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Diastereoisomer- and Enantiomer-Specific Accumulation, Depuration, and Bioisomerization of Hexabromocyclododecanes in Zebrafish (*Danio rerio*)

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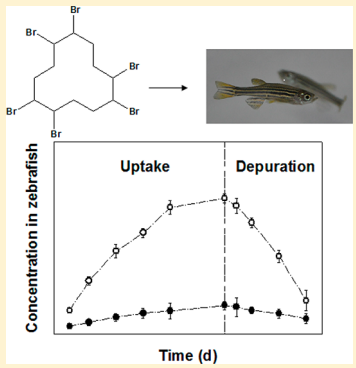
Supporting Information

ABSTRACT: In this study, zebrafish (*Danio rerio*) were exposed to two dietary concentrations of individual HBCD diastereoisomers (α-, β-, and γ-HBCD) for 42 days, followed by clean food for 21 days, to examine bioaccumulation, depuration, and enantiomer fractions (EFs) of HBCD diastereoisomers and to test the bioisomerization of HBCDs in fish. The depuration of α-, β-, and γ-HBCD in zebrafish followed the first-order process. Bioaccumulation parameters of the three diastereoisomers differed between low and high dose, suggesting that the bioaccumulation of them is concentration dependent. Calculated assimilation efficiencies (AEs), biomagnification factors (BMFs), and half-lives ($t_{1/2}$) of α-HBCD were the highest among the three diastereoisomers. Furthermore, the study showed that zebrafish could biotransform γ-HBCD to α-HBCD. The highest AE, BMF, and $t_{1/2}$ of α-HBCD and bioisomerization of γ-HBCD to α-HBCD could explain why α-HBCD appears to be dominant in biota samples. The EFs for α- and γ-HBCD in zebrafish estimated at different times of bioaccumulation and depuration were all significantly greater than those in corresponding food ($P < 0.05$), indicating selective enrichment of (+) α-enantiomer and (+) γ-enantiomer relative to (−) α-enantiomer and (−) γ-enantiomer, respectively.



INTRODUCTION

Hexabromocyclododecanes (HBCDs) are nonaromatic, brominated cyclic alkanes used primarily as additive flame retardants in polystyrene and textile products for fire-protection.[1] As the third most used brominated flame retardants in the world, after tetrabromobisphenol A and polybrominated diphenyl ethers, HBCDs are now ubiquitous organic contaminants, such as in air, sediment, animal tissue, even in human blood and milk.[2−5] In recent years, HBCDs have received widespread attention in the international community due to their stability, persistence, bioaccumulation, and toxicity.[1]

Technical HBCDs are produced by bromination of (1Z,5E,9E)-cyclododeca-1,5,9-triene (cis,trans,trans-CDT). This process yields a mixture consisting primarily of three diastereoisomers: α-HBCD (10−13%), β-HBCD (1−12%), and γ-HBCD (75−89%).[6] Moreover, α-, β-, and γ-HBCD are chiral and occur as racemates in the technical mixtures.[7] Structural dissimilarities among α-, β-, and γ-HBCD raise substantial differences in physical and chemical properties (polarity, dipole moment, and solubility in water),[7] which results in their distinctive environmental behavior and toxicological risks. For example, γ-HBCD predominates in commercial formulations and in sediments, whereas α-HBCD is the dominant isomer in biota, and β-HBCD always appears to be a very minor component.[7−10] Previous studies have indicated that the order of developmental toxicity of HBCD diastereoisomers in zebrafish is γ-HBCD > β-HBCD > α-HBCD,[11] and the cytotoxicity of individual HBCD diastereoisomers on Hep G2 cells with the order of γ-HBCD ≥ β-HBCD > α-HBCD is also diastereoisomer-specific.[12] Furthermore, there is also evidence for diastereoisomers-specific accumulation in aquatic and terrestrial organisms, especially the predominance of α-HBCD in higher trophic level species.[7,8,13] Therefore, in order to better understand the environmental fate of HBCDs, it is essential to get isomer specific data for the absorption, accumulation, metabolism, and depuration of HBCDs in biota. The present study aims to investigate diastereoisomer-specific accumulation, depuration, and chiral signatures of HBCDs as well as to test the hypothesis of bioisomerization of HBCD isomers in fish. By exposing adult zebrafish to two environmentally relevant doses of α-, β-, and γ-HBCD via the diet, we examined the bioaccumulation parameters and enantiomer fractions (EFs) of three HBCD diastereoisomers upon a 42-d exposure period, followed by a 21-d depuration period with untreated food.

