1 Developmental Toxicity of Three Hexabromocyclododecane
2 Diastereoisomers in Embryos of the Marine Medaka *Oryzias melastigma*

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1 **Abstract**

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

13

14 Key words: hexabromocyclododecanes; diastereoisomers; *Oryzias melastigma*; oxidative stress;
15 developmental toxicity

16

1 **Introduction**

2 Hexabromocyclododecanes (HBCDs), the third most widely used brominated flame
3 retardants, are extensively used in polystyrene insulation foam boards, textile products, and
4 electronic products. Attributed to their persistence and ecotoxicological concerns, HBCDs recently
5 have been listed by the Stockholm Convention as persistent organic pollutants for consideration of
6 elimination (www.pops.int). Nowadays, HBCDs can be found ubiquitously in various
7 environmental media including aquatic habitats, and HBCD concentrations in the ng/L range have
8 been detected in waters [1-3]. For example, 2100 ng/L of total HBCDs was recorded in the surface
9 water of the River Kuzuryu, Japan, which receives effluents from textile industries [4]. Owing to
10 their high octanol-water partition coefficient (K_{ow} , 5.625), HBCDs tend to build up heavily in
11 sediments, often in the $\mu\text{g/kg}$ dry weight range, and with exceptionally high concentrations
12 (i.e., 7800 $\mu\text{g/kg}$ dry weight) reported in the River Kuzuryu [4]. Aside from their significant
13 accumulation in water and sediments, relatively high concentrations of HBCDs are also detected
14 in several freshwater and marine fish species downstream of the source of the chemical and in
15 highly industrialized areas in Europe and China [5-7].

16 The commercially available HBCD (technical HBCD, tHBCD) is often characterized as a
17 mixture of mainly three diastereoisomers, i.e., α -, β - and γ -HBCD, and the composition of these
18 diastereoisomers in tHBCD varies in the range of 1-12% α -HBCD, 10-13% β -HBCD and 75-89%
19 γ -HBCD [8]. Stereo-structural difference of these three HBCD diastereoisomers results in
20 substantial differences in physicochemical properties (e.g., polarity, solubility and K_{ow}), which
21 may consequently lead to distinctive environmental fates [9]. Laboratory research shows that the
22 bioconcentration factor and biomagnification factor of α -HBCD in fish (e.g., zebrafish or mirror

1 carp) are much higher than those of β - and γ -HBCD [10, 11]. In addition, β - and γ -HBCD can be
2 biotransformed to α -HBCD in adult fish [10-12]. Indeed, α -HBCD is always the predominant
3 HBCD diastereoisomer detected in both freshwater and marine fish sampled from the field,
4 accounting for ~80-90% of total HBCDs [1, 3, 13, 14].

5 Although the composition of α -, β - and γ -HBCD in aquatic biota is very different from that
6 of tHBCD, tHBCD is used for most of the toxicological studies. For instance, studies using
7 tHBCD for exposure show that tHBCD induces the generation of reactive oxygen species (ROS)
8 and impacts the development of both freshwater and marine fish embryos [15, 16]. However, the
9 toxicity of individual HBCD diastereoisomers, which is important for risk assessment of HBCDs,
10 remains largely unknown. Du et al. [17] found that in the freshwater zebrafish *Danio rerio* the
11 order of developmental toxicity of these three HBCD diastereoisomers was
12 γ -HBCD> β -HBCD> α -HBCD. Based on this result, the bioconversion of β - and γ -HBCD to
13 α -HBCD in fish could be considered as a detoxification process and hence the toxicity data
14 obtained from tHBCD exposure in risk assessment might overestimate the toxicity of HBCDs.
15 Comparison studies on the toxicity of individual HBCD diastereoisomers using other aquatic
16 species, however, are very limited. Given the existence of inter-species variation, it is necessary to
17 conduct toxicological studies on more than one species to reduce uncertainty.

18 In addition, it is reported that the sensitivity of freshwater and saltwater species to
19 environmental pollutants could be significantly different [18, 19]. It is likely to be affected by
20 various biological and physicochemical factors, such as salinity, pH and ionic concentrations, and
21 could also be influenced by the mode of toxic action of the chemicals [18, 19]. In the case of
22 HBCDs, the lowest observable effect concentration (LOEC) of tHBCD to affect the heartbeat of

1 embryos is 5 µg/L in the marine medaka *Oryzias melastigma* and 50 µg/L in the freshwater
2 zebrafish [15, 16]. tHBCD causes bradycardia in zebrafish embryos but tachycardia in marine
3 medaka embryos. These findings suggest that freshwater and marine fish may have different
4 sensitivity and react differently to HBCDs [15, 16].

