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Exposure to a PBDE/OH-BDE mixture alters juvenile zebrafish (*Danio rerio*) development

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Abstract

Polybrominated diphenyl ethers (PBDEs) and halogenated phenolic compounds (e.g., hydroxylated BDEs (OH-BDEs)) are contaminants detected together frequently in human tissues, and are structurally similar to thyroid hormones (TH). THs partially mediate metamorphic transitions between life stages in zebrafish, making this a critical developmental window which may be uniquely vulnerable to chemicals disrupting thyroid signaling. In this study, zebrafish were exposed to 6-OH-BDE-47 (30 nM) alone or to a low (30 µg/L) or high dose (600 µg/L) mixture of PentaBDEs, 6-OH-BDE-47 (0.5–6 µg/L), & 2,4,6 tribromophenol (TBP) (5–100 µg/L) during juvenile development (9–23 days post fertilization; dpf) and evaluated for developmental endpoints mediated by TH signaling. Fish were sampled at three time points and examined for developmental and skeletal morphology, apical thyroid and skeletal gene markers, and modifications in swimming behavior (as adults). Exposure to the high mixture resulted in > 85% mortality within one week of exposure, despite being below reported acute toxicity thresholds for individual congeners. The low mixture and 6-OH-BDE-47 groups exhibited reductions in body length and delayed maturation, specifically relating to swim bladder, fin, and pigmentation development. Reduced skeletal ossification was also observed in 6-OH-BDE-47 treated fish. Assessment of thyroid and osteochondral gene regulatory networks demonstrated significantly increased expression of genes that regulate skeletal development and THs. Overall, these results indicate that exposures to PBDEs/OH-BDEs mixtures adversely impact zebrafish maturation during metamorphosis.

Keywords

PBDE; mixture; development; juvenile; zebrafish

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SUPPLEMENTAL DATA

The supplemental data includes additional methods description of analytical chemistry data, as well fish and water PBDE/OH-BDE concentration data, fish behavioral response data, and a description of the results of the high mixture fish treatment.

INTRODUCTION

PentaBDE was a commercial polybrominated diphenyl ether flame retardant formulation historically used in treating polyurethane foam in upholstered furniture [1]. PentaBDE consisted largely of diphenyl ethers with 4–6 bromine atoms, and was sold under several trade names, including DE-71. In the mid-2000s, PentaBDE was phased out from use due to concerns about its potential toxicity and persistence. Despite the removal of PentaBDE from the marketplace, human exposure to PBDEs has continued due to the abundance of existing products, recycling of treated materials and their recalcitrance in the environment [2,3].

PBDEs are oxidatively metabolized to form OH-BDEs and bromophenols in mammals [4], and these metabolites can have endocrine disrupting effects [5–7]. For example, oxidative metabolism of BDE-47 in human microsomes generates 6-OH-BDE-47, among other halogenated phenolic metabolites [4,8]. 2,4,6 TBP is used commercially as an antifungal agent, a reactive brominated flame retardant, and as an intermediate in the production of other brominated flame retardants [9,10]. In addition to these industrial uses, ortho-substituted OH-BDEs and bromophenols are naturally synthesized by some marine sponges and algae, or through interconversion with methoxylated BDEs (Me-O-BDEs). These natural sources provide additional environmental sources of these compounds [11,12]. As a result, there is widespread human exposure to PentaBDEs, 6-OH-BDE-47, and 2,4,6 TBP [13–16].

PBDEs and OH-BDEs share similar chemical structures with endogenous thyroid hormones, which may explain why endocrine/thyroid disruption has been observed in animal exposure studies [17]. Furthermore, 6-OH-BDE-47 and 2,4,6 tribromophenol have both been shown to bind to the thyroid transporter protein transthyretin in vitro, with higher affinity than the parent PBDEs [18,19]. 6-OH-BDE-47 was reported as a TR agonist that can also recruit coactivators [5]. Fish exposed to PBDEs experience thyroid dysregulation, as well as declines in reproductive fitness, neurotoxicity, and oxidative stress [20–22]. Hydroxylated BDE toxicity has also been examined in early life stage and adult zebrafish, and the data suggest hydroxyl-metabolites have enhanced toxicity relative to the parent chemicals [23,24]. Since both PBDEs and OH-BDEs are frequently detected in human serum and other biological matrices (breast milk, cord blood, etc.), it is likely that exposure to these mixtures occurs frequently [14,25]. In fact, a recent study found that exposure to mixtures of seven environmentally relevant OH-BDEs in vitro enhanced synergistic toxicity relative to a single compound exposure in tests of mitochondrial respiration and membrane potential [26]. However to date, we are not aware of any studies that have examined the effects of mixtures of PBDEs/OH-BDEs on fish development in vivo.

Juvenile animals represent a unique developmental life stage, whose maturation requires precise coordination of endocrine signaling. Zebrafish undergo sensitive developmental changes as they transition from larvae to juveniles and finally to adults [27–30]. While less dramatic than the amphibian metamorphosis, freshwater teleosts undergo extensive postembryonic remodeling with morphological and physiological changes [31]. These changes include formation of adult fins, ossification of fin rays, maturation of internal organs, scale formation, modification of the pigment patterns, and changes in body

proportions [27]. The endocrine system, and specifically thyroid hormones, are known to play an important role in these transitions [27,30]. Supporting this hypothesis, thyroid nuclear receptors increase in expression during hatching and during metamorphosis [27,30,32]. Additionally, thyroid deiodinase enzymes (Dio1, Dio2) have been found to increase in expression immediately before metamorphosis in multiple fish species [33]. Dio1 and Dio2 appear to be sensitive to exogenous stimuli as exposure to 6-OH-BDE-47 is demonstrated to upregulate deiodinase expression in zebrafish [34]. Furthermore, euthyroid status (and specifically deiodinases) are critical for normal skeletal development [35,36]. Since skeletal and developmental morphology are sensitive to alterations in thyroid status, these endpoints were selected for investigation in this study.

