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Toxicity profiling of flame retardants in zebrafish embryos using a battery of assays for developmental toxicity, neurotoxicity, cardiotoxicity and hepatotoxicity toward human relevance

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ABSTRACT

Following the voluntary phase-out of brominated flame retardants (BFRs) due to their environmental persistence and toxicity, the organophosphorus flame retardants (OPFRs) are emerging replacements. However, there is limited information on the potential human health effects of the OPFRs. Zebrafish embryos are a viable vertebrate model organism with many advantages for high throughput testing toward human hazard assessment. We utilized zebrafish embryos to assess developmental toxicity, neurotoxicity, cardiotoxicity and hepatotoxicity, of eight replacement OPFRs: (triphenyl phosphate [TPHP], isopropylated phenyl phosphate [IPP], 2-ethylhexyl diphenyl phosphate [EHDP], tert-butylated phenyl diphenyl phosphate [BPDP], trimethyl phenyl phosphate [TMPP], isodecyl diphenyl phosphate [IDDP], tris(1,3-dichloroisopropyl) phosphate [TDCIPP], and tris(2-chloroethyl) phosphate [TCEP]) and two BFRs (3,3′,5,5′- tetrabromobisphenol A [TBBPA] and 2,2′4,4′-brominated diphenyl ether [BDE-47]). To determine potential effects on teratogenicity, embryos were exposed to flame retardants (FRs) at 4 h post fertilization (hpf) to 4 days post fertilization (dpf) and morphological alterations and corresponding survival were evaluated at 2 and 4 dpf. Internal concentrations were measured in larvae used in this assay by liquid chromatography-mass spectrometry. Locomotor activity was assessed in larvae treated for 48 h (from 3 dpf to 5 dpf), followed by hepatotoxicity evaluation. Finally, alterations in heart rate and rhythmicity were assessed to determine cardiotoxicity in 48 hpf embryos exposed to compounds for 3 h. Results suggest that several OPFRs (BPDP, EHDP; IPP, TMPP; TPHP and TDCIPP) produced adverse effects in multiple target organs at concentrations comparable to the two BFRs. As these OPFRs have the capacity to disrupt an integrated vertebrate model, they potentially have the capacity to affect mammalian biology. Then, we compared the lowest effective levels (LEL) in zebrafish with estimated or measured human plasma concentrations using biomonitoring data (human plasma, breast milk, handwipe samples and house dust) and a high throughput toxicokinetic (HTTK) model. Results indicate that for some compounds, the nominal LELs were within the range of human exposures, while internal LELs in zebrafish are above internal exposures in humans. These findings demonstrate the value of the zebrafish model as a relevant screening tool and support the need for further hazard characterization of the OPFRs.

1. Introduction

For several decades, flame retardants (FRs) have been added to polymers and resins used in commercial products, including electronics, furniture and textiles. Until 2005, the polybrominated diphenyl ethers (PBDEs) were the primary FRs used in household products such as

Abbreviations: FR, flame retardant; BFR, brominated flame retardants; PBDEs, polybrominated diphenyl ethers; TPHP, triphenyl phosphate; IPP, isopropylated phenyl phosphate; EHDP, 2-ethylhexyl diphenyl phosphate; BPDP, tert-butylated phenyl diphenyl phosphate; TMPP, trimethyl phenyl phosphate; IDDP, isodecyl diphenyl phosphate; TDCIPP, tris(1,3-dichloroisopropyl) phosphate; TCEP, tris(2-chloroethyl) phosphate; TBBPA, 3,3′,5,5′- tetrabromobisphenol A; BDE, 47–2,2′4,4′-brominated diphenyl ether; Hpf, hours post fertilization; Dpf, days post fertilization; DRF, dose range finding; LEL, lowest effective level

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polyurethane foam and electronics. However, they were voluntarily phased-out due to concerns with their environmental persistence, bioaccumulation, and association with adverse human health effects such as impaired neurodevelopment, altered circulating hormone levels, and decreased fertility (Frederiksen et al., 2009; Harley et al., 2010; Herbstman et al., 2010; Meeker and Stapleton, 2010; Vuong et al., 2017). Over the last decade, there has been growing evidence of widespread exposure to a number of alternative FRs, such as the organophosphorus flame retardants (OPFRs), in house dust and furniture foam, including in infant products (Stapleton et al., 2009; Meeker and Stapleton, 2010; Stapleton et al., 2011), as well as in the urine of elementary school children (Mizouchi et al., 2015) and in hand wipe samples from children (Stapleton et al., 2014; Hoffman et al., 2015; Cowell et al., 2017). A recent study showed a high correlation between neurodevelopmental impairments in children with increased exposure to some of the OPFRs during pregnancy and childhood (Castorina et al., 2017). However, there is relatively sparse information regarding their safety.

The zebrafish model is an integrative model system, which is being used in a high content approach to predict adversity to biology in a developing vertebrate system (Sipes et al., 2011). There is increasing evidence linking the relevance of findings in zebrafish to mammalian models and humans (Sipes et al., 2011; Noyes et al., 2016; Bambino and Chu, 2017). Some distinguishing features of the zebrafish (*Danio rerio*) as a promising integrative tool include: 1) production of hundreds of offspring at weekly intervals and the small size of the embryos allow the develop of high throughput screenings using microwell plates, 2) direct compound exposure into the embryo medium, 3) ability to observe chemical effects due to transparency of the embryos and 4) importantly, many toxicity pathways are shared among fish and mammals due to their generally well-conserved development, cellular networks and organ systems (Kaufman et al., 2009; Noyes et al., 2016). Hence, the purpose of this study was to use the zebrafish model as an integrative tool in the assessment of relative activity of some of the alternative OPFRs to prioritize to further *in vivo* testing.

Recent findings suggest that the replacement OPFRs show comparable activity to some of the phased-out PBDEs *in vitro* and in alternative animal models, including zebrafish (Bailey and Levin, 2015; Behl et al., 2015; Jarema et al., 2015; Noyes et al., 2015; Oliveri et al., 2015; Cano-Sancho et al., 2017; Yan et al., 2017). In this study, these compounds have been evaluated for the first time using a system toxicity approach to include developmental toxicity, neurotoxicity, cardiotoxicity, and hepatotoxicity to better understand target organ toxicity of the replacement OPFRs compared to phased-out BFRs (BDE-47 and TBBPA). In addition to nominal concentrations (concentrations in the water that zebrafish were exposed to), internal concentration of compounds in larvae following the developmental toxicity assay was measured, and findings were contextualized with human biomonitoring data.

2. Materials and methods

2.1. Fish husbandry and egg production

Adult zebrafish were housed and maintained in accordance with standard procedures. Briefly, fish were maintained under a photoperiod of 14:10 h light:dark at 28.5 °C in water continuously filtered at pH 7–7.8, conductivity 500–800 μS and O_2 saturation at 60–90%. Adults were fed with ground dry pellets (Gemma 300, Skretting) and artemia (Catvis) twice a day each. Healthy mature zebrafish pairs were used for egg production. Embryos were collected in E3 embryo media containing 0.0001% methylene blue (Acros Organics, +96% purity) and 100 μg/mL ampicillin (Sigma-Aldrich) and kept in the incubator at 28.5 °C until they reached the stage specified below for each assay.

Zebrafish were maintained in accordance with the European Directive 2010/63 for the protection of animals used for scientific purposes and all experiments were approved by the ethical committee for animal experimentation of IIS Biodonostia (San Sebastián, Gipuzkoa, Spain).