MATERIALS AND METHODS

Chemicals and Reagents. Native α-HBCD, β-HBCD, and γ-HBCD were used as received from AccuStandard, Inc. (New

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Table 1. Concentration of HBCD Diastereoisomers in Seven Batches of Food

	α-low	α-high	β-low	β-high	γ-low	γ-high	control
nominal concentration (ng/g dw[a])	5	50	5	50	5	50	0
determined concentration (ng/g dw)	4.43 ± 0.15	45.76 ± 0.29	4.52 ± 0.49	47.48 ± 0.09	4.68 ± 0.25	44.82 ± 0.34	ND[b]

[a]dw, dry weight. [b]ND, below limit of detection. Values are presented as mean ± SD (n = 5).

Haven, CT, USA). d_{18}-α-HBCD, d_{18}-β-HBCD, and d_{18}-γ-HBCD were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). All solvents used in experiments (acetone, dichloromethane, n-hexane, methanol, and acetonitrile) were of HPLC grade and purchased from Tedia Company Inc. (Fairfield, OH, USA). Neutral silica gel (100−200 mesh) and alumina (60−100 mesh) were purchased from Fisher Scientific Inc. (Fair Lawn, NJ). Analytical grade sodium sulfate was obtained from China National Pharmaceutical Group Corporation (Shanghai, China).

Feed Preparation. The preparation of the fish food was performed as described by Haukas et al.[14] Commercial dried shrimps obtained from Damuzhi Company (Shanghai, China) were homogenized in a blender, mixed with acetone (approximately 1:1 by weight), and then spiked with α-, β-, or γ-HBCD. After 20 min of gentle stirring, the resulting mix was air-dried overnight to facilitate the evaporation of the solvent. Food was stored in the dark at 4 °C to prevent the possibility of phototransformation. Seven batches of food were prepared in this study. Six of the batches were spiked with a known amount of a particular HBCD diastereoisomer (Table 1). Two environmentally relevant concentrations (5 and 50 ng/g) were chosen as the exposure doses of each HBCD diastereoisomer. Control food in the seventh batch was prepared similarly using solvent without α-, β-, and γ-HBCD. Concentrations of HBCD diastereoisomers in food at different times were determined using the same analytical techniques used to determine concentrations in the zebrafish. The concentrations of α-, β-, and γ-HBCD in food did not change significantly over time during the uptake period. HBCD diastereoisomers in the control food were below our method detection limits (Table 1). Information on limits of detection and quantification can be found in the SI. In the six other batches of HBCD diastereoisomer-treated food, only the added HBCD diastereoisomer can be detected.

Experimental Animals. 90-day-old adult zebrafish (50−50% males and females) of the AB strain with fork length and body weight of 2.50 ± 0.56 cm and 0.105 ± 0.045 g (mean ± SD), respectively, were purchased from State Key Laboratory of Environmental Criteria and Risk Assessment, Chinese Research Academy of Environmental Sciences. The zebrafish were acclimated for two weeks in glass tanks (~50 L) containing aerated water at 25 °C. A 14:10 h light:dark cycle was maintained throughout the experiment. The feeding ceased 5 days before dosing.

Exposure and Samples. Each of the seven groups was kept in a 60 L aquarium. 100 zebrafish in each aquarium were fed with an individual diastereoisomers-treated or control food for a 42-d uptake period and then with untreated food for a 21-d depuration period. The daily feeding rate was 0.020 g food (dw)/g fish (ww), adjusted after each sampling day based on the overall weight of the remaining fish. The daily food was distributed equally twice a day in the morning and in the afternoon. During the experiment, fish feces in the aquarium were removed every day, and 80% of the volume was renewed once every 2 days. The aquariums were cleaned once a week. Sampling was done on days 0, 2, 7, 14, 28, and 42 during the uptake period and days 3, 7, 14, and 21 of the depuration period. The zebrafish were always sampled 24 h after the previous feeding to ensure that all food was consumed. At each sampling day, 10 zebrafish (5 males and 5 females) were selected randomly from each aquarium to determine their fork length and body weight and then stored at −20 °C for HBCD enantiomers analysis.