The goal of the present study was to investigate effects on developmental endpoints (pigmentation, fin maturation, skeletal and cartilage development) following juvenile exposure to either a mixture of PBDEs/OH-BDEs or a potent embryonic toxicant and TR agonist 6-OH-BDE-47 [5,23,24]. In addition, PBDE/OH-BDE impacts on whole organism function were evaluated using swimming behavior assays in juvenile animals. Swimming behavior is a functional test that depends on anatomical, physiological, and neurological function. Previous work in zebrafish larvae has observed neurobehavioral effects following exposures to these same compounds [37,38]. As thyroid hormones also regulate and direct many aspects of brain development [39,40], examining neurobehavioral effects can provide insight into neurological function. These developmental changes were compared between treatment groups to determine if any were the result of the mixture exposure or a single chemical exposure to 6-OH-BDE-47.

MATERIALS AND METHODS

Chemicals and reagents

DE-71, a commercial PentaPBDE mixture, and 1,3,7,8 Tebtetrabromodibenzo-p-dioxin (purchased to use as a standard to check for dioxin impurities in DE-71), were obtained from Wellington Laboratories. 6-hydroxy-2,2',4,4' tetrabromodiphenyl ether (6-OH-BDE-47; >99.9% purity) was purchased from Accustandard. 2,4,6 Tribromophenol (TBP; > 99% purity) was purchased from Sigma-Aldrich. Stable isotopically labelled surrogate standards ^{13}C -6-OH-BDE-47 and ^{13}C -6'-OH-BDE-100, and $^{13}\text{C}_{12}$ -2,2',3,4,5,5'-hexachlorodiphenyl ether (^{13}C -CDE-141) were purchased from Wellington Laboratories. 4'-fluoro-2,3',4,6-tetrabromodiphenyl ether (F-BDE-69) was purchased from Chiron. Hexane, dichloromethane, methanol, and acetone were HPLC grade (EMD Millipore Corporation). C18 solid phase extraction sorbent and 20 μM PTFE frits were purchased from Sigma Aldrich. Supelco Dual Layer Carbon Reversible Columns were purchased from Sigma Aldrich and used for DE-71 purification. Silica Solid Phase Extraction columns (Sep Pak, 1.0 g) were purchased from Waters. MS-222 (3-aminobenzoic acid ethyl ester methanesulfonate salt), Alcian Blue 8GX, Alizarin Red S, Trypsin, Sodium Borate, and Potassium Hydroxide were all purchased from Sigma-Aldrich. Neutral buffered formalin (10%) was purchased from VWR. TRIzol reagent and high capacity cDNA reverse transcription kits were purchased from Invitrogen and Applied Biosystems respectively.

Purification of DE-71

DE-71 was purified to remove brominated dioxin (PBDD) and dibenzofuran impurities (PBDF) using porous graphitic carbon to fractionate the non-coplanar PBDEs from the coplanar PBDD/DFs using the manufacturer's instructions (Supelco Dual Layer Carbon Reversible Columns). Solutions containing DE-71 dissolved in hexane were prepared and purified according to published methods [41,42]. Briefly, BDE mixtures were dissolved in hexane and passed through a porous graphite carbon column for fractionation of PBDE congeners from the PBDD/DFs. Following purification procedure, stocks were evaporated and reconstituted in DMSO for dosing fish water. Additional information regarding PBDE/OH-BDE analysis and QA/QC of fish and water samples can be found in the supplemental information.

PBDE/OH-BDE exposures

Approximately 1,300 9–10 day old zebrafish (*Danio rerio*, wild type; Tropical 5D strain; original source Dr. Robert Tanguay, Oregon State University) were randomly distributed between twelve 2.5 gallon (9.5L) glass aquaria (~110 fish/tank) and assigned to the following treatments (in triplicate tanks): control (< 0.001% DMSO), 6-OH-BDE-47 (30 nM 6-OH-BDE-47), low mixture (30 µg/L DE-71, 15 nM (4.9 µg/L) TBP, 1nM (0.5 µg/L) 6-OH-BDE-47), and high mixture (600 µg/L DE-71, 302 nM (99.9 µg/L) TBP, 12 nM (6.0 µg/L) 6-OH-BDE-47). The low mixture concentration was based on the medians reported in human serum levels of BDE-47 (ng/g lipid) and extrapolated to a molarity [14,43]. The high mixture concentration was approximately a twenty-fold increase from the low mixture concentration. The experimental concentrations of 6-OH-BDE-47 and 2,4,6 TBP were based off of previous work demonstrating endocrine disruption and range finding assays [34,44,45]. Fish received a semi-static exposure, with 50% water renewals every other day. All dosing water was mixed thoroughly for at least 6 hours in 4-L amber glass bottles prior to water changes. Water samples (4 mL) were collected before and immediately after water changes from each tank and stored at –20°C until extraction.

Fish were maintained at 28°C on a 14:10 light/dark photoperiod in dechlorinated freshwater supplemented with aquarium salt (600 µS). Water chemistry (pH, ammonia, nitrate, nitrite, hardness) was monitored daily using test drops (API Freshwater Master Test Kit). Beginning at 6dpf, larvae were fed 3–4 times daily with Zeigler Larval Diet (<50 microns), switching to larger particle size diets as the fish grew according to the manufacturer's recommendation (<100 microns, 100–150 microns, 150–250 microns, and 250–450 microns). Beginning at 7dpf, all larvae were supplemented with *Artemia* nauplii (Brine Shrimp Direct) once a day. Fish were exposed to aqueous treatments for 14 days (9 dpf–23 dpf) followed by a 22-day depuration period (45 dpf) in which fish were maintained in clean water containing no test chemicals. A subset of fish was randomly selected from each tank at the pre-selected sampling time points of 12 dpf, 23 dpf, and 45 dpf. These fish were then randomly allocated to a specific morphological or molecular endpoint analysis. Time points were selected in an effort to capture early and late metamorphic transitions following exposure. For all morphological scoring, images were randomized and the scorer was blinded to treatment. All fish care and experimental procedures were approved by Duke University's Institutional

Animal Care & Use Committee. The sampling and tissue pooling experimental design is summarized in Figure S1 and Table 1.

