

Determination of HBCD, PBDEs and MeO-BDEs in California sea lions (*Zalophus californianus*) stranded between 1993 and 2003

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Abstract

Blubber samples from male California sea lions (*Zalophus californianus*) stranded between 1993 and 2003 were analyzed for 27 polybrominated diphenyl ether (PBDE) congeners, three isomers of hexabromocyclododecane (HBCD) and 14 methoxylated polybrominated diphenyl ether (MeO-BDE) congeners. Total PBDEs ranged from 450 ng/g to 4740 ng/g wet mass and total HBCD ranged from <0.3 ng/g to 12 ng/g wet mass. The concentration of HBCD increased from 0.7 ng/g to 12.0 ng/g wet mass in sea lion blubber between 1993 and 2003. However, no significant temporal trend was observed for any of the other brominated compounds over this 10 year period. Only one of the 14 MeO-BDE congeners was detected in the blubber samples, 6-methoxy-2,2',4,4'-tetrabromodiphenyl ether (6-MeO-BDE 47), and concentrations ranged from <0.2 ng/g to 12 ng/g wet mass. A bromo-, chloro-heterocyclic compound, 1,1'-dimethyl-tetrabromo-dichloro-2,2'-bipyrrrole (DBP-Br₄Cl₂), previously reported in marine species along the Pacific coast, was also identified in the sea lion blubber. DBP-Br₄Cl₂ ranged from 44 ng/g wet mass to 660 ng/g wet mass and was present at concentrations rivaling the dominant PBDE congener, BDE 47 (2,2',4,4'-tetrabromodiphenyl ether). Concentrations of DBP-Br₄Cl₂ were positively correlated with 6-MeO-BDE 47 ($r = 0.7$; $p < 0.05$). Both of these compounds have been identified in marine algae and sponges, and studies suggest they are both produced from natural sources. This study demonstrates that brominated compounds from both anthropogenic and biogenic sources can accumulate to similar levels in marine mammals. In addition, HBCD concentrations appear to be increasing in California sea lion populations, whereas PBDE concentrations, between 1993 and 2003, were highly variable.
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1. Introduction

Many recent studies have shown that brominated compounds can bioaccumulate in freshwater and marine systems (Stapleton and Baker, 2003; Voorspoels et al., 2003; Wolkers et al., 2004; Morris et al., 2004; Remberger

et al., 2004; Tomy et al., 2004). Most of the brominated compounds measured are anthropogenic and synthesized for the purpose of flame-retarding consumer products such as furniture, carpets, TVs and computer components (WHO Environmental Health Criteria 162: Brominated Diphenyl Ethers; World Health Organization: Geneva, 1994; de Wit, 2002). Brominated flame retardants (BFRs) include compounds such as polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD), and tetrabromobisphenol-A (TBBPA). However, some brominated compounds measured in aquatic food webs are

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believed to be naturally produced by algae and/or sponges (Tittlemier et al., 1999; Vetter et al., 2001, 2002; Reddy et al., 2004; Teuten et al., 2005). Tittlemier et al. (1999) identified a mixed chloro-, bromo-heterocyclic compound in Pacific sea bird eggs that was recently shown to be naturally produced using radiocarbon dating (Reddy et al., 2004). Methoxylated polybrominated diphenyl ethers (MeO-BDEs), have been identified in Baltic Sea organisms (Haglund et al., 1997; Marsh et al., 2004) and dolphins from Australia (Vetter et al., 2002; Vetter et al., 2001) and the Mediterranean Seas (Pettersson et al., 2004) in concentrations as high as 3.8 µg/g blubber. These MeO-BDEs have also been isolated from marine sponges (*Dysidea* sp.) (Vetter et al., 2002) and suggest that naturally produced organohalogen compounds can bioaccumulate in a manner similar to anthropogenic organohalogen compounds like polychlorinated biphenyls (PCBs) and PBDEs. However, given the similarity in structures between MeO-BDEs and PBDEs, the possibility of metabolic formation of MeO-BDEs from PBDEs, cannot be ruled out.

Temporal studies have shown that the levels of PBDEs are increasing in environmental matrices (Ikonomidou et al., 2002; Norstrom et al., 2002; She et al., 2002; Noren and Meironyte, 2000), presumably due to increased use of these flame retardant chemicals in consumer products. In 2001, the global market demand for PBDEs was approximately 67,400 metric tons (Internet Communication, 2005). However, the European Union has phased out the use of two of the three commercial PBDE mixtures, pentaBDE and octaBDE. DecaBDE is the third mixture and is comprised almost entirely of decabromodiphenyl ether, BDE 209. The states of California and Maine, in the United States, have enacted legislation to prevent the use of pentaBDE and octaBDE in products sold within these states (Internet Communication, 2005). Additionally, the sole manufacturer of pentaBDE and octaBDE in the United States, Great Lakes Chemical Co., has voluntarily agreed to stop producing these mixtures (Tullo, 2003). Therefore, the use of these PBDE mixtures in consumer products is expected to decrease in the future and there is evidence that this is occurring in some locations (Prevedouros et al., 2004). HBCD, in contrast, had a global market demand of 16,700 metric tons, as reported in 2001 (Internet Communication, 2005), and is currently unregulated. Very few studies have reported HBCD measurements in environmental samples and little information is available on its fate and persistence in the environment. HBCD has been measured in food webs in Lake Ontario (Tomy et al., 2004) and in the North Sea (Morris et al., 2004), with biomagnification of HBCD demonstrated at both sites. A study in Sweden found that HBCD was increasing in guillemot eggs from the Baltic Sea at a rate of about 3% per year (Sellstrom et al., 2003; Bergman, 2004).

