF of 22±3.8[62]. The reason for the disparity between the calculated BCFs
393 in that study and our own here is not known. One explanation, however, may relate to differences
394 in metabolic activity in embryos at the different life periods sampled (120 hpf in this study
395 compared with 96 hpf in[62]); there is rapid rate of lipid reserve utilization between 96 and 120 hpf
396 and this may cause higher rates of metabolism of more readily biodegraded bisphenols, such as
397 BPS. Differences between the calculated BCF between the two studies may also relate to the
398 presence of residual BPS adsorbed to larval body surface. Thorough washing of larvae is
399 essential prior to chemical analysis to avoid residual bisphenol bound to the body surface that
400 will cause subsequent over estimations of BCFs.

401 Our findings show all the bisphenols tested are toxic to fish although at concentrations that
402 exceed those commonly measured in environmental compartments. Toxic potency and estrogenic
403 activity varied across the bisphenols and this probably relates principally to ability of individual
404 chemicals to bind to and activate the ERs. Differences in uptake and bioconcentration may also
405 play a role in the varied responses observed for the different analogues. Co-treatment with an ER
406 inhibitor indicated that estrogenic activity of BPA and all the analogues tested in our ERE-TG
407 zebrafish was mediated by the classical ER(s) signaling pathway.

408

409 **Table 1.** Measured water and whole body concentrations in 120 hpf zebrafish larvae for each
410 bisphenol.

411

	Nominal external water concentration (mg/L)	Measured external water concentration (mg/L)	Measured uptake per larvae (ng/larvae)	Estimated bioconcentration factor
BPA	0.1	0.09 (0.01)	0.31 (0.01)	3.1
	1.0	0.99 (0.02)	4.50 (0.42)	4.5
BPF	1.0	1.06 (0.06)	17.8 (1.3)	17.8
BPAF	0.1	0.09 (0.005)	0.53 (0.0035)	5.3
BPS	50	52.3 (0.03)	3.34 (0.15)	0.067

412

413 Data as shown are the mean of 3 replicates, each containing 5 larvae, repeated 3 times (SEM in
414 brackets)

415 *Larval volume was estimated to be 1 μL

416

417 **Table 2**. The 96 hpf mortality rate and 72 hpf hatching success rate of zebrafish larvae exposed to

418 bisphenol chemicals (SEM in brackets)

	Acute toxicity 96 hpf LC50 (mg/L)	Hatching success 72 hpf EC50 (mg/L)
BPAF	1.6 (0.09)	0.92 (0.06)
BPA	12 (0.22)	5.7 (0.33)
BPF	32 (0.55)	14 (0.41)
BPS	199 (7.6)	155 (15)

419

420

421 **Table 3.** Developmental abnormalities (%) observed in 96 hpf zebrafish larvae exposed to BPA,

422 BPF, BPAF and BPS (mg/L).

		Cardiac edema	Craniofacial abnormality	Tail development	Cranial haemorrhage	Yolk sac deformity
Control		-	-	-	-	-
BPA	1.0	-	-	-	-	-
	2.0	1.8 (1.8)	1.8 (1.8)	1.8 (1.8)	-	-
	5.0	37.4 (9.0)	-	-	-	-
	10.0	100.0	43.7 (11.7)	-	-	3.5 (3.5)
	12.5	100.0	100.0	-	10.0 (10.0)	-
BPF	1.0	1.7 (1.7)	-	-	-	-
	2.0	1.7 (1.7)	-	3.3 (1.7)	-	-
	5.0	10.0 (5.8)	3.3 (1.7)	1.7 (1.7)	1.7 (1.7)	-
	10.0	64.6 (7.3)	6.7 (4.4)	8.6 (3.6)	8.5 (4.4)	1.7 (1.7)
	20.0	100.0	11.7 (7.3)	11.7 (3.3)	36.7 (4.4)	21.7 (17.0)
	27.5	98.3 (1.7)	93.2 (3.4)	93.2 (14.0)	41.4 (0.70)	26.1 (11.0)
	35	100.0	90.0 (8.2)	90.0 (8.2)	40.0 (33.0)	40.0 (33.0)
BPAF	0.50	3.3 (1.7)	1.7 (1.7)	1.7 (1.7)	-	-
	0.75	3.3 (3.3)	1.7 (1.7)	-	-	-
	1.0	15.6 (8.7)	-	-	-	-
	2.0	34.6 (18.0)	2.8 (2.8)	2.8 (2.8)	-	-
BPS	10	-	-	-	-	-
	20	1.8 (1.8)	1.8 (1.8)	1.8 (1.8)	-	1.8 (1.8)
	50	-	-	-	-	-
	100	3.3 (1.7)	3.3 (1.7)	1.7 (1.7)	-	-
	200	94.1 (3.2)	96.3 (3.7)	82.2 (3.4)	3.7 (3.7)	-