2.2. Chemicals

Dimethyl sulfoxide (DMSO) (CAS 67-68-5, purity 99.9%) (vehicle control) was obtained from Scharlau, while Terfenadine (CAS 50679-08-8) (positive control for the cardiotoxicity assay) and 13-CIS-Retinoic acid (CAS 4759-48-2, purity > 98%) (positive control for the developmental toxicity assay based on Biobide internal validation) were obtained from Sigma-Aldrich. Tricaine (CAS 886-86-2) was obtained from Acros Organics. Flame retardants used in this study were: 2,2′4,4′-Tetrabromodiphenyl ether (BDE-47), *tert*-Butylphenyl diphenyl phosphate (BPDP), 2-Ethylhexyl diphenyl phosphate (EHDP), Isodecyl diphenyl phosphate (IDDP), Phenol, isopropylated, phosphate (3:1) (IPP), Tricresyl phosphate (TMPP), Triphenyl phosphate (TPHP), 3,3′,5,5′-Tetrabromobisphenol A (TBBPA), Tris(2-chloroethyl) phosphate (TCEP) and Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) (Supplemental Table 1). Information about lot numbers, purity and suppliers is also provided in supplemental table 2. Stock solutions of each chemical were prepared (experiment 1) or received (experiment 2) in DMSO and these were further diluted to the desired concentration in E3 media containing 10 mM HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid) (Sigma-Aldrich).

2.3. Experimental design

Details of the different assays performed are described below. We conducted first an experiment (experiment 1) and to validate our method and the robustness of the findings, we repeated the study independently (experiment 2). Both experiments were conducted blinded. The only difference between the two studies was that in experiment 1, Biobide received the compounds from the NTP in powder form and were then dissolved in DMSO; reference aliquots were prepared then at relevant concentrations. In experiment 2, Biobide received the chemicals already dissolved at 20 mM in DMSO and were also further diluted in-house to obtain the relevant final concentrations.

2.3.1. Assessment of developmental toxicity

To determine concentrations for use in the main study, a dose-range finding (DRF) study was first conducted at concentrations ranging from 0.1 to 1000 μM in the first experiment (or from 0.2 to 100 μM in the second experiment). Fertilized embryos (from transgenic line expressing CopGFP under the myocardium specific promoter *cmlc2*, (Letamendia et al., 2012)) at 4 h post fertilization (hpf) were placed in 24 well plates (5 embryos per well, 10 embryos per condition) with the corresponding chemical concentration. The use of this transgenic line allows a better visualization of the heart and, therefore, a more precise analysis of alterations as well as the presence or absence of heartbeat. A group of embryos treated with 0.5% DMSO (Hallare et al., 2006) was used as a vehicle control. Plates were incubated at 28.5 °C for 4 days and exposure solutions were renewed on the second day of treatment. Embryos were analyzed at 2 and 4 days post-fertilization (dpf) and the incidence of lethality and the presence of gross developmental defects were recorded.

Following the DRF, the main experiments were carried out and embryos were treated with 8 concentrations of interest (Supplemental Table 3). Only 5 concentrations were tested for TCEP in the second experiment since no toxicity was detected in the DRF in this case. Embryos were treated in a similar manner as described in the DRF above with the exception that a total of 15 embryos (instead of 10) were tested per experimental condition. In addition to vehicle control, a group of embryos were treated with 100 nM retinoic acid (positive control). Retinoic acid plays essential roles in early embryonic patterning and organogenesis in vertebrates and the alteration of its levels during development has shown to be teratogenic in mammals and

zebrafish (Tembe et al., 1996; Malvasi et al., 2009; Selderslaghs et al., 2009; Selderslaghs et al., 2012). Detailed morphology analysis of embryo, including malformations in the head, heart and tail, deformed body shape and the presence of edema (recorded as presence or absence) and lethality was performed at 2 and 4 dpf. Expected malformations induced by retinoic acid were observed in the conducted assays (results not shown). Percentage of altered and dead embryos was used for Effective Concentration 50% (EC50) and Lethal Concentration 50% (LC50) calculations applying a nonlinear regression test (sigmoidal dose-response curve) using the GraphPad Prism (GraphPad Software). A teratogenic Index (TI) was estimated as the ratio between LC50 and EC50. Two TIs were calculated, one per stage analyzed. Based on internal validation (data not shown) a TI $>$ 4, at least in one of the stages analyzed, was considered a clear indicator of teratogenic potential.

2.3.2. Internal concentration estimation

All larvae exposed to compounds at the LEL (lowest-effect-level) in developmental toxicity assay (at 4 dpf) were pooled after analysis into an Eppendorf tube, washed in E3 media and frozen at −80 °C until bioanalysis. On the day of analysis, larvae were defrosted, resuspended in Methanol (1 mL) and homogenized with vigorous agitation and ultrasounds (10 min each process). The extracts obtained after centrifugation at 15,000 rpm for 5 min were then analyzed using a Thermo Fisher Scientific -Dionex Ultimate 3000 ultra-performance liquid chromatography (UPLC) system (Dionex Softron GmbH, Part of Thermo Fisher Scientific Inc., Germany) coupled to a mass spectrometer (Exactive™, Thermo Fisher Scientific, Germany). Both devices were operated using Trace Finder and Xcalibur software. The UPLC system was equipped with a 2.1 × 100 mm, 2.0 mm (ACE C18-PFP, Hichrom Ltd., England) kept at 40 °C. A binary gradient mobile phase was used at a flow rate of 0.5 mL min^{-1} with solvent A (0.1% formic acid in water Type I) and solvent B (acetonitrile). The mass spectrometer was operated in electrospray positive mode (ESI, Thermo Fisher Scientific, Germany), while data acquisition was performed using the Parallel Reactions Monitoring mode. The source settings were as follows: spray voltage 3.500/5.500 V; capillary temperature 280 °C; sheath, auxiliary and sweep gas 40, 20 and 1 ad respectively; probe heater temperature 400 °C; S-Lens 60 V. The mass resolution was 35.000 and the error mass $<$ 2 ppm. The results were quantified using Trace Finder software. Recoveries of all compounds were within 80–120%. For internal concentration calculation the estimated volume of one larvae at 4 dpf was 0.4 µL.

For all OPFRs tested, only the parent compound corresponding to the CAS number was resolved in the chromatogram, except for IPP for which different peaks corresponding to TPHP, TPHP +1 propyl and TPHP +2 propyl groups were detected since it was known *apriori* some of the components of the isomeric mixture that IPP consisted of. Proportions of each component were unknown and therefore an approximation to determine internal dosing in larvae was performed. It was assumed that all components had a similar response factor in LC/MS and therefore, the signal of each of the 3 more abundant components (area) was added together as if there was a single compound.

2.3.3. Assessment of behavior (locomotor activity)

Wild-type AB embryos were obtained as described in Section 1 and kept at 28.5 °C until they reached 3 dpf. At this stage, larvae were dispensed in a 96 squared-well plate (one embryo per well) and exposed to 5 concentrations per compound that were selected based on the results obtained in the developmental toxicity assay (Supplemental Table 4). The LEL from the developmental toxicity assay was used as the highest concentration evaluated in the behavioral assessment. Larvae were visually checked under the stereoscope after tracking to look for the presence of morphological alterations. When malformations appeared in a few embryos ($<$ 20%), these embryos were removed from the analysis. But when malformations were common and linked to the treatment, analysis was performed but concentration/s causing malformations were not considered for conclusions. Higher concentrations were not tested to ensure that locomotor effects occurred in the absence of overt developmental toxicity. 16 embryos were treated per condition along with a group of vehicle controls (0.5% DMSO). After 48 h of incubation at 28.5 °C, plates were introduced in the Daniovision automated tracking system powered by Ethovision (Noldus, The Netherlands). Temperature was set at 28.5 °C and after 10 min of habituation, tracking, which consisted in two rounds of 10 min light and 10 min dark phases, started. Total duration of the tracking was 40 min. Several parameters were analyzed such as velocity, movement duration and frequency among others, but the total distance moved was selected as representative of locomotor activity. The mean of the total distance moved by embryos in each group was measured in two-minute time bins and treated *versus* control groups were compared using unpaired Student's *t*-test.