Sample Extraction and Cleanup. Each whole zebrafish was homogenized and dried with 10 g of anhydrous sodium sulfate (baked 6 h at 600 °C) prior to extraction. Detailed extraction and cleanup methods are provided in the SI. After solvent evaporation, each extract was spiked with 5 ng (5 μL of 1 ng/μL in acetonitrile) of each d_{18}-labeled HBCD as internal standards and then dissolved in 200 μL of acetonitrile.

Analytical Methods. The analysis of HBCD enantiomers was done according to the method described by Zhang et al.[15] with slight modifications. Briefly, HBCD enantiomers were determined using a Shimadzu Ultra Flow LC system (Shimadzu, Japan) equipped with a hybrid triple quadrupole−linear ion trap Applied Biosystem MSD Sciex 3200 QTRAP (Applied Biosystems, Foster City, CA, USA). Details about the separation of HBCD enantiomers are available in the SI. Mass spectrometry was operated in multiple reaction monitoring mode (MRM). The $[M - H]^- \rightarrow Br^-$ transitions at m/z 640.6 → 78.9 and 640.6 → 80.9 were monitored for HBCD enantiomers. The labeled HBCDs were monitored at m/z 658.6 → 78.9 and 658.6 → 80.9. Quantification results for each diastereoisomer were obtained by adding up both respective enantiomers. Additional information on quality assurance and quality control is available in the SI.

A hybrid quadrupole time-of-flight (Q-ToF) mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) was used to identify the possible metabolites of HBCD diastereoisomers. Q-ToF was operated with an electrospray source in negative ion mode. The instrument setting for the Q-ToF was as follows: capillary voltage 4.5 kV, nebulizer 0.6 bar, dry gas 6.0 L/min, dry temperature 180 °C, ion energy 10.0 eV, collision energy 10.0 eV. Twenty μL of each sample was injected directly into the mass spectrometer. Q-ToF chromatograms were acquired in a scan range from 50 to 1000 m/z.

Data Analysis. The whole-body growth rates were calculated by fitting the fish weight data to a linear growth model (eq 1)

$$W_t = W_0[1 + b^*t(\text{day})] \quad (1)$$

where W_t is the body weight at time t (days), W_0 is the body weight at t = 0, and b is the growth rate.[16] Concentrations of HBCD diastereoisomers and enantiomers in zebrafish from the low- and high-dose treatment were all corrected for lipid percent and growth dilution via multiplying the concentrations by the factor ($1+b^*t$). Data obtained during the depuration period was fitted to a first-order decay curve (eq 2)

$$C_t = C_0 e^{-kt} \quad (2)$$

where C_t is the concentration of target compounds at time t, C_0 is the concentration of target compounds at $t = 0$, and k (d^{-1}) is the depuration rate constant. Depuration rate (k) was calculated by eq 3

$$k = \frac{\ln C_0 - \ln C_t}{t} \quad (3)$$

Depuration half-life ($t_{1/2}$) was calculated by eq 4

$$t_{1/2} = \frac{\ln 2}{k} \quad (4)$$

The methods for calculating assimilation efficiencies (AEs) and biomagnification factors (BMFs) are described in the SI Equations S1 and S2.[16]

The enantiomer composition was expressed as EFs, preferred over enantiomer ratios for describing chiral signatures in environmental analysis and calculated from the peak areas of the enantiomeric pairs by eq 5.[17]

$$\mathrm{EF} = \frac{(+)A/(+)A_{\mathrm{IS}}}{(+)A/(+)A_{\mathrm{IS}} + (-)A/(-)A_{\mathrm{IS}}} \quad (5)$$

where $(+)A$, $(-)A$, $(+)A_{\mathrm{IS}}$, and $(-)A_{\mathrm{IS}}$ are the peak areas of the (+)- and (−)-enantiomers of each unlabeled HBCD and corresponding d_{18}-labeled HBCD, respectively.

RESULTS AND DISCUSSION

Biological Effects. No significant differences in whole body growth rates were found between treated and control zebrafish, indicating that HBCD diastereoisomers did not have any negative effects on the growth of zebrafish (SI Table S1). The lipid percentage in zebrafish also did not vary between treatments and did not change significantly in any treatment over time (SI Table S1). No mortality was observed in any group during the whole experiment.