423 Data as shown are the mean of 3 replicates (SEM in brackets). – = no abnormalities observed.

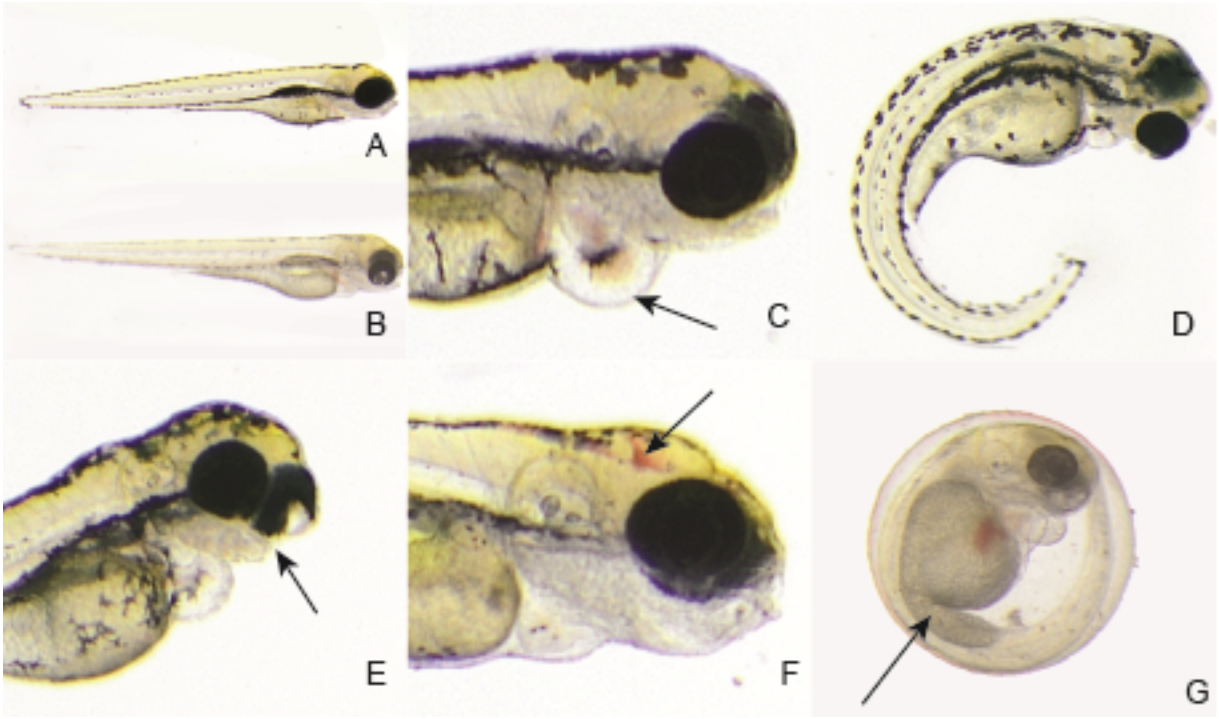

Figure 1. Examples of typical teratogenic responses of zebrafish larvae observed upon exposure to bisphenol chemicals: (A) normal, (B) pigment reduction, (C) cardiac edema, (D) spinal malformation, (E) craniofacial abnormality, (F) cranial haemorrhage, (G) yolk sac malformation.

