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# Toxic effects of polybrominated diphenyl ethers (BDE 47 and 99) and localization of BDE-99–induced *cyp1a* mRNA in zebrafish larvae

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ABSTRACT

Polybrominated diphenyl ethers (PBDEs) were once widely used as flame retardants in furniture and electronic products, and contamination persists in developing countries due to the dismantling of electronic waste. Our previous study confirmed that 2,2′,4,4′,5-pentabromodiphenyl ether (BDE-99) induced cytochrome P450 1A (Cyp1a) via aryl hydrocarbon receptor (Ahr)–mediated signaling in the zebrafish liver cell line (ZFL) *in vitro*. In this study, the toxicities of BDE-47 and BDE-99 at environmentally relevant concentrations (50 and 500 nM) were evaluated in newly hatched zebrafish (*Danio rerio*) larvae *in vivo*. A time-course study (8, 24, 48, and 96 h) was performed. BDE-99 was observed to cause yolk sac edema and pericardial edema after 72 h of exposure. Real-time polymerase chain reaction assay and whole-mount *in situ* hybridization assay confirmed *cyp1a* induction by BDE-99 in the liver and intestine. Continuous down-regulation of *trβ* by as much as 2.1-fold after 96 h and transient down-regulation of *ttr* by 7.1-fold after 24 h indicated the interference of BDE-99 in the thyroid hormone system. *cyp1a* induction was also observed in BDE-47–treated larvae, but cellular localization of *cyp1a* was not confirmed by whole-mount *in situ* hybridization. The induction of four *cyp1* genes (*cyp1a, cyp1b1, cyp1c1* and *cyp1c2*) by both BDE congeners warrants further study to understand the *in vivo* metabolism of BDE-47 and BDE-99 and the dioxin-like toxicity potencies of the OH-/MeO-PBDEs. The data obtained in this study will aid the characterization of molecular disorders caused by PBDEs in fish and help to delineate better models for toxicity assessment of environmental pollutants in ecological systems and in other vertebrates such as humans.

## 1. Introduction

Polybrominated diphenyl ethers (PBDEs) were once widely used in industry and consumer products as flame retardants. Their ubiquitous use since the 1970s and the fact that they are highly lipophilic, persistent, and bioaccumulative have resulted in widespread contamination. In the United States, the production of two major commercial PBDE mixtures, pentaBDE and octaBDE, was voluntarily ceased in 2004, and decaBDE was also phased out in 2009 due to its potential health risks [1]. The trends of PBDE levels detected worldwide differ. Temporal analysis of PBDE levels in North America revealed that the levels were climbing before around 2005 but then decreased, probably due to the rising public concerns and government strengthening of the regulation of disposal of electronic waste [2]. However, in East Asian countries, including China, PBDE levels began to increase in the 1970s and there was no sign of a decline in this trend until recently [3]. The faster increase of PBDEs from the 1990s in China was due to the blooming of the manufacturing industry, the extensive legal and illegal importation and recycling of PBDE-containing products from developed countries, and inappropriate disposal and dismantling of electronic waste [4].

Humans and ecosystems are exposed to PBDEs through many activities. Food consumption and absorption of house dust are the major pathways of PBDE exposure. In the case of children, hand-to-mouth activity and breast milk are two major exposure sources. The most abundant contaminants are 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) and 2,2′,4,4′,5-pentabromodiphenyl ether (BDE-99) in food, whereas decabromodiphenyl ether (BDE-209) is predominantly detected in indoor dust and in atmospheric particles [4]. In Hong Kong, the dietary intake of PBDEs to Hong Kong residents was estimated between 510 ng $day^{-1}$ for marine fish and 924 ng $day^{-1}$ for freshwater fish, which was higher than that calculated in other countries and raised a concern about PBDE contamination of fishery products [5]. The burdens of PBDEs in both marine and freshwater fish were reported to range from 3.0 ng/g to 5366.07 ng/g in lipid weight [6–10]. Bioaccumulation of PBDEs in aquatic species and further transfer to humans warrants a more careful risk assessment than those previously performed.

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In humans, endocrine disruptors are susceptible to cause obesity and diseases via various signaling pathways [11]. Human exposure to PBDEs is related to endocrine system disorder, neurodevelopment interference, and energy balance interruption [12]. A study of 207 pregnant women suggested that exposure to PBDEs (26.5 ng/g lipids) is associated with lower levels of thyroid-stimulating hormone (1.2 mU/L) during pregnancy, in which a 10-fold increase in PBDEs was associated with a 16.8% decrease in thyroid-stimulating hormone; these results have implications for maternal health and fetal development [13]. Both prenatal and childhood PBDE exposures were reported to be associated with poorer attention, fine motor coordination, and cognition in school-age children whose mother lived in California's agricultural Salinas [14]. PBDEs exposure in breast milk at 3 months postpartum were shown to be associated with increased anxiety in early childhood of 36 months age [15,16]. A survey on a U.S. population group showed involvement of PBDEs in the pathogenesis of diabetes and metabolic syndrome [17]. Nevertheless, the underlying mechanism has yet to be clarified.

Zebrafish are small tropical freshwater fish that are a useful model in toxicological studies [18–20]. Using zebrafish, both forward and reverse genetic techniques, coupled with large-scale, high-throughput screening, can help to identify the endpoints of toxicity and elucidate mechanisms derived by the toxicant. For instance, whole-mount *in situ* hybridization (WISH) and immunochemical analysis enable toxicologists to screen for chemical-induced abnormalities in the expression of specific genes in local tissues. Morpholino oligonucleotides provide manipulation of RNA at the embryonic stage. Gene alteration with CRISPR technology is also available and could be widely used [21].

Our previous *in vitro* study of the effects of BDEs in the zebrafish liver cell line (ZFL) model demonstrated that BDE-99 significantly induced *cyp1a*, *ugt1ab,* and an Ahr-dependent xenobiotic response element luciferase reporter system in a time-dependent manner [22,20]. We also confirmed the Ahr-mediated activation by BDE-99 in ZFL, but BDE-47 did not exhibit these effects in ZFL cell model [22]. Using zebrafish embryos and larvae, Chan and Chan [23] confirmed the impacts of BDE-47, bisphenol A, tetra-bromo bisphenol A on thyroid hormone related genes including receptors, transporters and a few metabolizing enzymes except the Phase I and II drug metabolizing enzymes. Kakutani et al. [24] recently also confirmed the induction of CYP1A and EROD by coplanar polychlorinated or brominated biphenyls in HepG2 cell, and the polymorphisms of CYP enzymes may provoke pathological conditions [25]. To better understand the CYP1A induction of these two BDE congeners in whole organism, the temporal and spatial expressions of *cyp1a* were assessed in zebrafish (*Danio rerio*) larvae *in vivo* from 2 h after hatching (8, 24, 48, and 96 h of chemical exposure) in this investigation. Exposure concentrations of 50 and 500 nM were chosen referring to the PBDEs levels detected in diverse water samples ranging from pg/L to ng/L [26–30]. RNA profiling for the genes tested in the *in vitro* ZFL cells [22] were also performed with real-time polymerase chain reaction (RT-PCR). Deformity of the larvae upon exposure was viewed under a stereomicroscope. WISH was used to confirm the tissue-specific *cyp1a* expression. This study accessed the disruption of PBDEs in the early life stages of zebrafish and provides new insight into the effects of BDEs in developing vertebrates, including humans.