2.3.4. Assessment of hepatotoxicity

Following evaluation of behavior, hepatotoxicity was assessed in the same fish. Plates were recovered from Daniovision and larvae were anesthetized with 0.12% tricaine and observed under the stereoscope. Liver in 5 dpf zebrafish larvae has a clearly recognizable periphery against the neighboring tissues. Normally zebrafish liver is clear, whereas after the treatment with hepatotoxic drugs, it becomes darker with a brown or gray coloration, indicating degeneration and/or necrosis (He et al., 2013). When liver opacity was observed, embryos were placed in a plate previously filled with 3% methylcellulose, laterally on their right side, and images of the liver region were taken using a stereoscope (Lumar V12 Zeiss, Germany) equipped with a digital camera (ICc1, Zeiss, Germany). Then, the optical density of a central area inside the liver was quantified on the pictures taken with ImageJ (NIH, Bethesda, MD) software. Statistical analysis was applied (one-way ANOVA and Dunnett's post-test) to compare treated *versus* control groups.

2.3.5. Assessment of cardiotoxicity

For cardiotoxicity evaluation, embryos from the transgenic line cmlc2:CopGFP were obtained as described in Section 1 and kept in an incubator at 28.5 °C until they reached 48–54 hpf. At this stage, embryos were dispensed in a 96 well plate (one embryo per well) and treated with 5 concentrations per compound (1, 3, 10, 30 and 100 µM). 20 embryos were treated per experimental condition. Embryos treated with 0.5% DMSO were used as the vehicle control; a group of larvae were treated with cardiotoxic drug Terfenadine as a positive control (Sorkin and Heel, 1985; Letamendia et al., 2012) at 5 µM. Then, plates were incubated at 28.5 °C for 3 h and heartbeat was analyzed as described in Letamendia et al. (2012). One video per embryo from a minimum of 7 embryos in each treated group were required for statistical analysis. As heartbeat followed a non-Gaussian distribution (skewness value −0.9, p $<$ 0,001), a Mann-Whitney *U* test was applied to compare treated *versus* control groups.

2.4. Modeling biomonitoring data in humans

The literature was searched for measured internal plasma concentrations for all compounds; however, limited data were available. Therefore, breastmilk, dust sample, and hand sample contamination concentrations were used for dose simulation in an HTTK model to estimate a child's internal plasma concentration from these exposures. Biomonitoring data were gathered from the public literature and converted into µM for plasma and serum, and a mg/kg dose for breast milk and dust samples. Multiple exposures are noted in the literature for some chemicals and exposure scenarios; however, for this analysis the study with the highest measured concentration was used. Supplemental Table 5 contains all values from the respective publications, including the ones not used for comparisons, which are easily identified in the table.

2.4.1. Human adult and child plasma, and human cord blood serum values

Concentrations of BDE47 (Wang et al., 2013) and TBBPA (Cariou et al., 2008) were obtained from adult plasma, BDE47 (Stapleton et al., 2012) from child plasma, and TBBPA (Cariou et al., 2008) from cord blood serum. All values were in ng/g-lipid which were converted into μM, assuming serum density is 1.06 kg/L. The minimum and maximum observed were used in the evaluation, as well as the reported mean for adult plasma, geometric mean for child plasma, and median for cord blood serum.

2.4.2. Breast milk samples

Breast milk samples, maximum median (when available) and maximum observed, in ng/g lipid (Kim et al., 2014) were converted into an estimated ingestion dose (mg/kg-day) by using an infant intake rate of 800 g lipid/day (Institute of Medicine (US) Committee on Nutritional Status During Pregnancy and Lactation, 1991) for a 4 kg (*i.e.*, 9 lb) infant.

2.4.3. Child handwipe samples

Child hand wipe samples (median, minimum, and maximum observed) in ng (Sugeng et al., 2017) were converted into an estimated ingestion dose (mg/kg) per time point using the following: hand wipe measurement × 0.5 transfer efficiency × 0.1 fraction of the hand contacted (Stapleton et al., 2008). The child weight (kg) was estimated using: (3 × age in years) + 7 (Luscombe et al., 2011). The age used for calculating biomonitoring dose was 15 months, which is based on the participants' age ranges, which included six 9–12-month-old and fifteen 13–18-month-old (Sugeng et al., 2017). Therefore, the estimated weight of a 15-month child is 10.75 kg. The contact is assumed to occur 18 times per hour over a 12-hour exposure period and does not account for handwashing (Stapleton et al., 2008).

2.4.4. House dust samples

House dust samples (median or geometric mean, minimum, and maximum observed) in ng per g of house dust (Castorina et al., 2017; Sugeng et al., 2017) were converted into an estimated ingestion dose (mg/kg/day) based on a child ingestion of 100 mg of dust/day (Stapleton et al., 2008). The age and weight were calculated as described above for the child hand wipe studies. When not stated, a 15-month-old child was used with a weight of 10.75 kg.

2.4.5. Estimation of internal plasma concentrations from breast milk and dust biomonitoring data

The High Throughput ToxicoKinetics (HTTK) R package (version 1.7) (Wambaugh et al., 2015; Pearce et al., 2017) was used in R (version 3.4.1) (R. Development Core Team 2017) to estimate a child's internal plasma concentrations from breast milk or dust exposure. Within this package, a generalized three-compartment toxicokinetic model, composed of the gut, liver, and rest of body with separate tissue and blood compartments was used. This model is governed by differential equations describing changes in chemical concentration over time. Notable model assumptions include, rapid oral absorption (1/h), 100% bioavailability, the chemical exits the body through hepatic clearance (using CL_{int}) and passive nonmetabolic renal clearance (glomerular filtration rate × f_{up}, being f_{up} fraction of the chemical unbound in plasma), and perfusion-limited tissues (Wambaugh et al., 2015). Model inputs include chemical-specific parameters such as acid dissociation constant, octanol/water partition coefficient, fraction of the chemical unbound in plasma (f_{up}), and intrinsic metabolic clearance (CL_{int}). The model assumed an average individual, not accounting for susceptible populations.

Chemical specific model inputs, as used in the HTTK package, are listed in Supplemental Table 5 (chem.physical_and_invitro.data tab). ADMET Predictor 7.2 (Simulations Plus Inc., Lancaster, CA, USA) was used to calculate and predict all of the chemical-specific parameters for most chemicals and converted into applicable model units (Sipes et al., 2017). Exceptions include TPHP and TDCIPP, which had measured CL_{int} and f_{up} already listed in the HTTK package. In addition, three chemicals, TBBPA, BDE47, and TCEP had estimated CL_{int} of zero from ADMET Predictor, using the method described in Sipes et al. (2017). Internal plasma concentrations were not calculated for TBBPA and BDE47, since measured plasma concentrations were available. The CL_{int} value for TCEP, found to be 1.37 nmol/min-g of liver (Chapman et al., 1991), was converted to μL/min-10^6 cells using 1 g of liver/$99 * 10^6$ cells.

Peak plasma concentration (μM) over 365 days was estimated for the various ingestion scenarios (breast milk, dust, hand wipe) using specific dosing protocols (Supplemental Table 5, exposure tab). For the breast milk exposure simulation, the calculated mg/kg-day dose was divided over a day with ingestion once every 3 h. For the dust exposure simulation, the calculated mg/kg-day dose was divided over a day with ingestion 18 times per hour over a 12-hour awake period followed by a 12-hour unexposed period. For the handwipe exposure simulation, the calculated mg/kg-contact dose was ingested 18 times per hour over a 12-hour awake period followed by a 12-hour unexposed period.

