BIOISOMERIZATION OF HBCD: RESULTS OF FEEDING EXPERIMENTS WITH PURE γ-HBCD ENANTIOMERS

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Abstract
1,2,5,6,9,10-hexabromocyclododecane (HBCD), a widely used flame retardant, is an ubiquitous contaminant in the environment. Still, only little is known about the distribution and behavior in the environment such as bioaccumulation or biotransformation of HBCD stereoisomers. In order to investigate enrichment and possible bioisomerization of HBCD stereoisomers, Mirror Carps were exposed to pure (+)- and (-)-γ-HBCD, randomly sampled after a predefined specific period and subjected to enantiomer-specific determination of α-, β-, and γ-HBCD. No evidence for the isomerization of HBCD stereoisomers was observed in Mirror Carp fillets.

Introduction
Brominated flame retardants are added to combustible polymers in electronic equipment, textiles, plastics and many other applications in order to improve their fire resistance.1,2,5,6,9,10-hexabromocyclododecane (HBCD) is worldwide one of the most widely used flame retardants in polystyrene foams, various plastic materials, upholstery textiles, adhesives and styrene-acrylonitrile resins[1-3]. Technical HBCD consists of a mixture of three diastereomeric pairs of enantiomers, termed (±)-α-,(±)-β-, and (±)-γ-HBCD (Fig. 1) with the γ-isomer as main component[4,5]. It is well known that HBCD stereoisomers rearrange at temperatures between 160 °C and 200 °C[6,7] and decompose above 220 °C[8,9]. Recently, it was shown that all six stereoisomers isomerize as shown in Figure 2[10,11]. The kinetic analysis revealed that the α-enantiomers are the energetically most favorable configurations and that under thermal stress the reactions (+)-γ-HBCD → (+)-α-HBCD and (-)-γ-HBCD → (-)-α-HBCD are the fastest ones.

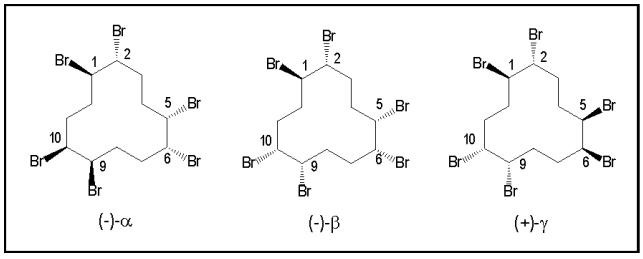

Figure 1. Structures of the first eluting HBCD diastereomers

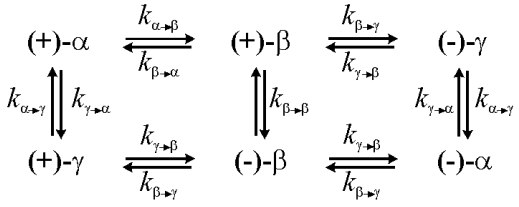

Figure 2. Pathways of thermal interconversion

In consequence of its usage as additive, HBCD tends to migrate into the environment and accumulate in biota. In the last years investigations have shown increasing concentrations of HBCD in the environment, especially in biota, with a dominance of the α- over the γ-diastereomer[9,12]. Generally, increasing trophic level goes along with increasing concentrations of α-HBCD[13,14,15]. In recent studies enantiomer fractions (EF) of HBCD stereoisomers in various biota were reported, indicating an enantio-specific enrichment of the first eluting stereoisomers (-)-α-, (-)-β-, and (+)-γ-HBCD[16,17,18] . Regarding the interconversion of HBCD isomers under thermal stress and the enantio-specific enrichment of HBCD in biota, it is important to know if these observations are a result of selective uptake of HBCD isomers only or at least partly also of bioisomerization. Evidence for the latter was reported for Rainbow Trout after exposure to racemic α-, β- and γ-HBCD[19]. The goal of this study was to provide evidence for or against the existence of bioisomerization of HBCD stereoisomers in fish. Mirror Carps were exposed to food fortified with enantiomerically pure (+)- and (-)-γ-HBCD, separately. In the following

preliminary, nevertheless decisive results of the dietary accumulation of (+)- and (-)-γ-HBCD in Mirror Carps are presented and the case for bioisomerization is discussed.

