ACCUMULATION OF HEXABROMOCYCLODODECANES (HBCDS) AND THEIR METABOLITES IN PUP AND ADULT HARBOUR SEALS FROM THE NORTHWEST ATLANTIC

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Abstract

The isomer-specific distribution of HBCDs was investigated in 56 liver samples of harbour seals (*Phoca vitulina concolor*; 50 pups and 6 adults) and 5 blubber samples of pups (n = 4) and one adult from the northwest Atlantic. In two liver samples, HBCDs were below the limit of quantification (< 1.5 ng/g lipid weight (lw) for each isomer). For all other samples, the sum of HBCDs ranged from 2.7 to 4805 ng/g lw in liver and from 2.0 to 25.3 ng/g lw in blubber. Median values were 23.5 ng/g lw and 11.1 ng/g lw for liver and blubber respectively. The α-HBCD isomer was the dominant isomer (mean percentage was 95%) in all liver samples. In blubber, γ-HBCD and β-HBCD were not found in any sample. Male pups and female pups were found to differ significantly in the concentration of α-HBCD in liver, however no statistical significant differences could be detected between male pups and adult males. Other factors, such as time of sampling, location, body length and body weight, were not able to explain the results found in the present study. Additionally, HO-HBCDs could be detected in some seal liver samples, indicating the capacity of harbour seals to (partially) metabolize HBCDs.

Introduction

Hexabromocyclododecanes (HBCDs) are additive brominated flame retardants (BFRs) which are widely used in household and commercial products, such as extruded and high-impact polyurethane foams (thermal insulation in buildings), upholstery textiles, and to a lesser extent, in electrical equipment[1-4]. HBCDs are associated with endocrine-disrupting, reproductive and developmental effects in animals[5-7].

Similar to PBDEs, HBCDs may enter coastal and marine waters from multiple sources and readily biomagnify in marine food webs[8,9]. Several recent studies have reported on the occurrence of the HBCD-isomers in marine ecosystems[10-13], but still insufficient information is available about their fate and environmental persistence. HBCD concentrations vary significantly by region, species and tissue. They have been reported in marine fish species from Asia[14], Arctic Canada[15], and various areas of Europe[16-18].

In contrast, information about HBCD concentrations in marine fishes from US waters is rather scarce [10,19]. HBCD concentrations reported in cetaceans from the eastern US coast[19-20] are one to two orders of magnitude lower than those detected in marine mammals from Europe[1,8,9,11], where the market demand for this compound is two- to three-fold higher than in Asia or the US[21] . However, increasing levels of HBCDs in Pacific dolphin species were reported for recent years[22].

Harbour seals (*Phoca vitulina concolor*) are common pinnipeds in the northwestern Atlantic. They have relatively long life spans, feed high in the food chains and are representative for near-shore contamination[23]. Consequently, they accumulate considerable amounts of pollutants in their tissues[10,24], leading to adverse effects on multiple levels, e.g. reproductive, developmental, immunological effects[25,26].

Currently, there are no restrictions on the global production or use of HBCDs, but the European Chemical Agency has recently recommended its strict control as a substance of very high concern. In view of the historical and ongoing dispersal of these compounds, there is a clear need for more information on the uptake/accumulation and biomagnification of HBCDs in marine food webs. Therefore, the aim of the present study was to investigate the accumulation of HBCDs in harbour seals from the northwestern Atlantic. Most animals investigated were pups, thereby giving the opportunity to assess the initial contamination in this species. Measured concentrations were compared with seals from other areas around the world and with fish species from the same region[10]. An attempt was made to identify HO-HBCDs as possible metabolites of HBCDs in marine mammal livers.

Materials and Methods

Collection of seals. Liver samples were collected from 56 harbor seals (6 adult males, 22 male pups and 28 female pups) that stranded along the northwest Atlantic coast (from Maine to New York) between 2000 and 2007. Seals were weighed, and standard length and axillary girth were measured. Age was estimated based on body size. Liver samples were stored in hexane and acetone rinsed aluminum foil at – 40°C until analysis. Blubber samples were collected from 5 harbour seals (1 adult male, 1 male pups and 3 female pups) from the same locations.