## 2. Materials and methods

### 2.1. Animal ethics statement and chemical license statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by Animal Experimentation Ethics Committee (AEEC) of the Chinese University of Hong Kong (Reference Number: (13–319) in DH/HA&P/8/2/1 Pt.31). The use of BDE-47 and BDE-99 were approved by the Environmental Protection Department of Hong Kong (Permit Number: HU-3 C0018/15/01).

### 2.2. Chemicals and solutions

BDE-47 (CAS 5436-43-1, 100% purity) and BDE-99 (CAS 60348-60-9, 99.2% purity) were purchased from AccuStandard Inc. (New Haven, CT). Benzo[a]pyrene (BaP, CAS B1760) was purchased from Sigma-Aldrich Co. (St. Louis, U.S.A). Working solutions of BDE-47, BDE-99 (50 and 500 nM) and BaP (500 nM) were diluted from stock solutions prepared in dimethyl sulfoxide with sea salted water (0.0375% sea salt in deionized distilled water). The vehicle control solution contained 0.005% dimethyl sulfoxide. A 4% (w./v.) paraformaldehyde solution was prepared in phosphate-buffered saline solution (PBS) with the pH adjusted to 7.4.

### 2.3. Production and maintenance of zebrafish (Danio rerio)

All procedures for maintaining zebrafish adults, obtaining eggs, and rearing embryos and larvae were carried out according to [31] and described in Chan and Chan [23]. A zebrafish line of AB obtained from Oregon (USA) was maintained in the laboratory at 28 °C with a light-dark period of 14:10 h. Adults were fed an artificial diet (48% protein, Mediaquafish, Japan Pet Drugs Co. Ltd, Tokyo, Japan) supplemented daily with live brine shrimp larvae. The zebrafish larvae were fed artificial diets of small micron size (ZM-100, ZM Fish Food, UK).

### 2.4. Zebrafish larvae exposure

Male and female zebrafish were incubated overnight at 28 °C, and spawning began when the lights were turned on the next morning. Embryos were collected 2 h after fertilization, washed with distilled water, and randomly picked and transferred to glass beakers for exposure. Each beaker contained 80 larvae and 25 mL BDE congener or BaP exposure solution. Exposures were carried out in triplicate at 28 °C with a light: dark period of 14: 10 h. Upon reaching the desired exposure time of 8, 24, 48, or 96 h, 20 larvae were randomly picked and transferred into 1.5-ml tubes. The larvae were killed by hypothermal shock in ice water for 20 min [32]. The larvae were then submitted to fixation, photography, RNA extraction, and RT-PCR or whole mount *in situ* hybridization (WISH) assays.

### 2.5. Fixation and photography

Zebrafish larvae were fixed in 4% (w./v.) paraformaldehyde and stored overnight at 4 °C. The larvae were then embedded in glycerol for photography. Photographic analysis was done with a stereomicroscope (Olympus SZX16).

### 2.6. RNA and RT-PCR analysis

Twenty whole larvae were homogenized in cold Takara RNAiso plus reagent (Takara, CAS 9109) with a sterile syringe. The following procedures, including total-RNA extraction, reverse transcription, and RT-PCR analysis, were performed as described in our previous study [20]. The expression of each target gene transcript was normalized to that of the house keeping gene EF1α, and fold-induction analysis was conducted using the $\triangle\triangle$Ct method. The sequences of primers used to quantify target gene expressions are shown in Table 1.

### 2.7. RNA probe synthesis

The antisense and sense zebrafish *cyp1a* (Accession No: NM_131879.1) probes were cloned by PCR amplification. Using cDNA from zebrafish larvae harvested 48 h after fertilization, a DNA fragment of 763 bp was amplified with forward primer of GGC TAA CGT AAT CTG CGG GA

**Table 1**
Nucleotide sequences of primer pairs used in RT-PCR assay.

| Oligos | Accession Number | Sequence (5′- > 3′) | | Amplicon Size (bp) |
|---|---|---|---|---|
| | | Forward | Reverse | |
| CYP1A | NM_131879.1 | GCATTACGATACGTTCGATAAGGAC | GCTCCGAATAGGTCATTGACGAT | 147 |
| CYP1B1 | NM_001145708.1 | TTGTCAGGTATCCAGAAATCCAG | GTGTCTTTGGTCGTGCTGTG | 184 |
| CYP1C1 | NM_001020610 | TGAGTGCTGATGGACGACAC | ACGCTTCTTACAGGGTTGGG | 220 |
| CYP1C2 | NM_001114849 | GAAAACAGCAGGGCGTTGAG | CAAAGGGTCCCGGAAGTCTC | 244 |
| CYP2Y3 | NM_001020822.1 | TGTTCACCGTGTCTCTGCTT | TGCAAACATGATGACAAGAGGATT | 231 |
| CYP3A65 | NM_001037438 | CCCGGTGGACTATGAAACCC | TCCGGTTCGCTCCAGTAATC | 207 |
| ef1α | NM_131263.1 | ACCTACCCTCCTCTTGGTCG | GGAACGGTGTGATTGAGGGA | 164 |
| Gapdh | NM_001115114.1 | TATAGCGAGAGGGACCCAGC | ACCCATGACAAACATGGGGG | 174 |
| rp18 | NM_200713.1 | TGGTGTGGCTATGAACCCTG | TGGACGGTCTTTGTTCCTCG | 159 |
| β-actin | NM_131031.1 | ACGAGAGATCTTCACTCCCCT | TGCCAACCATCACTCCCTGA | 189 |
| Hprt | NM_212986.1 | ATGGACCGAACTGAACGTCT | CTGTCATGGGAATGGAGCGA | 163 |
| dio I | NM_001007283.1 | TCTATCAGGGAGGCATCGGA | TTCCATCCGCCCCAATGTTT | 229 |
| dio II | NM_212789.3 | ACAAACAGGTGAAATTGGGCG | AGACCAGCAGGAAATCTGCC | 246 |
| dio III | NM_001177935.2 | ACAGCAACAAGATGTTCACGC | GTTGAGGATCAGCGGTCTCC | 188 |
| Trβ | NM_131340 | GGGTCATTTCAGGCCACGTA | GCAGAGCCTTACATCCTGCC | 215 |
| Ttr | NM_001005598.2 | GCCAGTGGGAAAGTGGACAT | TGTGGTGTACGAGAAAGGGC | 222 |
| ugt1ab | NM_213422 | CCACCAAGTCTTTCCGTGTT | GCAGTCCTTCACAGGCTTTC | 168 |
| ugt2a1 | NM_001191050 | GGCCCAGAAACTGGACATACC | GGGTTTACCCAGGACCTCAG | 250 |
| sult1-st1 | NM_182941 | CACCACAATGGACATGCCTGA | GGTGGTACCTGCTTTGGGG | 172 |
| sult1-st2 | NM_183347 | CCCTTCATGCGGAAAGGTAAA | TAAACAGATTTCCAAGTAGGCCA | 209 |
| sult1-st3 | NM_183348 | GGACAGCCTGAACCAGGAGACT | TCTGCGGCTGAGGTGGACAAT | 218 |
| sult1-st5 | NM_001199903 | GTGCGCATGCCGTTTTTAGA | CGGGCCACATATATAACCTTGC | 164 |