Materials and Methods

Chemicals. Technical HBCD was purchased from Fluka (Buchs, Switzerland). Ethanol (absolute, 99.8%) was obtained from chemsolute® (Th. Geyer GmbH & Co. KG, Renningen, Germany). Native and [$^{13}C_{12}$]-labeled α-, β-, and γ -HBCD standards (chemical purity > 98%) were provided by Wellington Laboratories, Inc. (Ontario, Canada) as racemic solutions in toluene. Hydromatrix was obtained from Varian, Inc. (Palo Alto, USA). Ammonium acetate, sea sand (washed and ignited), HPLC grade acetonitrile, methanol, dichloromethane, and n-hexane were obtained from J.T. Baker (Deventer, Netherlands). Picograde cyclohexane and ethyl acetate were bought from Promochem (Wesel, Germany). High-purity water was prepared with a Milli-Q system (Millipore, Bedford, USA). SPE cartridges (80 x 15 mm, J.T. Baker, Deventer, Netherlands) were filled with 1 g of activated Florisil (60–100 mesh, Sigma-Aldrich, Steinheim, Germany, baked at 400 °C for 24 h).

Fish. One year old Mirror Carps (*Cyprinus carpio morpha noblis*) with initial weights between 61.9 and 74.1 g were kept in three 800 L fish tanks (56 animals each) equipped with a separate warm water circulation system (22-24 °C) and the following conditions: concentration of ammonium 0.1 - 1.8 mg L^{-1}; nitrite: 0.28 - 5.14 mg L^{-1} and nitrate 61 - 700 mg L^{-1}. The dissolved oxygen was always at the level of saturation, the pH-value was between 5.5 and 7.6. Fish husbandry and feeding were performed by the Institute of Inland Fisheries Potsdam-Sacrow (Potsdam, Germany).

Food Preparation. The separation and characterization of γ-HBCD enantiomers was described elswhere[20]. Commercial fish food (DANA FEED A/S, Denmark, DAN-EX 1750 – fully extruded grower feed; 3 mm pellets, 50% protein, 17% lipid, 2.6% fiber) was treated separately with a known quantity of the respective γ-HBCD enantiomer as solution in ethanol (0.996 µg g^{-1}) in a 2 L round bottom flask. The solvent was slowly removed using a rotary-evaporator. The food was dried at 60 °C for 36 h, followed by blending with the same quantity of untreated food and homogenization. The control food was used untreated. The concentrations of each γ-HBCD enantiomer in the food were determined using the same technique described below for fish tissue. The resulting contents of γ-HBCD enantiomers were calculated to be 150 ng g^{-1}. The food was stored in the dark at -23 °C.

Exposure. Mirror Carps were randomly divided over the three tanks. After an acclimatization period of 7 days one group was fed with (+)-γ-HBCD fortified food the second group with the (-)-γ-HBCD fortified food while the control group received untreated food. The daily amount of feeding was equal to 1.1% of the mean body weight and the uptake phase lasted 107 days.

Sampling. Between six and eight specimens were randomly sampled from each group during the uptake phase on days 0, 14, 28, 42, 56, 73, 90 and 107.

Sample extraction and clean-up. Carps were eviscerated and heads, scales and skins were detached before cutting into fillets. Afterwards the fillets were reduced to small pieces and cryo-ground down to sub millimeter size through a 500 µm sieve on centrifugal mill (ZM 1000; Retsch GmbH, Haan, Germany) using liquid nitrogen for cooling. The cryo-ground material were lyophilized on a Lyovac GT2 (Finn-Aqua Santasalo – Sohlberg GmbH, Hürth, Germany), homogenized and stored at -20 °C until further processing.

In order to avoid cross-contamination, the centrifugal mill was cleaned after each usage. The fish powder was submitted to pressurized fluid extraction on a Dionex ASE™-200 instrument (Dionex Corporation, Sunnyvale, USA). Sample sizes ranged from 1.0 to 2.0 g and were spiked with 50 µL of a methanol solution containing 400 ng g^{-1} of $^{13}C_{12}$- labeled α-, β-, and γ-HBCD each. The cells were heated to 100 °C for 5 min and extracted with ethyl acetate at 140 bar. The flush volume was 60% over 3 static cycles. Extracts were collected in 60 mL vials and concentrated to 10 mL under a stream of nitrogen. Co-extracted lipid was removed using an automated GPC-system coupled to a UV detector (GPC VARIO, LCTech, Dorfen, Germany) and equipped with an automatic injector and a fraction collector. 6 mL of the fish extract were injected into an S-X3 Bio-Beads gel permeation column (500 mm x 40 mm, L x OD, 50 g of 200 – 400 mesh). A mixture of cyclohexane/ethyl acetate (1:1, v:v) was used as mobile phase with a flow rate of 4 mL min^{-1}. The fraction containing HBCD was collected in a 100 mL GPC-bottle, evaporated to dryness, re-dissolved in n-hexane and cleaned additionally on 1 g pre-treated Florisil (heated at 160 ºC for 24 h) with a mobile phase composed of n-hexane (5 mL) and n-hexane/dichloromethane (1:1, v:v, 13 mL). Extracts were concentrated to a dryness using a gentle stream of

nitrogen and re-dissolved in 300 µL of methanol for HPLC-MS/MS analysis. The lipid contents of the different fish samples were determined gravimetrically from portions of the extracts concentrated after GPC cleanup.