5 Given that the toxicity and toxic mechanisms of individual HBCD diastereoisomers in
6 marine organisms remain largely unknown, we chose the marine medaka *O. melastigma* as a
7 model species for toxicity investigation. *O. melastigma* is adopted by the International Life
8 Science Institute (ILSI, Washington DC, USA) Health and Environmental Science Institute for
9 embryo toxicity testing and is a very useful model for estuarine and marine ecotoxicological
10 research [20-22]. It has been demonstrated that tHBCD can cause developmental toxicity of *O.*
11 *melastigma* embryos by enhancing the generation of ROS and thus leading to apoptosis [16]. In
12 this study, our main purpose was to compare the relative toxicity of α -, β -, and γ -HBCD in *O.*
13 *melastigma* embryos. In addition, the transcription and expression of genes and proteins related to
14 oxidative stress and apoptosis were examined to compare the potential toxic mechanisms induced
15 by the three HBCD diastereoisomers.
16

1 **Materials and Methods**

2 **Chemicals and reagents.** tHBCD (>95% pure), from which α -, β - and γ -HBCD were purified,
3 was purchased from the Sinopharm Chemical Reagent Co. (Shanghai, China); α -HBCD, β -HBCD
4 and γ -HBCD, used as standards for LC-MS/MS analysis, were ordered from AccuStandard, Inc.
5 (New Haven, Connecticut, USA); [^{13}C]- α -HBCD, [^{13}C]- β -HBCD and [^{13}C]- γ -HBCD were
6 purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA); and the organic
7 solvents (acetonitrile, acetone, hexane and methanol, purity >99.5%) used for sample extraction,
8 high performance liquid chromatography (HPLC) separation, and liquid chromatography-tandem
9 mass spectrometry (LC-MS/MS) analysis were obtained from Tedia Inc. (Fairfield, OH, USA). All
10 other reagents were purchased from Sigma-Aldrich Chemical Co. (Saint Louis, MO, USA) except
11 where indicated.

12 **HPLC purification of α -HBCD, β -HBCD and γ -HBCD.** HPLC purification was performed on a
13 Varian Prostar 218 semi-preparative HPLC system (Varian Inc., Palo Alto, CA, USA) with a
14 photodiode array detector set at a wavelength of 250 nm. Separation of α -, β -, and γ -HBCD was
15 achieved with a 250 $\times$ 21.4 mm Varian Dynamax Microsorb 100-5 C18 column (5 μm in particle
16 size). The HPLC mobile phase consisted of two solvents: mobile phase A (10 mM ammonium
17 acetate in water:methanol, 50:50, v/v) and mobile phase B (100% methanol). The gradient
18 program used for separation was linear gradient from 80 to 100% B (0-20 min); hold isocratic at
19 100% B (20-30 min); linear gradient from 100-80% B (30-32 min); and re-equilibrate at 80% B
20 for 10 min. The mobile phase flow rate was 10 mL/min. The HPLC fractions containing α -, β -,
21 and γ -HBCD were collected on ice and immediately dried using a rotary evaporation at ambient
22 temperature. The dried samples were then redissolved in dimethyl sulfoxide (DMSO,

1 purity >99.5%) and desalted using the same reverse-phase C18 column for purification. After the
2 samples were loaded, the column was washed with water:methanol (15:85, v/v) for 10 min, and
3 the samples were eluted from the column using 100% methanol. The HPLC fractions containing
4 desalted α -, β -, and γ -HBCD were collected on ice and dried using rotary evaporation. Upon
5 reaching dryness, the samples were immediately stored at -80 °C to avoid decomposition. The
6 purity of the separated α -, β -, and γ -HBCD was checked using HPLC-ultraviolet spectrometry
7 (Supplementary Material) and the quantities were determined using LC-MS/MS.

8 **Medaka maintenance and embryo toxicity test.** Marine medaka *O. melastigma* were maintained
9 following previously described conditions [16, 22]. Briefly, they were maintained at 28 ± 2 °C in a
10 14-h light:10-h dark photoperiod in aerated artificial seawater at a salinity of 30‰ and a dissolved
11 oxygen concentration of 5.8 ± 0.2 mg/L. The fish were fed with *Artemia nauplii* twice daily.