Bioaccumulation and Depuration. All three diastereoisomers were detected in zebrafish from the respective low and high dose treatment. During the 42-d uptake period, the concentrations of α-, β-, and γ-HBCD increased over time and did not achieve steady state in the treated group (Figure 1). The shapes of uptake curves were mostly linear for the three diastereoisomers in low and high dose ($R^2 > 0.85$, Table 2), indicating continuous accumulation of them in zebrafish during the uptake period. The depuration of α-, β-, and γ-HBCD from zebrafish all followed roughly first-order kinetics ($R^2 > 0.90$, Table 2). AEs were calculated based on the concentrations of α-, β-, and γ-HBCD at days 7, 14, 21, and 28 of the uptake phase. Calculated AEs, BMFs, depuration rate constants (k), and half-lives ($t_{1/2}$) were highly variable among different diastereoisomers and between low and high dose treatments (Table 2).

Bioaccumulation parameters of α-, β-, and γ-HBCD were different when exposure concentration changed from low to high. In detail, AEs and BMFs of α-, β-, and γ-HBCD in low dose were all higher than those in high dose; $t_{1/2}$ of α- and β-HBCD were longer in low-dose treatment, whereas $t_{1/2}$ of γ-HBCD was longer in high-dose treatment. These results indicate that the bioaccumulation of HBCDs is concentration dependent. The octanol−water partition coefficient (K_{ow}) is widely regarded to be a useful indicator for the bioaccumulation of chemicals, and HBCD diastereoisomers with log $K_{\mathrm{ow}} > 5$ should be susceptible to bioaccumulation in fish.[18] Therefore we inspected the correlations between log K_{ow} and bioaccumulation parameters and found no clear correlations between them.

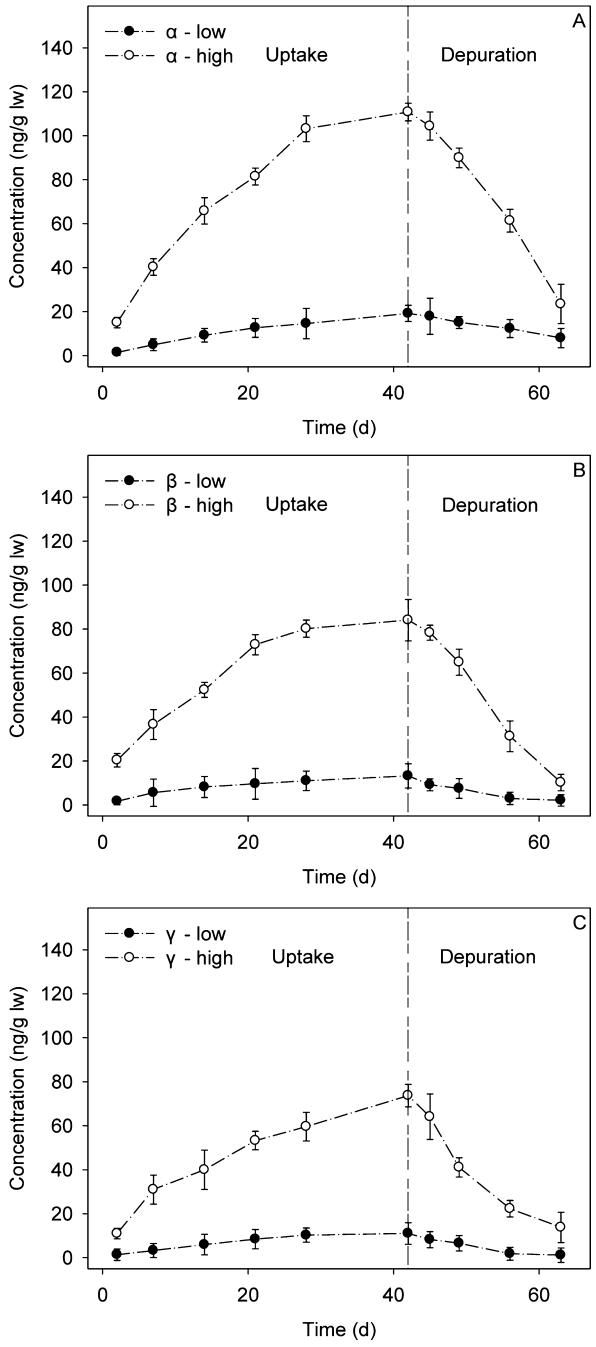

Figure 1. Accumulation and depuration of α-HBCD (A), β-HBCD (B), and γ-HBCD (C) in zebrafish. Concentrations in zebrafish are lipid based. The data are presented as the mean ± SD (n = 10).