Sample preparation. Seal liver (2 - 2.5 g) and blubber (0.2 – 0.3 g) were ground with sodium sulfate and spiked with internal standards (BDE 77, BDE 128, CB 143, ^{13}C-BDE 209 and ^{13}C-HBCDs). Samples were extracted for 2 hours by hot Soxhlet with a mixture of acetone/hexane (1/3, *v/v*). The extract was evaporated and cleaned by passing through 8 g of acid silica (H_2SO_4, 44%), from which pollutants were eluted with 20 ml hexane and 15 ml DCM[27,28]. Minor adaptations were required as PBDEs and PCBs were analysed by GC-ECNI/MS or GC-EI/MS and HBCDs by LC-MS/MS. The cleaned extract was evaporated to dryness, redissolved in 0.5 ml hexane and eluted from pre-packed silica cartridges with 6 ml hexane (for GC analysis) and 6 ml DCM (for LC analysis). Both fractions were evaporated to dryness and redissolved in 100 µl iso-octane or methanol, respectively.

LC analysis. The separation of α-, β-, and γ- HBCD isomers was achieved using a dual pump Agilent 1100 Series liquid chromatograph equipped with autosampler, vacuum degasser and an Agilent Zorbax Extended-C_{18} reversed phase analytical column (50 mm x 2.1 mm i.d., 3.5 µm particle size). A mobile phase of (a) water and (b) methanol at a flow rate of 200 µL/min was applied for elution of HBCD isomers; starting at 75% (b) then increased linearly to 100% (b) over 7 min; this was held for 12 min followed by a linear decrease to 75% (b) over 0.5 min and held for 10 min. The target analytes were baseline separated on the reversed phase C_{18} column with retention times of 7.0, 7.5, 7.8 min for α-, β- and γ- HBCD respectively. Mass spectrometric analysis was performed using an Agilent 6410 triple quadrupole mass spectrometer operated in the ES negative ion mode. MS/MS detection operated in the MRM mode was used for quantitative determination of the HBCD isomers based on m/z 640.6→ m/z 79 and m/z 652.6→ m/z 79 for the native and ^{13}C-labelled diastereomers, respectively.

Analysis of HO-HBCD. The LC analysis was similar to that for HBCD isomers. The following MRM transitions were monitored for the theoretical HO-HBCD isomers m/z 654.6→ m/z 79 and m/z 656.6→ m/z 79 in a window before the α-HBCD isomer.

Quality assurance and quality control. This was performed through the analysis of procedural blanks, a replicate sample and a standard reference material (SRM 1945, whale blubber, which has also indicative values for HBCDs). A value of 5.5 ± 0.9 ng/g ww was obtained, which compares well with the value of 6.4 ± 1.4 ng/g ww obtained by Keller et al.[29]. Procedural blanks of HBCDs were consistent (around 0.2 ng) (RSD < 20 %) and therefore the mean value of each analyte in the procedural blanks was used for subtraction. Method quantification limits (LOQs) for individual HBCD diastereomers were based on procedural blanks (3x SD) and the amount of sample taken for analysis (typically 2.5 g liver). LOQs were 1.5 ng/g lw for α-HBCD and 2 ng/g lw for β- and γ-HBCD.

Statistical analysis. Statistical analyses were conducted using the SPSS 15.0 statistical package. The level of statistical significance was defined at $p < 0.05$. Outliers in all groups, detected making boxplots and having concentrations of more than 3 times the median, were removed for further calculations. Non-parametric statistical tests were used since the data were found to have a non-normal distribution (Shapiro Wilk's statistical test). Differences in α-HBCD concentrations between the 3 age-gender groups (male pups, female pups and male adults) were investigated using Kruskal-Wallis. Spearman's correlation coefficients were calculated using GraphPad Prism 4 (GraphPad Software, Inc).

Results and Discussion

No HBCDs were detected in 2 out of 56 liver samples and one outlier of 4805 ng/g lw in liver was found. The majority of the liver samples (50 out of 56) had ΣHBCD levels < 100 ng/g lw, while 3 samples had HBCD levels ranging between 100 and 300 ng/g lw. The mean (SD) concentration of α-HBCD in liver samples was 37.8 (22.2) ng/g lw, with a range from < 1.5 to 277.8 ng/g lw.

Concentrations of HBCDs in blubber were all < 30 ng/g lw. The mean (SD) concentration of α-HBCD in the liver samples was 22.0 (14.2) ng/g lw, with a range from 4.2 to 27.4 ng/g lw In blubber, no γ-HBCD and β-HBCD were found in any sample.

Whereas commercial HBCD mixtures consist mainly of γ-HBCD (75–89%), α-HBCD (10–13%), and β-HBCD (1–12%)[30], stereoisomeric profiles of HBCDs in marine biota are dominated by α-HBCD, and selective enrichment of this isomer is observed with increasing trophic level in the food web[1]. Since α-HBCD was the dominant isomer, accounting for 95% of total HBCDs in all samples in the present study, all statistics were based upon this particular isomer.