12 The freshly fertilized eggs of *O. melastigma* were collected daily and visually sorted under a
13 dissecting microscope. As marine *O. melastigma* and freshwater *O. latipes* share high similarity in
14 development and morphology [20-22], embryo developmental stages of *O. melastigma* were
15 judged according to the morphology parameters used for staging *O. latipes* [23]. The embryos at
16 developmental stages 12-15 were sorted, when the blastoderm was expanding and the embryonic
17 shield was just visible but before clearly visible as a narrow streak [23]. The sorted embryos were
18 randomly distributed into sixteen groups and exposure was started immediately. For monitoring of
19 survival, development and heart rate, forty embryos were included in each replicate. The embryos
20 were exposed to 0, 20, 50, and 200 $\mu\text{g/L}$ (corresponding to 0, 31, 78, and 310 nM) of α -, β -, or
21 γ -HBCD in seawater. Each treatment consisted of four groups as replicates. The solvent control
22 groups received the same concentration of DMSO as the treated groups (0.01%, v/v). Each

1 exposure group was maintained in 60-mm crystallizing dishes containing 20 mL of exposure
2 media and the exposure solution was freshly prepared and renewed daily. The exposure
3 concentrations, toxicity endpoints selected for measurements, sample collecting time points and
4 sample replication numbers are indicated in Scheme 1.

5 **Toxicity endpoints for measurement.** To assess developmental toxicity, malformation of the
6 embryos was monitored daily under the microscope. Once the embryos were identified as
7 malformation or hatched, they were removed from the exposure group and kept separately in a
8 new container with exposure media being daily changed. The exposure was terminated 17 days
9 post fertilization (dpf) when most of the larvae were hatched and the survival rate, hatching
10 success rate, and hatch-out time were recorded. The number of abnormal embryos was counted for
11 calculation of malformation rate no matter how many abnormality appeared on each individual.
12 The heart rate and the distance between the sinus venosus (SV) and bulbus arteriosus (BA) region
13 of the heart (SV-BA distance) were measured, as described previously [24], to evaluate the effect
14 of HBCD diastereoisomers on embryo cardiac development (Figure S4, Supplementary Material).

15 To assess whether HBCD diastereoisomers stimulated the generation of ROS through the
16 induction of the inflammation genes TNF- α and IL-1 β , which then triggered the p53 signaling
17 pathway, activated caspases, and resulted in apoptosis [16], the concentrations of
18 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) in genomic DNA, the gene expression levels of
19 TNF- α , IL-1 β and p53, and the activities of caspase-3, caspase-8 and caspase-9 were measured.
20 Furthermore, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was
21 also conducted to detect DNA fragmentation resulting from apoptotic signaling cascades.

22 **Sample extraction and LC-MS/MS quantification of HBCD isomers.** To determine the actual

1 concentrations of HBCDs in the exposure media, 10-mL of each media sample was passed through a
2 C18 cartridge (Supelco, Bellefonte, PA, USA), which was preconditioned by methanol and water.
3 After washing the column with 10 mL H₂O for desatling, the extraction column were eluted with 9 mL
4 of methanol and the eluate was concentrated to 0.5 mL under N₂ flow.

5 To determine the concentrations of HBCDs accumulated in the embryos, each sample
6 containing twenty embryos was first spiked with the ¹³C-labeled α -, β -, and γ -HBCD isomers, and
7 then homogenized and extracted in 5 mL hexane:acetone (1:1, v/v) using ultrasonication for 30
8 min. After centrifugation, the supernatant was collected and the pellet was extracted again. The
9 resulting supernatant was combined with the first supernatant and then concentrated to 0.5 mL
10 under N₂ flow. At the end of the exposure (17 dpf) when most embryos were hatched, twenty
11 larvae per sample were collected and HBCDs in the hatched larvae were extracted by the same
12 method.

13 The quantification of the HBCD isomers was carried out on an Agilent 1290-6490 ultra
14 performance liquid chromatography (UPLC)-triple quadruple mass spectrometry system (Agilent
15 Technologies, Palo Alto, CA, USA) following conditions reported previously [16]. The α -, β -, and
16 γ -HBCD isomers and their corresponding [¹³C]-labeled internal standards were monitored in
17 multiple reaction monitoring mode with transition events of m/z 640.8 > 80.8 and m/z
18 652.8 > 80.8.

19 **Quantification of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG).** The genomic DNA of
20 thirty embryos per sample was isolated using a DNA Extractor[®] WB kit (Wako Pure Chemical
21 Industries, Osaka, Japan) following the manufacturer's protocol, except that 0.1 mM of
22 desferrioxamine was included in the solutions used for DNA extraction to minimize the artifactual

1 oxidation of DNA [25].

2 The extracted DNA was hydrolyzed using calf spleen phosphodiesterase, nuclease P1, snake
3 venom phosphodiesterase and alkaline phosphatase [16]. The 8-oxodG concentrations in the
4 resulting digestion mixture were quantified using the isotope-dilution method combined with
5 LC-MS/MS analysis following the conditions described previously [16].