Among the three diastereoisomers, α-HBCD had the highest AE, followed by β- HBCD, then γ-HBCD, regardless of the dose (Table 2). The BMFs among the diastereoisomers varied similarly to AEs except in high dose wherein the BMF of γ-HBCD was slightly higher than that of β-HBCD. Half-lives of α-HBCD were longer than those of β- and γ-HBCD (Table 2). The results further support the proposal of selective bioaccumulation of HBCD diastereoisomers and help explain why α-HBCD is the predominant diastereoisomer in higher-trophic-level organisms.[7,8] Zegers et al.[8] have proposed that biotransformation mediated by the cytochrome P450 systems is

Table 2. Bioaccumulation and Depuration Parameters of HBCD Diastereoisomers in Zebrafish through Dietary Exposure

group	R^2 for uptake curves	AE[a] (%)	BMF[b]	R^2 for linear relationships to determine k	depuration rate constant (k) (d^{-1})	half-life ($t_{1/2}$) (d)
α-low	0.97	54.67 ± 5.70	29.71	0.97	0.040	17.33
α-high	0.91	40.84 ± 1.45	12.33	0.91	0.071	9.76
β-low	0.90	47.71 ± 1.99	11.63	0.97	0.089	7.79
β-high	0.87	33.59 ± 4.34	7.34	0.94	0.100	6.93
γ-low	0.91	40.80 ± 2.17	7.61	0.97	0.114	6.08
γ-high	0.94	29.31 ± 2.55	7.76	0.99	0.083	8.35

[a]AE, assimilation efficiency. [b]BMF, biomagnification factor. Values are presented as mean ± SD (n = 5 for food and 10 for fish).

the most likely process to explain the exclusive accumulation of α-HBCD in biota. Indeed, the cytochrome P450 systems in liver microsomes from laboratory rats and a freshly dead harbor seal readily metabolized β- and γ-HBCD; on the contrary, α-HBCD did not appear to be metabolized. Although AEs and BMFs of β-HBCD were generally higher than those of γ-HBCD, the small amounts of β-HBCD (1−12%) and the dominance of γ-HBCD (75−89%) in commercial mixtures may be clues as to why β-HBCD is rarely detected in environmental samples.[19]

Law et al.[19] reported the bioaccumulation parameters of α-, β-, and γ-HBCD in juvenile rainbow trout and found that the AEs of three isomers were γ-HBCD > β-HBCD > α-HBCD, while the BMFs were α-HBCD > γ-HBCD > β-HBCD (Table 3). In a study where juvenile rainbow trout were exposed to

Table 3. Bioaccumulation and Depuration Parameters of HBCD Diastereoisomers in Juvenile Rainbow Trout after Dietary Exposure[19]

	AE[a] (%)	depuration rate constant (k) × 10^{-2}(d-1)	half-life ($t_{1/2}$) (d)	BMF[b]
α-HBCD	31.1	ND[c]	ND	9.2
β-HBCD	41.4	0.44 ± 0.2	157 ± 71	4.3
γ-HBCD	46.3	0.48 ± 0.2	144 ± 60	7.2

[a]AE, assimilation efficiency. [b]BMF, biomagnification factor. [c]ND, not determined.

technical grade HBCDs in food, Haukas et al.[14] found that the AEs of β-HBCD were the highest, and that of γ-HBCD the lowest. This inconsistency between these studies can be attributed to differences in dietary composition, HBCDs concentration in food and species characteristics.[20−22] Esslinger et al.[23] found that β-HBCD showed the most rapid microsomal degradation in rat liver, and their results are different from ours. This discrepancy may be due to different experiment conditions.

Our results show that there were no significant differences in the uptake of HBCD diastereoisomers between male and female zebrafish; therefore, the data in this study do not indicate gender differences.

Evidence of Bioisomerization. To test the possibility of bioisomerization, we screened the chromatograms of HBCD diastereoisomers in zebrafish. Chromatograms corresponding to HBCD diastereoisomers in zebrafish at day 28 of the uptake phase are shown in Figure 2. The other two isomers were not found in α- or β-HBCD groups. However, α-HBCD was detected in zebrafish exposed to γ-HBCD (Figure 2C). Because the contents of α-HBCD in most samples exposed to γ-HBCD were above the detection limit but below the limit of quantification, it was difficult to quantify the concentrations