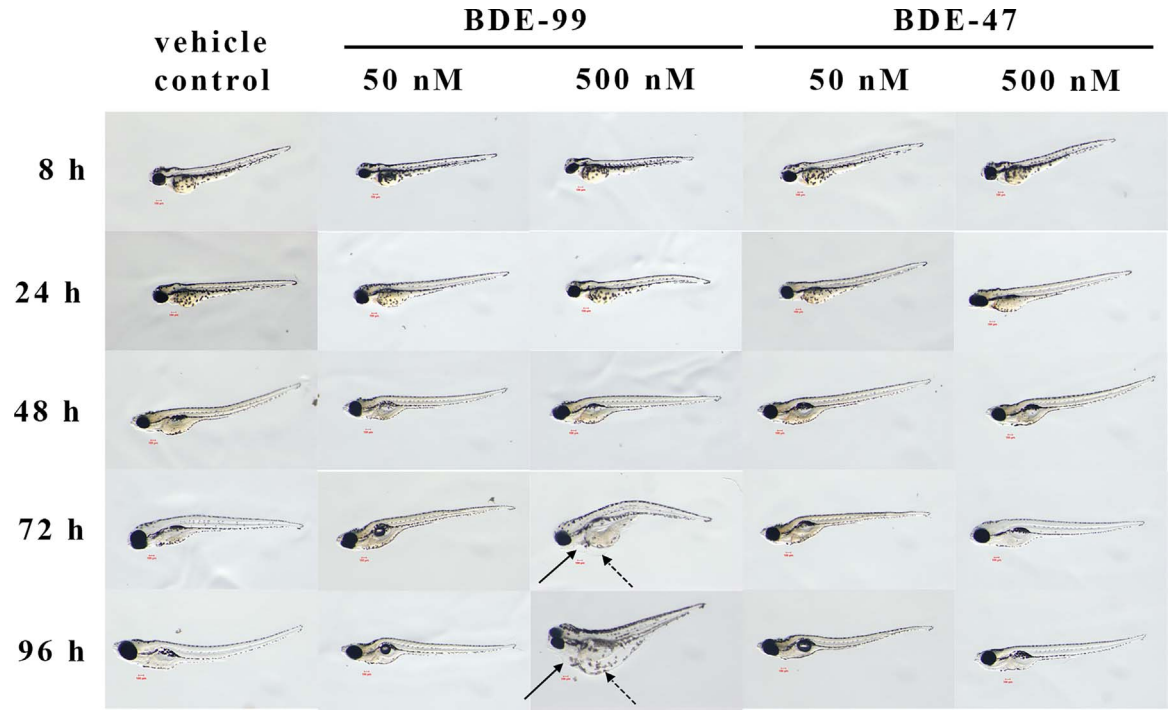


**Fig. 1.** Time-course physiological effect in newly hatched zebrafish larvae exposed to BDE-99 and BDE-47. Two-hour post-hatching (2 hph) larvae were exposed to BDE-99 and BDE-47 of different concentrations (50 nM and 500 nM) for 8 h, 24 h, 48 h, 72 h and 96 h. Paracardiac edema and yolk sac edema were observed in larvae exposed to BDE-99 and noted in solid line arrow and dotted line arrow, respectively. Images were captured from lateral view. All scale bars are 100 μm.

and reverse primer of CAC CTT CTC GCC TTC CAA CT. The PCR product was diluted 10-fold and was used as a template for probe synthesis, which contained a promoter for T7 RNA polymerase. An antisense zebrafish *cyp1a* probe of 450 bp was amplified with the following primers: forward, TGA GTT CGG GAA GAT CGT GG; reverse, ATA ATA CGA CTC ACT ATA GGG ATG AAG GAC TCC AGA AGCGG. A sense zebrafish *cyp1a* probe of 450 bp was amplified with the following primers: forward, ATA ATA CGA CTC ACT ATA GGG TGA GTT CGG GAA GAT CGT GG; reverse, ATG AAG GAC TCC AGA AGC GG. After amplification, 200 ng purified PCR products were put forward to digoxigenin-labeled RNA probe synthesis as manual suggested (Roche CAT# 11277073910).

### 2.8. WISH

The WISH protocol used in this study was modified from Thisse and Thisse [33]. Zebrafish larvae were fixed overnight in 4% paraformaldehyde/PBS at 4 °C and were then incubated in 3% $H_2O_2$/0.5% KOH medium for 20 min at room temperature until pigmentation completely disappeared. After washing once in PBS, the larvae were

**Table 2**
Number of larvae with edema deformity exposed to BDE-99 and BDE-47 at different concentrations (50 nM and 500 nM) for up to 96 h.

| Exposure | Edema (%) | | | |
|---|---|---|---|---|
| | 24 h | 48 h | 72 h | 96 h |
| vehicle control | 0.00 ± 0.00 | 0.42 ± 0.01 | 0.00 ± 0.00 | 0.42 ± 0.01 |
| 50 nM BDE-47 | 1.08 ± 0.01 | 1.08 ± 0.01 | 1.08 ± 0.01 | 0.67 ± 0.01 |
| 500 nM BDE-47 | 0.00 ± 0.00 | 0.42 ± 0.01 | 3.08 ± 0.02 | 1.67 ± 0.02 |
| 50 nM BDE-99 | 0.00 ± 0.00 | 4.00 ± 0.05 | 7.33 ± 0.06 | 6.67 ± 0.06 |
| 500 nM BDE-99 | 0.42 ± 0.01 | 11.5 ± 0.03** | 20.83 ± 0.02** | 37.33 ± 0.02** |

The data are shown as the means and SDs of triplicates. The symbol "**" indicates a significant difference from the control group ($p < 0.01$). The statistical results are derived from one-way ANOVA/Tukey's multiple range tests.