6 **Real-time quantitative polymerase chain reaction (qPCR).** Total RNA was extracted from ten
7 embryos (8 dpf) or larvae (newly hatched) per replicate. An equal amount of RNA was then
8 reverse-transcribed using mixtures of oligo (dT) primer (5'-TTTTTTTTTTTTTTTTTTT-3') and
9 random primers with moloney murine leukemia virus reverse transcriptase (BGI, Shenzhen, China)
10 to generate cDNA. qPCR was carried out on a CFX96TM Real-Time System (Bio-Rad
11 Laboratories, CA, USA) using SYBR Green I. The primers for qPCR of the p53, TNF- α , IL-1 β
12 and 18s rRNA genes were from the literature [24, 26]. The thermal cycle program consisted of an
13 initial denaturation step at 95°C for 3 min, followed by 50 cycles of 10 s at 95°C, and then 35 s at
14 60°C for 18s gene, 65°C for p53 gene and 70°C for TNF- α and IL-1 β genes. Dissociation curve
15 analysis was used to confirm that only the targeted PCR product was amplified and detected. Gene
16 expression levels were normalized to 18s rRNA expression levels. Two replicates of qPCR were
17 performed for each sample as technical replicate and four samples were performed for each
18 treatment. The fold change of the tested genes was analyzed using the $2^{-\Delta\Delta C_t}$ method.

19 **Caspase activity.** Crude enzymes were extracted from thirty embryos per sample and the
20 measurement of caspase-3, caspase-8, and caspase-9 activities was performed using caspase-3,
21 caspase-8, and caspase-9 colorimetric protease assay kits (Keygene Biotech Co., Nanjing, China)
22 following the manufacturer's instruction. The protein concentration was determined with reference

1 to standards of bovine serum albumin using the BCA assay (Thermo Fisher Scientific, MA, USA).

2 The enzyme activity was expressed as the fold change over the control samples.

3 **Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.** The

4 fragmented DNA of apoptotic cells was identified using the TUNEL method with the fluorescent

5 *in situ* cell death detection kit (Roche Diagnostic, Switzerland) following the manufacturer's

6 instruction. Briefly, twenty newly hatched larvae were fixed for 2 h at room temperature in 4%

7 paraformaldehyde in phosphate buffer saline (PBS, freshly prepared) [27]. The samples were then

8 washed twice in PBS and further permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate

9 (freshly prepared) for 10 min on ice, followed by two rinses in PBS. The samples were incubated

10 with the label solution mixture overnight at 4°C and then shaken for 1 h at 37°C. The labeling

11 reaction was stopped by washing the samples in PBS three times (5 min each) at room temperature

12 to remove excessive TdT and deoxynucleotides. After TUNEL staining, the larvae were

13 photographed by a fluorescent microscope with an excitation wavelength of 485 nm and emission

14 wavelength of 510 nm.

15 **Western blotting.** Total protein was extracted from ten larvae per replicate. The western blotting

16 assay for the relative quantification of P53 protein followed conditions reported previously [16].

17 Briefly, an equal amount of protein was used for analysis and pre-stained molecular weight

18 markers (Bio-Rad Laboratories, CA, USA) were run as standards on each gel. After sodium

19 dodecyl sulfate-polyacrylamide gel eletrophoresis separation and polyvinylidene difluoride

20 membrane transfer, the protein containing membrane was blocked with 5% non-fat dry milk,

21 incubated with the primary anti-p53 antibody (Bioss, Beijing, China) for 1h and washed three

22 times. After incubating with an anti-rabbit IgG-alkaline phosphatase conjugated secondary

1 antibody (Sigma-Aldrich Chemical Co., Saint Louis, MO, USA) at room temperature for 1 h, the
2 membrane was washed three times again, then rinsed twice with a buffer containing 100 mM Tris
3 (pH 9.5), 100 mM NaCl, and 10 mM MgCl₂, and incubated with NBT/BCIP solution (Roche,
4 Indianapolis, USA) until bands appeared.

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

8

1 **Results**

2 **Developmental toxicity.** Exposure of *O. melastigma* embryos to α -, β -, or γ -HBCD did not cause
3 significant effect on the survival rate and hatching rate (data not shown). The three
4 diastereoisomers all induced malformation of the hatched larvae, with yolk sac edema, pericardial
5 edema, and spinal curvature as the most frequently observed abnormalities (Figure S3,
6 Supplementary Material). At the highest exposure concentration (i.e., 200 μ g/L), the malformation
7 rates in both the α - and γ -HBCD exposure groups were significantly higher than that in the control
8 group ($p<0.05$, Figure 1).

