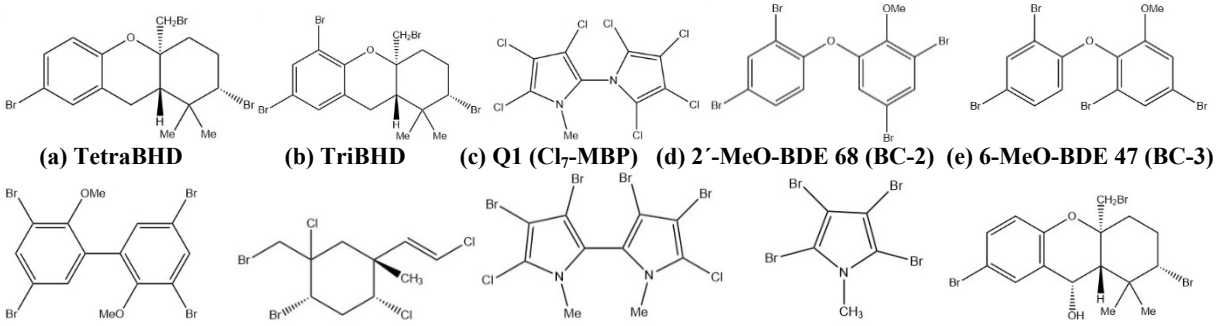
HALOGENATED NATURAL PRODUCTS WERE HIGHER CONCENTRATED THAN ANTHROPOGENIC POPS (PCBs, PBDEs) IN SPERM WHALES FROM THE MEDITERRANEAN SEA (ITALY)

Vetter W[1], Aechtler K[1], Schlienz A[1], Bartalini A[2], Muñoz-Arnanz J[3], Marsili L[2], Mazzariol S[4], Fossi MC[2], Jiménez B[3]

[1] University of Hohenheim, Institute of Food Chemistry (170b), 70593 Stuttgart, Germany
[2] Department of Environmental, Earth and Physical Sciences, University of Siena, Via Mattioli 4, Siena, Italy
[3] Department of Instrumental Analysis and Environmental Chemistry, Institute of Organic Chemistry, CSIC. Juan de la Cierva, 3, 28006 Madrid, Spain
[4] Department of Public Health, Comparative Pathology and Veterinary Hygiene, University of Padova, Viale dell'Università 16, 35020 Legnaro, PD, Italy

Introduction

Several man-made polyhalogenated organic compounds (e.g. polychlorinated biphenyls (PCBs), brominated flame retardants such as polybrominated diphenyl ethers (PBDEs) and chloropesticides (e.g. dichlorodiphenyltrichloroethane (DDT), chlordane, hexachlorocyclohexane, and toxaphene) are known major contaminants of marine biota. In addition, more than 4000 halogenated natural products (HNPs) have been identified to date, predominantly in marine organisms.[1] Known producers of HNPs are algae, sponges and marine bacteria.[1] Recent research has demonstrated that some HNPs are also often found at elevated concentrations in top predators of diverse marine foodwebs.[2] The structures of environmentally-relevant marine HNPs are varied and include polyhalogenated heterocycles but also aromatic compounds such as phenoxyanisoles and dimethoxybiphenyls (**Fig. 1**).[2,3]



(a) TetraBHD **(b) TriBHD** **(c) Q1 (Cl_7-MBP)** **(d) 2´-MeO-BDE 68 (BC-2)** **(e) 6-MeO-BDE 47 (BC-3)**

(f) 2,2´-diMeO-BB 80 (BC-1) **(g) MHC-1** **(h) Br_4Cl_2-DBP (BC-10)** **(i) TBMP** **(j) meroterpenoid**

Figure 1: Structures of relevant halogenated natural products (HNPs) quantified in this study except for meroterpenoid (see text)

As can be seen from these structures, polychlorinated, polybrominated, and mixed-halogenated compounds have been identified. Here we determined the quantities of HNPs and anthropogenic POPs (PCBs, PBDEs) in order to assess the major pollutant class in sperm whales (*Physeter macrocephalus*) stranded at different locations of the Italian coast (Mediterranean Sea).