This study was undertaken to examine the concentration of naturally produced brominated compounds (i.e., MeO-BDEs) and two anthropogenic brominated flame retardants, PBDEs and HBCD, in California sea lions

(*Zalophus californianus*) stranded along the California coast over the past 10 years. California sea lions are a marine mammal that inhabits coastal areas of the eastern North Pacific Ocean, and, because of its close association with urban near-shore areas, has been used in previous studies as a sentinel of environmental contamination by persistent organochlorine pollutants (Kajiwara et al., 2001; Debier et al., 2005; Ylitalo, 2005). The objective of this study was to determine if the concentrations of BFRs are changing in stranded sea lions over the past 10 years which would be reflective of the increased production and use of these compounds over the past 20 years. A second objective was to measure MeO-BDEs and determine if their concentrations were correlated to PBDE concentrations, which may support a metabolic source.

2. Materials and methods

2.1. Samples

Blubber samples from 25 male California sea lions were selected for analysis from the National Marine Mammal Tissue Bank (NMMTB) at the National Institute of Standards and Technology's (NIST's) environmental specimen bank facilities in Gaithersburg, MD and Charleston, SC. NIST maintains the NMMTB to provide archived samples for retrospective analysis and is run in conjunction with the National Marine Fisheries Service's (NMFS's) Marine Mammal Health and Stranding Response Program. All California sea lions samples banked in this program were collected by the Marine Mammal Center in Sausalito, California. Tissues from California sea lions that had recently stranded, or had been euthanized by the Marine Mammal Center, were sampled and cryogenically preserved according to established procedures (Zeisler et al., 1983; Geraci and Lounsbury, 1993). Tissue samples were only taken from stranded animals that were considered "freshly dead", which typically meant that the animal had died within the past 24 h. Morphometric information and histological analyses were performed on all animals for which samples were taken and included in the NMMTB. Teeth were also removed to determine age by counting growth layer groups (Rosas et al., 1993). One specimen has not been aged (B260 in Table 1) but it has been estimated at 4–8 years based on the size and development of the sagittal crest. All blubber samples were cryogenically homogenized according to NIST's standard procedure (Zeisler et al., 1983).

In addition to these 25 banked samples, an additional sample (labeled sample 2000D in Table 1) was analyzed with this sample set for a total of 26 samples. This sample was recently used in a NIST inter-laboratory comparison exercise for organochlorine compounds and contains blubber taken from two California sea lions of unknown sex that stranded in 2000. Males were selected for this study to avoid effects of lactation and reproduction (observed in females) on concentrations of contaminants that may interfere with the determination of temporal trends.

Table 1
Concentrations (ng/g wet mass) of Σ PBDEs, Σ HBCD, Σ MeO-BDEs and a brominated bipyrrole (DBP-Br₄Cl₂) in blubber of California sea lions with information on age, length, mass, lipid content, blubber thickness and date of stranding

Sample	% lipid	Mass (kg)	Length (cm)	Age (years)	Stranded	Blubber thickness (cm)	Σ PBDE	Σ HBCD	Σ MeO-BDE	DBP-Br ₄ Cl ₂
B052	13.3	69	175	8	10/27/93	0.7	2370	0.71	4.56	644
B059	79.2	121.5	242.5	12	05/05/96	5.5	451	<0.3	3.91	196
B078	20.2	199.6	126	11	08/20/96	12	936	1.51	11.62	579
B081	60.5	104	188	4.5	10/29/96	2.5	3245	1.33	<0.2	193
B084	8.2	139	210	11	10/26/96	0.9	1537	1.12	2.67	219
B088	55.4	78	164	7	11/06/96	1.8	1016	<0.3	<0.2	44
B091	18.9	144	221	8.5	11/11/96	2.2	733	<0.3	1.72	190
B094	36.0	220	167	9	12/05/96	2.9	1048	0.31	2.15	162
B115	30.3	63.5	165	10	04/28/97	0.5	1098	1.26	5.78	460
B126	54.9	134	208	14	05/22/97	1.9	1330	2.38	2.66	408
B139	64.0	39	141	3	08/07/97	1.4	2098	1.17	2.18	151
B142	6.3	135	198	14	09/02/97	0.8	878	<0.3	<0.2	48
B151	53.6	70	162	3.5	11/07/97	1.2	1454	2.67	1.83	64
B154	44.3	167	217	13	11/07/97	2.6	1910	1.54	1.45	81
B157	53.6	72	160	5	11/07/97	NM	1390	<0.3	3.32	167
B160	42.3	190	224	13	12/02/97	3.1	4739	6.56	3.89	286
B322	58.3	79	167	3	04/07/98	2.6	1166	0.63	1.99	66
B068	44.4	140	202	6	09/14/99	1.5	1449	2.15	3.93	218
B074	66.4	55.5	158	6	10/11/99	1.1	2739	3.18	6.14	259
2000D	7.0	55.5	NM	NM	07/01/00	NM	1704	6.75	4.31	577
B260	49.5	70	158	SA	07/29/00	1	2497	4.71	5.06	216
B266	51.0	126	172	4	08/20/00	2.5	1587	5.91	2.81	172
B249	61.0	112.5	218	6	09/20/00	1.1	1318	2.31	1.40	80
B500	62.7	186	222	17	08/01/02	3.2	1565	5.60	4.13	275
B503	67.8	35.5	130	3	08/01/02	1.5	1429	8.63	2.30	162
B497	69.0	99	167	5–8	01/01/03	2.6	1204	11.85	1.48	79

NM—indicates not measured.

SA—indicates sub-adult, age estimate approximately 4–8 years.