9 The heart rate of the *O. melastigma* embryos was recorded on both 5 and 8 dpf, two time
10 points that are within the critical period of embryo heart development [24]. In this period, the
11 atrio-ventricular region of the heart increases and the atrium and then ventricle lie adjacent to each
12 other [23] (Figure S4, Supplementary Material). The results showed that the heart rate of the
13 embryos was increased by exposure to α -, β -, or γ -HBCD generally in a concentration dependent
14 manner (Figure 2). Even at the lowest exposure concentration (i.e., 20 μ g/L), the heart rate of the
15 embryos on 8 dpf significantly increased from 139 ± 6 to 147 ± 5 , 146 ± 4 , and 144 ± 4 beats/min, upon
16 exposure to α -, β -, and γ -HBCD, respectively ($p<0.01$, Figure 2B).

17 To further examine the effects of HBCD diastereoisomers on the cardiac development of *O.*
18 *melastigma*, the SV–BA distance, which is used as an important index for the evaluation of
19 cardiac development [24], was measured in the developing embryos. The results showed that the
20 SV–BA distance of embryos was induced markedly by exposure to α -, β -, or γ -HBCD at 200 μ g/L
21 (Figure 3A, $p<0.05$), whereas exposure to other lower concentrations (e.g., 20 and 50 μ g/L) did
22 not have any significant effect (data not shown).

1 **Increased concentrations of 8-oxodG.** To assess whether HBCD diastereoisomers induced
2 oxidative stress and further led to oxidative DNA damage in *O. melastigma* embryos, the
3 concentrations of 8-oxodG, an oxidative product of deoxyguanine induced by various types of
4 ROS, were measured after 6 days of exposure. The concentration of 8-oxodG was 3.8 lesions/10⁶
5 deoxyguanine in the control and increased to 13.3, 8.1, and 13.5 lesions/10⁶ deoxyguanine,
6 respectively, following exposure to 200 µg/L of α-, β-, and γ-HBCD (Figure 3B).

7 **Induction of stress-responsive genes and proteins.** The expression of the inflammation genes
8 TNF-α and IL-1β and of the apoptotic gene p53 were analyzed for 8-dpf embryos and newly
9 hatched larvae. The mRNA transcription of the three genes was all significantly up-regulated at
10 the exposure concentrations of 50 or 200 µg/L in the two selected developmental stages in a
11 dose-dependent manner (Figure 4). In addition, the expression of p53 protein was up-regulated by
12 43, 18, and 31% of the control after exposure to 200 µg/L of α-, β-, and γ-HBCD, respectively,
13 which was consistent with the observation at transcription level.

14 **Induction of caspase activity.** To evaluate whether HBCD diastereoisomers induced apoptosis
15 via the caspase-dependent pathway, the activities of caspase-3, caspase-8, and caspase-9 were
16 evaluated on 8 dpf. The activity of caspase-3 was induced by 45, 73, and 37% at the exposure
17 concentration of 20 µg/L and by 45, 75, and 107% at the exposure concentration of 200 µg/L of α-,
18 β-, and γ-HBCD, respectively (Figure 5A). All the three diastereoisomers significantly induced the
19 activities of both caspase-8 and caspase-9 at the exposure concentration of 200 µg/L ($p < 0.05$,
20 Figure 5B-C).

21 **Induction of apoptosis.** After exposure to the three HBCD diastereoisomers at concentrations of
22 0, 20, 50, and 200 µg/L, the newly hatched larvae were fixed, stained using the fluorescent

1 TUNEL staining method, and then visualized under a fluorescence microscope. Larvae in the 50
2 and 200 $\mu\text{g/L}$ treatment groups for all the three diastereoisomers showed apoptotic signals in the
3 heart region (Figure 6). No obvious apoptotic hearts were observed in the control and 20 $\mu\text{g/L}$
4 treated groups (Figure 6).