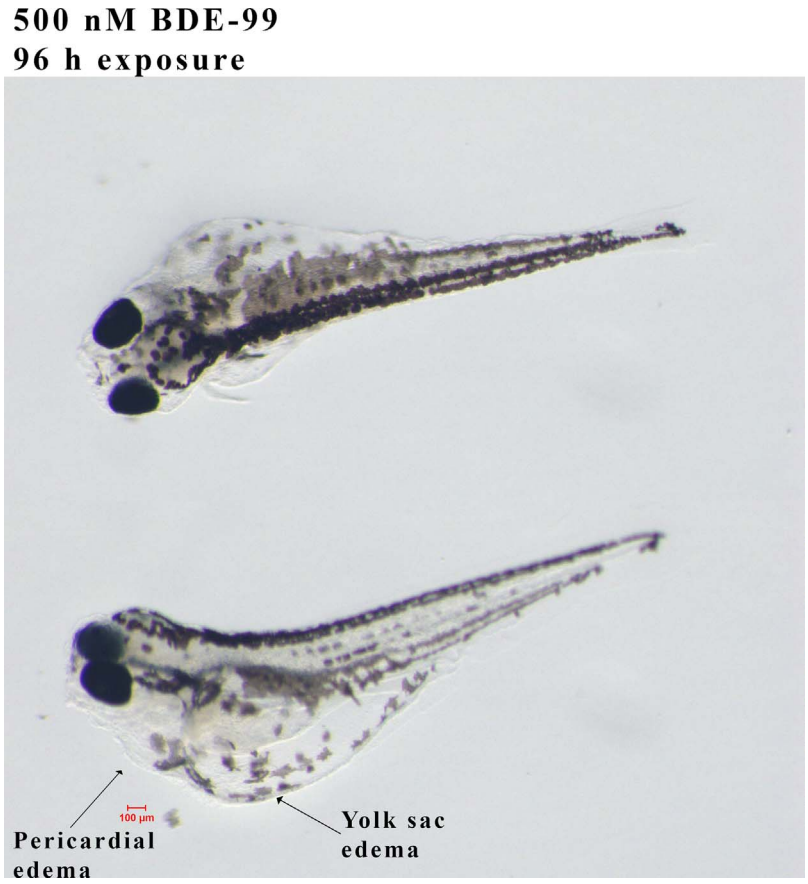


**Fig. 2.** Yolk sac edema and pericardial edema in newly hatched zebrafish larvae exposed to BDE-99 of 500 nM at 96 h. Two-hour post-hatching (2 hph) larvae were exposed to BDE-99 of 500 nM for 96 h. Paracardiac edema and yolk sac edema were observed. Larvae was pictured at dorsal view (upper section) and lateral view (lower section) with scale bar of 100 μm.

progressively dehydrated in 25% (v./v.), 50%, 75%, and 100% methanol for 5 min each at room temperature. All further steps were carried out at room temperature unless stated otherwise. The larvae were treated with proteinase K (10 μg/ml) for 30 min and then re-fixed in 4% paraformaldehyde for 30 min. After washing five times (5 min each) with PBST (PBS with Tween-20), the larvae were pre-incubated in the hybridization mix containing 50% formamide, 5× saline sodium citrate (SSC), pH 6.0 (adjusted with 1 M citric acid), 50 μg/ml yeast RNA, 50 μg/ml heparin, and 0.1% Tween-20 at 68 °C for 4 h. The larvae were then hybridized overnight in fresh hybridization mix containing 200 ng of digoxigenin-labeled RNA at the same temperature.

After hybridization, the larvae were rinsed one time in fresh hybridization mix for 10 min, three times in 2×SSC for 20 min, at 68 °C. Excess RNA was removed by incubating the larvae in 2×SSC containing 20 μg/ml RNase A and 10 U RNase T1 at 37 °C for 1 h. Excess RNase was then removed by washing the larvae with 2×SSC twice for 30 min each at 68 °C. The subsequent washings were 15 min twice in PBST. After pretreatment in PBST containing 2% BMB (Roche) for 30 min, and in PBST containing 2% BMB and 20% goat serum (blocking buffer) for 1 h, the larvae were incubated with fresh blocking buffer containing 1:5000 diluted anti-digoxigenin antibody (Roche) for 4 h. The excess anti-digoxigenin antibody was then removed by washing the larvae in PBST twice at room temperature for 30 min each, at 4 °C overnight, and 8 times at room temperature for 30 min each. The larvae were then incubated in alkaline tris buffer and transferred to BM purple staining solution. When the desired staining intensity was reached (around 24 h), the larvae were stored in 4% paraformaldehyde until photographed. The specimens were mounted in glycerol and photographed with an Olympus SZX16 camera.

### 2.9. Statistical analysis

Statistical analysis of real time PCR data was done on base-10 log-transformed data, then back-transformed for the graph. All statistical analyses were carried out with PRISM 6 with one-way analysis of variance set at a 95% confidence level and with Dunnett's test.