2.2. Materials

Chemical standards used in this study were purchased from either Cambridge Isotope Laboratories (Andover, MA, USA), Accustandard (New Haven, CT, USA) or Wellington Laboratories (Guelph, ON, Canada), with the exception of the MeO-BDEs and DBP-Br₄Cl₂. The MeO-BDE standards were a gift from Dr. Rob Letcher (University of Windsor, Windsor, ON, Canada) and Dr. Göran Marsh (Stockholm University, Sweden) and were synthesized by Dr. Marsh (Marsh et al., 2003). DBP-Br₄Cl₂ was a gift from Dr. Sheryl Tittlemier (Heath Canada, Ottawa, Ontario) (Reddy et al., 2004; Tittlemier et al., 2002). The solution of MeO-BDEs contained 14 congeners: 6'-MeO-BDE 17, 2'-MeO-BDE 28, 6'-MeO-BDE 49, 2-MeO-BDE 68, 6-MeO-BDE 47, 3-MeO-BDE 47, 5-MeO-BDE 47, 4'-MeO-BDE 49, 4-MeO-BDE 42, 6-MeO-BDE 90, 6-MeO-BDE 99, 2-MeO-BDE 123, 6-MeO-BDE 85, and 6-MeO-BDE 137. A PBDE solution containing 27 PBDE congeners (Suppl. Table 1) was prepared from solid material purchased from Accustandard (New Haven, CT, USA). The internal standards used for the analysis of tri-through octaBDEs, MeO-BDEs, and DBP-Br₄Cl₂ included ¹³C labeled 2,2',3,4,5-pentachlorodiphenyl ether (CDE 86); and ¹³C labeled 4,4'-dibromodiphenyl ether (BDE 15), purchased from Cambridge Isotope Laboratories (Andover, MA, USA). NonabDEs and decabromodiphenyl ether

(BDE 209) were quantified using ¹³C labeled decabromodiphenyl ether, also purchased from Cambridge Isotope Laboratories. ¹³C labeled γ -HBCD was used as an internal standard for the quantification of HBCD isomers and was purchased from Wellington Laboratories (Guelph, ON, Canada). All solvents used were HPLC grade.

2.3. Extraction and cleanup

A pressurized fluid extractor (PFE) was used to extract the blubber samples with dichloromethane. Approximately 1.5 g of sea lion blubber was mixed with pre-cleaned sodium sulfate and ground to a fine powder. Sodium sulfate was Soxhlet extracted with dichloromethane for 24 h and vacuum dried prior to use. This mixture was poured into 22 mL PFE cells and spiked with the internal standards described above. All samples, blanks and calibration solutions were extracted using the following program parameters: temperature 100 °C, heat time 5 min, static time 5 min, and pressure 13.8 MPa (2000 psig), for three cycles. Extracts were reduced in volume using nitrogen delivered by an automated evaporation system. The lipid content of the extracts was measured gravimetrically and then removed from the remaining extract using size exclusion chromatography (SEC) with a divinylbenzene-polystyrene column (10 μ m particle size, 100 Å pore size, 2.5 cm i.d. \times 60 cm, PL-Gel, Polymer Labs, Inc., Amherst, MA,

USA). Samples were eluted through the column using a mobile phase of 100% dichloromethane (DCM) at a flow rate of 10.0 mL/min. The collected eluent was reduced in volume and transferred into hexane. As a final clean up step the extracts were eluted through silica solid-phase extraction cartridges (Waters, Milford, MA, USA) with 20 mL of 20% (v/v) DCM in hexane, and again reduced in volume using nitrogen gas to 0.5 mL. Extracts in hexane were first analyzed for PBDEs using gas chromatography mass spectrometry. After PBDE analysis the extracts were reduced to dryness using nitrogen gas and then resolubilized in 100 μ L of methanol for LC/MS-MS measurement of HBCD.

2.4. Instrumental analysis

Extracts were analyzed for PBDEs, MeO-BDEs, and DBP-Br₄Cl₂ using gas chromatography (GC; Agilent GC 6890, Palo Alto, CA, USA) with a mass spectrometer (Agilent MS 5973, Palo Alto, CA, USA) operated in both electron capture negative ionization (GC/ECNI-MS) and electron impact ionization (GC/EI-MS). A 0.25 mm \times 15 m fused silica capillary column coated with a 5% (mole fraction) phenyl methylpolysiloxane column (DB-5MS, 0.25 μ m film thickness; J&W Scientific) was used for the separation of the analytes in the GC. On-column injection was employed in the GC and the injection port was set to track the oven temperature. The oven temperature was initially held at 80 °C for 2 min and then ramped to 140 °C at a rate of 12 °C/min, followed by a temperature ramp of 5 °C/min to a final temperature of 280 °C, which was held for an additional 20 min. The transfer line was maintained at 280 °C. The ion source and quadrupole were maintained at 200 °C and 100 °C, respectively. PBDEs, MeO-BDEs, and DBP-Br₄Cl₂ were measured using GC/ECNI-MS by monitoring m/z 79 and 81 (bromine ions) with the exception of BDE 209, which was monitored using ions 487 [C₆Br₅O⁻] and 409 [C₆Br₄O⁻] (Bjorklund et al., 2003). The chlorinated internal standard (CDE 86) was monitored using a molecular fragment [¹³C₁₂Cl₄O] at m/z 318 and 316. GC/EI-MS was used to confirm and quantify all analytes with the exception of HBCD. PBDEs were quantified using GC/EI-MS by monitoring the M⁺ and [M-2Br]⁺ ions for each homologue group. 6-MeO-BDE 47 was quantified in GC/EI-MS mode by monitoring the [M]⁺ ion at m/z 516 and 514. DBP-Br₄Cl₂ was quantified by monitoring the [M]⁺ ions at m/z 544 and 546. A five point calibration curve was used to quantify PBDEs and ranged in concentration from 0.06 ng/mL to 1000 ng/mL. A one point calibration curve was used for the quantification of MeO-BDEs and DBP-Br₄Cl₂ due to the limited availability of the standards.