Materials and methods

Samples. Samples (A-I) of nine male sperm whales (*Physetermacrocephalus*), which stranded at different points in Italy (seven east coast, Adriatic Sea, two west coast).

Sample cleanup. Lipids were gathered from samples (1.0-1.4 g; sample blanks without sample) by means of accelerated solvent extraction using α-PDHCH (107 ng) as internal standard and cyclohexane/ethyl acetate (46:54, w/w) as the solvent.[4] ASE extracts of sample H and I were cloudy and centrifuged before the next step. Lipids were removed by gel permeation chromatography using the same solvent. The polyhalogenated fraction was concentrated to 0.5 mL. Adsorption chromatography (3 g silica, deactivated with 30% water) was used prior to GC/ECNI-MS analysis. A group separation (PCB other compounds) on activated silica was performed according to Weichbrodt et *al.*[4] using additionally 2,3,6,7-tetrachloronaphthalin (TCN) as internal standards of fraction 1. Fraction 1 featured PCBs and Q1 while the fraction 2 ("organobromine fraction") contained all anthropogenic and naturally produced Br-containing compounds as well as chloropesticides. The lipid content of the samples ranged from 8.4-45.3%.

GC/ECNI-MS analysis. An Agilent 7890/5975C GC/ECNI-MS system was used in combination with a 7673 GC/SFC autosampler (Agilent Technologies, Waldbronn, Germany) using the parameters of Hauler *et al*.[5] Different full scan and selected ion monitoring mode (SIM) methods were peak verification and quantification.
Quality control. All samples were analyzed in duplicate. Recovery rates were >70% except for both duplicates of sample H (most likely partial loss due to centrifugation, see above). This sample was discussed separately and was not taken into account when overall-mean values were calculated. Recovery rate of sample E2 was also slightly below 70% and only data of sample E1 will be presented later. In all other cases, mean values of the duplicate samples will be reported. Duplicate analysis results of Q1, TetraBHD and PCB 138 varied less than 20%, which was considered good because samples were individually weighed (not fully homogeneous). The samples contained a sulfur artefact peak introduced during the sample cleanup, which eluted into fraction 1 and did not hamper evaluation. Limit of detection (LOD) ranged from 0.04 pg (TBMP) to 14 pg (Cl_6-DBP), but was typically at 0.1-1 pg.

Results and discussion

Halogenated natural products.

Except for Q1, all HNPs quantified in this study eluted into the "organobromine fraction", in which they were determined while Q1 (and PCBs) were analyzed in fraction 1 of the silica method (see experimental).

Polybrominated hexahydroxanthene derivatives (PBHDs).
GC/ECNI-MS chromatograms of the "organobromine fraction" (PCBs and Q1 separated) of the sperm whale samples were dominated by TetraBHD (**Fig. 2**). This is particularly remarkable as TetraBHD (**Fig. 1a**) has a very low GC/ECNI-MS response.[6]

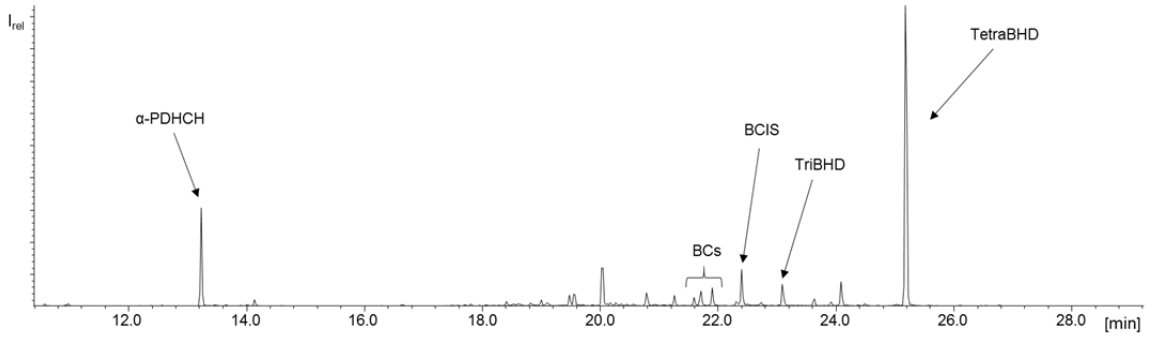


Figure 2: GC-ECNI-MS *full-scan* chromatogram of the "organobromine fraction" (PCBs and Q1 were separated on silica) in blubber of a sperm whale (*Physeter macrocephalus*) stranded at the Italian east coast of the Mediterranean Sea.

