Stereo-isomer specific bioaccumulation of hexabromocyclododecane (HBCD) in marine mammals

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Introduction

Hexabromocyclododecane (HBCD; CAS# 110-85-0; EINECS# 203-808-3) is the principal flame retardant in extruded (XEPS) and expanded (EPS) polystyrene foams used mainly as insulation material in the building industry[1]. With a world-wide production of 16,700 tons in 2001 of which the majority (9500 tons) was used on European market[1], it is recognised as a high production volume (HPV) chemical and a priority pollutant by the EU. HBCD is currently the third brominated flame retardant (BFR) in production volume. Four types of commercial HBCD mixtures are produced, which differ in melting points. All mixtures contain the isomers α-, β- and γ-HBCD, which are diastereomers[2]. The low melting point (LMP) mixture contains about 70% of the γ-isomer, while the HMP type contains about 90%. Environmental residues of HBCD have been reported recently[3-6]. Retrospective analyses of eggs of the guillemot (*Uria aalge*) from the Baltic Sea already demonstrated HBCD residues already in the early 1970s, but the levels started to increase sharply after 1980[5].

We investigated the total-HBCD levels in blubber of only female harbour porpoises (*Phocoena phocoena*) and common dolphins (*Delphinus delphis*) stranded on Western European coasts between Scotland (UK) and Galicia in Spain by GC/MS. The isomeric composition of HBCD in blubber was investigated by LC/MS in a selection of 19 samples, representing all sampling areas. The possible influence of cytochrome P450 mediated biotransformation on the changes in the isomer composition in the residues in blubber with respect to the commercial mixture was investigated by in vitro assays with hepatic microsomes of rat and harbour seal (*Phoca vitulina*).

Materials and Methods.

Sampling and age determination: Samples of stranded harbour porpoises (Phocoena phocoena) and common dolphins (*Delphinus delphis*) were collected as part of the national stranding programmes of the different regions. Blubber samples of complete vertical cross-sections were taken from the left side in front of the dorsal fin, following the ECS guidelines for gross post mortem examination tissue sampling[7]. Animals sampled ranged in decomposition state from extremely fresh (2a) to moderately decomposed (3). The BFR levels in different organs from a selection of animals showed that the lipid normalised concentration in blubber is good reflection of the concentrations in a number of other organs (liver, kidney, muscle), which is similar to the situation encountered for the PCBs. Age was determined on teeth samples collected from the middle of the lower jaw[7].

Analysis of HBCD. All isomer standards were obtained from Cambridge isotope laboratories (CIL). The principal tool for analysis was gas chromatography-mass spectrometry (GC-NCI/MS). HBCD was analysed with selective ion recording at the masses of the two naturally occurring bromine isotopes with m/z=79 and 81. The methods employed generally follow those described before for the PBDEs, with a minor alteration in the elution of the silica columns employed for clean-up after extraction and sulphuric acid treatment[8], i.e. elution was performed with 30 ml of an 85% pentane 15% diethyl ether mixture.
The diastereoisomeric composition of a selection of blubber samples from the field study and the in vitro assays was studied using a recently developed LC-MS method with electrospray negative ionisation single quadrupole mass spectrometry[3]. Selected ion monitoring was used allowing the monitoring of bromine containing molecules (m/z 79,81), the parent molecule (640, 642, 644) and mono-hydroxy HBCD (656,658,660). Separation was performed using a C18-column: (L * I.D.: 150 * 2.1 mm) and 0.25 ml min^{-1} 85% Methanol 15% 0.1 mM Sodium Acetate as mobile phase.

***In vitro* assays.** All assays were carried out in 25ml Erlenmeyer flasks in a shaking water bath at 37°C, being the body temperature of the experimental organisms. The flasks contained 1 ml 0,08 M phosphate buffer (pH 7.6) to which 100 µl microsomal suspension standardised at 10 mg total protein ml^{-1} The rat microsomes originated from male 14 weeks old Wistar rats that had been pre-induced with 0.1% w/v phenobarbital in drinking water for 7 days prior to sacrifice (Charles Tiver, Sulzfeld, Germany). These microsomes were a gift from Dr. Ilonka Meerts, department Toxicology of Wageningen University. For the assays, 10 µl of a of 200 µM 1:1:1 isomer HBCD-mix (rat) or 3 µl of either a 67 µM 1:1:1 isomer HBCD or 200µM of single HBCD isomers (harbour seal was added to microsomal suspensions of 1 ml. To all stock solutions,150 µM CB153 was added as persistent internal standard and 9µM BDE49 was added as appositive control, since it is rapidly metabolised. All assays were carried out in fourfold. After three minutes of pre-incubation, 100µl 11mM NADPH was added every ten minutes as electron donor for the biochemical reaction to each assay Erlenmeyer, but not to the references.