HBCD consists of three stereo isomers known as α -HBCD, β -HBCD and γ -HBCD. Typical GC/MS analyses are unable to resolve these three isomers; however, liquid chromatography combined with mass spectrometry (LC/MS-MS) has been shown to provide the required selectivity

(Budakowski and Tomy, 2003). An API 4000 triple quadrupole mass spectrometer (Applied Biosystems/Sciex, Concord, ON, Canada) was operated in negative mode electrospray ionization and coupled to an 1100 series HPLC system (Agilent, Palo Alto, CA, USA). The method used here for the quantification of HBCD is very similar to the method used by Budakowski and Tomy (2003), with the exception of the LC column used. In this study a C₃₀ YMC Carotenoid S-5, 4.6 \times 250 mm, HPLC column (Waters Co., Milford, MA, USA) was used for the separation of the three HBCD isomers and provided baseline separation of the alpha, gamma and beta isomers (elution order, respectively). The flow rate through the column was 0.5 mL/min using a gradient elution initially set at water/methanol (20:80 by volume), and then ramped to 100% methanol over 35 min. The injection volume was 5 μ L. The multiple reaction monitoring (MRM) transition was [M-H]⁻ \rightarrow Br⁻ at m/z 640.8 \rightarrow 78.8 for the three HBCD isomers and the MRM transition for the ¹³C labeled γ -HBCD internal standard was 652.8 \rightarrow 78.8. A four point calibration curve was used for the quantification of HBCD and ranged from 10 ng/mL to 100 ng/mL.

2.5. QA/QC

The PBDE limits of quantification using GC/ECNI-MS was determined by using a signal to noise (S/N) of 50, and ranged from 1 pg to 50 pg on column for the various congeners. Using an extraction mass of 1.5 g, an injection volume of 2 μ L and correcting for blank contamination, the method detection limits (MDL) ranged from 0.5 ng/g to 1.0 ng/g wet mass. The HBCD limits of quantification using LC/MS-MS was determined by using a signal to noise (S/N) of 100, which ranged from 5 pg to 25 pg on column. MDLs for HBCD were 0.3 ng/g wet mass for each HBCD isomer. PBDEs, MeO-BDEs, and DBP-Br₄Cl₂ were all quantified using both GC/ECNI-MS and GC/EI-MS. Results agreed well with each other for both GC/ECNI-MS and GC/EI-MS and were within 20% of each other. The results from the GC/ECNI-MS quantification are reported in Table 1 because the GC/ECNI-MS had greater sensitivity.

BDE 47 (2,2',4,4'-tetrabromodiphenyl ether), BDE 99 (2,2',4,4',5-pentabromodiphenyl ether) and BDE 209 were detected in laboratory blanks and ranged from 0.3 ng to 0.7 ng, <0.05 ng to 0.3 ng, and from 0.1 to 1.0 ng, respectively; however, levels were low enough to exclude the need for blank correction. Levels of MeO-BDEs, DBP-Br₄Cl₂, and HBCD were all below MDLs (0.2 ng/g, 0.2 ng/g and 0.3 ng/g wet mass for MeO-BDEs, DBP-Br₄Cl₂, and HBCD, respectively) in laboratory blanks. Replicate extractions were performed on two of the blubber samples and values for the replicates were within 20% of each other for Σ PBDEs, within 15% of each other for MeO-BDEs and DBP-Br₄Cl₂ and within 5% of each other for Σ HBCD (which is primarily α -HBCD). Marine mammal blubber standard reference material (SRM 1945), supplied by NIST, was used as a quality control material to ensure accuracy in

Table 2
Correlation matrix presenting all the California sea lion variables analyzed in this study

Variable	Σ HBCD	Σ PBDE	MeO-BDE	DBP-Br ₄ Cl ₂	% Lipid	Date stranded	Mass	Length	Age	Blubber thickness
PBDE	0.261	1								
MeO-BDE	-0.101	0.083	1							
DBP-Br ₄ Cl ₂	0.027	0.160	0.680*	1						
% Lipid	0.258	0.077	-0.279	-0.476	1					
Date stranded	0.800*	-0.024	-0.176	-0.258	0.419	1				
Mass	-0.121	-0.036	0.109	0.022	-0.252	-0.157	1			
Length	-0.174	0.033	-0.358	-0.151	-0.025	-0.138	0.510*	1		
Age	-0.143	-0.056	0.204	0.202	-0.263	-0.207	0.692*	0.619*	1	
Blubber thickness	0.104	-0.052	-0.241	-0.236	0.490	0.033	0.415	0.503	0.271	1

Values represent the correlation coefficient for the two variables. (Significant correlations, $p < 0.01$, indicated with an asterisk.)

measuring PBDE concentrations. PBDE concentrations measured in SRM 1945 ranged from 76% to 100% of the reference values reported for PBDEs in this SRM (Zhu and Hites, 2003; Stapleton et al., 2004a). HBCD method recovery was assessed by spiking three sodium sulfate blanks with approximately 25 ng of α -HBCD and carrying these spikes through the entire extraction method. Recoveries of α -HBCD ranged from 86% to 93%. All reported concentrations are corrected for recovery.