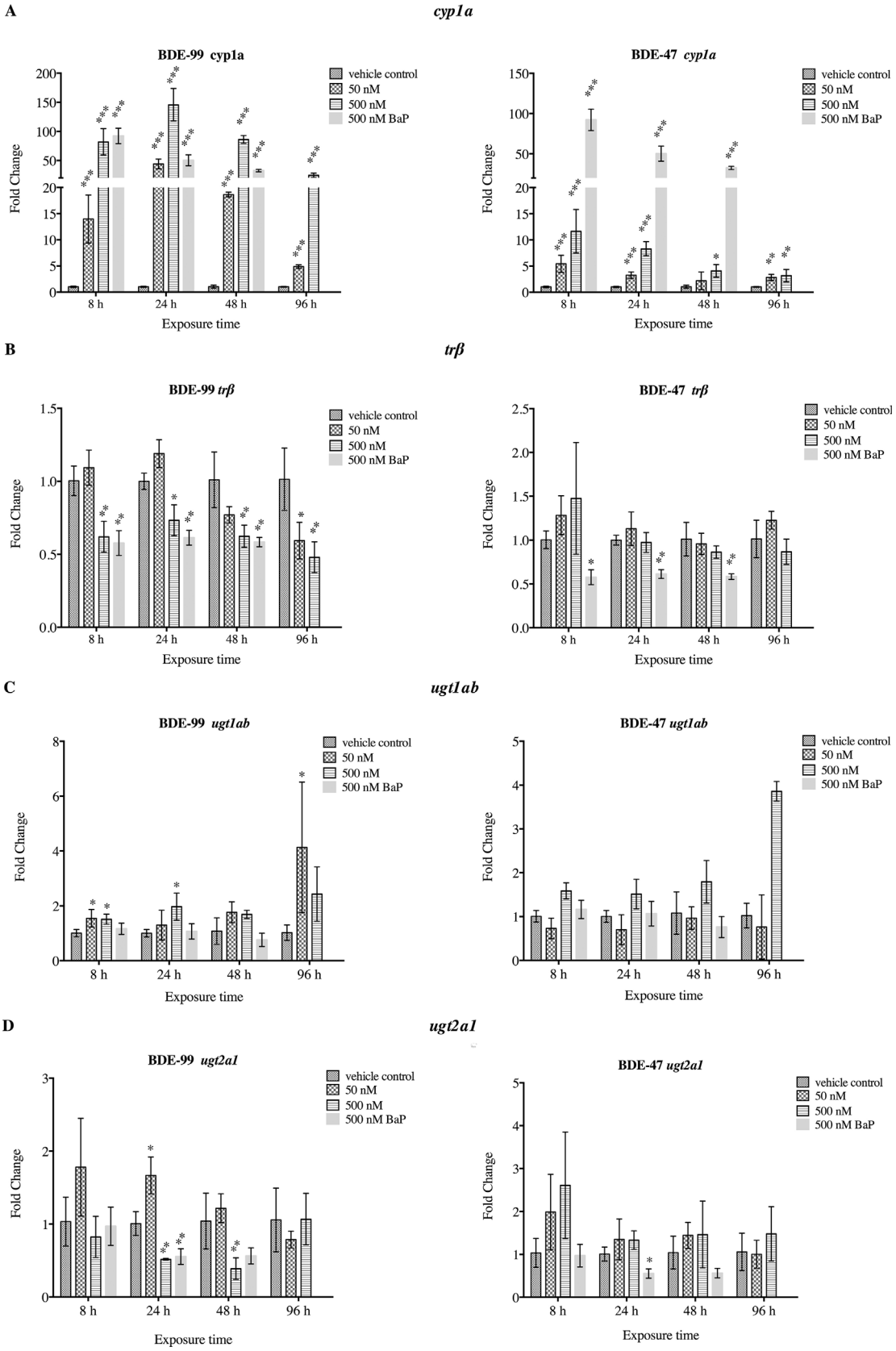


**Fig. 3.** Time-course (8 h, 24 h, 48 h, 96 h) dependent profiling of *cyp1a, trβ, ugt1ab* and *ugt2a1* in newly hatched zebrafish larvae exposed to BDE-99 and BDE-47. (A) *cyp1a*, (B) *trβ*, (C) *ugt1ab* and (D) *ugt2a1* were additionally profiled in larvae 8 h and 48 h. Data are shown as means with SD of triplicates. BaP of 500 nM exposure was added as a positive control of Cyp1a agonist. Statistical analysis was done on base-10 log-transformed data, then back-transformed for this graph. The statistical results were carried out with one-way ANOVA set at a 95% confidence level and with Dunnett's test. The symbol "*","**" and "***" indicates a significant difference from vehicle control group of $p < 0.05$, 0.01 and 0.001, respectively.

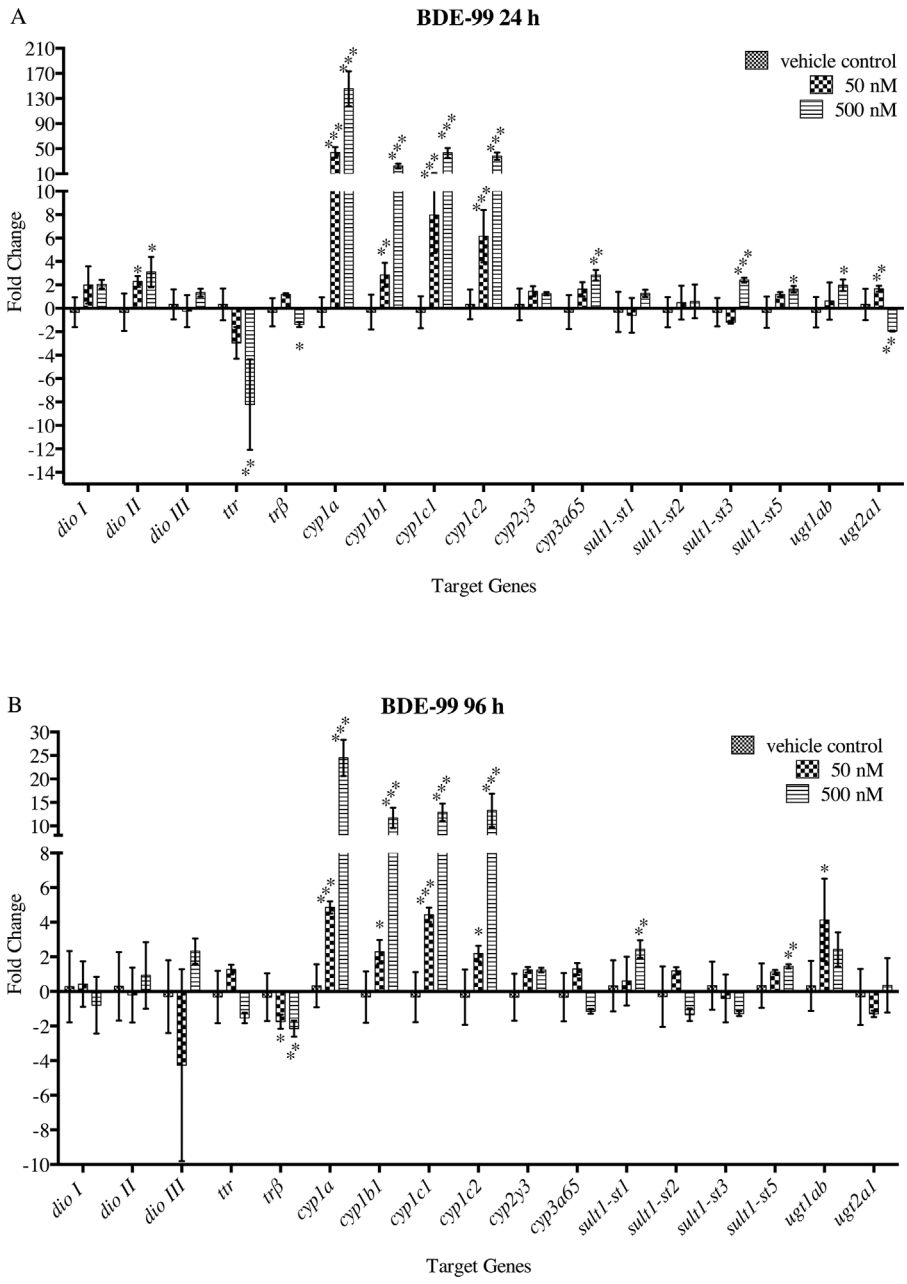


**Fig. 4.** Real time PCR results in newly hatched larvae exposed to BDE-99. Two hph larvae were exposed to at different concentrations of BDE-47 (50 nM and 500 nM) for 24 h and 96 h. Genes of deiodinases (*dio I/II/III*), transthyretin (*ttr*), thyroid hormone receptor β (*trβ*), cytochrome P450 enzymes (*cyp1a*, *cyp1b1*, *cyp1c1*, *cyp1c2*, *cyp2y3*, *cyp3a65*), sulfotransferases (*sult1-st1/2/3/5*) and UDP-glucuronosyltransferases (*ugt1ab*; *ugt2a1*) were profiled in whole larvae. Statistical analysis was done on base-10 log-transformed data, then back-transformed for this graph. The statistical results were carried out with one-way ANOVA set at a 95% confidence level and with Dunnett's test. The symbol "*","**" and "***" indicates a significant difference from vehicle control group of $p < 0.05$, 0.01 and 0.001, respectively.

## 3. Results

### 3.1. Physiological deformity

Neither concentration (50 or 500 nM) of either BDE-47 or BDE-99 was able to cause larval death during exposure. Both BDE congener-dependent and exposure concentration–dependent deformities were observed in this study (Fig. 1). The edema observed during exposure is summarized in Table 2.

For larvae exposed to 500 nM BDE-99, significant deformity of yolk sac edema and pericardial edema were observed after 48 h of exposure and grew more severe at later stages, leading to whole-body edema. Another sign was the failure to form an inflated swim bladder. The edema deformity was most serious at 96 h exposure as shown in Fig. 2. The edema also resulted in a curved body and bone deformity, but the details warrant further investigation with somites or bone parts. No significant deformity of edema was found in larvae exposed to 50 nM BDE-99, head appeared smaller than the controls after 48 h of exposure. After 72 h of exposure, more pigment was found around the bladder under stereomicroscopy. Smaller head and bladder with more pigment continued to be observed after 96 h of exposure.

For larvae exposed to 50 nM BDE-47, no significant edema was observed in BDE-47–treated larvae. An increase in bladder pigment similar to that seen in BDE-99–treated larvae was observed after 48 h of exposure. More red blood cells near the heart after 48 h of exposure were found in larvae dosed with both concentrations of BDE-47; this effect can be continuously found after exposure to 50 nM BDE-47 for 72 h.