PBDE Extractions & Analysis

Water samples—Water samples were analyzed using glass columns packed with 250 mg discovery sorbent (DSC-18; Supelco) containing Teflon frits (Supelco). Columns were conditioned using 3 mL hexane, 3 mL acetone, and 3 mL deionized water. Water samples (4 mL) were loaded onto the column, then spiked with internal standards (50 ng FBDE-69; 30 ng, 13C-6-OH-BDE-47), and washed with 3 mL deionized water (n= 3 samples/treatment/ time point). Tubes were then dried for 30 min under vacuum, and eluted with 2 mL acetone, 2 mL acetone:hexane, 6 mL hexane (F1), then 2 mL hexane:dichloromethane (DCM) (discarded), 8 mL DCM (F2) into tubes containing sodium sulfate to remove any excess water. Eluates were concentrated to 0.1 mL under nitrogen (F1), or to dryness (F2) and transferred to autosampler vials for GC-MS (F1) or LC-MS/MS analysis (F2). LC-MS/MS samples were reconstituted in methanol and spiked with a secondary standard, ¹³C-OH-BDE-100. GC-MS samples were spiked with a secondary internal standard (¹³C-CDE-141) to measure recovery of FBDE-69.

Fish Samples—Following euthanasia in 300 mg/L MS-222, fish carcasses randomly selected, pooled, and flash frozen in liquid nitrogen for subsequent PBDE analysis across each treatment (n=3). Samples were stored at -80°C until extraction. Earlier time points required additional fish samples to achieve sufficient tissue masses for extraction (n=40 fish vs n=10 fish at later time points; described in Table 1). Whole fish were homogenized using 0.1 mm glass beads in a bullet blender (Next Advance), and then transferred to 10 mL glass test tubes. Tissue homogenates were extracted with 5 mL 1:1 Hexane:Dichloromethane, then sonicated for 20 minutes, and extracts decanted into 15 mL glass tubes and the process was repeated three times. Volumes were combined, spiked with internal standards (50 ng FBDE-69; 30 ng 13C-6-OH-BDE-47) and concentrated to ~ 1 mL under nitrogen. Extracts were then cleaned using a Silica SPE (1 g; Waters Sep-Pak). Columns were conditioned using 8 mL hexane, then the sample was loaded. PBDEs were eluted with 8 mL hexane (fraction 1) and OH-BDEs/TBP were eluted with 8 mL DCM (fraction 2). Fractions were blown down to near dryness, solvent exchanged if necessary, and analyzed using GC-MS or LC-MS/MS.

Mass spectrometry analysis

Water samples and fish carcasses were analyzed for a suite of 32 PBDE congeners using gas chromatography mass spectrometry operated in electron capture negative ionization mode (GC/ECNI-MS) [46]. The operating conditions have been described previously [47]. OH-BDE and tribromophenol analyses of water and fish were performed using LC-MS/MS from a published method [48]. Further details can be found in the supplemental information.

Growth

Fish growth was assayed by recording length and mass of individual fish at each sampling point. Mass (mg wet weight (ww)) was determined by weighing a subset of sampled fish at each sampling time point using a microbalance (10 fish/tank, n=30/treatment). Length

measurements (mm) were recorded using ImageJ 1.47 software [49] from photomicrographs of laterally recumbent fish used in morphology assessments (10 fish/tank, n=30/treatment). Standard length (mm), distance from the snout to the caudal- most portion of the notochord (or the caudal peduncle in post-flexion larvae) was measured [31].

Fish growth rates for each tank were determined by fitting body mass measurements to the exponential model

$$\ln(\text{fish mass}) = b \times t + a$$

where b is the growth rate (slope; fish mass per time), t is the time in days, and a is a constant [50].

Developmental morphology endpoints

A morphology scoring system, modified from existing reports, was used to assess juvenile development [31,51] including standard length (mm) and mass (mg), as well as the identification of developmental markers (described in Table 2). Pigmentation pattern, swim bladder differentiation, and fin development were monitored at each sampling day. At each time point, 10 or 15 individuals per tank were examined (n=30 individuals/treatment for 9 dpf and 12 dpf sampling and n= 45 individuals/treatment for 23 dpf and 45 dpf sampling). The individual metric scores generated by these endpoints were then summed to create an “overall” development score that was used to compare differences across time. Fish were euthanized in 300 mg/L MS-222 and then placed on an agar mold, with lined grooves for positioning, and imaged in lateral recumbency using a Nikon Eclipse E600 microscope equipped with a Nikon DXM1200 digital camera and NIS-Elements 3.20.01 software (Nikon Instruments Inc.). Following imaging, fish specimens were placed in 10% neutral buffered formalin and stored at 4°C until staining.

Alcian blue and Alizarin red staining

These stains were performed by modifying an existing protocol [52]. Briefly, fixed fish were washed with 1x PBS with 10% TWEEN 20 (PBST) and then stained overnight in Alcian blue solution. Next, fish were dehydrated in a graded ethanol series, washed in 1xPBST, digested in trypsin enzyme solution (10 mg/mL trypsin in 30% saturated sodium borate solution), and bleached with 3% H₂O₂ in 1% KOH until eyes of specimens became transparent. Specimens were then stained overnight in Alizarin Red S solution and cleared in 0.5% KOH before transfer through a graded series of glycerol. Specimens were stored at 4°C in 100% glycerol until imaging. Fish were imaged on glass depression slides in left lateral recumbency.

Quantitative image analysis

The lateral aspects of Alcian blue and Alizarin red stained individuals were digitally photographed (Nikon Eclipse E600; Nikon DXM1200; NIS-Elements 3.20.01). Images (1 image per fish, n=15/treatment) were analyzed using ImageJ for standard length measurements, caudal fin area [53], and stain quantification.