5 **Concentrations of HBCD diastereoisomers in the embryos.** In the LC-MS/MS chromatograms,
6 only the HBCD diastereoisomer used for exposure was detected in the embryos, indicating that
7 there was no detectable bio-conversion among the three diastereoisomers in the embryos. The
8 recovery of α -, β -, and γ -HBCD from the embryo samples were 91%, 95% and 92%, respectively.
9 The concentrations of α -, β -, and γ -HBCD in embryos on 0 dpf (before exposure) were 1.7 ± 0.3 ,
10 4.5 ± 0.1 and 2.6 ± 0.1 ng/g ww, respectively (Figure 7). The concentrations of HBCD isomers in
11 embryos increased over time during the exposure period for all HBCDs treated groups, suggesting
12 continuous accumulation of HBCD isomers in medaka embryos through the water-borne exposure
13 (Figure 7). For the 20 $\mu\text{g/L}$ exposure groups, there was no significant difference in the
14 concentrations of the three HBCD diastereoisomers accumulated in the embryos up to 8 dpf
15 (Figure 7A), whereas for the two higher exposure concentrations (i.e., 50 and 200 $\mu\text{g/L}$), the
16 concentrations of α - and β -HBCD accumulated in the embryos were slightly higher than γ -HBCD
17 (Figure 7B-C). The concentrations of HBCDs in the larvae on 17 dpf were also measured and the
18 concentrations of α - and β -HBCD were also higher than γ -HBCD for the 50 and 200 $\mu\text{g/L}$ treated
19 groups (Figure S5, Supplementary Material).

20 The concentrations of HBCDs in the exposure media were determined right after media
21 renewal (0 h) and before next media renewal (24 h) (Table S1, Supplementary Material). After 24
22 h exposure, the concentrations of three HBCD diastereoisomers remained higher than 50% of the

- 1 initial concentrations. Nevertheless, there was no difference in the concentrations of α -, β -, and
- 2 γ -HBCD in the exposure media.
- 3
- 4

Discussion

The commercially available form of HBCD (tHBCD), consisting of ~90% γ -HBCD, 5% α -HBCD, and 5% β -HBCD, is widely used in toxicological studies of the brominated flame retardant on a variety of organisms, including fish and invertebrates in aquatic systems [15, 16, 28]. The development of zebrafish and *O. melastigma* embryos is affected by exposure to tHBCD [15-17]. 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 [16, 29, 30], the composition of α -, β -, and γ -HBCD accumulated in *O. melastigma* embryos exposed to tHBCD through water-borne pathway is identical to that of tHBCD, suggesting limited bioconversion among HBCD diastereoisomers [16]. The results in the present study also indicated no apparent bioconversion among α -, β -, and γ -HBCD. Nevertheless, in adult fish, e.g., zebrafish, mirror carp and rainbow trout, β - and γ -HBCD can be bioisomerized to α -HBCD [10-12]. In addition, *in vitro* work reveals that α -HBCD is more resistant to metabolic degradation by liver enzymes of both rats and adult trout than β - and γ -HBCD [31]. 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, α -HBCD in general accounts for ~80-90% of total HBCDs in both freshwater and marine fish tissues collected from the field, whereas β - and γ -HBCD are the minor fractions [6, 13]. HBCDs are maternally transferred to fish eggs [32], so the compositions of the three diastereoisomers in fish eggs are almost identical to those in other fish tissues, e.g., in fish fillet [6, 13]. Since α -HBCD but not γ -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

1 differentiate the developmental toxicity of individual HBCD diastereoisomers in fish embryos.

2 In the present study, we exposed *O. melastigma* embryos to three HBCD diastereoisomers
3 (i.e., α -, β -, and γ -HBCD) and compared their developmental toxicity. Considering all the
4 developmental toxicity endpoints collectively, including induction of heart rate, malformation rate
5 and SV-BA distance, α -, β -, and γ -HBCD in general exhibited very similar potency to induce
6 developmental toxicity in *O. melastigma* embryos. In contrast to Du et al. [17] who reported that
7 the rank order of developmental toxicity in zebrafish embryos was γ -HBCD > β -HBCD > α -HBCD,
8 the hatching success of *O. melastigma* embryos was not affected by any of the three
9 diastereoisomers. In addition, the heart beat of zebrafish embryos was reduced at 48 and 96 hpf by
10 both α - and β -HBCD, whereas it was first reduced at 48 hpf and then increased at 96 hpf by
11 γ -HBCD [17]. In contrast, in *O. melastigma* embryos, the three diastereoisomers all caused
12 significant tachycardia effect at the two developmental stages (i.e., 5 and 8 dpf). The different
13 response of freshwater zebrafish and marine *O. melastigma* to the exposure of HBCDs highlights
14 species-specific and possibly freshwater and seawater species differences in toxicity evaluation.

15 tHBCD can induce oxidative stress in aquatic organisms and subsequently results in
16 developmental toxicity [15, 33]. Induction of antioxidant biomarkers, e.g. glutathione
17 *S*-transferase, superoxide dismutase, and DNA damage, are observed in the clam *Venerupis*
18 *philippinarum* upon exposure to tHBCD [28]. The measurement of 8-oxodG, a robust biomarker
19 for oxidative stress, indicated that all the three HBCD diastereoisomers induced the generation of
20 ROS in the *O. melastigma* embryos (Figure 3B).