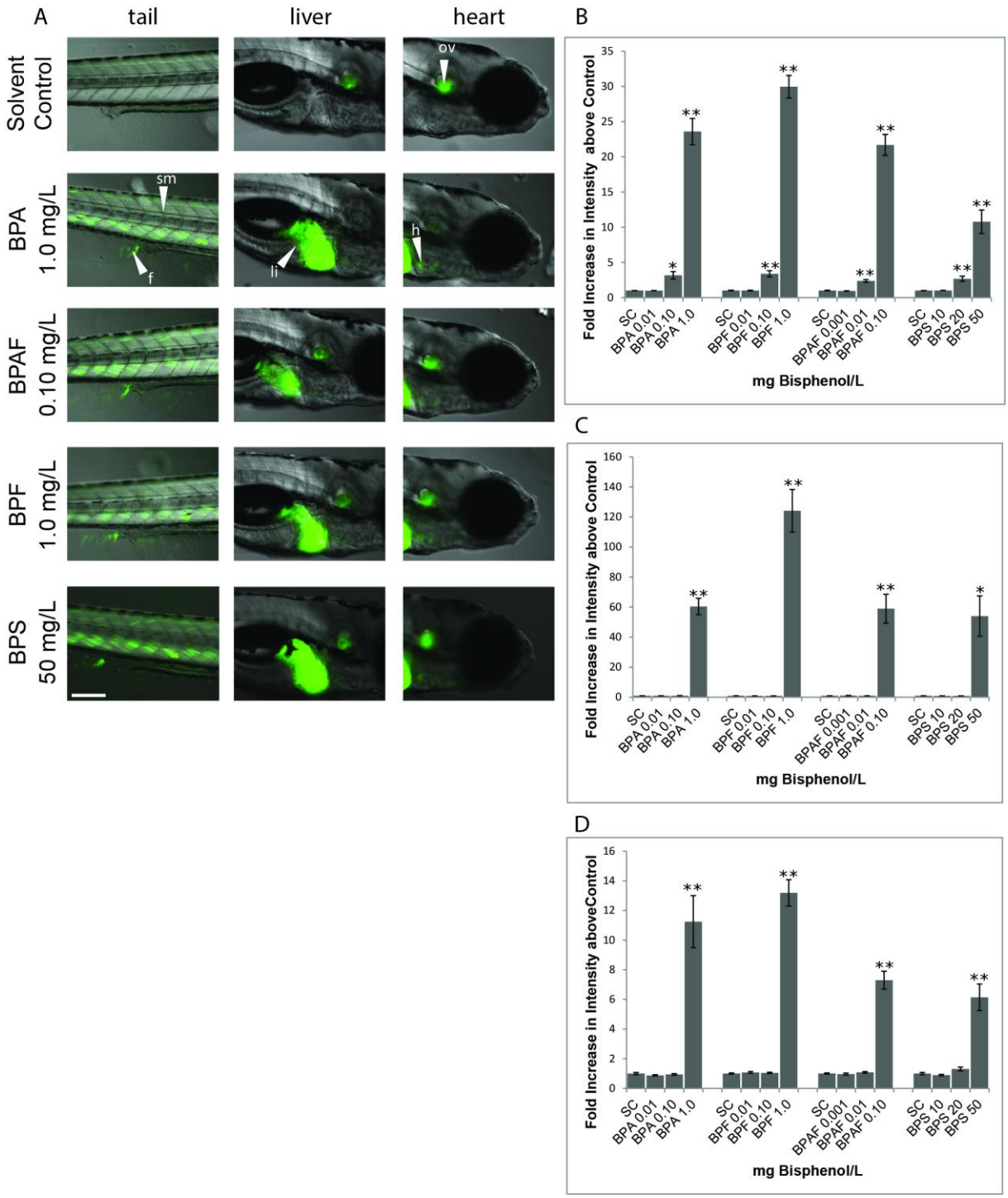

Figure 2. (A) Images of 120 hpf ERE-TG zebrafish larvae exposed to 1.0 mg/L BPA, 0.10 mg/L BPAF, 1.0 mg/L BPF and 50 mg/L BPS (images for exposures to lower concentrations not shown). Fluorescence observed in otic vesicle (ov), heart (h), liver(li), somite muscle (sm) and fin (f). Scale bar represents 200 µm; (B-D) GFP induction (fluorescence) in 120 hpf ERE-TG

zebrafish larvae exposed to bisphenol chemicals measured by fold increase above control in the (B) heart, (C) liver, (D) tail somites. Data are reported as mean ±SEM (asterisk indicate significant difference compared with the control, * $p < 0.05$ and ** $p < 0.01$.