HPLC-ESI(-)-MS/MS. Determination of HBCD in fish samples was performed on a LC–MS/MS system with electrospray negative ionization (ESI-). In detail, an Agilent 1100 series HPLC binary pump system (Agilent Technologies, Waldbronn, Germany) equipped with a vacuum degasser, a thermostated autosampler and a thermostated column compartment was coupled with an API 4000^{TM} triple-stage-quadrupole mass spectrometer from Applied Biosystems / MDS SCIEX (Foster City, California / Concord, Ontario, Canada).

The enantio-specific quantification of HBCD was performed using a combination of a Zorbax XDB-C_{18}, (Agilent Technologies, Waldbronn, Germany) and a chiral NUCLEODEX β-PM (Macherey-Nagel GmbH & Co, Düren, Germany) analytical column (both columns: 5 µm particle size, 200 x 4.6 mm) maintained at 15 °C[21]. The mobile phase for the isocratic separation of the HBCD isomers consisted of a mixture of 10 mM ammonium acetate buffer and acetonitrile:methanol (90:10, v:v) in the ratio of 90:10. The flow rate was set to 300 µL min^{-1}. The transitions monitored for native HBCD were 640.6 → 79.0 and 652.6 → 79.0 for the $^{13}C_{12}$ labeled HBCD. The first and third quadrupoles were set to unit resolution. Source parameters were as follows: ion spray voltage, -4500 V; declustering potential, -30 V; desolvation temperature, 450 °C; ion source gas 1, 40 arbitrary units (a.u.); ion source gas 2, 30 a.u.; curtain gas, 20 a.u.; collision energy, -40 eV and collision gas, 4 a.u.. Data were collected and processed with the Analyst 1.4.1 software package (Applied Biosystems / MDS SCIEX).

Results and Discussion

Mirror Carps were selected for the feeding experiment in respect to their easy maintenance in closed aquatic circulation systems, their good food acceptation as well as their growth rates. The exposure period was chosen on basis of experiences reported in the literature[19]. The idea behind the use of pure enantiomers of γ-HBCD is to observe a clear increase of the respective single α-HBCD-enantiomer (Fig. 2) in case of bioisomerization. Due to the HBCD background levels any decisive conclusions from feeding experiments for the isomerization in vivo would require a significant increase of the respective α-enantiomer to be formed from the fed γ-enantiomer.

Effects on fish health. The exposure to pure (+)- and (-)-γ-HBCD did not seem to affect the health of carps under the applied conditions. There was no significant difference between the two exposure groups and the control group at any point in lipid or water content of the fillets, livers and perivisceral fat body. The mortality (one specimen of the (-)-γ-HBCD-group died on day 77) and behavior of the carps were not affected by (+)- and (-)-γ-HBCD exposure. Fishes of all three groups showed similar specific growth rates (SGR, Eq. 1)[22] over the whole feeding period (Table 1) with a middle daily specific growth rate of 1.300 ± 0.009%. The calculated food conversion ratio was 1.150 ± 0.014 kg food / kg growth.

Table 1: Specific growth rate[22] (SGR) and growth of the fish groups during the feeding period

Growth parameter/	trial day						
Feeding Group	**14**	**28**	**42**	**56**	**73**	**90**	**107**
(+)-γ-HBCD							
SGR, %	0.665	1.40	1.48	1.37	1.43	1.36	1.30
% growth/fish	9.76	34.9	25.9	15.5	32.0	19.5	18.3
mean growth/fish, g	8.00	31.4	31.5	23.7	56.4	45.3	50.9
(-)-γ-HBCD							
SGR, %	0.665	1.35	1.57	1.45	1.30	1.51	1.25
% growth/fish	9.76	32.9	32.4	16.9	14.2	51.1	0.00
mean growth/fish, g	8.00	29.6	28.8	26.7	26.4	108	0.00
control							
SGR, %	0.665	1.68	1.36	1.40	1.55	1.28	1.20
% growth/fish	9.76	45.7	10.8	23.7	41.8	1.70	13.9
mean growth/fish, g	8.00	41.1	14.2	34.4	75.1	4.33	35.9

$$SGR\% = 100 \cdot \left(\frac{\ln W_d - \ln W_0}{d} \right) \qquad \textbf{(1)}$$