21 Reactive oxygen species attack DNA, generate oxidative DNA damage (e.g. 8-oxodG), which
22 can be detected and repaired by certain DNA repair enzymes, and meanwhile activate the p53

1 pathway to promote cell cycle arrest or apoptosis [34]. Our results showed that in correspondence
2 with the increasing concentrations of 8-oxodG, the expression of both p53 gene and protein was
3 up-regulated upon exposure to the three HBCD diastereoisomers (Figure 4E-F). Activation of p53
4 triggers the mitochondria-mediated apoptotic pathway, subsequently resulting in cytochrome c
5 release and activation of caspases [35]. The activities of caspases, particularly caspase-3, were
6 significantly and similarly induced by α -, β -, and γ -HBCD in *O. melastigma* embryos (Figure 5),
7 whereas the potency of γ -HBCD to induce caspase-3 activity was much higher than α - and
8 β -HBCD in zebra fish [17]. The apoptotic cells around the heart area in both HBCDs treated
9 zebrafish and *O. melastigma* embryos [15-16, this study] suggested that the generation of ROS,
10 induction of DNA oxidative damage and increase of caspase enzymes occurred likely in the heart.
11 Consistently, the heart rate and SV-BA distance were significantly increased (Figure 2 and Figure
12 3) in HBCDs treated embryos, suggesting that HBCDs induced developmental toxicity in the
13 cardiovascular system of *O. melastigma* embryos.

14 tHBCD exposure up-regulated the transcription of TNF- α (tumor necrosis factor- α) and
15 interleukin-1 β (IL-1 β) and resulted in the generation of ROS in *O. melastigma* embryos [16].
16 TNF- α can stimulate ROS production by several mechanisms, including its effect as an agonist to
17 increase the product of nicotinamide adenine dinucleotide phosphate oxidase and O₂ and thus
18 promote the formation of ROS and its role in the changes of mitochondria redox state [36, 37].
19 IL-1 β , an inflammatory cytokine, also has been implicated in ROS generation [38]. Here we
20 showed that α -, β -, and γ -HBCD had similar potency in the induction of expression of TNF- α and
21 IL-1 β genes (Figure 4), which was consistent with the measurement of 8-oxodG. Transcription of
22 CYP1A or COX-1/COX-2 genes in *O. melastigma* embryos was not up-regulated upon α -, β -, or

1 γ -HBCD exposure (data not shown), indicating that the AhR/ARNT (aryl hydrocarbon
2 receptor/AHR nuclear translocation protein) pathway and COX genes mediated inflammatory
3 process were not involved in the induction of oxidative stress.

4 HBCDs are readily bioavailable and can be bioaccumulated in aquatic organisms through the
5 food web [39]. Relatively high concentrations of HBCDs are detected in fish species from Europe
6 and China [5-7]. For instance, ~10,000 ng/g ww of HBCDs are found in fish samples caught from
7 the Skerne River in England [5]. In addition, field studies show that HBCD concentrations in fish
8 eggs are higher than or similar to those found in fish muscles [6, 13]. We showed that the
9 concentrations of three HBCD diastereoisomers accumulated in *O. melastigma* embryos reached
10 approximately 6000, 10,000 and 50,000 ng/g ww on 8 dpf with exposure concentrations of 20, 50
11 and 200 $\mu\text{g/L}$, respectively (Figure 7). At these concentrations, significant increases in the
12 malformation rate, tachycardial effect, and apoptosis were observed in *O. melastigma* embryos for
13 all three diastereoisomers. Therefore, environmentally realistic concentrations of HBCDs,
14 α -HBCD as the major component, are likely to cause developmental toxicity in fish embryos. On
15 the other hand, in the future studies it is important to examine the effects of HBCD
16 diastereoisomers through maternal transfer on the development of embryos of next generation,
17 which is more likely to mimic the real world scenarios.

18 Exposure to waterborne HBCD diastereoisomers resulted in slightly different concentrations
19 of the isomers in *O. melastigma* embryos, particularly at high exposure concentrations (e.g. 50 and
20 200 $\mu\text{g/L}$). For example, after being exposed to 200 $\mu\text{g/L}$ of α -, β -, and γ -HBCD for 8 days, the
21 embryos accumulated approximately 30% more α - and β -HBCD than γ -HBCD. Nevertheless, the
22 modest difference in the concentrations of HBCDs accumulated in the *O. melastigma* embryos

1 might not have a significant impact on the relative toxicity of α -, β -, and γ -HBCD.