Craniofacial skeletal components and digital landmarks were identified according to published work [54,55]. Images with uneven background illumination or color cast were adjusted using background correction or a white balancing plugin [56]. Images were then quantified for bone and cartilage using the color deconvolution plugin for ImageJ [57] using new stain vectors developed from non-experimental fish stained with only one dye at a time. The color deconvolution plugin generates individual images of single stained areas from a co-stained individual. Each of these separated images was thresholded and the stained area (μm^2) was calculated. If insufficient clearing of the surrounding tissues resulted in staining of unneeded areas, then Auto Local Threshold with the Sauvola method (radius of 50, Parameter 1 of 0.05) was used [58]. Data regarding Alcian or Alizarin area were normalized as total stained skeletal area (Alcian + Alizarin area) to remove allometric effects of body size. In addition, an area measurement of the caudal fin was recorded using ImageJ using previously described methods [53].

RNA isolation and quantitative real-time PCR

On each sampling day, whole fish were sampled by euthanasia in 300 mg/L MS-222 followed by rapid freezing in liquid nitrogen. Total RNA was extracted from individual whole fish using TRIzol reagent following the manufacturer's recommendation. The concentration of each RNA sample was determined using a Nanodrop 1000 Spectrophotometer (Thermo Scientific). Total RNA was converted to cDNA using the High Capacity cDNA reverse transcription kit (Life Technologies) according to the manufacturer's methods. Approximately 10 ng of cDNA was analyzed in a 20 μL qPCR reaction using Taqman Gene Expression assays with an Applied Biosystems 7300 Real-Time PCR system. Genes encoding the following proteins were selected for quantitative real time PCR analysis of whole body homogenates on each sampling day ($n=6-9$; mean SEM): Deiodinases 1 and 2 [*dio1*, *dio2*], thyroid hormone receptors [*tra*, *tr β*], SRY box containing gene [*sox9a*, *sox9b*], osterix/sp7 transcription factor [*osx/sp7*], runt related transcription factor [*runx2b*]. Table S1 contains a summary of the gene information. Beta-2-microglobulin (*b2m*) was selected as a reference (i.e., housekeeping) gene due to its stable expression between treatments (data not shown). Expression values are reported as the expression ratio relative to control samples within each sample day normalized to *b2m* using the $2^{-\text{Ct}}$ method[59].

Juvenile behavior assays: novel tank diving test and startle habituation tap test

Zebrafish neurobehavioral tasks are a sensitive platform for detecting chemical impacts of toxicants on swimming behavior in both larval and adult animals. Upon reaching 45 dpf, approximately 30 fish per treatment (control, 30 nM 6-OH-BDE-47, or low mixture; due to mortality there were no high mixture treated fish) were tested on two behavioral procedures (novel tank diving test and startle habituation test) to quantify any neurobehavioral effects from toxicant exposure. The 45 dpf time point was selected as the earliest age when fish had achieved sufficient size to be reliably tracked by the Noldus Image Analysis program EthoVision (Wageningen). No fish exhibiting fin malformations were used in behavioral analyses.

The behavioral procedures, novel tank diving, and the startle habituation, have been described in detail previously [60]. The novel tank diving test measures the species-specific swimming pattern of initial bottom dwelling following introduction to a new environment with later exploration of higher levels of the water column. The task is designed to measure predator avoidance behavior (bottom dwelling), similar to measures of thigmotaxis in rodent models [61,62]. Here, the initial dive is characterized as predator avoidance activity, and the later fuller exploration of the tank is thought to be driven by food seeking. Deviation from this dive/explore pattern is considered a maladaptive response.

One day after completion of the dive test, animals were tested for sensorimotor function and habituation learning using a tap startle test as previously described [63]. Briefly, fish were placed individually into swimming arenas, and once per minute for ten minutes the bottoms of the arenas received a mechanical tap stimulus. This task examines the swimming activity of fish before (5-seconds prior) and after (5-seconds post) the delivery of a mechanical stimulus to the swimming chamber. This stimulus is repeated over time, resulting in the fish habituating to this stimulus and demonstrating an attenuated response.

Statistical analysis

Statistical analyses were conducted using Graphpad Prism v. 6.0. PBDE/OH-BDE levels in fish (nmol/g ww) and water (µg/L) are presented as mean ± SEM and were analyzed using a one-way ANOVA with Dunnett's post-hoc test. Changes in gene expression were evaluated with a two-way ANOVA and Tukey's test. For mortality, survival curves were analyzed using a log-rank test; statistical significance was established using a Bonferroni correction for multiple survival curve comparisons. Morphology score data were control normalized and analyzed using a Kruskal-Wallis Test and a Dunn's post hoc test.

For behavioral data, Superanova (SAS) software was used for statistical analysis. For all behavioral tests, no significant effects were observed between tanks of animals, so data were combined. All behavioral data were log transformed prior to analysis. For the novel tank diving test, the mean distance from tank floor and mean distance traveled (cm) was calculated for each minute of the five minute task. For the tap startle test, the mean distance traveled for the 5 sec before and after each stimulus delivery was calculated. The tap activity data were log transformed prior to analysis. A mixed design ANOVA with a between subjects factor of chemical treatment and repeated measures of trial or time was used to assess statistical significance with a Dunnett's post hoc test to determine differences between control and each of the exposure groups. Level of significance for all analyses was p value < 0.05.

RESULTS

PBDE/OH-BDE analysis in water and fish tissues

Measured concentrations of PBDEs in the aqueous exposures were generally lower than our reported nominal concentrations (Figure S1), and these concentrations decreased over the 14 day exposure period. The average water concentration in the low mixture was approximately 48–66% of the target nominal concentration for BDE-47, -99, -100 -153, and -154

(dominant congeners in the DE-71 mixture). For example, the average concentration of BDE-47 in the water was 3.1 ± 1.0 $\mu\text{g/L}$ for the low mixture group and the nominal concentration was 11.4 $\mu\text{g/L}$. The average concentration of 6-OH-BDE-47 in the 30 nM 6-OH-BDE-47 treatment group was measured at 36.7 ± 9.5 nM. The concentrations of 2,4,6-tribromophenol was below the targeted nominal concentration (15 nM) and was measured at 3 ± 4 nM in the low mixture treatment.