Influence of age and gender. To explore possible age-gender effects, the liver samples of 52 harbour seals were divided into 3 groups: male pups (n = 20), female pups (n = 26) and male adults (n = 6). Differences in α-HBCD concentrations were found between these 3 groups (X^2 (df = 2) = 7.898; p = 0.019) and more specifically between the male pups and the female pups (X^2 (df = 1) = 7.190; p = 0.007) with median values of 33.8 and 13.6 ng/g lw respectively. Harbour seal pups gain much in weight and length in the first months of their lives as they have to be prepared to survive on their own. Since the exact age of the pups was not known, the influence of growth on the concentration of α-HBCD was checked using body weight and body length data of the pups. However, no correlations could be found between levels of α-HBCD and body weight or body length for male pups or female pups (Spearman correlations; all p > 0.05) (Fig 1).

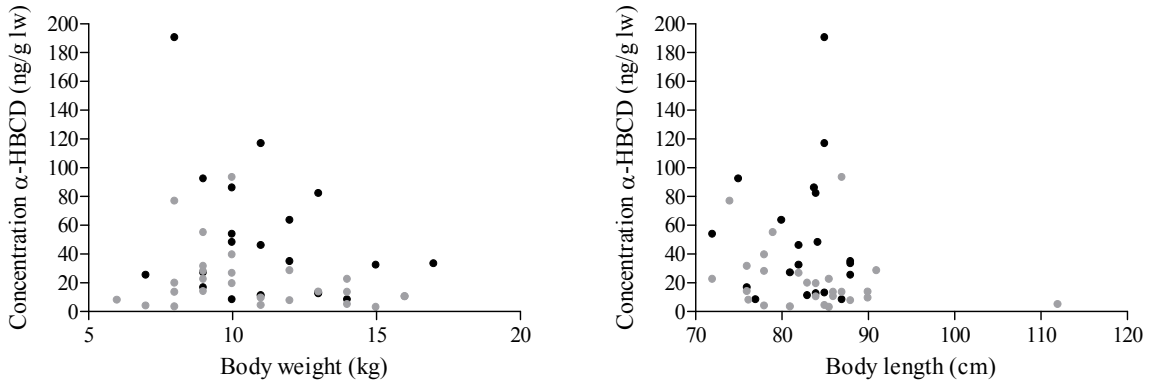

Fig 1. Relationship between α-HBCD concentrations (ng/g lw) and (A) body weight (kg) or (B) body length (cm) for male pups (●) and female pups (●).

The comparison between adult males and male pups revealed no significant differences (X^2 (df = 1) = 2.905; p = 0.088) indicating that possible bioaccumulation and biomagnification of α-HBCD with age is countered by a sufficient metabolism and elimination in adults. In the present study, the median concentration in adult males (23.4 ng/g lw) was lower than the median in pups (33.8 ng/g lw), leaving pups as a vulnerable and sensitive age class. On the other hand, it is obvious that the concentration of α-HBCD in pups is largely responsible for the body burden of this compound in the adult stage.

Spatial and temporal trends. Samples analyzed in the present study were from different areas and from 2001 – 2006. To assess potential spatial trends, concentrations of α-HBCD in liver of male (female) pups from the northern part of the sampling area were compared with male (female) pups from the southern part. However, no statistical significant differences were found between male pups from north (n = 8) and south (n = 12) (X^2 (df = 1) = 0.054; p = 0.817) or between female pups from north (n = 15) and south (n = 11) (X^2 (df = 1) = 1.617; p = 0.203). Sample sizes for each year from 2001 – 2006 were too small to make comparisons. Therefore,

concentrations were plotted against the year of sampling (Fig 2). Results show that concentrations of α-HBCD were slightly decreasing from 2001 to 2006, although these trends were not statistically significant for both male pups or female pups.

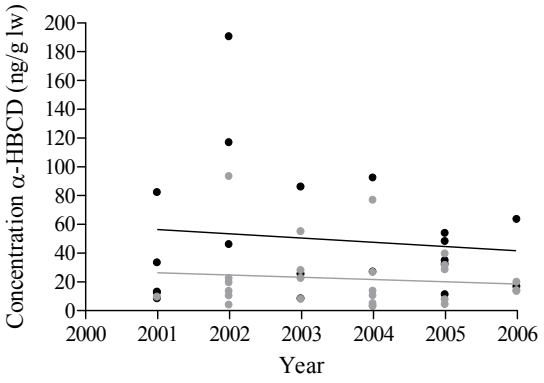

Fig 2. Slight, but not significant, decrease in the concentrations of α-HBCD from 2001 to 2006 for male pups (●) and female pups (●).