Accordingly, concentrations of TetraBHD were particularly high and ranged from 14,600-77,700 ng/g lipids. The mean TetraBHD concentration was 34,300 ng/g lipids (**Table 1**). TriBHD (**Fig. 2**), typically found together with TetraBHD in samples,[6] was more than one order of magnitude lower concentrated (mean: 1430 ng/g lipids, range: 940-2770 ng/g lipids TriBHD) (**Table 1**). TriBHD and TetraBHD are typical HNPs of the Mediterranean Sea,[6] most likely produced by the sponge *Scalarispongia scalaris*.[7] The ratio of Tetra- to TriBHD in the samples was relatively constant at ~24. Compared to that, fish from the Mediterranean Sea was about 7x higher contaminated with TriBHD than TetraBHD.[8] Likewise, Melcher *et al.* found more TriBHD (530-770 ng/g lipids) than TetraBHD (210-370 ng/g lipids) in sardines from the Mediterranean Sea.[6] However, Covaci *et al.* reported that TetraBHD was more abundant (>10-fold) in Mediterranean deep sea fish, and that PBHDs represented 90-99% of all organobrominated compounds[7]. Sperm whales are known to predate in deep water, which may be the explanation for the predominance of TetraBHD (96%). Interestingly, the GC-ECNI-MS chromatograms showed a peak at the retention time between TriBHD and TetraBHD (24.1 min). *m/z* 79 (Br^-) and Br_2^- fragment ions (*m/z* 158) confirmed the presence of at least two Br substituents. The potential M^- at *m/z* 480 (tribrominated, verified by *m/z* 401, M-Br]$^-$ with dibromo isotope pattern) was 16 u higher than the one of TriBHD (*m/z* 464). The high concentrations of TriBHD and TetraBHD made it assumable that the additional compound was also produced by sponges. One option could be the additional presence of oxygen compared to TriBHD or $C_{16}H_{19}Br_3O_2$. A corresponding hexahydroxanthene derivative (**Fig. 1j**), a brominated meroterpenoid[9] is known to be produced by *Cacospongia sp*. (*Scalarispongia scalaris*). Yet, hydroxylated compounds are less lipophilic, which makes it less likely that the compound is accumulated in the lipids.

Table 1: Concentrations (ng/g lipids) of halogenated natural products in sperm whales (*Physeter macrocephalus*) from Italy, Mediterranean Sea with mean values as well as minimum (Min) and maximum (Max) values in the corresponding sample

ng/g lipids	TetraBHD	TriBHD	Q1	BC-2	BC-3	BC-1	MHC-1	∑DBPs	∑HNPs
Mean	34,300	1430	530	86	120	100	7	170	36,800
Min	14,600	940	250	40	70	40	<NG	80	16,100
sample	G	G	G	G	G	G	H / I	E	G
Max	77,700	2770	1360	140	170	160	16	370	82,500
sample	F	F	F	F	A	A	F	I	F

Polyhalogenated 1´-methyl-1,2´-bipyrroles (PMBPs). Next to PBHDs, Q1 (**Fig. 1c**) was the third-most abundant HNP in the samples (Q1 was already detectable in the full scan GC/ECNI-MS chromatograms of *P. macrocephalus*; note that Q1 is not visible in Fig. 2 because it was previously separated by column chromatography). Similarly to TetraBHD, sample G featured the lowest Q1 levels of ~250 ng/g lipids and sample F the highest Q1 concentration of 1360 ng/g lipids while the mean value was 530 ng/g lipids Q1 (Table 1). Six further polyhalogenated methylbipyroles (PMBPs) were also detected (both most abundant isotope peaks present in the correct ratio), but could not be quantified due to the lack of reference standards, i.e. two Br_2Cl_5-MBPs, three Br_3Cl_4-MBPs (highest in sample H) and traces of Br_7-MBP in six samples. Based on GC-ECNI responses, Q1 > $BrCl_6$-MBPs while others were detected only in traces.