Zebrafish exposed to the low mixture treatment accumulated PBDE congeners, with detectable levels after four days of exposure (12 dpf) (Figure S2). Concentrations of the PBDEs in the tissues varied depending on the congener, timepoint, and treatment condition. After two weeks of exposure (23 dpf), concentrations of PBDEs/OH-BDEs significantly increased relative to the 12 dpf measurements for both the 30 nM 6-OH-BDE-47 and low mixture treatment groups. At 23 dpf in the low treatment group, BDE-47 levels were 82.2 ± 11.4 nmol/fish and BDE-99 levels were 15.2 ± 5.1 nmol/fish. After ~ 3 weeks of depuration, concentrations were significantly decreased relative to the 23 dpf measurements, but still elevated relative to the control fish. In the 30 nM 6-OH-BDE-47 treatment group, tissue concentrations increased from 0.21 ± 0.20 nM at 12 dpf to 0.97 ± 0.39 nM at 23 dpf. We were unable to detect 6-OH-BDE-47 or TBP in fish tissues at 45 dpf following the depuration period.

Fish survival

Fish were exposed to the various treatments from approximately 9 dpf to 23 dpf (14 days total) followed by a 22-day depuration period in which fish were maintained in clean water containing no test chemicals (Figure S1). Low overall mortality (3–12%) was observed in the low mixture, 6-OH-BDE-47, and control groups with no statistical differences between each treatment group. Conversely, a significant increase in cumulative mortality was observed among the high mixture treatment group (Figure S4). By the end of the exposure period (23 dpf), approximately 85% of the fish in the high mixture treatment had died. The onset of mortality occurred between 5–9 days into the exposure period (14–19 dpf), with no significant mortality occurring at our first sampling date (12 dpf). Due to the high mortality, the high mixture treatment has been omitted from all further results due to survivor bias and a greatly reduced sample size (additional high mixture treatment data is described in supplemental information).

Fish growth

No significant differences in length or mass were observed in the subsample of individuals examined at the initiation of exposure (9–10 dpf). Fish mass increased from ~ 1 mg at 9–10 dpf to ~ 30 mg by 45 dpf. With the first sampling (12 dpf) we observed no significant effects of treatment on length or mass; however, on the second sampling time point (23 dpf), we observed a significant reduction (9–11%) in the length of fish in the 6-OH-BDE-47 and low mixture treated animals (Figure 1). This reduction in length ($\sim 19\%$) persisted after exposure ceased and was observed at 45 dpf, but was only statistically significant in the 6-OH-BDE-47 treated individuals. No significant differences were observed in mass at the other sampling time points (Figure 1). Calculated growth rates (based on mass) from 9–45 dpf were $0.10 \pm 3.0\text{E-}3$, $0.10 \pm 3.0\text{E-}3$, $0.11 \pm 4.0\text{E-}3$ mgday^{-1} for the control, low mixture, and

30 nM 6-OH-BDE-47 treatment groups, respectively. There were no statistically significant differences between growth rates among treatments groups.

Morphology

Within each sampling time point, growth heterogeneity was apparent among groups, but significant differences in growth rates among treatments were not detected. Growth heterogeneity in zebrafish development, has been reported even for fish raised in isolation but under identical conditions [31]. Decreased morphological scores (sum of all morphological endpoints including caudal, fin ray, pigmentation, and swim bladder score) were observed for overall development at the first 2 sampling time points in the low mixture group suggestive of effects, although the difference was not quite statistically significant at $p < 0.05$ (Figure 2; $p = 0.056$ in the low mixture at 12 dpf). The morphology score in the low mixture group was ~10% lower than the controls at 12 dpf, and ~18% lower relative to the controls at 23 dpf. A Mann Whitney statistical test between the control and low mixture treatment indicated a significant decrease in morphology score at 23 dpf ($p = 0.05$). However, by 45 dpf the overall morphology scores for all treatment groups were roughly equivalent, indicating recovery of any delayed maturation.

In terms of specific morphological endpoints, the greatest change with maturation was observed at the end of the 14 day exposure period. Swim bladder maturation was significantly reduced (~35% score reduction from controls) in the 30 nM 6-OH-BDE-47 treated fish at 23 dpf (Figure 3). Decreased caudal fin, fin ray, and pigmentation scores were also noted at the 23 dpf time point for the low mixture and 30 nM 6-OH-BDE-47 treated groups, but these differences were not statistically different from controls (Figure 3). By the end of the recovery period at 45 dpf, morphology scores for fin ray, swim bladder, and pigmentation were similar between control and treated groups (Figure S5), while there was still a slight decrease in caudal score at 45 dpf.

Cartilage and bone development

Results from the quantitative image analysis of skeletal staining are shown in Figure 4. The ratio of Alizarin Red area/total area (i.e., % bone) significantly increased as fish aged, which is expected given that ossification increases with age. A significant decrease in the ratio of bone area/total area was observed for the 6-OH-BDE-47 treatment group compared to DMSO controls at 12 dpf (Figure 4), indicating reduced bone mineralization. No significant effects on the ratio of cartilage or bone area relative to total area were noted for the other treatments across sampling time points.

Specific deformities to the caudal fin were observed in ~15 % of the low mixture treatment group (Figure 5). The incidence of caudal deformities showed a weak correlation with length (greater percent incidence in smaller fish), but this difference was not statistically significant. Alcian blue and Alizarin red staining revealed normal vertebral centra with uniform intervertebral spacing. However, caudal fins lacked formation of the lepidotrichia. In addition, hypural cartilage plates, normally forming anchor points for lepidotrichia were absent in affected individuals. Caudal fin area was significantly reduced (26–38%) in the 30 nM 6-OH-BDE-47 treated animals at 23 dpf (Figure S6), but not quite significant for the low

mixture treatment ($p=0.10$). There was also a decreasing trend for caudal area at 45 dpf for the low mixture treatment, but these results were not statistically significant ($p=0.12$). Importantly, the caudal area measurements are distinct from the caudal scoring, as areal measurements were taken in stained fish specimens that did not exhibit overt deformities, whereas the scoring was completed in freshly anaesthetized animals (based on criteria in Table 1 and on the shape of the caudal fin).

