



# Developmental toxicity of three hexabromocyclododecane diastereoisomers in embryos of the marine medaka *Oryzias melastigma*



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## ABSTRACT

The composition of major hexabromocyclododecane (HBCD) diastereoisomers, i.e.  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCDs, in marine biota is different from that of the commercially available form (technical HBCD), which is used extensively for toxicological studies. To properly evaluate the impact of HBCDs, the embryos of *Oryzias melastigma* were used to examine the developmental toxicity of the individual diastereoisomers. Results showed that HBCD diastereoisomers at the environmentally realistic concentrations in the embryos induced malformation rate and heartbeat, and caused the appearance of apoptotic heart. In addition,  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCDs had similar potency to stimulate the generation of reactive oxygen species, consequently leading to apoptosis in *O. melastigma* embryos. The order of the developmental toxicity of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCDs in *O. melastigma* embryos was different from that in zebrafish embryos studied previously, which highlighted the importance of using species from both fresh and salt water for toxicity assessment.

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## 1. Introduction

Hexabromocyclododecanes (HBCDs), the third most widely used brominated flame retardants, are extensively used in polystyrene insulation foam boards, textile products, and electronic products. Attributed to their persistence and ecotoxicological concerns, HBCDs recently have been listed by the Stockholm Convention as persistent organic pollutants for consideration of elimination ([www.pops.int](http://www.pops.int)). Nowadays, HBCDs can be found ubiquitously in various environmental media including aquatic habitats, and HBCD concentrations in the ng/L range have been detected in waters (Law et al., 2006a; Wu et al., 2010; He et al., 2013). For example, 2100 ng/L of total HBCDs was recorded in the surface water of the River Kuzuryu, Japan, which receives effluents from textile industries (Oh et al., 2014). Owing to their high octanol-water partition coefficient ( $K_{ow}$ , 5.625), HBCDs tend to build up heavily in sediments, often in the  $\mu\text{g/kg}$  dry weight range, and with exceptionally high concentrations (i.e., 7800  $\mu\text{g/kg}$  dry weight) reported in the River Kuzuryu (Oh et al., 2014). Aside from their significant accumulation in water and sediments, relatively high concentrations of HBCDs are also detected in several freshwater and marine fish species

downstream of the source of the chemical and in highly industrialized areas in Europe and China (Allchin and Morris, 2003; Xian et al., 2008; Koppen et al., 2010).

The commercially available HBCD (technical HBCD, tHBCD) is often characterized as a mixture of mainly three diastereoisomers, i.e.,  $\alpha$ -,  $\beta$ - and  $\gamma$ -HBCDs, and the composition of these diastereoisomers in tHBCD varies in the range of 1–12%  $\alpha$ -HBCD, 10–13%  $\beta$ -HBCD and 75–89%  $\gamma$ -HBCD (Koeppen et al., 2007). Stereo-structural difference of these three HBCD diastereoisomers results in substantial differences in physicochemical properties (e.g., polarity, solubility and  $K_{ow}$ ), which may consequently lead to distinctive environmental fates (MacGregor and Nixon, 2004). Laboratory research shows that the bioconcentration factor and biomagnification factor of  $\alpha$ -HBCD in fish (e.g., zebrafish or mirror carp) are much higher than those of  $\beta$ - and  $\gamma$ -HBCDs (Zhang et al., 2014a; Du et al., 2012a). In addition,  $\beta$ - and  $\gamma$ -HBCDs can be biotransformed to  $\alpha$ -HBCD in adult fish (Zhang et al., 2014a; Du et al., 2012a; Law et al., 2006b). Indeed,  $\alpha$ -HBCD is always the predominant HBCD diastereoisomer detected in both freshwater and marine fish sampled from the field, accounting for ~80–90% of total HBCDs (Law et al., 2006a; He et al., 2013; Li et al., 2012; Xia et al., 2011).

Although the composition of  $\alpha$ -,  $\beta$ - and  $\gamma$ -HBCDs in aquatic biota is very different from that of tHBCD, tHBCD is used for most of the toxicological studies. For instance, studies using tHBCD for exposure show that tHBCD induces the generation of reactive oxygen species (ROS) and impacts the development of both freshwater and marine fish embryos (Deng et al., 2009; Hong et al., 2014). However, the toxicity of individual HBCD diastereoisomers, which is important for risk assessment of

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HBCDs, remains largely unknown. Du et al. (Du et al., 2012b) found that in the freshwater zebrafish *Danio rerio* the order of developmental toxicity of these three HBCD diastereoisomers was  $\gamma$ -HBCD >  $\beta$ -HBCD >  $\alpha$ -HBCD. Based on this result, the bioconversion of  $\beta$ - and  $\gamma$ -HBCDs to  $\alpha$ -HBCD in fish could be considered as a detoxification process and hence the toxicity data obtained from tHBCD exposure in risk assessment might overestimate the toxicity of HBCDs. Comparison studies on the toxicity of individual HBCD diastereoisomers using other aquatic species, however, are very limited. Given the existence of inter-species variation, it is necessary to conduct toxicological studies on more than one species to reduce uncertainty.

In addition, it is reported that the sensitivity of freshwater and saltwater species to environmental pollutants could be significantly different (Leung et al., 2001; Wheeler et al., 2002). It is likely to be affected by various biological and physicochemical factors, such as salinity, pH and ionic concentrations, and could also be influenced by the mode of toxic action of the chemicals (Leung et al., 2001; Wheeler et al., 2002). In the case of HBCDs, the lowest observable effect concentration (LOEC) of tHBCD to affect the heartbeat of embryos is 5  $\mu\text{g/L}$  in the marine medaka *Oryzias melastigma* and 50  $\mu\text{g/L}$  in the freshwater zebrafish (Deng et al., 2009; Hong et al., 2014). tHBCD causes bradycardia in zebrafish embryos but tachycardia in marine medaka embryos. These findings suggest that freshwater and marine fish may have different sensitivity and react differently to HBCDs (Deng et al., 2009; Hong et al., 2014).

Given that the toxicity and toxic mechanisms of individual HBCD diastereoisomers in marine organisms remain largely unknown, we chose the marine medaka *O. melastigma* as a model species for toxicity investigation. *O. melastigma* is adopted by the International Life Science Institute (ILSI, Washington DC, USA) Health and Environmental Science Institute for embryo toxicity testing and is a very useful model for estuarine and marine ecotoxicological research (Chen et al., 2009, 2011; Kong et al., 2008). It has been demonstrated that tHBCD can cause developmental toxicity of *O. melastigma* embryos by enhancing the generation of ROS and thus leading to apoptosis (Hong et al., 2014). In this study, our main purpose was to compare the relative toxicity of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCDs in *O. melastigma* embryos. In addition, the transcription and expression of genes and proteins related to oxidative stress and apoptosis were examined to compare the potential toxic mechanisms induced by the three HBCD diastereoisomers.

## 2. Materials and methods

### 2.1. Chemicals and reagents

tHBCD (>95% pure), from which  $\alpha$ -,  $\beta$ - and  $\gamma$ -HBCDs were purified, was purchased from the Sinopharm Chemical Reagent Co. (Shanghai, China);  $\alpha$ -HBCD,  $\beta$ -HBCD and  $\gamma$ -HBCD, used as standards for LC–MS/MS analysis, were ordered from AccuStandard, Inc. (New Haven, Connecticut, USA); [ $^{13}\text{C}$ ]- $\alpha$ -HBCD, [ $^{13}\text{C}$ ]- $\beta$ -HBCD and [ $^{13}\text{C}$ ]- $\gamma$ -HBCD were purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA); and the organic solvents (acetonitrile, acetone, hexane and methanol, purity >99.5%) used for sample extraction, high performance liquid chromatography (HPLC) separation, and liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis were obtained from Tedia Inc. (Fairfield, OH, USA). All other reagents were purchased from Sigma-Aldrich Chemical Co. (Saint Louis, MO, USA) except where indicated.