2.6. Statistical analyses

Variables such as lipid content, blubber thickness, age, mass, length and year of stranding can all have an effect on the contaminant loadings in the sea lion blubber. To try and examine the effect of these variables on contaminant levels within the sea lion tissues, individual correlation analyses were run among all variables and contaminant levels and are presented in the correlation matrix (Table 2). A significant correlation ($R = 0.80$, $p < 0.01$) was observed for the effect of date of stranding on HBCD levels. To test for co-variance, multiple linear regression was performed on the log transformed HBCD data considering age, lipid, mass, and length as independent variables. Statistical significance was set at $p \leq 0.01$. Samples with contaminant concentrations below the MDL were assigned a value equivalent to the MDL for the statistical analyses.

3. Results and discussion

3.1. Lipid measurements

Lipid content was highly variable among blubber samples, ranging from 6% to 79%, and was not correlated with the concentrations of any of the brominated compounds. A low lipid measurement (<50%) in blubber may indicate that some of the animals were nutritionally stressed prior to stranding which would deplete their lipid stores. Blubber thickness was measured in the sea lion tissues (Table 1) and was positively correlated to % lipid (Table 2), reinforcing this idea. This variance has been observed previously in stranded California sea lions (Kannan et al., 2004). Due to this variability in the blubber lipid content, and the lack of a correlation between lipid and contaminant levels, the

concentrations are reported in all tables and figures in terms of ng/g wet mass.

3.2. HBCD

HBCD was detected in 81% of the samples analyzed and ranged in concentration from <0.3 ng/g to 12 ng/g wet mass (Table 1), and from <0.4 ng/g to 96 ng/g lipid. α -HBCD was the dominant isomer detected in all samples and ranged from 62% to 100% of the total HBCD measured in all sea lion samples. Tomy et al. (2004) observed a higher rate of biomagnification for the α -HBCD isomer compared to the γ -HBCD isomer in a Lake Ontario food web. Zegers et al. (2005) reported a predominance of α -HBCD in harbor porpoises (*Phocoena phocoena*) and common dolphins (*Delphinus delphis*) from the European Coast. Results from in vitro studies conducted using harbor seal liver microsomes have also suggested that α -HBCD is more resistant to P450 mediated biotransformation than the β - and γ -HBCD stereoisomers (Zegers et al., 2005), and that β - and γ -HBCD may be absent in some species due to efficient metabolism.

The HBCD levels measured in sea lions in this study are lower than levels measured in fish, porpoises and seals from the North Sea (Morris et al., 2004); fish from the Cinca River in Spain (Eljarrat et al., 2004) and peregrine falcon eggs in Sweden (Lindberg et al., 2004). This trend may reflect the greater demand for HBCD in Europe relative to North and South America, which was reported at 9500 metric tons and 2800 metric tons, respectively, in 2001 (Internet Communication, 2005). This trend is also reflected in the study by Tomy et al. (2004) which measured HBCD in a Lake Ontario food web and found that the HBCD levels in Lake Ontario fish are lower than levels measured in fish from European waters. No significant trends were observed between Σ HBCD and mass, length, lipid or age of the sea lions (Table 2). The only significant trend was between Σ HBCD and date of stranding, which will be discussed below.

3.3. PBDEs

These sea lion blubber samples were analyzed for 27 individual PBDE congeners ranging from tribromodiphenyl

ethers through decabromodiphenyl ether (BDE 209). BDE 209 was not present (<1.0 ng/g wet weight) in any of the samples examined. Tribromo- through heptabromodiphenyl ethers were detected in all sea lion samples analyzed and ranged in total concentration from 450 ng/g to 4740 ng/g wet mass (Table 1) or from 570 ng/g to 24,240 ng/g lipid. The geometric mean was 1470 ng/g wet mass, or 3900 ng/g lipid. These Σ PBDE levels are some of the highest levels ever reported for marine mammals and are higher than levels measured in killer whales (*Orcinus orca*) from the Northeastern Pacific (Rayne et al., 2004), northern fur seals (*Callorhinus ursinus*) from the Pacific coast of Japan (Kajiwara et al., 2004), grey seals (*Halichoerus grypus*) from the North Sea (Kalantzi et al., 2005) and polar bear (*Ursus maritimus*) and beluga whales (*Delphinapterus leucas*) from the Arctic (Wolkers et al., 2004). However, they are comparable to levels measured in male Atlantic white-sided dolphins (*Lagenorhynchus acutus*) (Tuerk et al., 2005), harbor porpoises from England and Wales (Law et al., 2002), and San Francisco harbor seals (*Phoca vitulina*) (She et al., 2002). No correlation was observed between Σ PBDEs and HBCD in these sea lion samples.

BDE 47 (2,2',4,4'-tetrabromodiphenyl ether) was the dominant congener in all sea lions measured and represented $55\% \pm 8\%$ of the total PBDE burden (Fig. 1). BDE 100 (2,2',4,4',6-pentabromodiphenyl ether), and BDE 99 (2,2',4,4',5-pentabromodiphenyl ether) contributed $20\% \pm 7\%$ and $12\% \pm 3\%$, respectively. This congener pattern is very similar to BDE congener patterns observed in other marine mammals (Wolkers et al., 2004; She et al., 2002; Rayne et al., 2004; Tuerk et al., 2005). Generally, BDE 99 is found in the highest percentage in the pentaBDE commercial mixtures, followed by BDE 47 and BDE 100 (Sjodin et al., 1998). The fact that BDE 100 is generally higher in concentration than BDE 99 in these sea lion samples may indicate that sea lions metabolize BDE 99 to some degree. In vivo laboratory exposures have shown that the