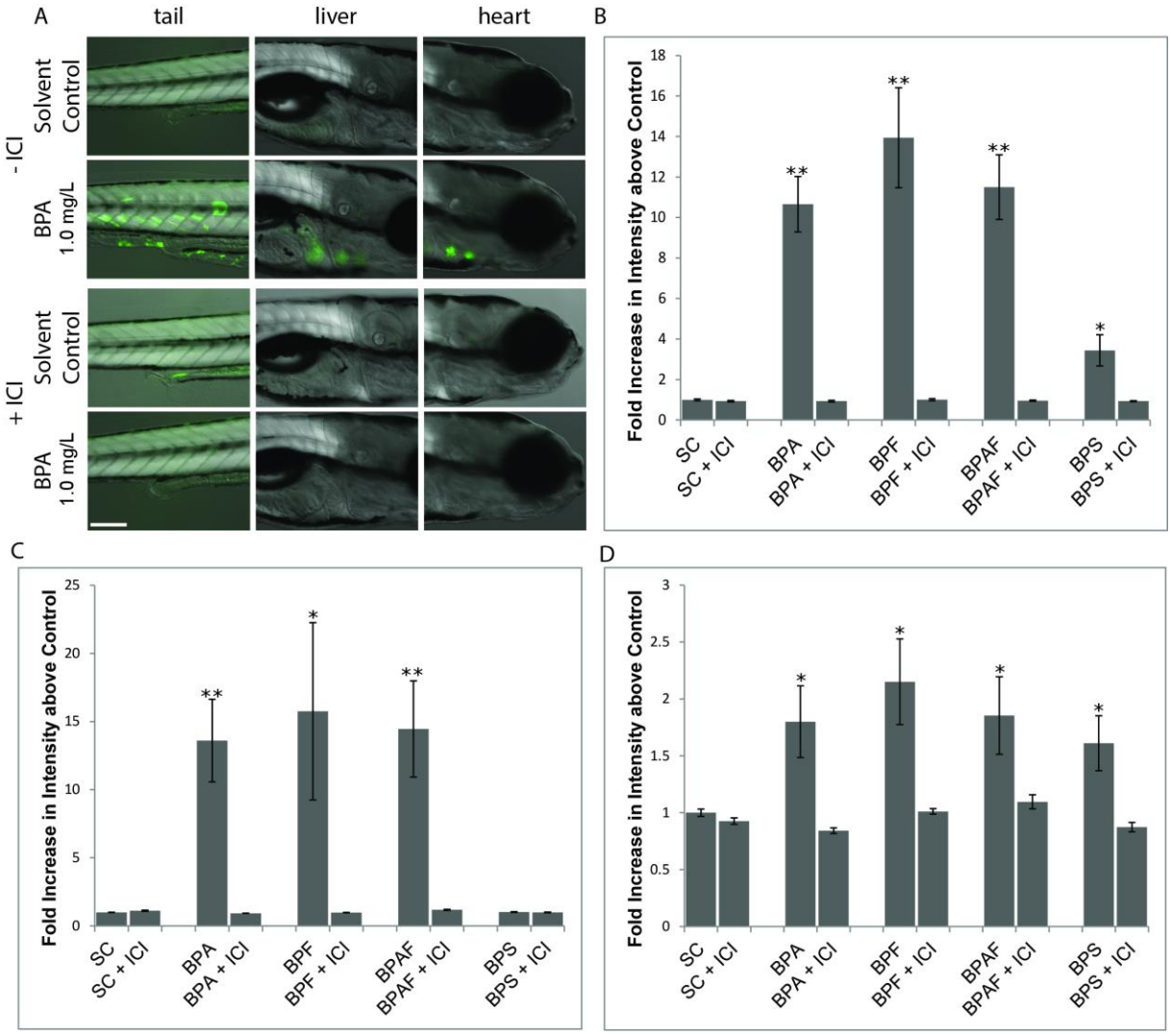

Figure 3. (A) Images of 120 hpf ERE-TG zebrafish larvae exposed to 1.0 mg/L BPA in the presence and absence of estrogen inhibitor ICI 182 780, scale bar represents 200 µM. (B-D) GFP induction (fluorescence) in 120 hpf ERE-TG zebrafish larvae exposed to bisphenol chemicals in combination with estrogen inhibitor ICI 182 780 measured as fold increase in GFP above control in the (B) heart, (C) liver, (D) tail somites. Bisphenol concentrations were 1.0 mg/L for BPA and BPF, 0.10 mg/L for BPAF and 20 mg/L for BPS; when included ICI 182 780 concentration was 607 µg/L. Data are reported as mean ±SEM (asterisk indicate significant difference compared with the control, * $p < 0.05$ and ** $p < 0.01$.

ASSOCIATED CONTENT

Supporting Information.

Methodology for LC-MS used for chemical detection of bisphenolic chemicals in exposure medium and fraction taken up in 120 hour post-fertilisation (hpf) zebrafish larvae (Tables S1-S4). Data showing full dose response curves for effects on mortality (Figure S1) and hatching rate (Figure S2) of 96 hpf zebrafish larvae on exposure to different bisphenolic chemicals. This information is available free of charge via the Internet at http://pubs.acs.org.

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