2 **Conclusions.** The environmentally realistic concentrations of HBCD diastereoisomers in *O.*

3 *melastigma* embryos were likely to cause developmental toxicity, e.g. increase of malformation

4 rate and heartbeat rate. α -, β -, and γ -HBCD had similar potency to induce the expression of the

5 inflammation genes TNF- α and IL-1 β , stimulate the generation of ROS, and enhance the

6 expression of p53 and the activities of caspases, consequently leading to apoptosis of *O.*

7 *melastigma* embryos. The different response of *O. melastigma* and zebrafish embryos to the

8 exposure of HBCD diastereoisomers highlighted the importance of using species from both fresh

9 and salt water for the toxicological studies of environmental pollutants.

10

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9

10

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2

1 **Figure Legends**

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

8
9 **Figure 2.** HBCD diastereoisomer exposure increased the heart rate in *O. melastigma* embryos on
10 both 5 (A) and 8 (B) days post fertilization. The *O. melastigma* embryos were exposed to different
11 concentrations of HBCD diastereoisomers (0, 20, 50, and 200 µg/L). The values are presented as
12 the mean ± SD (n = 20). The values that are significantly different from the control are indicated
13 by asterisks (one-way ANOVA, followed by LSD *post hoc* test: * $p < 0.05$, ** $p < 0.01$, *** $p <$
14 0.001).

15
16 **Figure 3.** HBCD diastereoisomer exposure induced sinus venosus-bulbus arteriosus (SV-BA)
17 distance (A) and the concentrations of 8-oxodG (B) in *O. melastigma* embryos. The *O.*
18 *melastigma* embryos were exposed to 200 µg/L of α -, β -, and γ -HBCD. The SV-BA distance was
19 measured as the direct distance between the centers of SV and BA on 8 days post fertilization
20 (dpf). The structure of cardiovascular systems of embryos, including sinus venosus, atrium,
21 ventricle and bulbus arteriosus, was shown in Figure S4 (Supplementary Material). The
22 concentrations of 8-oxodG were quantified by LC-MS/MS on 6 dpf. The values are presented as

1 the mean $\pm$ SD (n = 8 for SV-BA; n = 4 for 8-oxodG). Data presentation is same as Figure 2.

2

3 **Figure 4.** Induction of the gene expression of TNF- α (A, B), IL-1 β (C, D) and p53 (E, F) in *O.*

4 *melastigma* embryos on 8 days post fertilization and in newly hatched larvae after exposure to 0,

5 20, 50, and 200 μ g/L of HBCD diastereoisomers. The values are presented as the mean $\pm$ SD (n =

6 4). Data presentation is same as Figure 2.

7

8 **Figure 5.** Induction of the activities of caspase-3 (A), caspase-8 (B) and caspase-9 (C) in *O.*

9 *melastigma* embryos after exposure to 0, 20, and 200 μ g/L of α -, β -, and γ -HBCD. The values are

10 presented as the mean $\pm$ SD (n = 4). Data presentation is same as Figure 2.

11

12 **Figure 6.** Induction of apoptosis in the heart region of newly hatched larvae of *O. melastigma*

13 detected using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.

14 The embryos were exposed to 0 (A), 20 (B, E, H), 50 (C, F, I) and 200 μ g/L (D, G, J) of α - (B, C,

15 D), β - (E, F, G), and γ -HBCD (H, I, J) and the newly hatched larvae were fixed in 4%

16 paraformaldehyde in phosphate buffer saline (PBS), washed twice in PBS, permeabilized with 0.1%

17 Triton X-100 in 0.1% sodium citrate and incubated with the label solution mixture. The labeling

18 reaction was stopped by washing the samples in PBS, and the larvae were photographed by a

19 fluorescent microscope with an excitation wavelength of 485 nm and emission wavelength of 510

20 nm. Red arrows indicated the heart region of *O. melastigma* larvae.

21

22 **Figure 7.** Concentrations of HBCDs in *O. melastigma* embryos after exposure to 20, 50 and 200

1 $\mu\text{g/L}$ of α -, β -, and γ -HBCD for 0, 2, 5 and 8 days. The values are presented as the mean $\pm$ SD (n =
2 4).

3

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

6

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Scheme 1

A

	($\mu\text{g/L}$)	0	20	50	200
	(nM)	0	31	78	310
Malformation observation (n=4)		✓	✓	✓	✓
Heart beat (n=20)		✓	✓	✓	✓
SV-BA distance (n=8)		✓	✓	✓	✓
HBCDs concentration (n = 4)		✓	✓	✓	✓
8-oxodG (n=4)		✓			✓
Gene expression (n=4)		✓	✓	✓	✓
Caspase activities (n=4)		✓	✓		✓
TUNEL (n=20)		✓	✓	✓	✓

B