3. Results and discussion

Two distinct set of experiments (called experiment 1 & 2 as noted in the methods above) were conducted to validate our method and to assess the robustness of our findings. Since there was high concordance between the experiments, for simplicity to the reader, we report findings for the experiment 1 in the main text, and all data for experiment 2 is reported in supplemental material. While discussing our findings, we highlight important differences between findings between both the experiments when noticed.

3.1. Effects of flame retardants on developmental toxicity

To evaluate developmental toxicity, embryo exposure started at 4 hpf (Fig. 1A). Specific concentrations tested per chemical (Supplemental Table 3) were selected based on previous range finding study (data not shown). Based on the analysis performed at 2 and 4 dpf, percentages for mortality and evidence of morphological alterations were determined for each tested concentration. To determine the teratogenic potential of each compound a Teratogenic Index (TI) was calculated as the ratio between LC50 (as a measure of general embryotoxicity) and EC50 (reflecting teratogenic effects). A TI numerical threshold to classify teratogenicity has been typically defined by a training set of known *in vivo* positive and negative compounds (Brannen et al., 2010; Selderslaghs et al., 2012). While compounds with a TI (also calculated as LC50/EC50) higher than 2 can be consider teratogenic (Selderslaghs et al., 2012), based on our internal validation we decided to increase our cutoff to 4 to ensure an accurate capture of teratogenicity.

In general, similar results were obtained in the two experiments performed (Table 1 and Supplemental Table 6). A LC50 was not obtained for BDE-47 and IDDP since these chemicals precipitated prior to inducing mortality (for BDE-47 at ≥25 μM in the first and ≥30 μM in the second experiments, and for IDDP at ≥150 μM in the first and ≥50 μM in the second experiments). Therefore, a TI value could not be estimated in these two cases. For the other tested compounds, TI could be accurately calculated at least at 4 dpf. No precipitation was noted for TMPP in the first experiment up to 100 μM (maximum concentration tested) while it precipitated at concentrations ≥30 μM in the second one. As a result, we were able to calculate a TI at 2 dpf only in the first but not in the second experiment. The nominal LEL at which toxicity was induced was comparable between both experiments as well as between several of the OPFRs and the two BFRs (Table 1 and Supplemental Table 6) even though precipitation was detected at different treatment concentrations in certain cases. Although we are not sure about the exact reason for the differences in compound precipitation

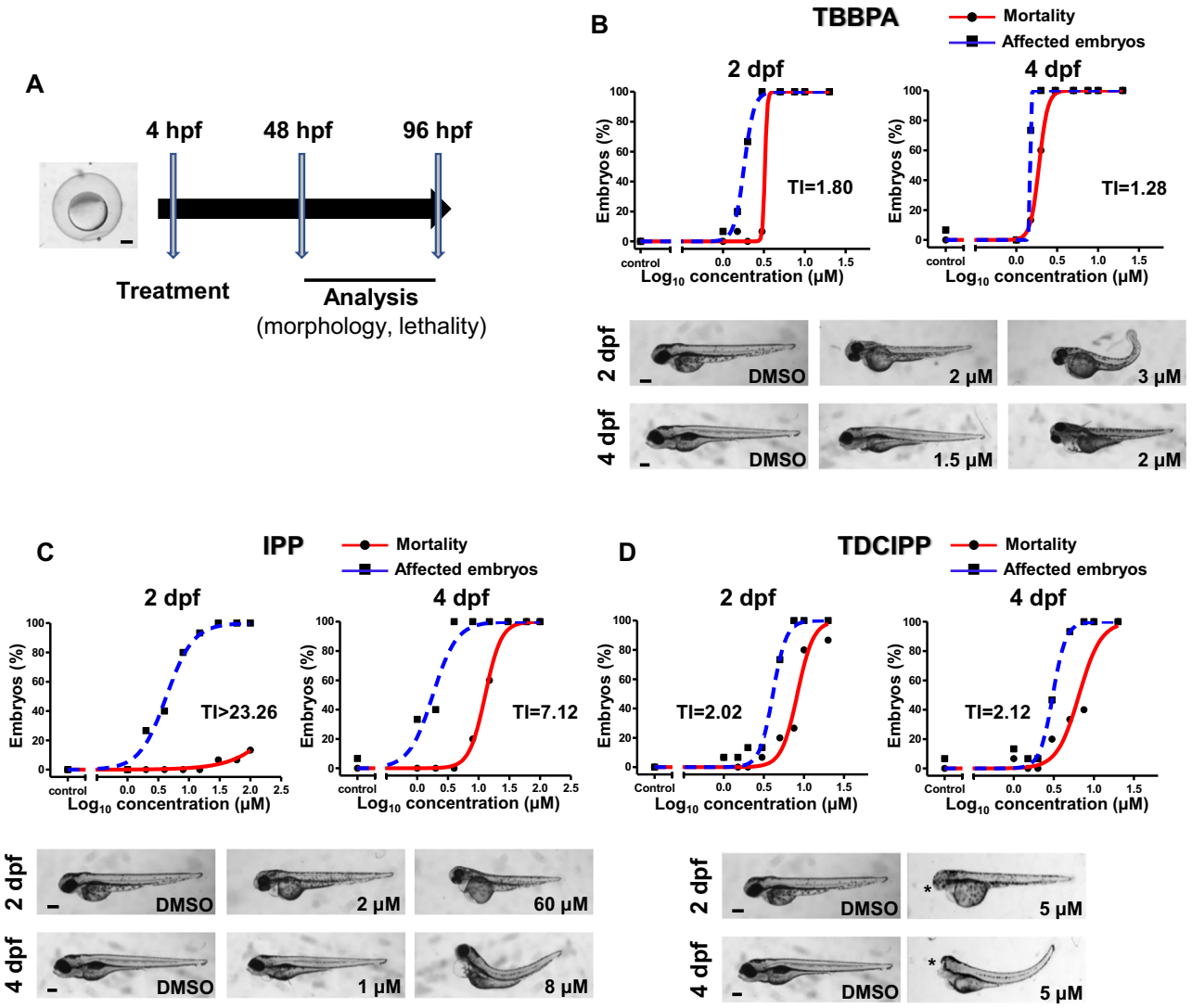

Fig. 1. Developmental toxicity assay and examples of the results obtained for the some of the BFRs and OPFRs (Experiment 1). A) Schematic depicting a summary of the experimental design of developmental toxicity assay. B, C, D) Results obtained for the BFR TBBPA (B) and for the replacement OPFRs IPP (C) and TDCIPP (D). Sigmoidal dose-response curves were calculated at 2 and 4 dpf for the percentage of affected and dead embryos. TI values (LC50/EC50) are indicated. Representative bright field pictures of the embryos treated at the stages and concentrations indicated are also shown. Asterisks point to the absence of eyes. Scale bar, 200 μM in all pictures.

rate across the two experiments, some potential reasons may include that some of them had different lot numbers and suppliers (see supplemental Table 2) and were shipped in different forms (original powder for experiment 1 and in DMSO stock solution for experiment 2). While this is a common practice that different researchers think that they are testing the "same" compound (based on CAS#), these are some factors that may influence results.