Exploring tissue differences. To examine tissue differences, this study analyzed HBCDs in blubber samples of 5 harbour seals (4 pups, 1 adult male) as well. In all blubber samples, α-HBCD was the only HBCD isomer detected. As liver-blubber pairs were from a male pup, female pups and an adult male, age and sex are confounding factors (Fig 3).

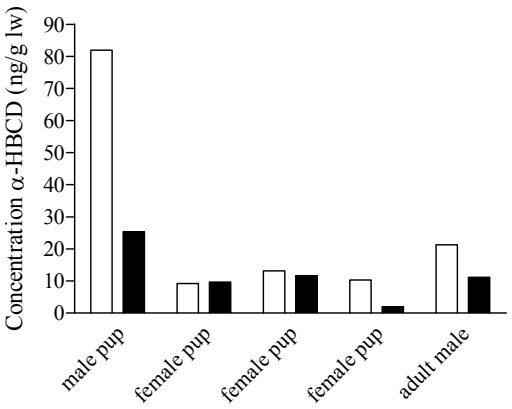

Fig 3. Comparisons between liver (□) and blubber (■) of 5 harbour seals.

Comparisons with worldwide data and prey. The presence of HBCDs in the most common prey of NE Atlantic harbour seals has been recently reported[10]. Total HBCDs were measured by GC in 87% of the fish samples (Atlantic herring (n = 20), alewife (n = 10), and Atlantic mackerel (n = 10)) at concentrations ranging from 2.4 to 38.1 ng/g lw (overall mean 17.2 ± 10.2 ng/g, lw)[10]. These species are all highly migratory and feed across an extended spatial range in the western Atlantic (from Newfoundland, Canada to South Carolina).

The HBCD levels reported in liver of harbour seals are higher than those recently reported in blubber of bottlenose dolphins from the Florida coast (mean 7.38 ng/g lw), but lower than levels in muscle of Atlantic sharpnose shark (54.5 ng/g lw) and bull shark (77.7 ng/g lw)[19]. In harbour porpoises from UK waters, a sharp increase in HBCD levels was observed from about 2001 onward[12] followed by a leveling-off since 2006[13]. In Japan, HBCD concentrations in cetaceans appear to exceed those of PBDEs[31]. Stapleton et al.[32] reported that HBCD concentrations were increasing exponentially in California sea lions between 1993 and 2003, possibly indicating a shift toward greater usage of HBCD in the US. Elevated levels of HBCD reported in marine mammals are suggestive of biomagnification[8,11,16,19,20], but the transfer of HBCD through the food chain has not been fully investigated[1]. Biomagnification of HBCD from forage fish to predator fishes was reported in a Lake Ontario[33] and Lake Winnipeg food web[34], from fish to seals in a Norwegian Arctic food chain[18], and from fish to cetaceans in an eastern Canadian Arctic food web[15].

HO-HBCDs. In vitro studies using harbour seal liver microsomes suggest that α-HBCD is resistant to P450 metabolism[11], whereas Leonards et al.[35] reported recently a lack of biomagnification of α-HBCD from fish to harbour seals, suggesting that seals can metabolize α-HBCD *in vivo*. In the present study, HO-HBCD were tentatively identified using LC-tandem MS with two specific MRM transitions examining the molecular ion arising from the substitution of an H-atom by OH (*m/z* 656.4 and m/z 654.4) to the bromide ion (*m/z* 78.9). Additionally to what has been reported so far on HBCD hydroxy-metabolites, liver tissues containing high levels of α-, β- and γ-HBCD showed a similar 'three-peak' profile in the metabolite-region (5.2-6 min). However, only one peak (5.2 min) was observed consistently, eluting ~1.5 minutes before the α-HBCD isomer, similarly to that reported recently by Tomy et al.[36] This peak was tentatively identified as an OH-HBCD isomer. Concentrations of OH-HBCD were estimated based on α-HBCD as a standard, because this isomer eluted closest to the tentatively-identified OH-HBCD and we assumed equal response factors between α-HBCD and OH-HBCD. Concentrations of OH-HBCD ranged from 2.1 to 15.0 ng/g lw, with a mean of 6.3 ± 4.4 ng/g lw for the harbour seals where it was detected (n = 6). In the near future, the identity of the OH-HBCD isomer(s) by LC-TOF/MS will be confirmed, as well as measuring their presence in other biotic samples.

In general, the discrepancy between various studies regarding biotransformation of HBCDs in marine mammals and in view of the increasing global use of this BFR compound, there is a need for more studies on the loading, kinetics, biotransformation and biomagnification potential of HBCD in marine ecosystems.

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