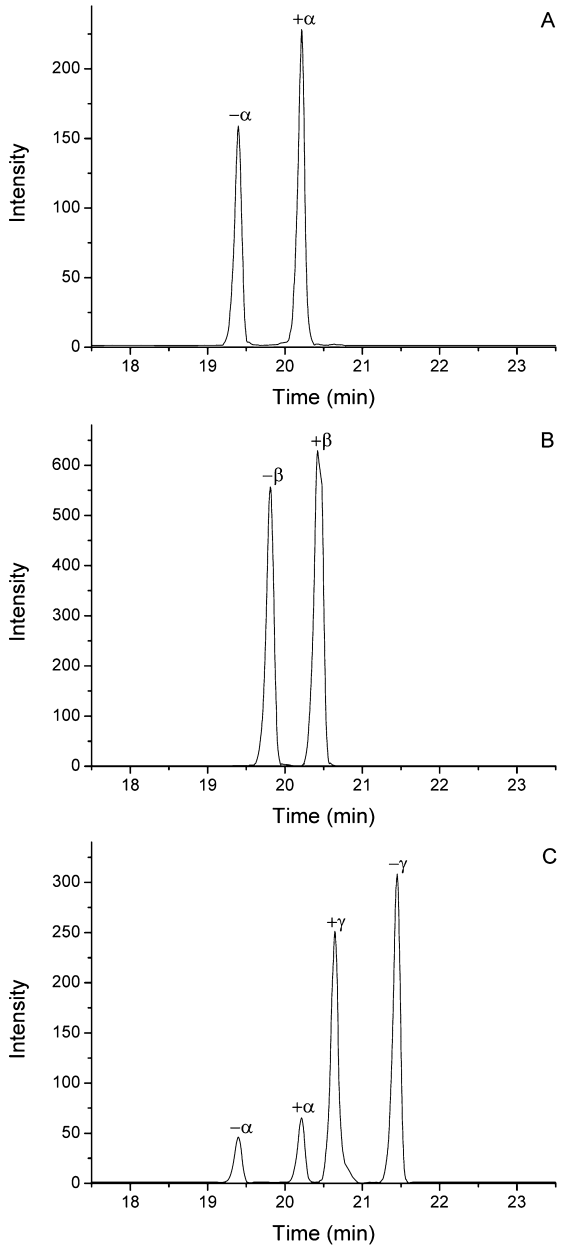

Figure 2. Chromatographic separation of HBCD enantiomers in zebrafish from α-high group (A), β-high group (B), and γ-high group (C). The elution order of those enantiomers was according to Heeb et al.[37]

of α-HBCD. Taking into account the lack of α-HBCD traces in the β-HBCD groups, the results suggest that zebrafish may have the capability to bioisomerize of γ-HBCD to α-HBCD. Law et al.[19] first reported that juvenile rainbow trout have the capacity

to bioisomerize β- and γ-HBCD, whereas α-HBCD appears to resist bioisomerization. By exposing mice to γ-HBCD, Szabo et al.[24] also observed bioisomerization from γ-HBCD to β-HBCD in liver and brain tissues as well as bioisomerization from γ-HBCD to α- and β-HBCD in fat and feces. However, no evidence for bioisomerization of HBCD diastereoisomers was observed in mirror carps after dietary exposure to pure γ-HBCD.[25] Different metabolic mechanisms and experimental conditions could explain the inconsistency in the bioisomerization potential of various species. The isomerization mechanism is not unclear but has been investigated. Koppen et al.[26] have investigated the thermally induced isomerization of γ-HBCD and consider that such isomerization in biota is most likely catalyzed by some unknown enzymes. A simulation study of the interconversion of HBCD diastereoisomers by statistical thermodynamics showed that the interconversion from (+) γ-HBCD to (+) α-HBCD is the dominating reaction.[27] Bioisomerization of γ-HBCD to α-HBCD could be one of the reasons explaining the dominance of α-HBCD in the majority of biota samples.

Enantiomer Fractions of HBCD Diastereoisomers. The enantiomers of HBCD diastereoisomers in standards, food, and zebrafish were determined using the method described in the Experimental Section. The EFs of HBCD diastereoisomers in six batches of treated food were not significantly different from HBCD diastereoisomers standards ($P < 0.05$) (SI Table S2). Additional information on statistic analysis is shown in the SI.