### 3.2. Both BDE congeners induced CYP1 family mRNA expressions

BaP here added as a positive control of Cyp1a agonist, up-regulated *cyp1a* expression by 92.2-fold at 8 h and the up-regulation decreased to 32.6-fold at 48 h exposure. Results of gene expressions time were absent at 96 h exposure when larvae under 500 nM of BaP exposure didn't survive at all. BDE-99 significantly induced *cyp1a* throughout the

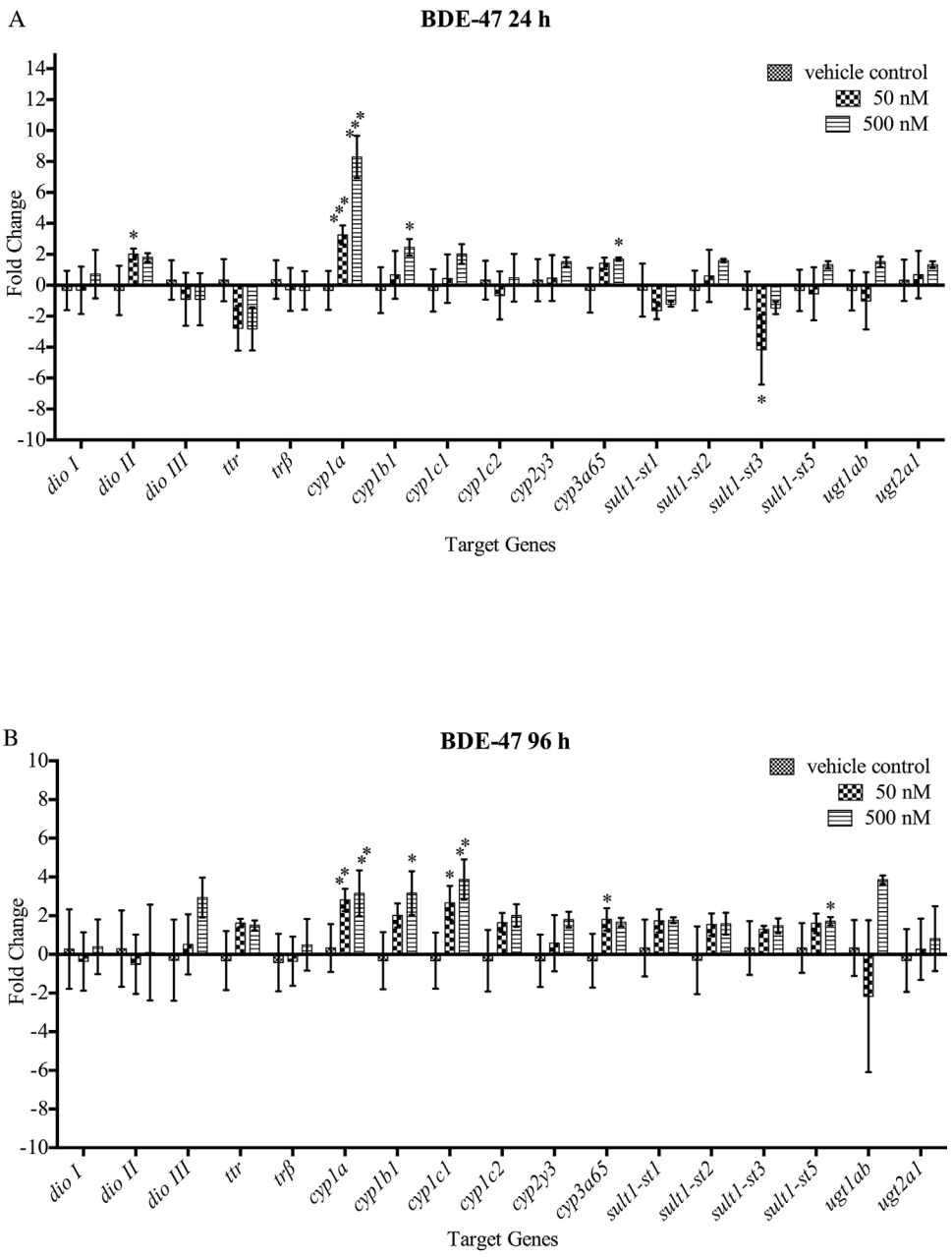


**Fig. 5.** Real time PCR results in newly hatched larvae exposed to BDE-47. Two hph larvae were exposed to at different concentrations of BDE-47 (50 nM and 500 nM) for 24 h and 96 h. Genes of deiodinases (*dio I/II/III*), transthyretin (*ttr*), thyroid hormone receptor β (*trβ*), cytochrome P450 enzymes (*cyp1a*, *cyp1b1*, *cyp1c1*, *cyp1c2*, *cyp2y3*, *cyp3a65*), sulfotransferases (*sult1-st1/2/3/5*) and UDP-glucuronosyltransferases (*ugt1ab*; *ugt2a1*) were profiled in whole larvae. Statistical analysis was done on base-10 log-transformed data, then back-transformed for this graph. The statistical results were carried out with one-way ANOVA set at a 95% confidence level and with Dunnett's test. The symbol "*","**" and "***" indicates a significant difference from vehicle control group of $p < 0.05$, 0.01 and 0.001, respectively.

exposure in a dose-dependent manner, in which the induction was higher at earlier exposure times and decreased at later stages. The inductions in larvae treated with 500 nM were comparable after 8 h (82.0-fold), 24 h (145.8-fold), and 72 h (86.1-fold), but decreased to 24.5-fold after 96 h. The 50 nM concentration also caused significant up-regulation of *cyp1a* after 8 h (13.0-fold), 24 h (44.1-fold), 48 h (18.6-fold) and 96 h (4.9-fold). BDE-47 exposure led to an up-regulation of *cyp1a* throughout the exposure as well. *cyp1a* was induced in the larvae treated with 500 nM by 11.7-fold at 8 h, 8.3-fold at 24 h, 4.1-fold at 48 h and 3.2-fold at 96 h exposure, and with 50 nM by 5.4-fold at 8 h, 3.3-fold at 24 h, 2.2-fold at 48 and 2.8-fold at 96 exposure, respectively.

RNA profiling of other five *cyp* genes (*cyp1a, cyp1b1, cyp1c1, cyp1c2, cyp2y3,* and *cyp3a65*) was performed at two time points of 24 h and 96 h, respectively (Fig. 4 and Fig. 5). Significant up-regulations of the other *cyp1* family genes (*cyp1b1, cyp1c1, cyp1c2*) were found in BDE-99 treated larvae of both concentrations, with a greater induction by 500 nM at both time points (Fig. 4). The induction was higher at 24 h than 96 h. In larvae exposed to BDE-47, *cyp1b1* was significantly induced by 500 nM treatment (2.5-fold at 24 h and 3.2-fold at 96 h), and *cyp1c1* were induced at 96 h (2.7-fold by 50 nM and 3.9-fold by 500 nM) (Fig. 5). Moreover, the induction of *cyp* genes under test was around 10 timers higher in treated larvae of BDE-99 than BDE-47, which was similar to that of *cyp1a* induction.