Gene expression

Gene expression results varied across time and treatment as shown in Figure 6. Expression of *Sox9b* was significantly increased 2.2 fold at 12 dpf in the low mixture treatment compared to DMSO controls. *Sox9a* expression was also significantly increased (4.4 fold, $p<0.05$) compared to control in the low mixture treatment at 23 dpf. Osterix (*osx*) gene expression decreased 38% relative to control levels in the low mixture group at 12 dpf, however these changes were not statistically significant. No further changes were observed in *osx* expression at later time points. *TRa* expression was significantly increased at 12 dpf (3.6 fold, $p<0.05$) in the low mixture group, with no effects at the later time points. There was also an increase in *TRβ* expression (2.2 fold, $p<0.05$) at 45 dpf in the 30 nM 6-OH-BDE-47 treatment, with no changes at earlier sampling times. Other gene expression trends included decreases in *dio* expression and increases in *runx2* expression, but these changes were not statistically significant. Temporal expression for some genes varied over time (*osx*), likely in accordance with normal developmental changes during maturation.

Behavior

Dive test—Spatial location in a novel environment and total distance traveled in that environment were used to quantify behavior during the novel tank exploration procedure. Location in the tank ($F(4,119)=26.547$ $p<0.0001$) and total distance traveled ($F(4,119)=3.772$ $P<0.005$) changed as a function of time spent in the environment, which is consistent with the typical dive/explore swim pattern common during this test. However, there was no significant main effect of exposure on the dive response or swimming speed (distance from floor, total distance traveled per minute; Figure S7).

Tap test—The tap test measures habituation to a repeated startle stimulus by examining swimming activity before and after the delivery of a mechanical tap stimulus. There was a significant reduction in startle activity (net difference distance swum pre/post tap stimulus) with increased tap number ($F(1,87)=122.1$ $p<0.0001$), in all groups. This is indicative of the typical behavior response pattern during this test. There was no significant main effect of exposure on startle response to the tap stimulus ($F(2, 87)=1.95$, $p=0.15$). There was a significant interaction with pre/post tap delivery and tap number ($F(4, 87)=7.705$ $p<0.0001$) (Figure S8), with toxicant-effects only emerging after the tap. This indicates that the alterations in locomotion quantified here are not generalized hypoactivity, rather a specific toxicant-induced attenuation of movement following a tap. There appeared to be a lessened startle response in the mixture-exposed fish. Importantly, there were no significant differences between control and treated fish in terms of total swimming distance, indicating their ability to swim as well as control animals (swimming activity was not impaired).

DISCUSSION

Few studies have examined toxicological effects specifically during juvenile fish life stages (relative to larval and adult life stages), particularly for environmental mixtures. Furthermore, morphological effects and bone development are infrequently measured endpoints in developmental studies, despite their sensitivity to developmental perturbations [31]. PBDEs and their metabolites elicit developmental toxicity, in addition to impacts on thyroid and reproductive function in fishes [21,22]. While most studies have determined toxicity of individual PBDE congeners, few have assessed direct toxicity of mixtures. The present study was unique in that it examined the effects of exposure to a mixture of PBDEs and their metabolites on zebrafish morphology, bone development, behavior, and gene expression during juvenile maturation. Juvenile maturation is a unique exposure period since developing zebrafish undergo a range of morphological transitions. For example, Bar-Ilan et al. (2013) found delayed metamorphosis and stunted growth, when zebrafish (0–23 dpf) were exposed simultaneously to TiO₂ nanoparticles and light [64]. Fish failed to develop fin rays, had poorly organized pigmentation, and the caudal fin failed to transform from the larval tail to the organized tail present in juvenile animals [64]. Similar phenotypes indicative of delayed metamorphosis were observed following exposure to the low dose mixture in the present study, which utilized a similar exposure period (9–23 dpf).

One unanticipated finding was the high acute toxicity in high mixture treatment to zebrafish juveniles, resulting in >85% mortality within 8 days of exposure in all replicate tanks. The mortality was not expected since the reported lethal concentration values (LC₅₀) in zebrafish larvae for individual BDE congeners have been reported as 4,200, 5,200, and 8,400 µg/L for BDE-47, 99, and 100 respectively [65], and the LC₅₀ for 6-OH-BDE-47 was estimated to be 960 µg/L [23]. Water concentrations of BDE-47 & 6-OH-BDE-47 were measured at the study onset in the high mixture treatment to be ~112 µg/L and 22 µg/L, respectively, which are 22–37x lower than the reported larval LC₅₀ value for these chemicals. In a study of juvenile zebrafish exposed to 1,000 µg/L DE-71 from 4–8 weeks of age, mortality was only 15% of the treated animals [66]; however, mortality was eliminated after purification of the DE-71 (i.e., the PBDD/DFs were removed) [66]. The authors also reported that DE-71 treated animals exhibited reduced body weight and length, similar to results observed in the present study [49]. However, this study also used purified DE-71 and thus we did not expect to see any mortality in the high mixture group. In a follow up study, Kuiper et al., [67] found that adult zebrafish (8 mo old) exposed to 16–500 µg/L of analytically purified DE-71 for 30 days showed no enhanced mortality or significant effects on growth, thyroid hormone levels, or reproduction. However, Kuiper et al. [67] reported juvenile toxicity when offspring from these exposed parents were raised to adulthood with continued exposure to DE-71, suggesting that juvenile fish are likely more susceptible to PBDE exposures. Therefore, we hypothesize that the unique exposure window (9–23 dpf) and the mixture of OH-BDEs/PBDEs, likely contributed to the enhanced mortality observed in the high mixture treatments. There is also evidence to suggest that some OH-BDEs can impact mitochondrial function and ATP production, which could have also contributed to the observed acute toxicity [24,26].