### 2.2. HPLC purification of $\alpha$ -HBCD, $\beta$ -HBCD and $\gamma$ -HBCD

HPLC purification was performed on a Varian Prostar 218 semi-preparative HPLC system (Varian Inc., Palo Alto, CA, USA) with a photodiode array detector set at a wavelength of 250 nm. Separation of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCDs was achieved with a 250  $\times$  21.4 mm Varian Dynamax Microsorb 100–5 C18 column (5  $\mu\text{m}$  in particle size). The HPLC mobile

phase consisted of two solvents: mobile phase A (10 mM ammonium acetate in water:methanol, 50:50, v/v) and mobile phase B (100% methanol). The gradient program used for separation was linear gradient from 80 to 100% B (0–20 min); hold isocratic at 100% B (20–30 min); linear gradient from 100 to 80% B (30–32 min); and re-equilibrate at 80% B for 10 min. The mobile phase flow rate was 10 mL/min. The HPLC fractions containing  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCDs were collected on ice and immediately dried using a rotary evaporation at ambient temperature. The dried samples were then redissolved in dimethyl sulfoxide (DMSO, purity >99.5%) and desalted using the same reverse-phase C18 column for purification. After the samples were loaded, the column was washed with water:methanol (15:85, v/v) for 10 min, and the samples were eluted from the column using 100% methanol. The HPLC fractions containing desalted  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCDs were collected on ice and dried using rotary evaporation. Upon reaching dryness, the samples were immediately stored at  $-80^\circ\text{C}$  to avoid decomposition. The purity of the separated  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCDs was checked using HPLC–ultraviolet spectrometry (Supplementary Material) and the quantities were determined using LC–MS/MS.

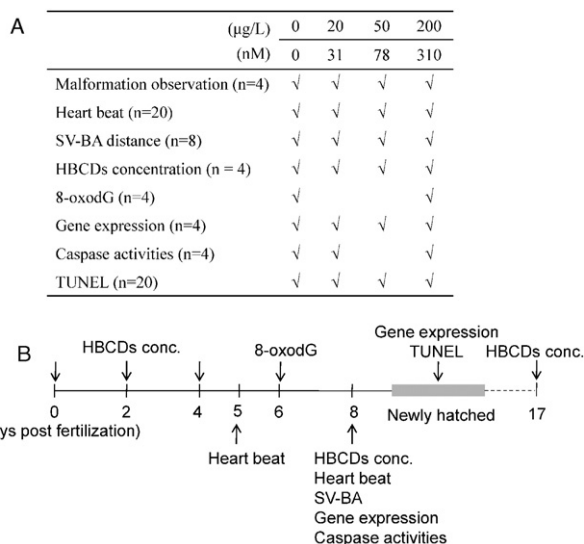
### 2.3. Medaka maintenance and embryo toxicity test

Marine medaka *O. melastigma* were maintained following previously described conditions (Hong et al., 2014; Kong et al., 2008). Briefly, they were maintained at  $28 \pm 2^\circ\text{C}$  in a 14-h light:10-h dark photoperiod in aerated artificial seawater at a salinity of 30‰ and a dissolved oxygen concentration of  $5.8 \pm 0.2$  mg/L. The fish were fed with *Artemia nauplii* twice daily.

The freshly fertilized eggs of *O. melastigma* were collected daily and visually sorted under a dissecting microscope. As marine *O. melastigma* and freshwater *Oryzias latipes* share high similarity in development and morphology (Chen et al., 2009, 2011; Kong et al., 2008), embryo developmental stages of *O. melastigma* were judged according to the morphology parameters used for staging *O. latipes* (Iwamatsu, 2004). The embryos at developmental stages 12–15 were sorted, when the blastoderm was expanding and the embryonic shield was just visible but before clearly visible as a narrow streak (Iwamatsu, 2004). The sorted embryos were randomly distributed into sixteen groups and exposure was started immediately. For monitoring of survival, development and heart rate, forty embryos were included in each replicate. The embryos were exposed to 0, 20, 50, and 200  $\mu\text{g/L}$  (corresponding to 0, 31, 78, and 310 nM) of  $\alpha$ -,  $\beta$ -, or  $\gamma$ -HBCD in seawater. Each treatment consisted of four groups as replicates. The solvent control groups received the same concentration of DMSO as the treated groups (0.01%, v/v). Each exposure group was maintained in 60-mm crystallizing dishes containing 20 mL of exposure media and the exposure solution was freshly prepared and renewed daily. The exposure concentrations, toxicity endpoints selected for measurements, sample collecting time points and sample replication numbers are indicated in Scheme 1.

### 2.4. Toxicity endpoints for measurement

To assess developmental toxicity, malformation of the embryos was monitored daily under the microscope. Once the embryos were identified as malformation or hatched, they were removed from the exposure group and kept separately in a new container with exposure media being daily changed. The exposure was terminated 17 days post fertilization (dpf) when most of the larvae were hatched and the survival rate, hatching success rate, and hatch-out time were recorded. The number of abnormal embryos was counted for calculation of malformation rate no matter how many abnormalities appeared on each individual. The heart rate and the distance between the sinus venosus (SV) and bulbus arteriosus (BA) region of the heart (SV–BA distance) were measured, as described previously (Huang et al., 2011), to evaluate the effect of HBCD diastereoisomers on embryo cardiac development (Figure S4, Supplementary Material).



**Scheme 1.** Exposure concentrations (A) and sampling schedule (B) for the measurement of individual endpoints.

To assess whether HBCD diastereoisomers stimulated the generation of ROS through the induction of the inflammation genes TNF- $\alpha$  and IL-1 $\beta$ , which then triggered the p53 signaling pathway, activated caspases, and resulted in apoptosis (Hong et al., 2014), the concentrations of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) in genomic DNA, the gene expression levels of TNF- $\alpha$ , IL-1 $\beta$  and p53, and the activities of caspase-3, caspase-8 and caspase-9 were measured. Furthermore, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was also conducted to detect DNA fragmentation resulting from apoptotic signaling cascades.

#### 2.5. Sample extraction and LC-MS/MS quantification of HBCD isomers

To determine the actual concentrations of HBCDs in the exposure media, 10-mL of each media sample was passed through a C18 cartridge (Supelco, Bellefonte, PA, USA), which was preconditioned by methanol and water. After washing the column with 10 mL H<sub>2</sub>O for desalting, the extraction column was eluted with 9 mL of methanol and the eluate was concentrated to 0.5 mL under N<sub>2</sub> flow.

To determine the concentrations of HBCDs accumulated in the embryos, each sample containing twenty embryos was first spiked with the <sup>13</sup>C-labeled  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCD isomers, and then homogenized and extracted in 5 mL hexane:acetone (1:1, v/v) using ultrasonication for 30 min. After centrifugation, the supernatant was collected and the pellet was extracted again. The resulting supernatant was combined with the first supernatant and then concentrated to 0.5 mL under N<sub>2</sub> flow. At the end of the exposure (17 dpf) when most embryos were hatched, twenty larvae per sample were collected and HBCDs in the hatched larvae were extracted by the same method.

The quantification of the HBCD isomers was carried out on an Agilent 1290–6490 ultra performance liquid chromatography (UPLC)–triple quadruple mass spectrometry system (Agilent Technologies, Palo Alto, CA, USA) following conditions reported previously (Hong et al., 2014). The  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCD isomers and their corresponding [<sup>13</sup>C]-labeled internal standards were monitored in multiple reaction monitoring mode with transition events of  $m/z$  640.8 > 80.8 and  $m/z$  652.8 > 80.8.

#### 2.6. Quantification of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG)

The genomic DNA of thirty embryos per sample was isolated using a DNA Extractor® WB kit (Wako Pure Chemical Industries, Osaka, Japan) following the manufacturer's protocol, except that

0.1 mM of desferrioxamine was included in the solutions used for DNA extraction to minimize the artifactual oxidation of DNA (Ravanat et al., 2002).

The extracted DNA was hydrolyzed using calf spleen phosphodiesterase, nuclease P1, snake venom phosphodiesterase and alkaline phosphatase (Hong et al., 2014). The 8-oxodG concentrations in the resulting digestion mixture were quantified using the isotope-dilution method combined with LC-MS/MS analysis following the conditions described previously (Hong et al., 2014).