Brominated methoxy diphenyl ethers (MeO-PBDEs). 2´-MeO-BDE 68 (BC-2, **Fig. 1d**) and 6-MeO-BDE 47 (BC-3, **Fig. 1e**) were detected in all samples. BC-2 (mean: 86 ng/g lipids, range: 40-140 ng/g lipids) was slightly but generally lower concentrated than BC-3 (mean: 120 ng/g lipids, range: 70-170 ng/g lipids). Petterson *et al.* also found higher abundance of BC-3 (7-630 ng/g lipids, semi-quantitative levels) in livers of cetaceans from the Mediterranean Sea.[10] Barón *et al.*[11] analyzed MeO-BDEs in dolphins (2004-2011, five species) and concentrations ranged from not detectable to 2510 ng/g lipids (mean value higher than in the present study). Reasons could be different habitats (Alborán Sea vs. Italy), but also feed and food chain position.

Tetrabrominated dimethoxylated biphenyls (diMeO-BB 80, BC-1). BC-1 (**Fig. 1f**) concentrations were comparable with MeO-BDEs (maximum 160 ng/g lipids, mean value 100 ng/g lipids). The two samples from the west coast of Italy (H and I) had similar levels (68 and 67 ng/g lipids BC-1), which were slightly lower than the mean. Literature BC-1 concentrations in marine mammals varied from 12-800 ng/g lipids.[12]

Mixed halogenated monoterpene 1 (MHC-1). MHC-1 (**Fig. 1g**) concentrations in samples from the east coast were only between 2.4-16 ng/g lipids (**Table 1**). Yet, MHC-1 was not detected in samples H and I from the west coast of Italy. Assumedly the abundance of the natural producer *Plocamium cartilagineum* was higher in the Adriatic Sea (east coast). Absence/presence of MHC-1 remained the only general difference between both sites. MHC-1 concentrations were in the range of those reported for fish in the Mediterranean Sea (not detected - 52 ng/g lipids[8]). Concentrations of MHC-1 in dolphins (1.1-130 ng/g lipids)[11] were higher.

Polyhalogenated 1,1`-dimethyl-2,2`-dipyrroles (PDBPs). Cl_6-DBP, Br_5Cl-DBP, Br_6-DBP, Br_4Cl_2-DBP (BC-10) were available as standards and could be quantified. Highest PDBP concentrations were determined for Cl_6-DBP ranging from 45-330 ng/g lipids. Noteworthy, Br_4Cl_2-DBP (BC-10, **Fig. 1h**), which is frequently the most abundant PDBP homolog in marine mammals,[13] was much lower concentrated (10-24 ng/g lipids). However, Cl_6-DBP has a very low GC-ECNI-MS response and without a reference standard its relevance is most likely underrated. Recently, Cl_6-DBP was also found to dominate in dolphins from the Great Barrier Reef.[14] Br_6-DBP (8.3-15 ng/g lipids) and Br_5Cl-DBP (1.4-4.1 ng/g lipids) contributed only 6% and 1.2% to the total-PDBP content. Further homologues detected were three Br_2Cl_4-DBPs and one Br_3Cl_3-DBP. An additional isomer of Br_4Cl_2-DBP (BC-10) as well as Br_5Cl-DBP and Br_6-DBP were also detected.

Polyhalogenated 1-methylpyrroles (PMBs). PMPs were detected in form of TBMP (**Fig. 1i**) in four samples at 0.2 (samples F, G, I) up to 2.0 ng/g lipids (sample E). Further PMPs as described by Hauler *et al.*[5] could not be identified.

Anthropogenic compounds.

Polychlorinated biphenyls (PCBs). PCB 153 and PCB 138 showed similar mean concentrations of ~3700 ng/g lipids (**Table 2**). PCB 180 concentrations were about half the one of PCB 153 (**Table 2**). Other isomers were quantified with the response of PCB 180 (heptaCBs) and mean of PCB 153 and PCB 138 (hexaCBs). The highest sumPCB level of 39,200 ng/g lipids was detected in samples F. Both samples were ~twice as high contaminated with PCBs as the other samples of *P. macrocephalus*.