$\ln W_d$: natural log of final weight of fish, g
$\ln W_0$: natural log of initial weight of fish, g
d: trial day

Bioaccumulation and bioisomerization. The fed γ-HBCD enantiomers were significantly increased in every carp fillet after two weeks of exposure. None of the γ-HBCD enantiomers reached steady-state within the uptake period. Figure 3 shows the enrichment of (+)-γ-HBCD in the corresponding feeding group after 42 d. In the course of interpretation the background contamination of the commercial fish food and the young fishes at the beginning of the experiment need to be considered. Due to these background contaminations the chromatograms show all six HBCD isomers, the fed γ-HBCD enantiomer as well as traces of the other five isomers.

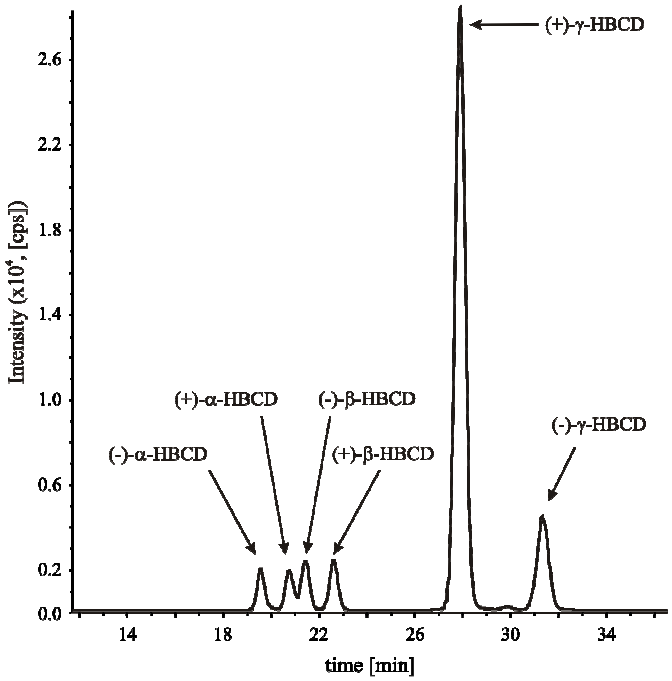

Figure 3. LC separation of the HBCD enantiomers from carp fillet extracts ((+)-γ-HBCD group) on day 42

This background contamination does not significantly interfere with the interpretation of results. Figure 4 depicts the content of (+)-γ- (Fig. 4A) and (-)-γ-HBCD (Fig. 4B) in both feeding groups. Contents of the control group were subtracted from those of the exposed groups. Data were also corrected for the growth. The contents of all HBCD isomers in both feeding groups remain constant until day 14 followed by an increase of the content of (+)- and (-)-γ-HBCD, respectively, It remains unclear why depuration initiates at day 73, while the feeding rate was kept constant. Metabolism or excretion might occur however bioisomerization to the respective α-isomer does not take place as is clearly seen from the stable (±)-α-HBCD contents (Fig. 4C). A similar depuration phenomenon was observed by Law et al.[19] during their exposure experiments of Rainbow Trout with racemic α-, β-, and γ-HBCD. After an uptake phase of 56 days the depuration of HBCD diastereomers was investigated over 112 days, during which all fish were fed untreated food. In course of the depuration phase (day 63 of experiment) a decrease of the (±)-β- and (±)-γ-HBCD concentrations was observed and at the same time the increase of the (±)-α-HBCD content. This was interpreted tentatively as possible evidence for bioisomerization under the applied conditions. It should be taken into account that different fish species might exhibit different metabolism pathways and rates.