#### 2.7. Real-time quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from ten embryos (8 dpf) or larvae (newly hatched) per replicate. An equal amount of RNA was then reverse-transcribed using mixtures of oligo (dT) primer (5'-TTTTTTTTTTTTT-3') and random primers with moloney murine leukemia virus reverse transcriptase (BGI, Shenzhen, China) to generate cDNA. qPCR was carried out on a CFX96™ Real-Time System (Bio-Rad Laboratories, CA, USA) using SYBR Green I. The primers for qPCR of the p53, TNF- $\alpha$ , IL-1 $\beta$  and 18s rRNA genes were from the literature (Huang et al., 2011, 2012). The thermal cycle program consisted of an initial denaturation step at 95 °C for 3 min, followed by 50 cycles of 10 s at 95 °C, and then 35 s at 60 °C for 18 s gene, 65 °C for p53 gene and 70 °C for TNF- $\alpha$  and IL-1 $\beta$  genes. Dissociation curve analysis was used to confirm that only the targeted PCR product was amplified and detected. Gene expression levels were normalized to 18s rRNA expression levels. Two replicates of qPCR were performed for each sample as technical replicate and four samples were performed for each treatment. The fold change of the tested genes was analyzed using the 2- $\Delta\Delta C_t$  method.

#### 2.8. Caspase activity

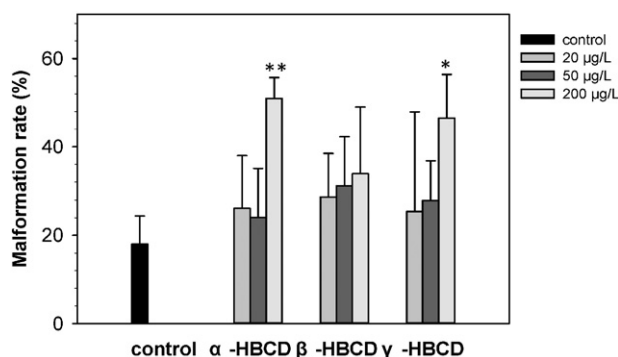
Crude enzymes were extracted from thirty embryos per sample and the measurement of caspase-3, caspase-8, and caspase-9 activities was performed using caspase-3, caspase-8, and caspase-9 colorimetric protease assay kits (Keygene Biotech Co., Nanjing, China) following the manufacturer's instruction. The protein concentration was determined with reference to standards of bovine serum albumin using the BCA assay (Thermo Fisher Scientific, MA, USA). The enzyme activity was expressed as the fold change over the control samples.

#### 2.9. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

The fragmented DNA of apoptotic cells was identified using the TUNEL method with the fluorescent in situ cell death detection kit (Roche Diagnostic, Switzerland) following the manufacturer's instruction. Briefly, twenty newly hatched larvae were fixed for 2 h at room temperature in 4% paraformaldehyde in phosphate buffer saline (PBS, freshly prepared) (Li et al., 2009). The samples were then washed twice in PBS and further permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate (freshly prepared) for 10 min on ice, followed by two rinses in PBS. The samples were incubated with the label solution mixture overnight at 4 °C and then shaken for 1 h at 37 °C. The labeling reaction was stopped by washing the samples in PBS three times (5 min each) at room temperature to remove excessive TdT and deoxynucleotides. After TUNEL staining, the larvae were photographed by a fluorescent microscope with an excitation wavelength of 485 nm and emission wavelength of 510 nm.

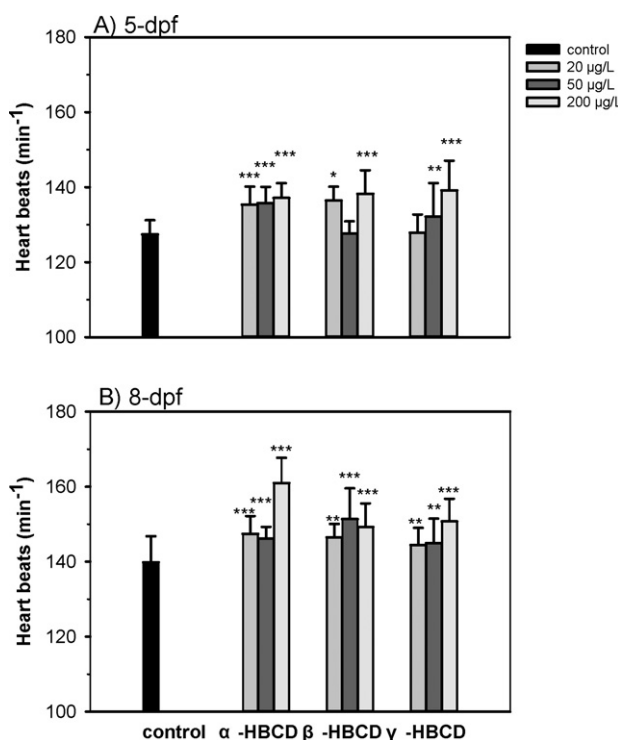
#### 2.10. Western blotting

Total protein was extracted from ten larvae per replicate. The Western blotting assay for the relative quantification of P53 protein followed conditions reported previously (Hong et al., 2014). Briefly, an equal amount of protein was used for analysis and pre-stained molecular



**Fig. 1.** HBCD diastereoisomer exposure induced malformation in *O. melastigma* embryos. The *O. melastigma* embryos were exposed to different concentrations of HBCD diastereoisomers (0, 20, 50, and 200 µg/L). The major malformation included yolk sac edema, pericardial edema and spinal curvature. The values are presented as the mean  $\pm$  SD ( $n = 4$ ). The values that are significantly different from the control are indicated by asterisks (one-way ANOVA, followed by LSD *post hoc* test: \* $p < 0.05$ ; \*\* $p < 0.01$ ).

weight markers (Bio-Rad Laboratories, CA, USA) were run as standards on each gel. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis separation and polyvinylidene difluoride membrane transfer, the protein containing membrane was blocked with 5% non-fat dry milk, incubated with the primary anti-p53 antibody (Bioss, Beijing, China) for 1 h and washed three times. After incubating with an anti-rabbit IgG-alkaline phosphatase conjugated secondary antibody (Sigma-Aldrich Chemical Co., Saint Louis, MO, USA) at room temperature for 1 h, the membrane was washed three times again, then rinsed twice with a buffer containing 100 mM Tris (pH 9.5), 100 mM NaCl, and 10 mM MgCl<sub>2</sub>, and incubated with NBT/BCIP solution (Roche, Indianapolis, USA) until bands appeared.



**Fig. 2.** HBCD diastereoisomer exposure increased the heart rate in *O. melastigma* embryos on both 5 (A) and 8 (B) days post fertilization. The *O. melastigma* embryos were exposed to different concentrations of HBCD diastereoisomers (0, 20, 50, and 200 µg/L). The values are presented as the mean  $\pm$  SD ( $n = 20$ ). The values that are significantly different from the control are indicated by asterisks (one-way ANOVA, followed by LSD *post hoc* test: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).

## 2.11. Data analysis

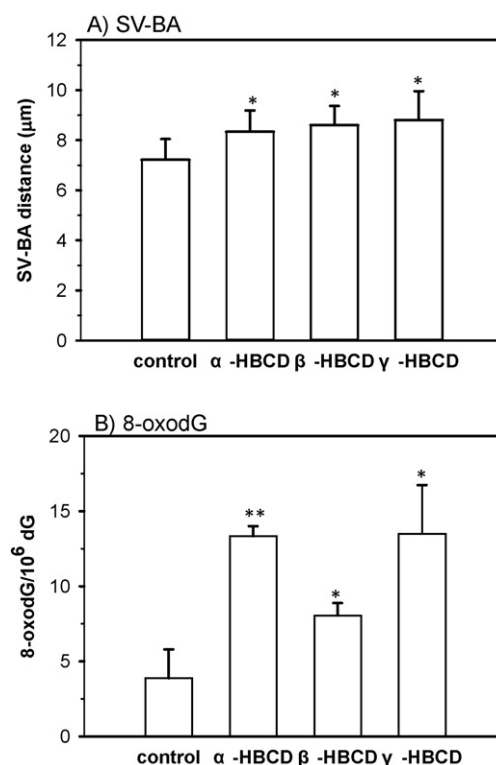
Data were analyzed using SPSS Statistics 19.0 (IBM Software, Columbus, OH, USA) for significance of differences by one-way analysis of variance (ANOVA) combined with a least significant difference (LSD) *post hoc* test.