### 3.3. BDE-99, rather than BDE-47, induced cyp1a in liver and intestine

The small size of zebrafish larvae makes it difficult to dissect and harvest the specific tissue or organ to determine where *cyp1a* induction occurred, so WISH assay was performed to localize the *cyp1a* induction in whole-mount larvae. Only the treatment groups with 500 nM concentrations and 24 h of exposure time for both BDE congeners, which resulted in the maximal *cyp1a* induction, were used in the WISH assay to investigate the responding cellular site. Compared to the vehicle control in which no *cyp1a* signal was detected, *cyp1a* induction by BDE-99 occurred in the liver and intestine (Fig. 6). In contrast to the great induction measured with RT-PCR assay, no *cyp1a* signal was detected in

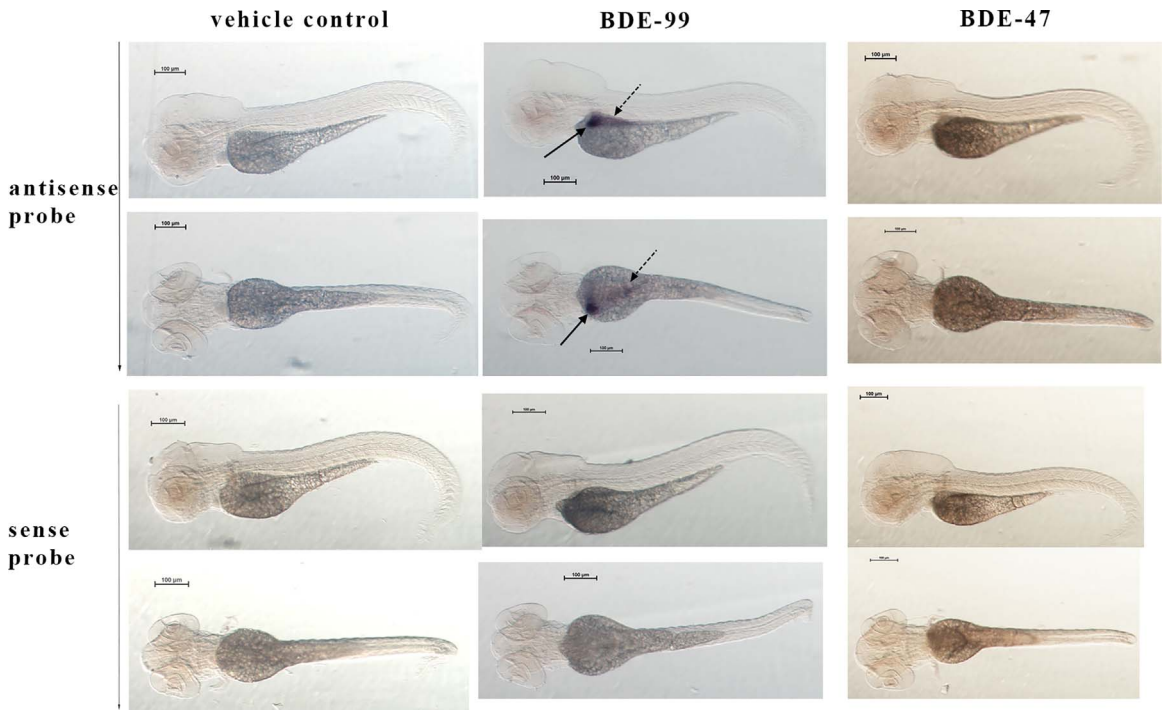


**Fig. 6.** *In situ* hybridization results of *cyp1a* mRNA in newly hatched zebrafish larvae. Larvae harvested 2 h after hatching were exposed to BDE-99 and BDE-47 of 500 nM for 24 h, as shown by blue coloration in *in situ* hybridization with whole mount. Larvae were captured in left view and dorsal view, respectively. The *cyp1a* probe was detected as shown (solid line arrow: liver; dotted line arrow: intestine). Sense probe was used as negative control. All scale bars are 100 μm.

*cyp1a* probe–hybridized larvae with BDE-47 treatment.

### 3.4. Down-regulation of *trβ* and *ttr* by BDE-99 indicated interference on thyroid hormone system

Gene expressions of *trβ*, *ugt1ab* and *ugt2a1* were measured at four time points (Fig. 3B–D) in BDE congener and BaP individually treated larvae with exception of BaP exposure at 96 h, when larvae under exposure didn't survive at all. For the other genes of interest, real time PCR were done at 24 h and 96 h (BDE-99: Fig. 4; BDE-47: Fig. 5). Continuous down-regulations of *trβ* at all four time points by 1.6-fold (8 h), 1.4-fold (24 h), 1.6-fold (48 h) and 2.1-fold (96 h) were detected in BDE-99 treated larvae of 500 nM dose (Fig. 3B). Comparable inhibition of *trβ* at all measured three time points were observed in larvae of 500 nM BaP exposure. No expression change of *trβ* was caused by BDE-47.

BDE-99 exposure of 50 nM induced *ugt1ab* in zebrafish larvae at 8 h by 1.5-fold and no up-regulation was detected until continuous exposure went to 96 h by 4.1-fold (Fig. 3C), and a slight up-regulation was measured in 500 nM treatment at 8 h (1.5-fold) and 24 h (2.0-fold, p value = 0.0484). No expression change of *ugt1ab* was caused by BDE-47 or by BaP.

In BDE-99 treated larvae, *ugt2a1* expression was induced by 50 nM dose at 24 h by 1.7-fold, while inhibited by 500 nM dose (1.9-fold at 24 h, 2.6-fold at 96 h) (Fig. 3D). BaP also inhibited *ugt2a1* at 24 h by 1.8-fold. No expression change of *ugt2a1* was caused by BDE-47.

Both BDE congeners showed mild effects on the deiodinase mRNAs (*dio I/II/III*), except a slight up-regulation of *dio II* by BDE-99 (2.3-fold at 50 nM, 3.1-fold at 500 nM) at 24 h and BDE-47 (2.0-fold at 50 nM) exposure time. For sulfotransferase genes (*sult1's*), BDE-99 of 500 nM continuously induced *sult1-st5* isoform at both time points by 1.6-fold (24 h) and 1.3-fold (96 h), respectively. Slight up-regulations by 500 nM BDE-99 were observed for *sult1-st1*at 96 h (2.4-fold) and *sult1-st3* at 24 h (1.9-fold). Slight down-regulation of *sult1-st3* (3.0-fold by 50 nM at 24 h) and up-regulation of *sult1-st5* (1.7-fold by 500 nM at 96 h) were measured in BDE-47 treated larvae. For *ttr*, a decrease was observed in BDE-99 treated larvae at 24 h exposure by 7.1-fold at 500 nM dose.

## 4. Discussion and conclusions

We previously used ZFL cells to investigate liver-specific molecular mechanisms of BDE-47 and BDE-99 *in vitro* and BDE-99 was found to induce both Cyp1a mRNA and enzymatic activity [22]. Xenobiotic response element-driven luciferase reporter gene activation confirmed Ahr-mediated activation involvement. However, BDE-47, did not exhibit the above effects. In this study, the toxicities of BDE-47 and BDE-99 were further assessed in newly hatched zebrafish *in vivo*.