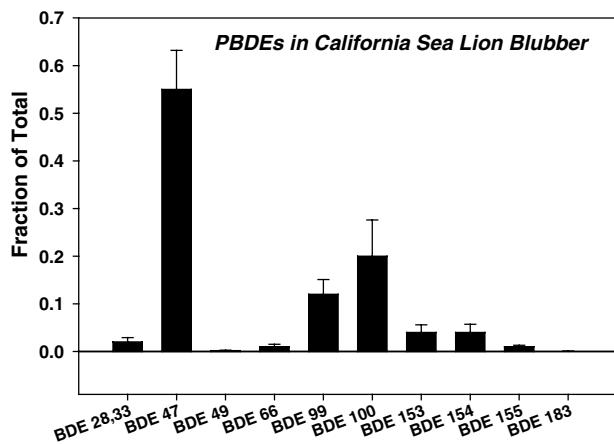


Fig. 1. Average PBDE congener composition in California sea lion blubber samples ($n = 26$). Error bars represent the standard deviation.

common carp debrominates BDE 99 to form BDE 47 (Stapleton et al., 2004b). It is possible that sea lions may possess a similar metabolic capacity to debrominate BDE 99 which results in an increased accumulation of BDE 47 within their tissues and a relative depletion of BDE 99. In contrast, we cannot rule out the possibility that this congener pattern in the sea lion tissue reflects the pattern in their source of exposure, primarily their diet.

3.4. Methoxylated PBDEs

California sea lion blubber samples were analyzed for 14 individual methoxylated polybrominated diphenyl ethers (MeO-BDEs). Only one MeO-BDE was positively identified, 6-MeO-BDE 47 (Fig. 2). Concentrations ranged from <0.2 ng/g to 12 ng/g wet mass and are comparable to levels of MeO-BDEs measured in grey and ringed seals (*Phoca hispida*) from the Baltic Sea (Haglund et al., 1997) and dolphins from the Mediterranean Sea (Pettersson et al., 2004). If MeO-BDEs were formed from the metabolism of PBDEs, it would most likely occur via the cytochrome P450 system. Cytochrome P450s have been shown to metabolize PCBs and PBDEs to hydroxylated analogues

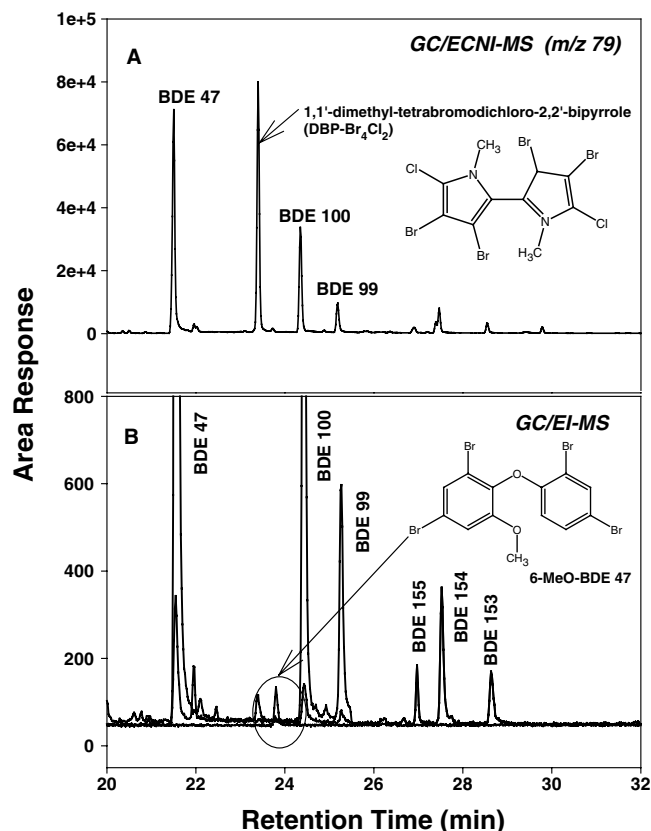


Fig. 2. Chromatograms from the analysis of California sea lion blubber analyzed by GC/ECNI-MS in which a bipyrrrole, referred to as DBP- Br_4Cl_2 , is identified (A), and GC/EI-MS analysis to confirm the presence of MeO-BDEs (B). The GC/EI-MS chromatogram overlays the ion chromatograms for m/z 486 (BDE 47), m/z 516 (6-MeO-BDE 47), m/z 404 (BDEs 100 and 99) and m/z 484 (BDEs 155, 154, and 153).

(Hakk and Letcher, 2003). If PBDEs were metabolized to MeO-BDEs, the first step would be the oxidation of a PBDE congener by a CYP enzyme followed by enzyme-mediated methylation. Oxidation of PCBs and PBDEs via CYP enzymes has typically been observed in the *meta*- and *para*-positions relative to the diphenyl ether bond (Hakk and Letcher, 2003; Orn and Klasson-Wehler, 1998; Letcher et al., 2000). 6-MeO-BDE 47, which was identified in the sea lion blubber, has a methoxy group present in the *ortho*-position, which lends support to a biogenic origin. In addition, Malmvarn et al. (2005) recently identified 6-MeO-BDE 47 in the red algae *Ceramium* sp. and blue mussels in the Baltic Sea and concluded that this compound was produced either from the red algae itself or associated microflora and/or microfauna. Teuten et al. (2005) also recently identified 6-MeO-BDE 47 and 2-MeO-BDE-68 in True's beaked whales (*Mesoplodon mirus*) and determined it was of a non-anthropogenic origin based on its radiocarbon content.