However, it is difficult to obtain unequivocal evidence for a potential bioisomerization from information on the contents of HBCD enantiomers alone. Thus, EF-values (enantiomer fractions), representing the chiral signature, were calculated relative to the first eluting HBCD enantiomer (E_1) of α- and γ-HBCD:

$$EF = \frac{[E_1]}{[E_1]+[E_2]}$$

The EF-values can range from 0 to 1.0, with 0.5 representing the theoretical racemic mixture. Any significant deviation from this value indicates a shift in enantiomeric composition. Figure 5 depicts the time series of the concentrations of (±) α-, and (±) γ-HBCD in the different feeding groups in terms of EF-values as measured in the Mirror Carp fillets. While a bioaccumulation of the fed γ-HBCD enantiomer is considerable, the EF_α remain constant with respect to the combined uncertainty (u_c, Fig. 5) of its determination and similar regardless of the type or exposure. Therefore, no evidence for bioisomerization of HBCD stereoisomers was observed during this experiment.

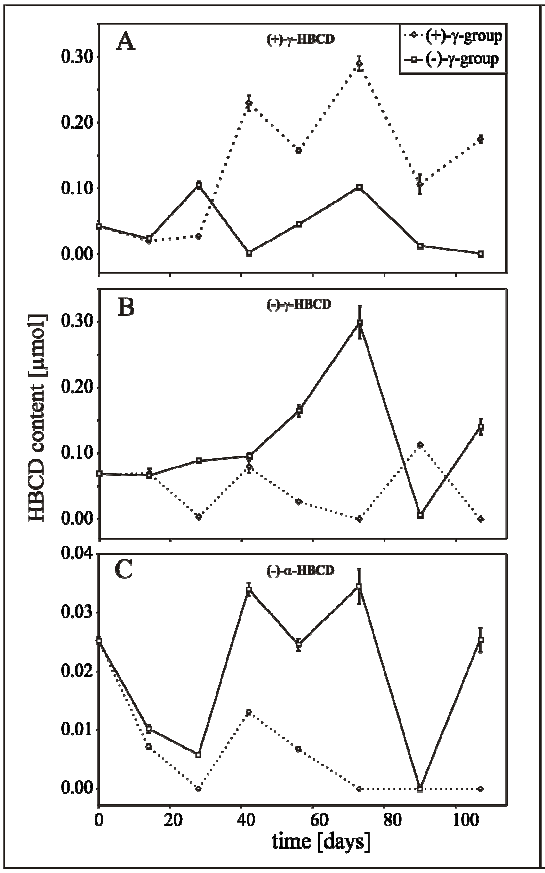

Figure 4: Absolute contents of the HBCD-enantiomers (**A**: (+)-γ-HBCD; **B**: (-)-γ-HBCD; **C**: (-)-α-HBCD); mean ± S.D. (n = 4)

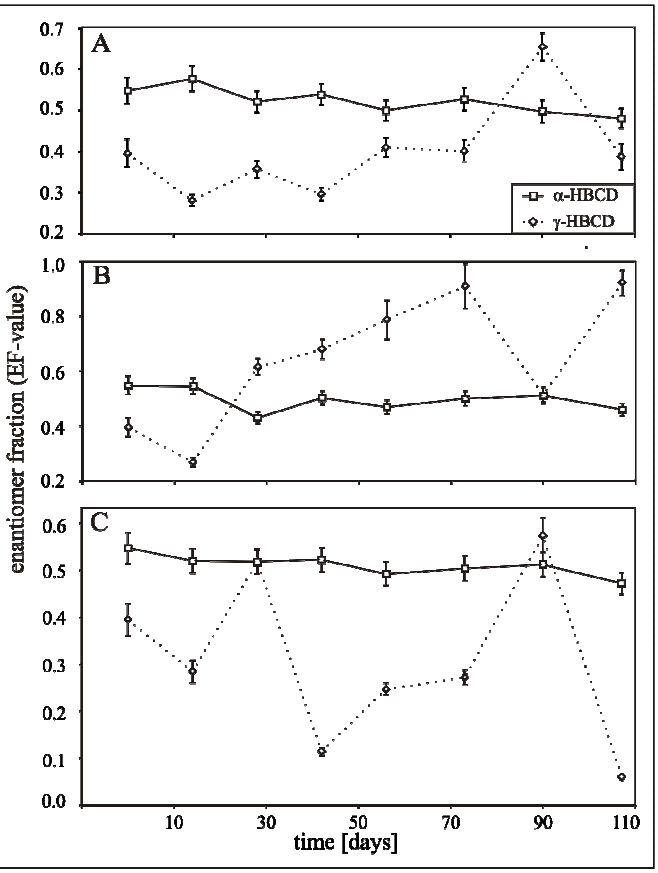

Figure 5: Development of EF_α and EF_γ values from Mirror Carp fillets during the feeding period (**A**: control group; **B**: (+)-γ-HBCD group; **C**: (-)-γ-HBCD group; mean ± u_c)

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