Regarding their teratogenic potential, the highest TI values (higher than 4) were obtained for some of the OPFRs tested, specifically for IPP, TMPP (at 2 dpf, experiment 1) and BPDP (IPP > TMPP > BPDP) (Fig. 1, Supplemental Fig. 1, Table 1 and Supplemental Table 6). TPHP had a TI close or higher than 4 (based on results from experiment 1 and 2), thereby ranking 4th. These findings are comparable to results obtained in a previous study (Behl et al., 2015) where an independent testing protocol and a different method of analysis (point of departure) were used. Although high TI values were not obtained for TDCIPP in this study (Fig. 1D), a great variability in both the sensitivity of the response between individuals as well as in the toxicity phenotype induced was observed for this OPFR. A substantial reduction, or even absence, in the eye size as well as cyclopia was specifically caused by this chemical in approximately 50% of embryos at concentrations where malformations were induced, suggesting that TDCIPP could affect pathways implicated in eye development. Alterations in dorsoventral patterning has been described in zebrafish treated early (2-cell stage) with TDCIPP (Dasgupta et al., 2017). A recent publication noted alterations in eye development following exposure to this FR (Dasgupta et al., 2018). Changes in the expression of genes involved in brain and retinal development and a decrease in the ocular area and pigmentation were observed in response to TDCIPP treatment. Although these are preliminary findings in our screen, it is an important outcome to follow-up with more extensive studies. It is also important to know that typical rodent studies are not designed to evaluate vision unless there is a specific trigger to do so; hence findings such as these may have gone unnoticed using conventional testing strategies. This illustrates an example of how the zebrafish may serve as a powerful complementary tool to rodent studies.

These results suggest that some of the OPFRs may have greater or equal teratogenic potential compared with the representative BFRs tested based on the TI values obtained or in specific morphological alterations induced. IPP can be considered to be the most active

Table 1
Summary of the results obtained in the first experiment after the evaluation of developmental toxicity in zebrafish embryos. A comparison between nominal concentration and estimated internal concentrations for LEL at 4 dpf is also shown. The numbers in the brackets indicate the 95% confidence intervals of the EC50 and LC50 values given or if these intervals were very wide or interrupted. *Point out compounds that precipitated and concentrations at which precipitation occurred. In light yellow values probably overestimated due to compound precipitation. High TI values, indicative of high teratogenic potential, are shown in red.

Test item	NOAEL (μM)		EC50 (μM)		LC50 (μM)		TI		Internal concentration	LEL (μM)	
	2 dpf	4 dpf	2 dpf	4 dpf	2 dpf	4 dpf	2 dpf	4 dpf		Nominal	Internal
BDE47*	>25	2	–	12.01 (8.44 to 17.11)	–	>25	–	>2.08		4	1040
BPDP	8	4	11.45 (10.56 to 12.42)	4.75 (0.086 to 263.1)	84.15 (80.72 to 87.72)	15.24 (12.33 to 18.84)	7.35	3.21		8	1222
EHDP	>20	3	–	5.06 (4.89 to 5.24)	–	9.78 (Very wide)	–	1.93		5	2880
IDDP*	>150	20	–	77.23 (57.77 to 103.2)	–	>150	–	>1.94		40	665.1
IPP	1	<1	4.30 (3.66 to 5.05)	1.80 (1.31 to 2.47)	>100	12.82 (11.97 to 13.73)	>23.26	7.12		1	4.21
TMPP	8	2	11.48 (11.40 to 11.56)	3.00 (2.78 to 3.24)	143.8 (107.2 to 192.9)	9.52 (9.46 to 9.57)	12.53	3.17		4	1078
TPHP	2	1	3.84 (3.41 to 4.33)	1.72 (1.61 to 1.84)	15.11 (very wide)	5.15 (Interrupted)	3.93	2.99		1.5	335.2
TBBPA	1.5	1	1.81 (1.76 to 1.86)	1.48 (Very wide)	3.26 (Very wide)	1.90 (1.88 to 1.92)	1.80	1.28		1.5	20.68
TCEP	400	400	521.2 (462.8 to 587.0)	415.2 (Very wide)	>1000	977.6 (Very wide)	>1.92	2.35		600	342.7
TDCIPP	3	2	4.11 (3.68 to 4.58)	3.08 (2.79 to 3.40)	8.29 (7.15 to 9.61)	6.53 (5.07 to 8.40)	2.02	2.12		3	76.68

compound for zebrafish in developmental toxicity assay since it showed the highest TI (7.12 and 12.78 in the first and second experiment respectively at 4 dpf) and the lowest effective concentration (1 or 0.5 μM in the first and second experiment respectively at 4 dpf). Further studies to evaluate the developmental toxicity effects of this FR, including developmental neurotoxicity in rodents, are underway at the National Toxicology Program (Behl et al., 2015).

3.2. Internal concentration analysis

Although in most studies in the literature nominal concentrations (concentrations in the well at the beginning of exposure) are reported, they do not always reflect the internal exposure due to differences in chemical uptake based on physicochemical properties and kinetics (Berghmans et al., 2008; de Koning et al., 2015). Hence, we evaluated internal concentrations in 4 dpf larvae exposed to LEL following assessment of developmental toxicity. Except TCEP that showed a larvae internal concentration below the nominal concentration (around 57%–87%), all other tested chemicals showed an accumulation/selective absorption of various orders of magnitude (Table 1 and Supplemental Table 6). Accumulation of highly lipophilic compounds (with high logP) in zebrafish embryos, including some OPFRs, have been previously described (Dishaw et al., 2014; Wang et al., 2015). Therefore, while toxicity was noted at low nominal concentrations for most of the FRs tested, the equivalent internal exposures were generally up to 200-fold higher compared to nominal concentrations (Table 1, Fig. 4). However, one of the OPFRs tested, IPP, was toxic at lower concentrations (4.21 μM in experiment 1). Although internal concentrations are an approximation due to the unknown proportion of each of derivatives that conforms this mixture (see supplemental methods), these results confirm that IPP is probably the most active compound for zebrafish in developmental toxicity assay. It is important to note that since this was a screening study with a goal to prioritize compounds for further testing, in most cases we only measured levels of the major parent compound inside the larvae; the presence of other isomers that could be present in the mixtures were not evaluated. Since compounds have different physicochemical properties, they are expected to have different rates of uptake. This has important implications for the accumulation of these compounds for both, human exposure and ecotoxicity. Further studies are warranted to define the toxicokinetic profiles of these compounds to better characterize hazard associated with exposure.

3.3. Effects of flame retardants in behavior alteration

Because of their structural similarities to organophosphate pesticides, which have been shown to be neurodevelopmentally toxic (Slotkin et al., 2006), neurotoxicity has been the main concern of alternative OPFRs. For the detection of neurotoxicity, potential alterations in locomotor activity caused by FRs were evaluated in 5 dpf larvae. Analysis was performed after 48 h of incubation with chemicals only in hatched and not malformed larvae. Although more specific neurotoxic effects are expected to be detected after shorter periods of incubation, larvae used in this assay were also analyzed for the detection of hepatotoxicity, for which longer exposures are necessary (He et al., 2013). Therefore, acute neurotoxicity as well as effects more related with the inhibition of processes that occur late in nervous system development and maturation are expected to be detected with this assay. Combining results of experiments 1 and 2, alterations in locomotor activity were produced by most of the flame retardants evaluated (Table 2, Supplemental Table 7, Fig. 2, Supplemental Fig. 2). Since the highest concentration evaluated in the behavioral assay was the LEL obtained in developmental toxicity, it was difficult to discriminate between general systemic toxicity *versus* specific neurotoxicity when hypoactivity was the alteration caused only at the highest concentration tested. This occurred for BPDP, IDDP, TMPP, TPHP, TCEP and TDCIPP and then, their effects were not considered specifically neurotoxic but a consequence of general toxicity. However, for BDE-47, there was a clear concentration-dependent decrease in activity only in the dark phase with no differences detected in the light phase, suggesting that this compound altered neurobehavior (Fig. 2B). For EHDP, we noted a concentration-dependent hyperactivity under light condition and absence of responsiveness to light changes in both our experiments (Fig. 2C). Moreover, larvae treated with this chemical also manifested corkscrew movements and loss of equilibrium. Therefore, BDE-47 and EHDP were neuroactive/neurotoxic for zebrafish larvae.