## 3. Results

### 3.1. Developmental toxicity

Exposure of *O. melastigma* embryos to  $\alpha$ -,  $\beta$ -, or  $\gamma$ -HBCD did not cause significant effect on the survival rate and hatching rate (data not shown). The three diastereoisomers all induced malformation of the hatched larvae, with yolk sac edema, pericardial edema, and spinal curvature as the most frequently observed abnormalities (Figure S3, Supplementary Material). At the highest exposure concentration (i.e., 200 µg/L), the malformation rates in both the  $\alpha$ - and  $\gamma$ -HBCD exposure groups were significantly higher than that in the control group ( $p < 0.05$ , Fig. 1).

The heart rate of the *O. melastigma* embryos was recorded on both 5 and 8 dpf, two time points that are within the critical period of embryo heart development (Huang et al., 2011). In this period, the atrio-ventricular region of the heart increases and the atrium and the ventricle lie adjacent to each other (Iwamatsu, 2004) (Figure S4, Supplementary Material). The results showed that the heart rate of the embryos was increased by exposure to  $\alpha$ -,  $\beta$ -, or  $\gamma$ -HBCD generally in a concentration dependent manner (Fig. 2). Even at the lowest exposure concentration (i.e., 20 µg/L), the heart rate of the embryos on 8 dpf significantly



**Fig. 3.** HBCD diastereoisomer exposure induced sinus venosus–bulbus arteriosus (SV–BA) distance (A) and the concentrations of 8-oxodG (B) in *O. melastigma* embryos. The *O. melastigma* embryos were exposed to 200 µg/L of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCDs. The SV–BA distance was measured as the direct distance between the centers of SV and BA on 8 days post fertilization (dpf). The structure of cardiovascular systems of embryos, including sinus venosus, atrium, ventricle and bulbus arteriosus, was shown in Figure S4 (Supplementary Material). The concentrations of 8-oxodG were quantified by LC–MS/MS on 6 dpf. The values are presented as the mean  $\pm$  SD ( $n = 8$  for SV–BA;  $n = 4$  for 8-oxodG). Data presentation is same as Fig. 2.



increased from  $139 \pm 6$  to  $147 \pm 5$ ,  $146 \pm 4$ , and  $144 \pm 4$  beats/min, upon exposure to  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCDs, respectively ( $p < 0.01$ , Fig. 2B).

To further examine the effects of HBCD diastereoisomers on the cardiac development of *O. melastigma*, the SV–BA distance, which is used as an important index for the evaluation of cardiac development (Huang et al., 2011), was measured in the developing embryos. The results showed that the SV–BA distance of embryos was induced markedly by exposure to  $\alpha$ -,  $\beta$ -, or  $\gamma$ -HBCD at 200  $\mu\text{g/L}$  (Fig. 3A,  $p < 0.05$ ), whereas exposure to other lower concentrations (e.g., 20 and 50  $\mu\text{g/L}$ ) did not have any significant effect (data not shown).

### 3.2. Increased concentrations of 8-oxodG

To assess whether HBCD diastereoisomers induced oxidative stress and further led to oxidative DNA damage in *O. melastigma* embryos, the concentrations of 8-oxodG, an oxidative product of deoxyguanine induced by various types of ROS, were measured after 6 days of exposure. The concentration of 8-oxodG was 3.8 lesions/ $10^6$  deoxyguanine in the control and increased to 13.3, 8.1, and 13.5 lesions/ $10^6$

deoxyguanine, respectively, following exposure to 200  $\mu\text{g/L}$  of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCDs (Fig. 3B).

### 3.3. Induction of stress-responsive genes and proteins

The expression of the inflammation genes TNF- $\alpha$  and IL-1 $\beta$  and of the apoptotic gene p53 was analyzed for 8-dpf embryos and newly hatched larvae. The mRNA transcription of the three genes was all significantly up-regulated at the exposure concentrations of 50 or 200  $\mu\text{g/L}$  in the two selected developmental stages in a dose-dependent manner (Fig. 4). In addition, the expression of p53 protein was up-regulated by 43, 18, and 31% of the control after exposure to 200  $\mu\text{g/L}$  of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCDs, respectively, which was consistent with the observation at transcription level.

### 3.4. Induction of caspase activity

To evaluate whether HBCD diastereoisomers induced apoptosis via the caspase-dependent pathway, the activities of caspase-3, caspase-8, and caspase-9 were evaluated on 8 dpf. The activity of caspase-3 was

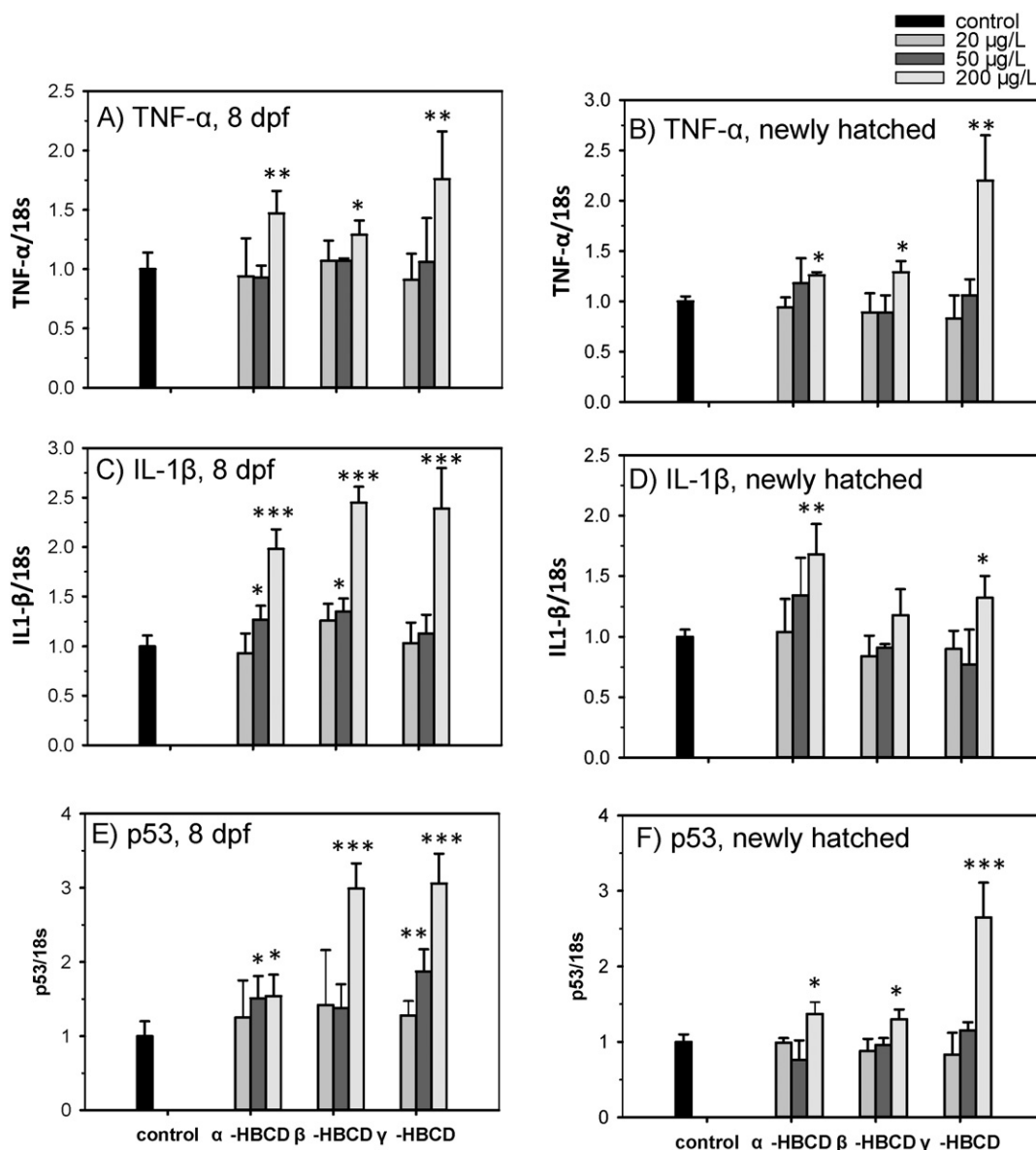


Fig. 4. Induction of the gene expression of TNF- $\alpha$  (A, B), IL-1 $\beta$  (C, D) and p53 (E, F) in *O. melastigma* embryos on 8 days post fertilization and in newly hatched larvae after exposure to 0, 20, 50, and 200  $\mu\text{g/L}$  of HBCD diastereoisomers. The values are presented as the mean  $\pm$  SD ( $n = 4$ ). Data presentation is same as Fig. 2.