Results and discussion.

The (Σ-) HBCD levels in harbour porpoises from the Scottish and the Irish coasts of the Irish Sea and in the North Western part of Scotland were significantly higher (P ANOVA on ^{10}log-transformed HBCD concentrations < 0.001; and P< 0.05 in pair-wise Tukey post-hoc tests for the different areas) than the levels in all other areas except for the Southcoast of Ireland (SI). The relationships between age and HBCD levels were not significant for the different sampling areas. Since there is no currently known HBCD producer near the Irish Sea or in NW Scotland, industries using BFRs are the most likely sources. However, a single source in the Irish Sea would already suffice to explain our results, since measurements on the transport of radio-isotopes from the Sellafield nuclear plant have demonstrated that the outflow of the Irish Sea is mainly through the Northern Channel along the Scottish Northwest coast.[9; 10]. The values of the lipid-normalised levels of HBCD for harbour porpoises stranded on the coasts of the Irish Sea and NW Scotland with median values of 4 and 5 ug g^{-1} lipid were of a similar magnitude as the levels in pike (*Esox lucius*) caught just downstream of a textile industry along the Swedish river Viskan[11] , and in eel (*Anguilla anguilla*) and brown trout (*Salmo trutta*) caught near a sewage treatment plant at Newton Aycliffe along the UK river Skerne, to which the sewer outlet of an HBCD production plant was also connected[12]. In areas not directly connected to a local HBCD source, such as several fish species from the Lake Ontario[6], and bird eggs from Scandinavia[4; 5]; the range of levels was similar to the ranges in our other sampling areas.

The high temperatures used in GC/MS analysis prevent the discrimination between the different HBCD-isomers, since they change to mainly the thermodynamically most stable α-isomers at temperatures > 160°C[2]. Therefore, we also used our recently developed LC/MS method[3] to analyse the isomer composition of the HBCD residue in blubber of a selection of 10 harbour porpoises and 9 common dolphins. To our great surprise, all samples contained exclusively the α-isomer, despite the dominance of the γ-isomer in all commercial mixtures.

One of the processes that might explain this compositional difference between the commercial mixtures and the residues in top-predators, are different rates of enzyme-mediated biotransformation. The initial step in the biotransformation by higher organisms of organohalogen compounds that lack any functional group is very often the oxidation by the cytochrome P450 system[13]. This initial step can be studied very elegantly by in vitro assays with liver microsomes. Since microsomes from harbour porpoise or dolphins were not at our disposal, we had to revert to liver microsomes of laboratory rats and a harbour seal from the Wadden Sea that could be sampled within a few hours after it died[14]. In the four replicate preparations of (phenobarbital induced) rat microsomes with NADPH, the peaks of the β- and γ-isomers of HBCD in an artificial 1:1:1 mixture showed a highly significant decrease, but the peak of the α-isomer had not become significantly smaller even after 90 minutes incubation at 37°C.

In the incubations with harbour seal microsomes, we saw an average decrease ± sdev. of the parent isomers of 69±16% for the β-isomer and of 60±10% for the γ-isomer after 90 minutes, but also here α-HBCD was not significantly biotransformed after 90 min. incubation, with an average decrease of only 17±14%. In this case, we also looked for the formation of new peaks. When these peaks appear only after the addition of NADPH added as fuel for enzymatic reactions, they are indicative for metabolites. For β-HBCD,

three bromine-containing metabolites could be observed in the m/z = 79+81 trace, while for γ-HBCD two of such metabolites were observed. All metabolites eluted prior to the parent compound, indicating an increased polarity. The quantities of the metabolites formed were insufficient to obtain their full-scan mass-spectra. To maintain a maximum of sensitivity at the cost of some selectivity, we therefore chose selective ion recording at the masses of the supposed molecular ions of the metabolites formed. Since mono-hydroxy metabolites are the most likely initial reaction products of HBCD biotransformation by the cytochrome P450 system, we assumed that an H-atom would be replaced by an –OH group, and thus a small mass range at 16 mass units above the mass range of the molecular ions of the parent HBCD isomers was selected for this purpose. The m/z=655-660 LC-MS trace of β-HBCD showed two peaks with the same retention times as the first and third eluting peaks in the bromine isotope trace. The second metabolite peak in the bromine ion trace of β-HBCD did not show such a corresponding peak in the m/z = 655-660 trace, suggesting that this was either not a mono-hydroxy metabolite, or a metabolite without a stable molecular ion. For γ-HBCD, there was a complete match in retention times between the two new peaks appearing after biotransformation in the bromine isotope trace and those in the 655-660 trace. A peak with a similar retention time as $M_{\gamma,2}$ in the in vitro experiments was also found in residues in some blubber samples, indicating that at least one of these metabolites may still accumulate to a certain degree in lipid-rich tissues of marine mammals.

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