Table 2
Summary of the results obtained in the behavior assay (first experiment). The type of effect detected and the LEL are indicated. *Probably not specific effect.

Test item	Behavior alteration	
	Effect	LEL (μM)
BDE47*	Reduced activity	1
BPDP	*Reduced activity/toxic	10
EHDP	Hyperactivity	10
IDDP*	Reduced activity	80
IPP	Not detected	–
TMPP	Not detected	–
TPHP	*Reduced activity/toxic	2
TBBPA	Not detected	–
TCEP	*Reduced activity/toxic	1000
TDCIPP	Not detected	–

BDE-47 is a well-known developmentally neurotoxicant in mammals (reviewed in Costa and Giordano, 2007) and its neurotoxic effect has been also reported in zebrafish (Chen et al., 2012; Jarema et al., 2015; Noyes et al., 2015). In fact, a lower activity in the dark was also the primary effect of BDE-47 reported by Chen et al. (2012) and Jarema et al. (2015) while Noyes et al. described a decreased activity in both light and dark phases. Nevertheless, none of them detected the loss of responsiveness to light changes that was observed in this study at similar tested concentrations. Extensive behavioral changes following acute exposure to EHDP were also described by Jarema et al. (2015) at low micromolar range as noted in the present study. These independently conducted studies show strong concordance in patterns of results, thereby encouraging the use of zebrafish in neurodevelopmental toxicity and highlight the importance of including a behavioral assessment while evaluating development toxicity to identify compounds for which the nervous system is a suspected target.

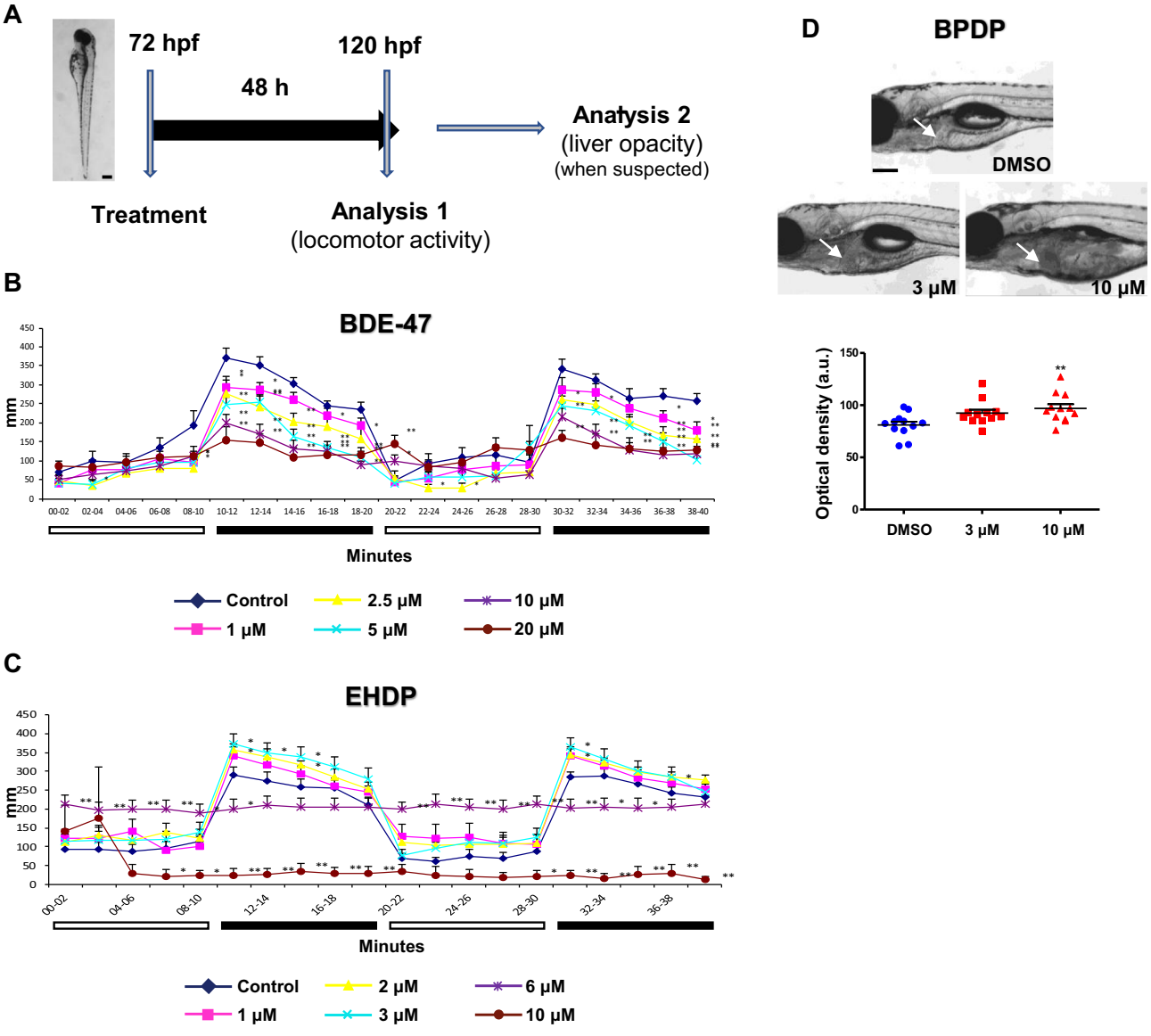

Fig. 2. Behavior alteration and hepatotoxicity assay and examples of the results obtained for the phased-out BDE-47 and the replacement EHDP in the behavior assay and for the OPFR BPDP in the hepatotoxicity assay (Experiment 1). A) Schematic depicting a summary of the experimental design for both assays. B, C) Behavior profile of 5 dpf larvae treated with BDE-47. (B) and EHDP (C). Graphs represent the mean of the distance moved by embryos treated with each chemical in 2 min' time bins (*$p < 0.05$; **$p < 0.01$, unpaired Student's *t*-test). White and black rectangles indicate the periods of light and dark respectively. (D) Representative bright field pictures of embryos untreated and treated with BPDP at the indicated concentrations. Arrows point to the position of the liver. Mean ± SEM of the liver optical density from 12 embryos per experimental group is represented in the graph. (**$p < 0.01$, one-way ANOVA and Dunnett's post-test). Scale bar for all pictures represents 200 μM.

Table 3
Summary of the results obtained in cardiotoxicity and hepatotoxicity assays. The type of effect detected is indicated for cardiotoxicity assay. Hepatotoxicity was only analyzed when suspected after visual inspection. For BDE-47, the maximum concentration that could be tested is indicated between brackets.

Test item	Cardiotoxicity		Hepatotoxicity	
	Effect	LEL (μM)	Effect	LEL (μM)
BDE47	Not detected (30 μM)	–	Not observed	–
BPDP	Bradycardia/ Atrial failure	10	Yes	10
EHDP	Bradycardia	30	Yes	10
IDDP	Bradycardia	100	Not observed	–
IPP	Bradycardia/ Atrial failure	100	Not observed	–
TMPP	Bradycardia/ Atrial failure	30	Not observed	–
TPHP	Bradycardia/ Atrial failure	10	Not observed	–
TBBPA	Arrhythmia/ Ventricular failure	3	Not observed	–
TCEP	Not detected	–	Not observed	–
TDCIPP	Not specific	–	Not observed	–

3.4. Effects of flame retardants on hepatotoxicity

After behavior analysis, embryos were visualized for the detection of liver opacity that was suspected in larvae treated with BPDP and EHDP. This effect was confirmed after optical density quantification (Fig. 2D, Table 3, Supplemental Table 8) in both experiments conducted. Other toxicity manifestations (mainly edemas) at concentrations at which hepatotoxicity was detected were also present at the time of analysis.