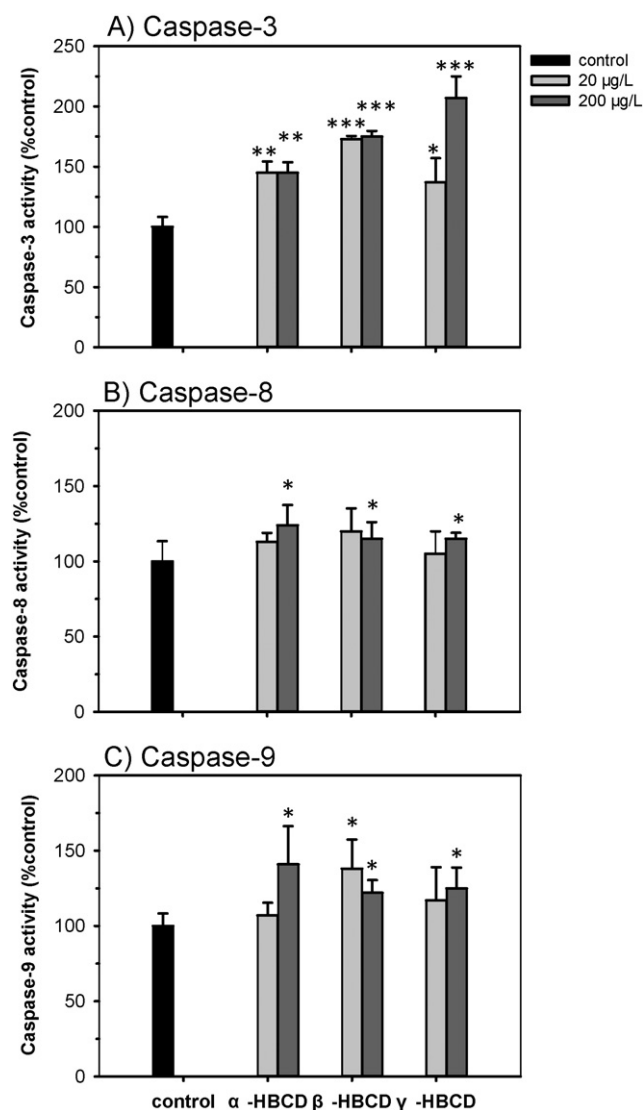


Fig. 5. Induction of the activities of caspase-3 (A), caspase-8 (B) and caspase-9 (C) in *O. melastigma* embryos after exposure to 0, 20, and 200 µg/L of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCDs. The values are presented as the mean  $\pm$  SD ( $n = 4$ ). Data presentation is same as Fig. 2.

induced by 45, 73, and 37% at the exposure concentration of 20 µg/L and by 45, 75, and 107% at the exposure concentration of 200 µg/L of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCDs, respectively (Fig. 5A). All the three diastereoisomers significantly induced the activities of both caspase-8 and caspase-9 at the exposure concentration of 200 µg/L ( $p < 0.05$ , Fig. 5B–C).

### 3.5. Induction of apoptosis

After exposure to the three HBCD diastereoisomers at concentrations of 0, 20, 50, and 200 µg/L, the newly hatched larvae were fixed, stained using the fluorescent TUNEL staining method, and then visualized under a fluorescence microscope. Larvae in the 50 and 200 µg/L treatment groups for all the three diastereoisomers showed apoptotic signals in the heart region (Fig. 6). No obvious apoptotic hearts were observed in the control and 20 µg/L treated groups (Fig. 6).

### 3.6. Concentrations of HBCD diastereoisomers in the embryos

In the LC–MS/MS chromatograms, only the HBCD diastereoisomer used for exposure was detected in the embryos, indicating that there was no detectable bio-conversion among the three diastereoisomers in the embryos. The recovery of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCDs from the embryo

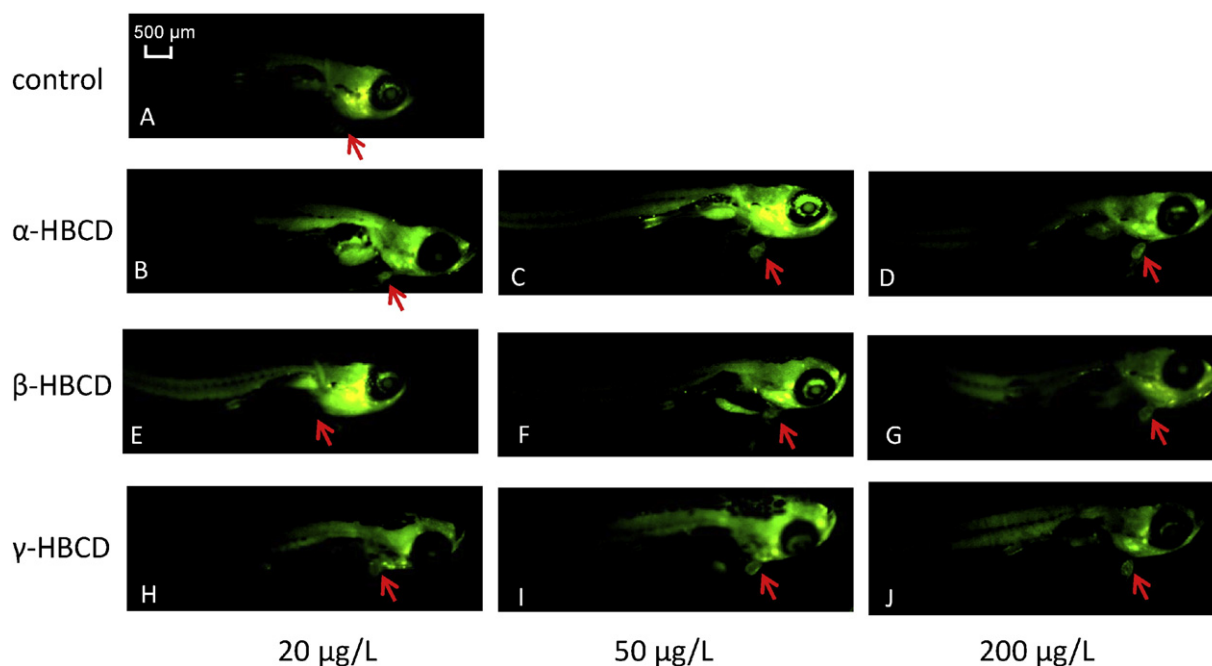
samples was 91%, 95% and 92%, respectively. The concentrations of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCDs in embryos on 0 dpf (before exposure) were  $1.7 \pm 0.3$ ,  $4.5 \pm 0.1$  and  $2.6 \pm 0.1$  ng/g ww, respectively (Fig. 7). The concentrations of HBCD isomers in embryos increased over time during the exposure period for all HBCDs treated groups, suggesting continuous accumulation of HBCD isomers in medaka embryos through the water-borne exposure (Fig. 7). For the 20 µg/L exposure groups, there was no significant difference in the concentrations of the three HBCD diastereoisomers accumulated in the embryos up to 8 dpf (Fig. 7A), whereas for the two higher exposure concentrations (i.e., 50 and 200 µg/L), the concentrations of  $\alpha$ - and  $\beta$ -HBCDs accumulated in the embryos were slightly higher than  $\gamma$ -HBCD (Fig. 7B–C). The concentrations of HBCDs in the larvae on 17 dpf were also measured and the concentrations of  $\alpha$ - and  $\beta$ -HBCDs were also higher than  $\gamma$ -HBCD for the 50 and 200 µg/L treated groups (Figure S5, Supplementary Material).