Fish models especially those of embryonic and larval stages have long term showed benefits and sensitivity to xenobiotic compound exposure studies [34,35,23,36,37,38,39]. Generally, the main deformities caused by heavy metals on fish are spinal ones [37,38], while polycyclic aromatic hydrocarbons (PAHs) and planar (dioxin-like) halogenated aromatic hydrocarbons usually cause cardiovascular defects and edemas [34–36]. Information on developmental toxicity of nonplanar halogenated aromatic hydrocarbons, such as PBDEs, were limited. In current study, BDE-99 exposure on developing zebrafish larvae caused yolk sac and pericardial edema, while BDE-47 caused more red blood cells nearby the heart. Data from Usenko et al. [40,41] found that BDE-99 exposure in 2 hpf wildtype (Tropical 5D) zebrafish embryo for 5 days resulted in curved body but not the deformity of edema as observed in this study, and both BDE-47 and BDE-99 increased *cyp1a* in 120 hpf exposure time point. The differences could be from the zebrafish strains applied, exposed developmental stages and concentrations used.

In this study, more red blood cells (RBCs) nearby the heart in BDE-47 treated larvae than control group indicated the interference of BDE-47 with erythropoiesis. TCDD was reported to interfere with cardiovascular system in developing zebrafish by producing a decrease in blood flow and a small delay in the migration of RBCs from the intermediate cell mass to the dorsal mesentery and dorsal aorta [42]. Whether the observation in current study stands for increased

formation of RBCs, or delaying RBCs to distant vascular as did TCDD remains to be verified.

The significant up-regulation of *cyp1* genes in current study indicated the *in vivo* metabolism of BDE-47 and BDE-99 in newly hatched zebrafish larvae. Bräunig et al. [43] demonstrated that cyp1a, cyp1b, cyp1c, ahr2, and EROD all showed with enhanced activity or gene expression levels in zebrafish larvae following the administrations of PAH-containing sediments and b-naphthoflavone. On the other side, the induction of *Cyp1a* might not be related to the physiological defect of edema in yolk sac and pericardial area observed in BDE-99 treated larvae as indicated by TCDD and other AHR agonists, but ARNT1-AHR2 interactions might have mediated such toxic effects [44–50]. The direct link of the deformity to *Cyp1a* and *Ahr* activations in current study requires further investigation through the employment of transgenic fish models. Other pathways also accounted for the edema deformity. COX2b-thromboxane pathway was recently identified in pericardial edema formation after TCDD exposure in developing zebrafish [51]. Moreover, water permeability function failure and renal failure, also noted as glomerular filtration barrier function failure, also accounted for the edema defects [52,53,51].

Besides the great induction of *Cyp1a* mRNA expression, we also observed that other *Cyp1* isoforms (*cyp1b1, cyp1c1* and *cyp1c2*) were up-regulated in zebrafish larvae whole tissue RNA extract under BDE-99 treatment as they were also induced by BDE-47 although to a lesser extent. Compared with our previous study [22] that only BDE-99 induced *Cyp1a* isoform in ZFL cells *in vitro,* tissue-specific responses of *cyp* gene induction that not limited to liver might exist [54,55] and tissue-specific detection of *cyp1* genes, for instance, with *in situ* hybridization assay, would help localize the gene expression in further study. On the other side, these data at transcript level support the oxidative metabolism of BDE-47 and BDE-99 in exposed newly hatched larvae, with regard to the fact that parent PBDEs don't contain a functional group like the hydroxyl group in THs. Oxidative metabolism of BDE-47 and BDE-99 and their toxicity potencies has been recently investigated, including *in vitro* studies in human liver microsomes [56–58] and *in vivo* zebrafish exposure [59,60]. BDE-99 could be de-brominated to BDE-47 [40], while no oxidative metabolites of BDE-99 was reported in zebrafish yet. For further study, it will be necessary to identify the bioaccumulation and biotransformation of BDE-99 and BDE-47 in zebrafish larvae. The main concern for the HO- and MeO-PBDE metabolites is that they may pose more ecological risks to wildlife [61–66]. Dioxin-like toxicity through AHR-mediated pathway has been recently identified to be the most sensitive molecular pathway for these PBDE metabolites. 2-MeO-BDE-68 and 6-MeO-BDE-47 as predominant PBDE analogues detected in consumption fish from eastern China, were found to induce significant dioxin-like responses in H4IIE-*luc* cells [63]. An *in vitro* avian AHR based luciferase study also reported that HO-/MeO-PBDEs activate avian AHR-mediated pathways in a congener- and species- specific manner, and 5-Cl-6-HO-BDE-47 was the most potent among the tested HO-/MeO-PBDEs [66]. Additional investigations should be conducted to evaluate the toxic potencies of these OH- and MeO-PBDEs, which have been detected in other environmental media, including human blood.

The decrease of *ttr* and *trβ* transcripts by BDE-99 treatment in developing zebrafish larvae indicated its disrupting effect on HPT axis and thyroid hormone action. After hatching, the zebrafish larvae begin postembryonic development in a yolk-sac, which marks an important switch from endogenous to exogenous nutrient acquisition, and many organs continue to develop and differentiate in preparation for the transition. The yolk-sac transition takes 3–5 days, and the critical role of TH for zebrafish larvae metamorphosis was reported by Brown [67]. In zebrafish, the thyroid gland is formed by 40 h after fertilization [68], and the uptake of iodide is reported to be initiated as early as 3 days after fertilization [67]. THs would be ready for synthesis during the period, given the fact that substantial amounts of TRs, especially TRβ, were expressed during early development in zebrafish [69]. In this study, the decreased *ttr* by BDE-99 was accompanied by the decrease of *trβ* mRNA. The result was similar to that reported by Liu and Chan [69], in which 48 h of amiodarone exposure (50 nM), a TR antagonist, in newly-hatched zebrafish larvae decreased *trβ*.

Moreover, the hydroxylated BDE-47 compound, namely 6-OH BDE-47, was recently studied in both RT-PCR and ISH assays to reduce *trβ* in the periventricular zone of zebrafish embryonic brain, and increase *dio I* and *dio III* in both the periventricular zone of the brain and pronephric duct, which had the possibility of affecting neurodevelopment [70,71]. Their findings combine the current data highlighted the detection of *in vivo* metabolites of BDE-99 as well as the localized response at the HPT axis caused in further study.

To summarize, the exposure of BDE-99 in newly-hatched zebrafish larvae caused yolk sac edema and pericardial edema. RT-PCR and WISH assays confirmed *cyp1a* induction in the liver and intestine. Continuous down-regulation of *trβ* and transient down-regulation of *ttr* indicated the interference of BDE-99 on the thyroid hormone system. *cyp1a* induction and *ttr* down-regulation were also observed in BDE-47–treated larvae, but cellular localization could not be confirmed via WISH. The induction of four *cyp1* genes (*cyp1a, cyp1b1, cyp1c1* and *cyp1c2*) by both BDE congeners warrants further study to understand the *in vivo* metabolism of BDE-47 and BDE-99 and the dioxin-like toxicity potencies of the OH-/MeO-PBDEs. The data obtained in this study will aid the characterization of molecular disorders caused by PBDEs in fish and help to delineate better models for toxicity assessment of environmental pollutants in ecological systems and other vertebrates such as humans.

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