The potential adverse effects of OPFRs in liver have not been adequately tested using *in vivo* models. Although this is the first time that hepatotoxicity is being reported for BPDP and EHDP in zebrafish, changes in metabolism and liver gene expression for other OPFRs such as TDCIPP and TPHP, have been previously described (Du et al., 2016; Liu et al., 2016). This suggests the need for more in-depth evaluation of the liver as a potential target of toxicity for this class of compounds.

3.5. Cardiotoxicity of tested flame retardants

The results obtained following cardiotoxicity evaluation are shown in Table 3, Supplemental Table 8, Fig. 3 and Supplemental Fig. 3. Similar results were obtained in the two experiments performed, although LEL at which cardiotoxicity was initially detected was different for some of the FRs tested. Representative results of the BFR TBBPA and the OPFR TMPP, including the results of the positive control (Terfenadine), are shown in Fig. 3. Only 2 compounds, BDE-47 and TCEP were negative in this assay. The possible cardiotoxicity of EHDP and IDDP was not confirmed since bradycardia was the only phenotype observed and the slower heart rate can be a consequence of general toxicity. This was clear for TDCIPP since lethality was also caused at the same doses at which bradycardia was detected. Then, this compound was not considered cardiotoxic. A specific pattern of cardiotoxicity which showed bradycardia induction first followed by cardiac arrest only in the atrium at higher concentrations, was noted in four of the non-halogenated OPFRs tested (BPDP, IPP, TMPP and TPHP) at

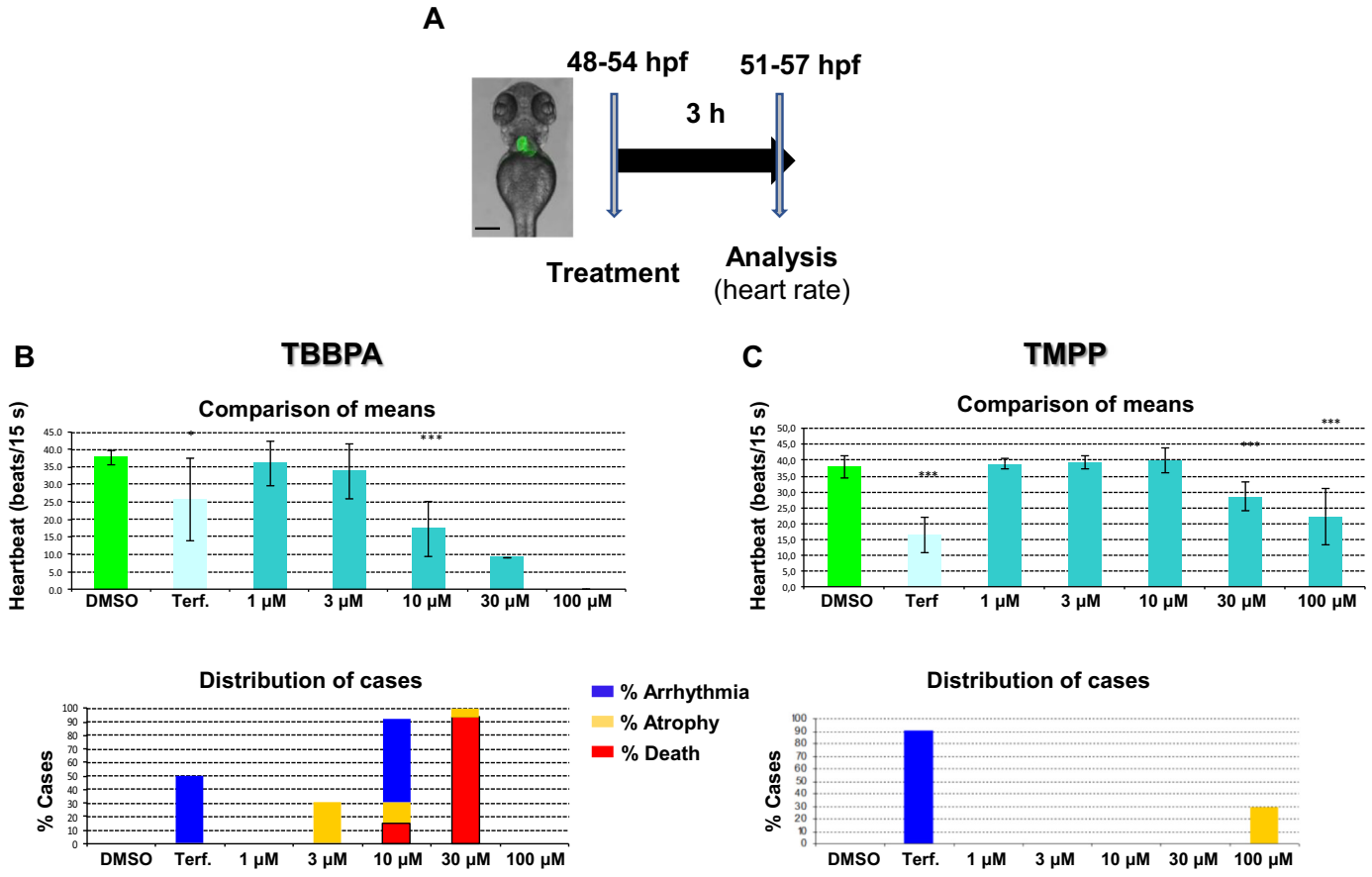

Fig. 3. Examples of cardiotoxicity results obtained for the BFR TBBPA and the OPFR TMPP (Experiment 1). (A) Schematic depicting a summary of the experimental design. B, C Upper graphs show the mean ± S.D. of the heartbeat rate obtained for TBBPA (B) and TMPP(C) (***$p < 0.001$, Mann-Whiteney U test). In the lower graphs, bars represent the percentage of embryos with altered heartbeat rhythmicity, atrophy, or death. Terf.: 5 μM Terfenadine. Scale bar represents 200 μM.