The concentrations of HBCDs in the exposure media were determined right after media renewal (0 h) and before next media renewal (24 h) (Table S1, Supplementary Material). After 24 h exposure, the concentrations of three HBCD diastereoisomers remained higher than 50% of the initial concentrations. Nevertheless, there was no difference in the concentrations of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCDs in the exposure media.

## 4. Discussion

The commercially available form of HBCD (tHBCD), consisting of ~90%  $\gamma$ -HBCD, 5%  $\alpha$ -HBCD, and 5%  $\beta$ -HBCD, is widely used in toxicological studies of the brominated flame retardant on a variety of organisms, including fish and invertebrates in aquatic systems (Deng et al., 2009; Hong et al., 2014; Zhang et al., 2014b). The development of zebrafish and *O. melastigma* embryos is affected by exposure to tHBCD (Deng et al., 2009; Hong et al., 2014; Du et al., 2012b). Although fish embryos and larvae could metabolize certain pollutants, e.g. benzo[a]pyrene, and express some metabolic enzymes, e.g. CYP2B6, CYP3A5 and UGT1A1 (Hong et al., 2014; Hornung et al., 2007; Jones et al., 2010), the composition of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCDs accumulated in *O. melastigma* embryos exposed to tHBCD through water-borne pathway is identical to that of tHBCD, suggesting limited bioconversion among HBCD diastereoisomers (Hong et al., 2014). The results in the present study also indicated no apparent bioconversion among  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCDs. Nevertheless, in adult fish, e.g., zebrafish, mirror carp and rainbow trout,  $\beta$ - and  $\gamma$ -HBCDs can be bioisomerized to  $\alpha$ -HBCD (Zhang et al., 2014a; Du et al., 2012a; Law et al., 2006b). In addition, in vitro work reveals that  $\alpha$ -HBCD is more resistant to metabolic degradation by liver enzymes of both rats and adult trout than  $\beta$ - and  $\gamma$ -HBCDs (Abdallah et al., 2014). Therefore, the stereoisomer-selective biotransformation of HBCDs in adult fish can result in totally different isomer composition in fish body from that of tHBCD. Indeed,  $\alpha$ -HBCD in general accounts for ~80–90% of total HBCDs in both freshwater and marine fish tissues collected from the field, whereas  $\beta$ - and  $\gamma$ -HBCDs are the minor fractions (Xian et al., 2008; Li et al., 2012). HBCDs are maternally transferred to fish eggs (Nyholm et al., 2008), so the compositions of the three diastereoisomers in fish eggs are almost identical to those in other fish tissues, e.g., in fish file (Xian et al., 2008; Li et al., 2012). Since  $\alpha$ -HBCD but not  $\gamma$ -HBCD is the predominant fraction of HBCDs in fish eggs, exposure of fish embryos to waterborne tHBCD might not be appropriate for evaluating the developmental toxicity of HBCDs. It is important to differentiate the developmental toxicity of individual HBCD diastereoisomers in fish embryos.

In the present study, we exposed *O. melastigma* embryos to three HBCD diastereoisomers (i.e.,  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCDs) and compared their developmental toxicity. Considering all the developmental toxicity endpoints collectively, including induction of heart rate, malformation rate and SV–BA distance,  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCDs in general exhibited very similar potency to induce developmental toxicity in *O. melastigma* embryos. In contrast to Du et al. (Du et al., 2012b) who reported that the rank order of developmental toxicity in zebrafish embryos was



**Fig. 6.** Induction of apoptosis in the heart region of newly hatched larvae of *O. melastigma* detected using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. The embryos were exposed to 0 (A), 20 (B, E, H), 50 (C, F, I) and 200  $\mu\text{g/L}$  (D, G, J) of  $\alpha$ - (B, C, D),  $\beta$ - (E, F, G), and  $\gamma$ -HBCDs (H, I, J) and the newly hatched larvae were fixed in 4% paraformaldehyde in phosphate buffer saline (PBS), washed twice in PBS, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate and incubated with the label solution mixture. The labeling reaction was stopped by washing the samples in PBS, and the larvae were photographed by a fluorescent microscope with an excitation wavelength of 485 nm and emission wavelength of 510 nm. Red arrows indicated the heart region of *O. melastigma* larvae.

$\gamma$ -HBCD >  $\beta$ -HBCD >  $\alpha$ -HBCD, the hatching success of *O. melastigma* embryos was not affected by any of the three diastereoisomers. In addition, the heart beat of zebrafish embryos was reduced at 48 and 96 hpf by both  $\alpha$ - and  $\beta$ -HBCDs, whereas it was first reduced at 48 hpf and then increased at 96 hpf by  $\gamma$ -HBCD (Du et al., 2012b). In contrast, in *O. melastigma* embryos, the three diastereoisomers all caused significant tachycardia effect at the two developmental stages (i.e., 5 and 8 dpf). The different response of freshwater zebrafish and marine *O. melastigma* to the exposure of HBCDs highlights species-specific and possibly freshwater and seawater species differences in toxicity evaluation.

tHBCD can induce oxidative stress in aquatic organisms and subsequently results in developmental toxicity (Deng et al., 2009; Zhang et al., 2008). Induction of antioxidant biomarkers, e.g. glutathione S-transferase, superoxide dismutase, and DNA damage, is observed in the clam *Venerupis philippinarum* upon exposure to tHBCD (Zhang et al., 2014b). The measurement of 8-oxodG, a robust biomarker for oxidative stress, indicated that all the three HBCD diastereoisomers induced the generation of ROS in the *O. melastigma* embryos (Fig. 3B).

Reactive oxygen species attack DNA, generate oxidative DNA damage (e.g. 8-oxodG), which can be detected and repaired by certain DNA repair enzymes, and meanwhile activate the p53 pathway to promote cell cycle arrest or apoptosis (Harris and Levine, 2005). Our results showed that in correspondence with the increasing concentrations of 8-oxodG, the expression of both p53 gene and protein was up-regulated upon exposure to the three HBCD diastereoisomers (Fig. 4E–F). Activation of p53 triggers the mitochondria-mediated apoptotic pathway, subsequently resulting in cytochrome c release and activation of caspases (Elmore, 2007). The activities of caspases, particularly caspase-3, were significantly and similarly induced by  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCDs in *O. melastigma* embryos (Fig. 5), whereas the potency of  $\gamma$ -HBCD to induce caspase-3 activity was much higher than  $\alpha$ - and  $\beta$ -HBCDs in zebra fish (Du et al., 2012b). The apoptotic cells around the heart area in both HBCDs treated zebrafish and *O. melastigma* embryos [15–16, this study] suggested that the generation of ROS, induction of DNA oxidative damage and increase of caspase enzymes

occurred likely in the heart. Consistently, the heart rate and SV–BA distance were significantly increased (Figs. 2 and 3) in HBCDs treated embryos, suggesting that HBCDs induced developmental toxicity in the cardiovascular system of *O. melastigma* embryos.

tHBCD exposure up-regulated the transcription of TNF- $\alpha$  (tumor necrosis factor- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) and resulted in the generation of ROS in *O. melastigma* embryos (Hong et al., 2014). TNF- $\alpha$  can stimulate ROS production by several mechanisms, including its effect as an agonist to increase the product of nicotinamide adenine dinucleotide phosphate oxidase and  $\text{O}_2$  and thus promote the formation of ROS and its role in the changes of mitochondria redox state (Mariappan et al., 2007; Madamanchi et al., 2005). IL-1 $\beta$ , an inflammatory cytokine, also has been implicated in ROS generation (Hensley et al., 2000). Here we showed that  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCDs had similar potency in the induction of expression of TNF- $\alpha$  and IL-1 $\beta$  genes (Fig. 4), which was consistent with the measurement of 8-oxodG. Transcription of CYP1A or COX-1/COX-2 genes in *O. melastigma* embryos was not up-regulated upon  $\alpha$ -,  $\beta$ -, or  $\gamma$ -HBCD exposure (data not shown), indicating that the AhR/AhRNT (aryl hydrocarbon receptor/AhR nuclear translocation protein) pathway and COX genes mediated inflammatory process were not involved in the induction of oxidative stress.