The EFs for α-, β-, and γ-HBCD in zebrafish at different stages of experiment are presented in Figure 3. For α-HBCD, whether in low dose or in high dose, its EFs in zebrafish at different times were significantly greater than those in corresponding food ($P < 0.05$). This trend was also observed with γ-HBCD. The results demonstrate that there must be a selective enrichment of the (+) α-enantiomer relative to the (−) α-enantiomer and a selective enrichment of (+) γ-enantiomer compared to the (−) γ-enantiomer in zebrafish in our laboratory. This enantioselective accumulation of α- and γ-HBCD may arise from selective uptake or metabolism of one of the enantiomers.[28] The EFs for β-HBCD in zebrafish showed no significant change as compared to corresponding food, suggesting no enantioselective enrichment of β-HBCD was observed in zebrafish (Figure 3B). Although the toxicological significance of the enantioselective enrichment for HBCD diastereoisomers is so far not known, changes in EFs indicate an enantiospecific shift during the biologically mediated process.[13] A significant enrichment of (+) α-enantiomer and (+) γ-enantiomer was observed in bib and whiting liver, while sole showed a slight enantiomer-selective enrichment of (−) α-enantiomer.[7] Tomy et al.[13] found the preferential accumulation of (−) α-enantiomer relative to (+) α-enantiomer in narwhal, beluga, and walrus, whereas the EFs of γ-isomer in these animals were not significantly different from standard values. Several other studies on chiral signatures of HBCDs in bird eggs and fish demonstrate that the enantiomer-specific profiles are variable among different species and tissues, especially in the case of α-HBCD.[29−31] The enantiomers of a chiral compound possess the same physical and chemical properties but may vary with each other or with the racemate on biological and toxicological effect.[32−34] A previous study clearly showed that all (+)-enantiomers of α-, β-, and γ-HBCD were significantly more toxic than corresponding (−)-enantiomers on Hep G2 cell.[12] Therefore, further research on enantiomer-specific environmental fate and toxicological information of HBCDs should be encouraged.

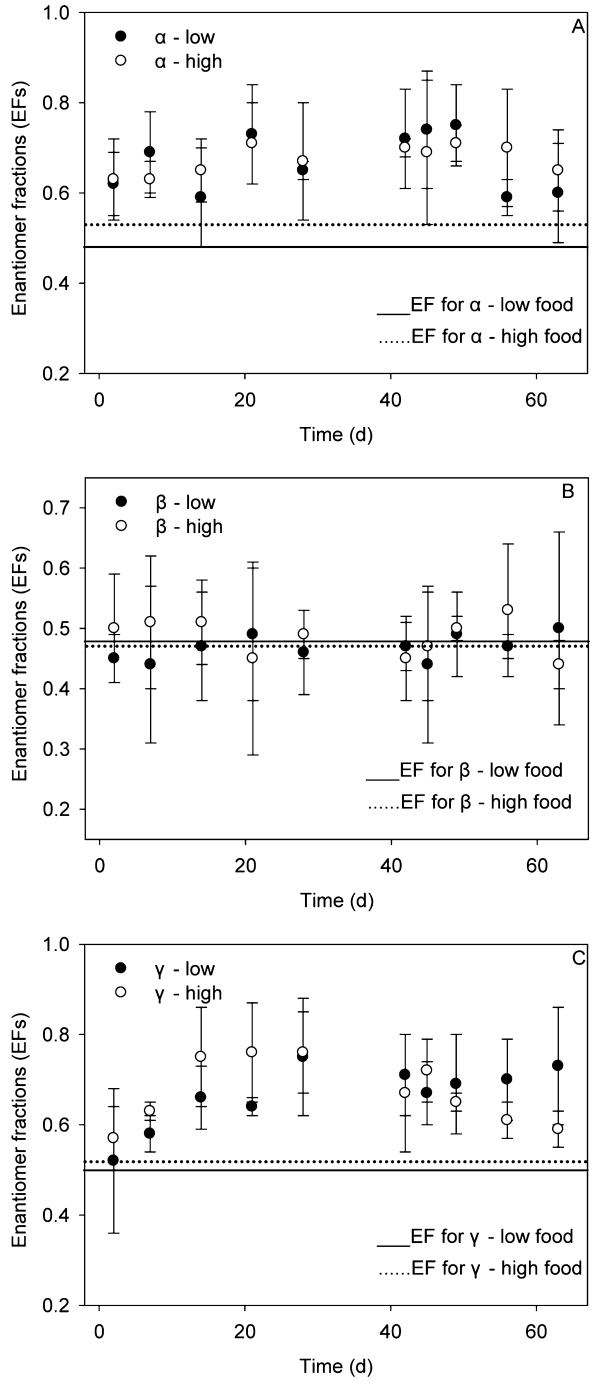

Figure 3. Enantiomer fractions (EFs) of α-HBCD (A), β-HBCD (B), and γ-HBCD (C) in zebrafish over time. The data are presented as the mean ± SD (n = 10).