Thyroid disruption is a common endpoint of interest for PBDEs/OH-BDEs and has been examined following DE-71 exposure in a few previous studies. A 14 day aqueous DE-71 exposure (10 µg/L) in young fish (0–14 dpf) reduced thyroxine (T4) in pooled fish tissues and increased mRNA expression of thyroid related genes (*dio1*, *dio2*, *TSH*) but had no effect on *TR* gene expression [67]. In contrast, our results demonstrated a significant increase in *TRa* gene expression at 12 dpf, and an increase in *TRβ* expression at 45 dpf; however, no effects were observed on *dio* gene expression at any time point. The differential responses we observed may reflect differences in life stage and exposure conditions, including the presence of 6-OH-BDE-47 and 2,4,6 TBP in the exposure mixture along with DE-71. In addition to effects on thyroid homeostasis, long term exposure to DE-71 can elicit reproductive effects through altered sex ratios, decreased gamete quality, and altered steroidogenesis [68–70]. Reproductive endpoints were not evaluated in the current work, so further studies are needed to discern potential effects of PBDE/OH-BDE mixtures on reproductive maturation.

There is still a limited understanding of how environmental chemicals influence cartilage and skeletal development. Although bone is an endocrine target tissue with potential sensitivity to many chemicals, few studies have examined bone effects following xenobiotic exposures [71]. Thyroid hormones are involved in skeletal development, influencing both chondrogenic and osteogenic pathways [72–74]. Thyroid hormone acts primarily via *TRa* which is highly expressed in bone tissue. The skeletal response to TH is not fully understood, but it does involve several other endocrine factors including growth hormone, insulin-like growth factor, fibroblast growth factor, and other signaling pathways [35]. Formation of early skeletal structures requires strict coordination of gene regulatory network to direct proliferation, expansion, and differentiation of mesenchymal precursor cells towards osteoblasts capable of bone matrix secretion [75,76]. In zebrafish, this process occurs through either membranous, endochondral and/or perichondral ossification [72,75,76]. Ossified structures are subsequently maintained through remodeling involving bone desorption by osteoclasts followed by deposition of new mineral matrix through osteoblast activities [54,77]. A 23% decrease in bone staining was observed at 12 dpf in fish treated with 30 nM 6-OH-BDE-47. However, these changes were not accompanied by detectable differences in *Osx* (*osterix*) gene expression; an osteoblast-specific transcription factor required for osteoblast differentiation [77,78]. This suggests that 6-OH-BDE-47 mediated alterations in bone mineralization may be due to disruption of a different bone mineralization pathway or to effects down stream of *Osx*. No changes in bone or cartilage staining were observed in the low mixture treated fish, or for other sampling time points, suggesting early juvenile development may be more sensitive to alterations in osteochondral development.

Exposure of zebrafish to the low mixture treatment also resulted in a highly specific tail phenotype. Importantly, this phenotype was not observed in the 6-OH-BDE-47 treatment, suggesting that some effects were not driven by the metabolites alone. Approximately ~15% of the low mixture treated fish evaluated for morphology exhibited caudal malformations with an absence of visible hypural cartilage and highly stunted lepidotrichia at the blastema site. This fin phenotype appeared specific to the caudal fin, as there were no notable other fin or vertebral malformations. While less dramatic, other xenobiotics including the brominated

flame retardant TBECH and 2,3,7,8 tetrachlorodibenzodioxin (TCDD), appear to impact cartilage formation in both zebrafish and medaka (*Oryzias latipes*) [67,68]. These effects occurred in concert with significant reductions in *sox9a* and *sox 9b* expression [79,80]. *Sox9* is essential for chondrocyte differentiation and cartilage formation, and directly regulates other proteins that form cartilage matrix protein (*col2a1*) [77]. *Sox9* expression can have stimulatory or inhibitory functions on cartilage development depending on the state of cell differentiation, therefore it is reasonable to assume alterations in *sox9* signaling could promote or inhibit cartilage formation, depending on developmental stage [77,81]. Contrary to observations with TBECH and TCDD, we observed a significant increase in *sox9b* at 12 dpf and *sox9a* at 23 dpf in the low mixture group. This discrepancy in the directionality of *sox9* gene expression may represent treatment effects on chondrogenic gene expression and bone formation that vary with time and treatment.

Vascular supply to affected structures may also play a role in the observed caudal deformities. Embryonic exposure to TCDD resulted in reduced red blood cell perfusion rates that contributed to lower jaw deformities in zebrafish [82]. Caudal tail malformations have also been observed in larval zebrafish exposed to the brominated flame retardant, Tetrabromobisphenol A, which were linked to changes in matrix metalloproteinase expression [83]. In addition, differential timing for onset of exposure must be considered. A combination of 9 and 10 day old fish were used at the start of this experiment. The earliest caudal fin progenitor cells start infiltrating the ventral side of the caudal bud around 8–9 dpf where they initiate formation of the eventual adult fin [84–86]. Since this timing aligns with the onset of exposure, younger individuals may be more grossly impacted. A greater incidence of caudal malformations was observed in smaller fish, but this correlation with fish length was not statistically significant. Additionally, we cannot directly test this hypothesis due to our pooling of 9 and 10 dpf fish just before their distribution to study tanks. Since other fin formation was normal (pectoral, dorsal, anal, pelvic) the onset time of exposure may be especially important since caudal fin differentiation precedes that of other fins [54,84,87].

While effects on some developmental morphologic endpoints were observed at 12 and 23 dpf following exposure to the low dose mixture, there were no treatment related differences in morphology score at the time of behavioral testing at 45 dpf, and the overall behavioral response of treated fish appeared to be normal. No significant behavioral effects in the dive test, either with regard to diving response, its habituation or swimming speed were observed. A modest reduction in motor response resulting from a tap startle was observed in the low mixture group; however, this difference was diminished as the session progressed and the control fish showed diminished response with habituation. This later developmental exposure may be past the window for producing lasting neurobehavioral impairment. Previous work from our laboratory showed persisting neurobehavioral impairments after early developmental exposure (0–6 dpf) to 6-OH-BDE-47 [37], but the exposure period of the current study was later in life. The limited test battery only found modest behavioral effects with juvenile exposure, but it does contribute to the body of literature examining PBDE/OH-BDE impacts on fish neurobehavioral responses.