HBCDs are readily bioavailable and can be bioaccumulated in aquatic organisms through the food web (Tomy et al., 2004). Relatively high concentrations of HBCDs are detected in fish species from Europe and China (Allchin and Morris, 2003; Xian et al., 2008; Koppen et al., 2010). For instance, ~10,000 ng/g ww of HBCDs is found in fish samples caught from the Skerne River in England (Allchin and Morris, 2003). In addition, field studies show that HBCD concentrations in fish eggs are higher than or similar to those found in fish muscles (Xian et al., 2008; Li et al., 2012). We showed that the concentrations of three HBCD diastereoisomers accumulated in *O. melastigma* embryos reached approximately 6000, 10,000 and 50,000 ng/g ww on 8 dpf with exposure concentrations of 20, 50 and 200  $\mu\text{g/L}$ , respectively (Fig. 7). At these concentrations, significant increases in the malformation rate, tachycardial effect, and apoptosis was observed in *O. melastigma* embryos for all

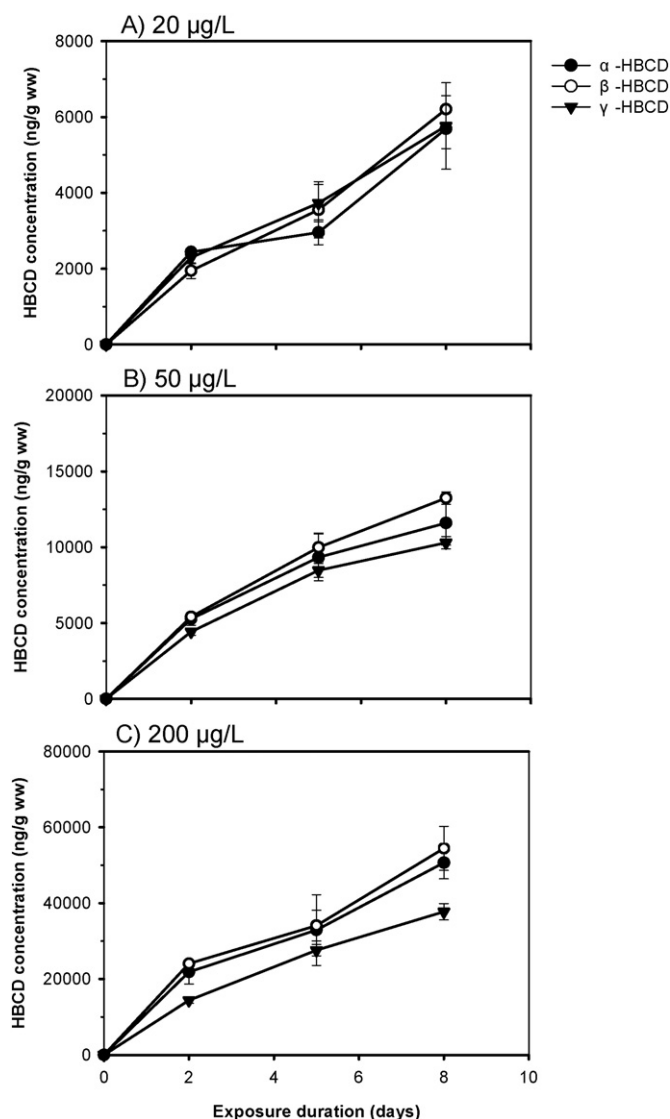


Fig. 7. Concentrations of HBCDs in *O. melastigma* embryos after exposure to 20, 50 and 200 µg/L of α-, β-, and γ-HBCDs for 0, 2, 5 and 8 days. The values are presented as the mean ± SD (n = 4).

three diastereoisomers. Therefore, environmentally realistic concentrations of HBCDs, α-HBCD as the major component, are likely to cause developmental toxicity in fish embryos. On the other hand, in the future studies it is important to examine the effects of HBCD diastereoisomers through maternal transfer on the development of embryos of next generation, which is more likely to mimic the real world scenarios.

Exposure to waterborne HBCD diastereoisomers resulted in slightly different concentrations of the isomers in *O. melastigma* embryos, particularly at high exposure concentrations (e.g. 50 and 200 µg/L). For example, after being exposed to 200 µg/L of α-, β-, and γ-HBCDs for 8 days, the embryos accumulated approximately 30% more α- and β-HBCDs than γ-HBCD. Nevertheless, the modest difference in the concentrations of HBCDs accumulated in the *O. melastigma* embryos might not have a significant impact on the relative toxicity of α-, β-, and γ-HBCDs.

## 5. Conclusions

The environmentally realistic concentrations of HBCD diastereoisomers in *O. melastigma* embryos were likely to cause developmental toxicity, e.g. increase of malformation rate and heartbeat rate. α-, β-, and γ-HBCDs had similar potency to induce the expression of the

inflammation genes TNF-α and IL-1β, stimulate the generation of ROS, and enhance the expression of p53 and the activities of caspases, consequently leading to apoptosis of *O. melastigma* embryos. The different response of *O. melastigma* and zebrafish embryos to the exposure of HBCD diastereoisomers highlighted the importance of using species from both fresh and salt water for the toxicological studies of environmental pollutants.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.marpolbul.2015.11.009>.

## References

- Abdallah, M.A., Uchea, C., Chipman, J.K., Harrad, S., 2014. Enantioselective biotransformation of hexabromocyclododecane by *in vitro* rat and trout hepatic sub-cellular fractions. *Environ. Sci. Technol.* 48, 2732–2740.
- Allchin, C.R., Morris, S., 2003. Hexabromocyclododecane (HBCD) diastereoisomers and brominated diphenyl ether congener (BDE) residues in edible fish from the River Skerne and Tees, UK. *Organohalogen Compd.* 61, 41–44.
- Chen, X.P., Li, L., Wong, C.K.C., Cheng, S.H., 2009. Rapid adaptation of molecular resources from zebrafish and medaka to develop an estuarine/marine model. *Comp Biochem Phys C* 149, 647–655.
- Chen, X.P., Li, L., Cheng, J.P., Chan, L.L., Wang, D.Z., Wang, K.J., Baker, M.E., Hardiman, G., Schlenk, D., Cheng, S.H., 2011. Molecular staging of marine medaka: a model organism for marine ecotoxicity study. *Mar. Pollut. Bull.* 63, 309–317.
- Deng, J., Yu, L., Liu, C., Yu, K., Shi, X., Yeung, L.W., Lam, P.K., Wu, R.S., Zhou, B., 2009. Hexabromocyclododecane-induced developmental toxicity and apoptosis in zebrafish embryos. *Aquat. Toxicol.* 93, 29–36.
- Du, M., Lin, L., Yan, C., Zhang, X., 2012a. Diastereoisomer- and enantiomer-specific accumulation, depuration, and bioisomerization of hexabromocyclododecanes in zebrafish (*Danio rerio*). *Environ. Sci. Technol.* 46, 11040–11046.
- Du, M., Zhang, D., Yan, C., Zhang, X., 2012b. Developmental toxicity evaluation of three hexabromocyclododecane diastereoisomers on zebrafish embryos. *Aquat. Toxicol.* 112–113, 1–10.
- Elmore, S., 2007. Apoptosis: a review of programmed cell death. *Toxicol. Pathol.* 35, 495–516.
- Harris, S.L., Levine, A.J., 2005. The p53 pathway: positive and negative feedback loops. *Oncogene* 24, 2899–2908.
- He, M.J., Luo, X.J., Yu, L.H., Wu, J.P., Chen, S.J., Mai, B.X., 2013. Diastereoisomer and enantiomer-specific profiles of hexabromocyclododecane and tetrabromobisphenol A in an aquatic environment in a highly industrialized area, south China: vertical profile, phase partition, and bioaccumulation. *Environ. Pollut.* 179, 105–110.
- Hensley, K., Robinson, K.A., Gabbita, S.P., Salsman, S., Floyd, R.A., 2000. Reactive oxygen species, cell signaling, and cell injury. *Free Radic. Biol. Med.* 28, 1456–1462.
- Hong, H.Z., Li, D.M., Shen, R., Wang, X.H., Shi, D.L., 2014. Mechanisms of hexabromocyclododecanes induced developmental toxicity in marine medaka (*Oryzias latipes*) embryos. *Aquat. Toxicol.* 152, 173–185.
- Hornung, M.W., Cook, P.M., Fitzsimmons, P.N., Kuehl, D.W., Nichols, J.W., 2007. Tissue distribution and metabolism of benzo[a]pyrene in embryonic and larval medaka (*Oryzias latipes*). *Toxicol. Sci.* 100, 393–405.
- Huang, Q., Fang, C., Wu, X., Fan, J., Dong, S., 2011. Perfluorooctane sulfonate impairs the cardiac development of a marine medaka (*Oryzias latipes*). *Aquat. Toxicol.* 105, 71–77.
- Huang, Q.S., Fang, C., Chen, Y.J., Wu, X.L., Ye, T., Lin, Y., Dong, S.J., 2012. Embryonic exposure to low concentration of bisphenol A affects the development of *Oryzias latipes* larvae. *Environ. Sci. Pollut. Res.* 19, 2506–2514.
- Iwamatsu, T., 2004. Stages of normal development in the medaka *Oryzias latipes*. *Mech. Dev.* 121, 605–618.
- Jones, H.S., Panter, G.H., Hutchinson, T.H., Chipman, J.K., 2010. Oxidative and conjugative xenobiotic metabolism in zebrafish larvae *in vivo*. *Zebrafish* 7, 23–30.
- Koeppen, R., Becker, R., Emmerling, F., Jung, C., Nehls, I., 2007. Enantioselective preparative HPLC separation of the HBCD-stereoisomers from the technical product and