Metabolites of HBCD Diastereoisomers. By comparison of Q-ToF chromatograms for samples between control and HBCD diastereoisomers-treated group, a new peak at m/z 339 (Figure 4) was found in all HBCD diastereoisomers-treated group. Then multiple reaction monitoring (MRM) was performed to acquire fragment ion information of m/z 339 by gradually increasing the collision energy. The possible metabolite of HBCD diastereoisomers was assigned as monohydroxy-dibromocyclododecadiene (OH-DBCDi,

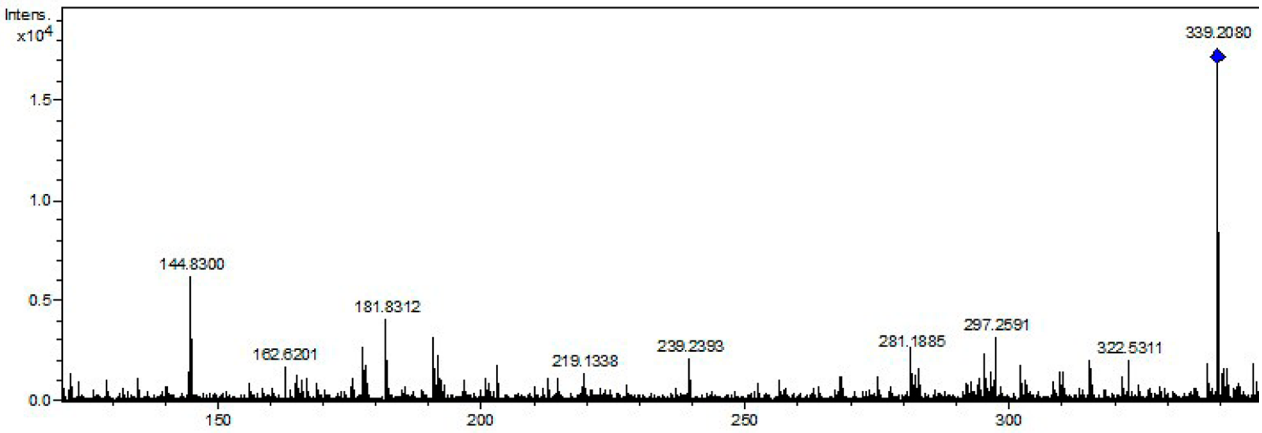

Figure 4. Q-ToF mass spectrum of the possible metabolite of HBCD diastereoisomers.

$C_{12}H_{18}Br_2O$, MW = 339) based on the fragmentation pattern, which showed ions at *m/z* 322, 239, 181, 162, and 144 (Figure 4). No authentic standard of OH-DBCDi could be obtained for this study. The Q-ToF chromatogram of the parent γ-HBCD as an example is provided in SI Figure S2. Zebrafish is a complex system with various metabolic enzymes. HBCDs in zebrafish go through a variety of metabolic processes from different organs and tissues. Thus, there should be different metabolites in zebrafish. Several previous studies have identified different metabolites of HBCDs in environmental media. Brandsma et al.[35] have identified a series of hydroxylated metabolites of HBCDs in wildlife and Wistar rats. Esslinger et al.[23] have characterized the patterns of hydroxy-HBCDs in the rat liver microsomes and assigned individual hydroxy-HBCDs to their respective parent HBCD diastereoisomers. Davis et al.[36] have demonstrated that tetrabromocyclododecene, dibromocyclododecadiene, and cyclododecatriene are the biodegradation products of HBCDs in wastewater sludge and freshwater aquatic sediment. Furthermore, lower brominated HBCD derivatives (pentabromocyclododecenes and tetrabromocyclododecadienes) formed via stepwise elimination of HBr were observed in human milk.[5] In this study, possibly due to the method unsuitable for metabolites extraction, we did not find other metabolites except for monohydroxy-dibromocyclododecadiene. Besides, low concentration of metabolites in fish and ion signal suppression because of matrix effects may contribute to the nondetection.[19] To fully elucidate the metabolic mechanism of HBCD diastereoisomers in fish, metabolic pathways and metabolites identification are our next research priorities.

■ ASSOCIATED CONTENT

Supporting Information

Details on experimental methods and data analysis. Additional information regarding growth parameters and enantiomer fractions. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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