Limitations of the present study include the growth heterogeneity among individuals, making treatment related effects more challenging to determine [31,51]. Age (i.e., dpf) or length are not necessarily good indicators of fish maturation, which is why morphological markers may be more sensitive endpoints. Temperature, fish density, and water quality can also directly impact the rate of development, potentially confounding results. While we made every effort to keep water quality and temperature equivalent among tanks, we did experience mortality in some treatments, which altered fish density within tanks. Our analyses also utilized whole fish homogenates for gene expression analyses and whole-mount area analyses to look at gross level changes in cartilage and bone staining. It is possible that tissue specific responses were occurring but undetected using the present design and methods. Furthermore, larger sample sizes are needed to overcome the heterogeneity among fish when examining individual growth metrics and cartilage and bone staining.

In conclusion, altered morphological development and toxicity was observed in juvenile animals following exposure to a mixture of flame retardant chemicals and their potential metabolites. This work comes with several caveats, including high mortality in one of the treatments. Future studies should attempt to measure thyroid hormone levels in tissues, to anchor phenotypic developmental effects with endocrine disruption at the tissue level. These results were different from what might have been predicted from tests in larval animals and from tests with single compounds, which emphasizes the need for additional mixture testing in toxicological screenings. This work also highlights the importance of studying intermediate life stages, and chemical mixtures, since embryonic and adult studies may not be predictive of toxicity when exposure occurs in developing juvenile life stages.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACE	Acetone
DMSO	Dimethyl Sulfoxide
DCM	Dichloromethane
DE-71	PentaBDE commercial mixture
FR	Flame Retardant

F1	Fraction 1
F2	Fraction 2
HEX	Hexane
PBDE	Polybrominated Diphenyl Ether
OH-BDE	Hydroxylated Polybrominated Diphenyl Ether
TH	Thyroid Hormone
hpf	Hours Post Fertilization
dpf	Days Post Fertilization
Dio	deiodinase enzyme
T₄	Thyroxine
T₃	Triiodothyronine
TR	Thyroid receptor

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Figure 1.

Juvenile fish length (A) and mass (B) at various times over study duration. Significant reduction in length was observed in treated groups at 23 dpf, with persisting reductions in the 30 nM 6-OH-BDE-47 treatment at 45 dpf. Data are mean $\pm$ SEM (n=30/treatment). Asterisks indicate significant differences from controls (p<0.05).

Figure 2.

Morphology scores across time and treatment relative to controls. The overall morphology score is a sum of individual morphology endpoint scores as described in Table 2. Data are mean $\pm$ SEM ($n=30$ fish 12 dpf, and $n=45$ fish 23 and 45 dpf). No values were statistically significant at the $p<0.05$ level.

Figure 3.

Summary of individual morphological endpoints following end of the exposure period (23 dpf). Data are presented as mean $\pm$ SEM (n=45/treatment). Decreasing trends in morphology scores were observed for caudal fin, pigmentation, and dorsal/anal fin ray score but these were not statistically significant. There was a significant reduction in swim bladder score for the 30 nM 6-OH-BDE-47 treatment group. Asterisks indicate significant differences from control group ($p < 0.05$).

Figure 4.

ImageJ quantitation of blue and red pixel area (Alcian blue or Alizarin red staining, respectively) relative to total stained area (μm^2) in 12 dpf, 23 dpf, and 45 dpf intact juvenile fish. A significant reduction in bone area was observed in fish treated with 30 nM 6-OH-BDE-47 at 12 dpf (panel B), but statistically significant differences were not observed at other time points. Data are mean $\pm$ SEM ($n=15/\text{treatment}$). Asterisks indicate significant differences from controls ($p<0.05$).

Figure 5.

Representative caudal fins stained with Alcian blue and Alizarin red to illustrate cartilage and bone development. A subset of treated individuals (~15% low mixture group) exhibited caudal malformations when the hypural cartilage failed to form and resulted in a bundle of stunted lepidotrichia at the blastema site (arrowheads). This caudal malformation was in the absence of other fin or vertebral malformations

Figure 6.
Summary of gene expression data (mean $\pm$ SEM n=6-9/treatment) across time and treatment. Asterisks indicate significant differences from controls (p<0.05).

Table 1
Sampling Regimen for Study End Points Evaluated

Overview of the sampling set-up for each endpoint evaluated in this study. Importantly, after the first sampling time (12 dpf), the high mixture treatment group contained fewer individuals for sampling due to mortality. Fish were evenly sampled among the replicate treatment tanks for each collection.

<u>Study End Point</u>	<u>Sample Size</u>	<u>Sampling Times</u>
<i>PBDE/OH-BDE Uptake</i>	N=3 tanks (10–40 fish pooled per tank)	9dpf, 12dpf, 23 dpf, 45 dpf
<i>Morphology</i>	N= 30–45 fish/treatment	12 dpf, 23 dpf, 45 dpf
<i>Alcian Blue/Alizarin Red Examinations</i>	N= 15 fish/treatment	12 dpf, 23 dpf, 45 dpf
<i>mRNA expression</i>	N=6–9 individual fish/treatment	12 dpf, 23 dpf, 45 dpf
<i>Behavior</i>	N=30 fish/treatment	45 dpf

Table 2

Summary of morphological parameters and scoring system used to assess development and fish maturation. Scoring systems were modified from previous work describing postembryonic zebrafish development [31,51]

<u>Morphology Score</u>	<u>Description</u>	<u>Numeric Score</u>	<u>References</u>
Swim Bladder Differentiation	Single chambered (0) Intermediate (1) Two Chambered (2)	0–2	[31]
Caudal Fin Differentiation	Transitioning from larval paddle caudal fin (1) to the adult forked form (5)	1–5	[51]
Body Pigmentation	Transitioning from larval (melanophores) to juvenile (iridophores, xanthophores, iridiphores)	1–5	[51]
Anal/Dorsal Fin Differentiation	Absent (0), forming/bud (1), or formed (2)	0–2	Modified from [51]
Fin Rays	Absence fin rays (0) Presence of fin rays only caudally (1) Presence fin rays in caudal/anal/dorsal fins (2)	0–2	Modified from [51]
Overall Score		Max 16	