There was no correlation between levels of 6-MeO-BDE 47 and BDE 47 ($p < 0.01$), nor were there any correlations with other PBDE congeners. Levels of 6-MeO-BDE 47 were, however, positively correlated with another brominated compound observed in all GC/ECNI-MS chromatograms (Fig. 2), which was determined to be DBP-Br₄Cl₂. This compound was identified by acquiring full-scan EI and ECNI mass spectra and comparing it to the full-scan and GC retention times of a synthetic standard of DBP-Br₄Cl₂. Fig. 3 presents the full scan EI spectra obtained for DBP-Br₄Cl₂ in the sea lion extract, which compares very well to the spectra obtained by Tittlemier et al. (1999). DBP-Br₄Cl₂ is a mixed bromo-, chloro-heterocyclic compound which has only been identified in marine species. It has been found in Pacific seabird eggs at levels 1.5–2.5 times higher than levels in Atlantic seabird eggs (Tittlemier et al., 1999), and here we have identified DBP-Br₄Cl₂ in another Pacific marine organism, the California sea lion. Concentrations of DBP-Br₄Cl₂ ranged from

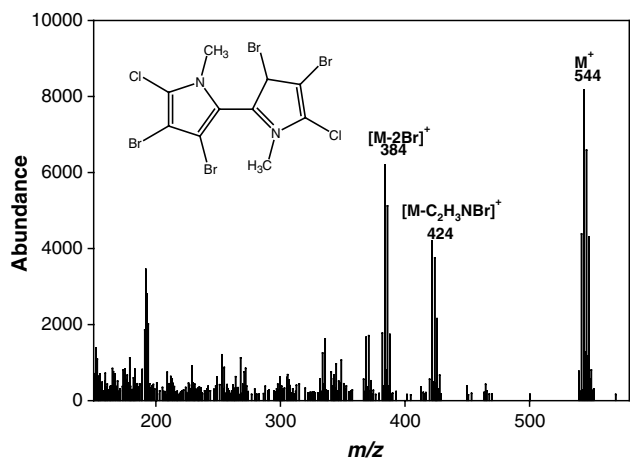


Fig. 3. EI full mass spectrum of DBP-Br₄Cl₂ in sea lion blubber extract.

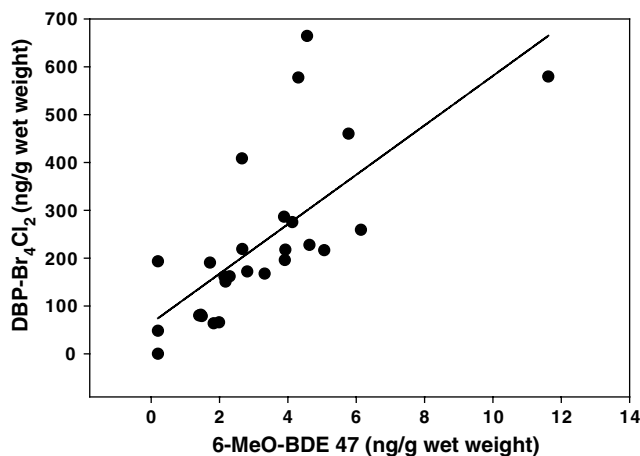


Fig. 4. Correlation between 6-MeO-BDE 47 and 1,1'-dimethyl-3,3',4,4'-tetrabromo-5,5'-dichloro-2,2'-bipyrrole (DBP-Br₄Cl₂) in California sea lion blubber. A value of 0.2 ng/g wet weight was used for the three samples in which the concentration of 6-MeO-BDE 47 was <0.2 ng/g.

44 ng/g to 660 ng/g wet mass and were comparable to the levels of BDE 47. Reddy et al. (2004) recently isolated DBP-Br₄Cl₂ from a marine mammal and performed radiocarbon dating which suggested a biogenic origin. DBP-Br₄Cl₂ and 6-MeO-BDE 47 concentrations in California sea lions were significantly correlated (Fig. 4; $r = 0.7$; $p < 0.05$). The lack of a correlation between 6-MeO-BDE 47 and BDE 47, and the positive correlation between DBP-Br₄Cl₂ and 6-MeO-BDE 47, suggests that the latter compounds may both accumulate from natural sources. No information is available to determine if there are any potential toxicity concerns associated with exposure to these biogenic brominated compounds.

3.5. Contaminant trends

The California sea lions in this study were stranded between 1993 and 2003 (Table 1), which provided an opportunity to examine the changes in the accumulation of brominated compounds in the blubber of sea lions during this time. The number of sea lion samples that were collected and banked were not uniform every year between 1993 and 2003 and therefore there are limitations on the temporal trend analyses conducted here. However, we can make some general observations on contaminant trends based on the available samples. No significant temporal trend was observed in the concentrations of 6-MeO-BDE 47, DBP-Br₄Cl₂ or ΣPBDEs (Fig. 5). Additionally, no temporal trend was evident in the concentrations of individual PBDE congeners. Previous studies have shown that ΣPBDEs in San Francisco harbor seal blubber were increasing over the period 1989 to 1998 (She et al., 2002), in Arctic ringed seals from 1981 to 2000 (Ikonou et al., 2002), in Great Lakes herring gull eggs (*Larus argentatus*) from 1981 to 2000 (Norstrom et al., 2002) and in northern fur seals collected in Japan between 1972 to 1998 (Kajiwara et al., 2004). The majority of these studies