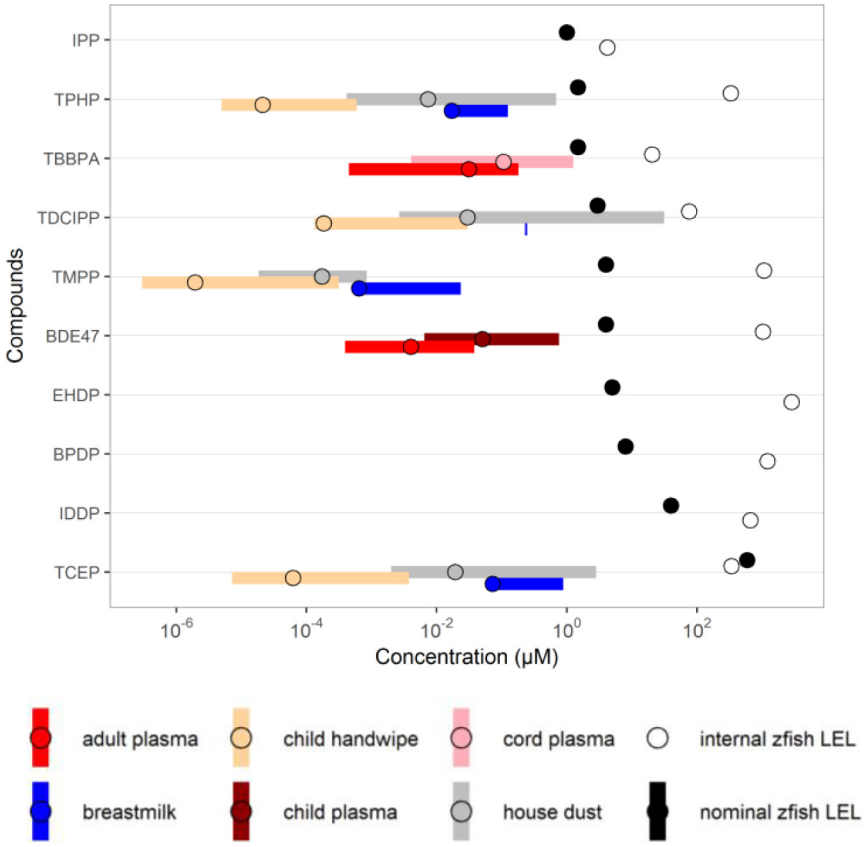

Fig. 4. Comparison of estimated and measured FR concentrations in human plasma and lowest effective levels (LELs) in zebrafish embryos as shown in Table 1. The LELs in zebrafish embryos plotted for the most potent effect (in terms of nominal concentration) and the corresponding measured internal zebrafish embryo concentration were plotted as black and white circles, respectively. Child exposure to contaminated breastmilk, child handwipe and house dust was evaluated using the HTTK R-package (version 1.7) and converted into an internal human child plasma concentration and plotted (along with unit-converted measured values of adult plasma, child plasma and cord plasma, for reference) as the colored circles and bars. Colored circles from the human biomonitoring data or estimated internal concentrations are using geometric mean, median, or maximum mean data, as noted in the Methods. The lower and upper range of the unit-converted (plasma) and HTTK-estimated internal concentrations were calculated using the lowest and highest reported values, respectively. Biomonitoring data for TDCIPP in breastmilk was based on one sample, where the internal plasma concentration is represented by a blue line.

concentrations between 10 and 100 μM (Supplemental Movies 1 and 2). Finally, TBBPA was the most active compound in this assay since cardiotoxicity was observed as low as 3 μM. Arrhythmia 2:1, a phenotype indicative of ERG (ether-a-go-go-related gene) inhibition (Langheinrich et al., 2003) and related to long QT syndrome in humans, was caused by the treatment with this FR (Supplemental Movie 3).

Alterations in heart development as well as defects in heartbeat have been previously described in zebrafish caused by some non-halogenated OPFRs as TPHP, isopropylated triaryl phosphate (ITP) and cresyl diphenyl phosphate (CDP) (McGee et al., 2013; Du et al., 2015). These findings are in agreement with previous published studies that have shown the OPFRs to be bioactive in human stem cell derived cardiomyocytes (Sirenko et al., 2017). These results indicate that cardiotoxicity may be a relevant target for the non-halogenated OPFRs. Further characterization of possible cardiotoxic effects of OPFRs in mammals is warranted.

3.6. Relevance of findings in zebrafish to biomonitoring data in humans

To provide the toxicology community with values typically used for hazard assessments, *in vitro* activity concentrations are converted to human daily dose equivalents using high throughput toxicokinetic and compared to dose exposure estimates (Rotroff et al., 2010; Wetmore et al., 2012; Wetmore et al., 2013; Sipes et al., 2017). The basis for this assumes that the *in vitro* well concentration is equivalent to human plasma concentrations. Converting zebrafish LELs to a human daily dose is more complicated due to the kinetics of the zebrafish. Therefore, for a more straightforward assessment, we estimated or found measured human plasma concentrations and directly compared these with the zebrafish LEL concentrations (internal and nominal). Human biomonitoring data were unit-converted using HTTK modeling to estimate internal plasma concentrations in children and consisted of breast milk, handwipes, and house dust, plasma and cord-blood serum levels of FRs (Cariou et al., 2008; Stapleton et al., 2012; Stapleton et al., 2014; Kim et al., 2014; Castorina et al., 2017; Sugeng et al., 2017) for which data was available (TPHP, TBBPA, TDCIPP, TMPP, BDE-47 and TCEP) (Fig. 4). Within the confines of the model assumptions (see methods section for model assumptions), for some of the compounds the LEL of nominal concentration was within the upper bounds of the range of estimated human child exposure from hand-mouth-contact through house dust (TDCIPP and TPHP) and measured human cord plasma (TBBPA) (Fig. 4). Internal concentrations of these chemicals are up to 200-fold higher in the fish as indicated by the open circles in Fig. 4. While there is little to no information on the compartmentalization, accumulation and toxicokinetic profile of these compounds in humans, these findings have important implications with regards to their potential to be present at higher concentrations in different tissues within the body, thereby suggesting the need for further targeted kinetic evaluations.

The comparison of human biomonitoring data with exposures in zebrafish includes some noteworthy challenges as follows: 1) high degree of variability in the range of human exposures (see Supplemental Table 5 for variability ranges between different cohorts), 2) presence of multiple isomeric mixtures in the environment, thereby posing a challenge while estimating exposures to single components, 3) insufficient sampling for human biomonitoring data, not necessarily adequately reflecting the extent of potential individual differences in susceptibility, and 4) the comparison in estimates are largely governed by the model assumptions.

In spite of the challenges noted above, these comparisons provide valuable context between toxicity outcomes noted in zebrafish, other *in vitro* models (Behl et al., 2015) and current human exposures. Studies

are currently underway at the NTP to evaluate the effects of some of these compounds in rodent studies. Further toxicokinetic studies in developing and adult zebrafish are warranted to understand the dynamics of these chemicals in relation to human exposure as well as to other aquatic organisms to determine the potential human and ecological hazards that these chemicals might pose.

4. Conclusion

In this study we demonstrated the utility of the zebrafish as an integrative tool to screen for a class of compounds and showed how these findings may be related to human exposure. This is the first time that a systems toxicity approach has been used to compare OPFRs with representative BFRs in zebrafish, and findings contextualized with human biomonitoring data. In general, some of the non-halogenated aromatic OPFRs (BPDP, IPP, TPP, EHDP, TMPP) showed comparable toxicity to the phased-out BDE-47 and to TBBPA. A unique cyclopia was noted following exposure to TDCIPP, distinct from all other FRs tested in the study. IPP manifested a high teratogenic potential; neurotoxicity was clearly seen following exposure to BDE-47 and EHDP. Additionally, many of the non-halogenated OPFRs induced a characteristic cardiotoxic effect. Overall, it appears that the FRs affected multiple pathways during zebrafish development thereby resulting in multiple target organs of toxicity. Generally, the results from both of our independently conducted blinded experiments largely corroborated with each other. Interestingly, these findings are also consistent with previous developmental toxicity data in the literature (Behl et al., 2015; Jarema et al., 2015) that used different protocols and zebrafish strains, thereby emphasizing the robustness of this model as a reliable screening tool for potential toxicants.

Finally, in comparing toxicity outcomes in the zebrafish with human biomonitoring data, our results suggest that in some cases, nominal concentrations at which we noted toxicity in zebrafish were within the upper range of potential human exposure. Some of these compounds accumulate in the zebrafish up to ~200 fold higher, which has important implications for other aquatic organisms and ecotoxicity. Further in-depth studies are warranted to better understand their toxicokinetic profiles to characterize hazard in humans and wildlife.

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Conflict of interest

Ainhoa Alzualde, Aintzane Alday, Arantza Muriana and Celia Quevedo work for Biobide, a company that provide services using zebrafish as experimental model.

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