- their absolute structure elucidation using X-ray crystallography. *Chirality* 19, 214–222.
- Kong, R.Y.C., Giesy, J.P., Wu, R.S.S., Chen, E.X.H., Chiang, M.W.L., Lim, P.L., Yuen, B.B.H., Yip, B.W.P., Mok, M.O.L., Au, D.W.T., 2008. Development of a marine fish model for studying in vivo molecular responses in ecotoxicology. *Aquat. Toxicol.* 86, 131–141.
- Koppen, R., Becker, R., Esslinger, S., Nehls, I., 2010. Enantiomer-specific analysis of hexabromocyclododecane in fish from Etnefjorden (Norway). *Chemosphere* 80, 1241–1245.
- Law, K., Halldorson, T., Danell, R., Stern, G., Gewurtz, S., Alae, M., Marvin, C., Whittle, M., Tomy, G., 2006a. Bioaccumulation and trophic transfer of some brominated flame retardants in a Lake Winnipeg (Canada) food web. *Environ. Toxicol. Chem.* 25, 2177–2186.
- Law, K., Palace, V.P., Halldorson, T., Danell, R., Wautier, K., Evans, B., Alae, M., Marvin, C., Tomy, G.T., 2006b. Dietary accumulation of hexabromocyclododecane diastereoisomers in juvenile rainbow trout (*Oncorhynchus mykiss*) I: bioaccumulation parameters and evidence of bioisomerization. *Environ. Toxicol. Chem.* 25, 1757–1761.
- Leung, K.M.Y., Morritt, D., Wheeler, J.R., Whitehouse, P., Sorokin, N., Toy, R., Holt, M., Crane, M., 2001. Can saltwater toxicity be predicted from freshwater data? *Mar. Pollut. Bull.* 42, 1007–1013.
- Li, D., Lu, C.L., Wang, J., Hu, W., Cao, Z.F., Sun, D.G., Xia, H.F., Ma, X., 2009. Developmental mechanisms of arsenite toxicity in zebrafish (*Danio rerio*) embryos. *Aquat. Toxicol.* 91, 229–237.
- Li, H., Zhang, Q., Wang, P., Li, Y., Lv, J., Chen, W., Geng, D., Wang, Y., Wang, T., Jiang, G., 2012. Levels and distribution of hexabromocyclododecane (HBCD) in environmental samples near manufacturing facilities in Laizhou bay area, east China. *J. Environ. Monit.* 14, 2591–2597.
- MacGregor, J., Nixon, W., 2004. Determination of hexabromocyclododecane (HBCD) diastereomers using a generator column method. 439C–138. Final Report. Wildlife International, Easton, MD, USA.
- Madamanchi, N.R., Vendrov, A., Runge, M.S., 2005. Oxidative stress and vascular disease. *Arterioscler. Thromb. Vasc. Biol.* 25, 29–38.
- Mariappan, N., Soorappan, R.N., Haque, M., Sriramula, S., Francis, J., 2007. TNF- $\alpha$ -induced mitochondrial oxidative stress and cardiac dysfunction: restoration by superoxide dismutase mimetic tempol. *Am. J. Physiol. Heart Circ. Physiol.* 293, H2726–H2737.
- Nyholm, J.R., Norman, A., Norrgren, L., Haglund, P., Andersson, P.L., 2008. Maternal transfer of brominated flame retardants in zebrafish (*Danio rerio*). *Chemosphere* 73, 203–208.
- Oh, J.K., Kotani, K., Managaki, S., Masunaga, S., 2014. Levels and distribution of hexabromocyclododecane and its lower brominated derivative in Japanese riverine environment. *Chemosphere* 109, 157–163.
- Ravanat, J.L., Douki, T., Duez, P., Gremaud, E., Herbert, K., Hofer, T., Lasserre, L., Saint-Pierre, C., Favier, A., Cadet, J., 2002. Cellular background level of 8-oxo-7,8-dihydro-2'-deoxyguanosine: an isotope based method to evaluate artefactual oxidation of DNA during its extraction and subsequent work-up. *Carcinogenesis* 23, 1911–1918.
- Tomy, G.T., Budakowski, W., Halldorson, T., Whittle, D.M., Keir, M.J., Marvin, C., Macinnis, G., Alae, M., 2004. Biomagnification of alpha- and gamma-hexabromocyclododecane isomers in a Lake Ontario food web. *Environ. Sci. Technol.* 38, 2298–2303.
- Wheeler, J.R., Leung, K.M.Y., Morritt, D., Sorokin, N., Rogers, H., Toy, R., Holt, M., Whitehouse, P., Crane, M., 2002. Freshwater to saltwater toxicity extrapolation using species sensitivity distributions. *Environ. Toxicol. Chem.* 21, 2459–2467.
- Wu, J.P., Guan, Y.T., Zhang, Y., Luo, X.J., Zhi, H., Chen, S.J., Mai, B.X., 2010. Trophodynamics of hexabromocyclododecanes and several other non-pbde brominated flame retardants in a freshwater food web. *Environ. Sci. Technol.* 44, 5490–5495.
- Xia, C.H., Lam, J.C.W., Wu, X.G., Sun, L.G., Xie, Z.Q., Lam, P.K.S., 2011. Hexabromocyclododecanes (HBCDs) in marine fishes along the Chinese coastline. *Chemosphere* 82, 1662–1668.
- Xian, Q., Ramu, K., Isobe, T., Sudaryanto, A., Liu, X., Gao, Z., Takahashi, S., Yu, H., Tanabe, S., 2008. Levels and body distribution of polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecanes (HBCDs) in freshwater fishes from the Yangtze River, China. *Chemosphere* 71, 268–276.
- Zhang, X., Yang, F., Zhang, X., Xu, Y., Liao, T., Song, S., Wang, H., 2008. Induction of hepatic enzymes and oxidative stress in Chinese rare minnow (*Gobiocypris rarus*) exposed to waterborne hexabromocyclododecane (HBCD). *Aquat. Toxicol.* 86, 4–11.
- Zhang, Y.W., Sun, H.W., Ruan, Y.F., 2014a. Enantiomer-specific accumulation, depuration, metabolism and isomerization of hexabromocyclododecane (HBCD) diastereomers in mirror carp from water. *J. Hazard. Mater.* 264, 8–15.
- Zhang, H., Pan, L.Q., Tao, Y.X., 2014b. Antioxidant responses in clam *Venerupis philippinarum* exposed to environmental pollutant hexabromocyclododecane. *Environ. Sci. Pollut. Res.* 21, 8206–8215.