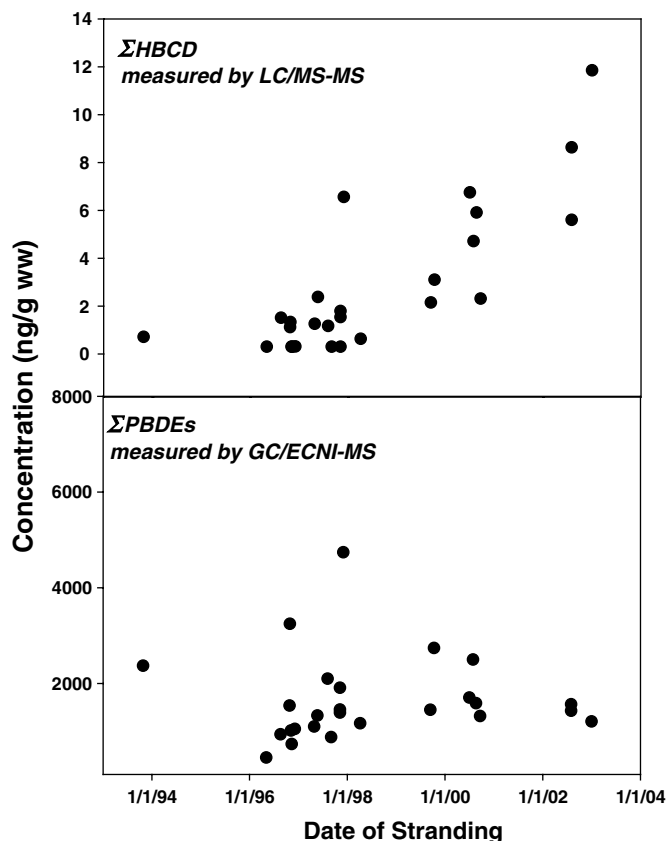


Fig. 5. Concentration (ng/g wet weight) of Σ HBCD and Σ PBDEs in blubber tissues recovered from stranded California sea lions. A value of 0.3 ng/g was used for the five samples in which HBCD was less than detection.

that have observed a temporal increase in Σ PBDEs have examined samples collected, or archived, starting in the 1970s to 1980s and continuing up through the year 2000. Kajiwara et al. (2004) observed a 150 fold increase in Σ PBDEs in fur seals from 1972 to 1994, but then an apparent decrease in concentration from 1994 to 1998. Sellstrom et al. (2003) observed an increase in Σ PBDEs in guillemot eggs from the Baltic Sea from 1970 to the mid 1980s, followed by a significant decrease up to 2001. Additionally, studies from Sweden have shown that PBDEs in human breast milk increased from 1970 to 1997, but then decreased from 1997 to 2000 (Betts, 2002). Based on these latter studies, it may be possible that PBDEs have reached a peak concentration in sea lion blubber. A recent study on Atlantic dolphins sampled between 1993 and 2000 also found no temporal trend for Σ PBDEs (Tuerk et al., 2005). Differences in PBDEs levels among the sea lions may also be a consequence of the sea lions feeding in different locations up and down the California coast that may have different PBDE loadings.

In contrast, Σ HBCD increased ($r^2 = 0.63$; $p < 0.01$) in California sea lion blubber between 1993 and 2003. Multiple linear regression was performed on the data set which included Σ HBCD, % lipid, age, mass, and length. The only significant ($p < 0.01$) independent variable was the date of

stranding. Concentrations of HBCD appear to be increasing almost exponentially (Fig. 5); however, the number of samples in this study may be too limited to make an accurate estimation of the HBCD accumulation rate in sea lion blubber. If we assume this increase is exponential, then the doubling time of HBCD in sea lion blubber between 1993 and 2003 is approximately 2 years. Doubling times calculated for PBDEs between 1970 to 2000 in various media have been reported at about 5 years for human blood, milk, and tissues (Hites, 2004), 3 years for Great Lakes herring gull eggs (Norstrom et al., 2002) and from 4 to 9 years for Arctic ringed seals (Ikonomou et al., 2002). Sellstrom et al. (2003) reported an increase in HBCD in guillemot eggs from the Baltic Sea from the early 1980s, but concentrations have remained relatively stable from 1991 to 2001.

It is unclear at this time why HBCD levels appear to be increasing in sea lion blubber while PBDE levels are variable. According to the Bromine Science and Environmental Forum (www.bsef.com), the world market demand for HBCD increased from 15,900 metric tons to 16,700 metric tons between 1999 and 2001, while the demand for penta-BDE decreased from 8500 metric tons to 7500 metric tons. This trend in the sea lion tissues may be related to the relative production and use of HBCD compared to penta-BDE. To the authors' knowledge, this is the first report of increasing HBCD concentrations in a marine mammal population.

In field studies, HBCD was observed to have a biota/sediment accumulation factor of about 15 (Sellstrom et al., 1998), suggesting it is fairly bioaccumulative. Only a few studies have examined the potential toxicity and metabolism of HBCD (Darnerud, 2003; Birnbaum and Staskal, 2004). In general, HBCD appears not to be acutely toxic to rats, mice or fish, but chronic exposure can lead to increased liver weights, thyroid hyperplasia and increase intragenic recombination in in vitro assays (Darnerud, 2003; Birnbaum and Staskal, 2004; Ronisz et al., 2004). It also has potential neurotoxicological effects by inhibiting the uptake of the neurotransmitters dopamine, glutamate, and γ -amino-*n*-butyric acid in rat brain tissue (Mariussen and Fonnum, 2003). Considering the apparent rate of increase in HBCD concentrations in sea lions, and the potential toxicity concerns, more studies on the loadings and concentrations of HBCD in other environmental media may be warranted.

This study has shown that biogenic brominated compounds can be found at levels comparable to anthropogenic persistent organic pollutants. There was no correlation between PBDEs and MeO-BDEs, which suggests that MeO-BDEs in California sea lions are not primarily due to metabolism of PBDEs. This work demonstrates that HBCD appears to be increasing in sea lion blubber whereas PBDE concentrations appear to be stable. Future monitoring should be conducted to determine if PBDE burdens will decrease in the future now that penta-BDE and octaBDE will no longer be produced as flame retardants in the U.S.

4. Disclaimer

Certain commercial equipment, instruments, or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.marpolbul.2005.09.045](https://doi.org/10.1016/j.marpolbul.2005.09.045).

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