Table 2: Concentrations (ng/g lipids) of PCBs and PBDEs and sum values of POPs and HNPs in sperm whales (*Physeter macrocephalus*) from Italy, Mediterranean Sea with mean values as well as minimum (Min) and maximum (Max) values in the corresponding sample

ng/g lipids	PCB 138	PCB 153	PCB 180	∑PCBs	BDE 47	Sum PBDEs	∑PCBs + PBDEs	Ratio ∑HNPs/ ΣPCB+PBDEs
Mean	3680	3770	1580	18,300	260	490	18,800	1.96
Min	1300	1250	550	6900	90	260	7150	2.25
sample	E	E	E	E	G	E	E	G
Max	8430	9250	3840	39,200	590	900	40,100	2.06
sample	F	I	F	F	F	F	F	F

Polybrominated diphenyl ethers (PBDEs). One tetraBDE (BDE 47), two pentaBDEs (BDE 99 and BDE 100) and two hexaBDEs (BDE 153 and BDE 154) were identified due to t_R and the commercial BDE-Mix 39. A distinct predominance of BDE 47 (86-590 ng/g lipids) was found in all samples.[15] The same five PBDEs and BDE 28 were also detected by Baron *et al*. in dolphins from the Mediterranean Sea, predominance also BDE 47.[11] Highest PBDE levels were found in samples F and I. Sample H was also high in PBDEs although the recovery rate was low. Sample H was a juvenile animal which are frequently lower contaminated. Samples H and I originated from Tyrrhenian and Ligurian Sea which may be higher polluted with PBDEs than the Adriatic Sea.

Further anthropogenic polyhalogenated compounds. *p,p´*-DDT metabolites *p,p´*-DDE and *p,p´*-DDD, as well as *trans*-chlordane, *cis*-chlordane and *trans*-nonachlor and HCB were also detected but not quantified in this study due to the focus on HNPs.

SumHNPs versus SumPOPs in the sperm whale (*P. macrocephalus*) samples. SumHNP concentrations were generally higher than 10,000 ng/g lipids (>10 ppm) and the maximum level was >80 ppm. These levels are among the highest reported to date in marine mammals. This indicates that the Mediterranean Sea is a rich source of HNPs whose abundance should be studied more in detail. Previously, Petterson *et al*. analyzed cetaceans from the Mediterranean Sea on PBDEs and MeO-BDEs and mentioned the presence of several additional peaks from unidentified brominated compounds in GC-ECNI-MS ion chromatograms.[10] These were most likely TetraBHD, TriBHD, BC-1, along with PMBPs and PDBPs, i.e. HNPs quantified in the present study. Concentrations of HNPs were clearly in the same range as anthropogenic POPs (**Table 2**). Likewise, sample F was usually the one with the highest concentrations of both HNPs and POPs.

References

1. Gribble GW (2000) Environ. Sci. Pollut. Res. 7, 37–49.
2. Vetter W (2006) Rev. Environ. Contam. Toxicol. 188, 1–57.
3. Bidleman TF, Andersson A, Jantunen LM, Kucklick JR, Kylin H, Letcher RJ, Tysklind M, Wong F (2019) Emerg. Contam. 5, 89-115.
4. Weichbrodt M, Vetter W, Luckas B (2000) J. AOAC Int. 63, 1334-1343.
5. Hauler C, Rimkus G, Risacher C, Knölker H-J, Vetter W (2014) Sci. Total Environ. 490, 994–1001.
6. Melcher J, Janussen D, Garson MJ, Hiebl J, Vetter W (2007) Arch. Environ. Contam. Toxicol. 52, 512-518.
7. Covaci A, Losada S, Roosens L, Vetter W, Santos FJ (2008) Environ. Sci. Technol. 42, 8654-8660.
8. Hiebl J, Melcher J, Gundersen H, Schlabach M, Vetter W (2006) J. Agric. Food Chem. 45, 2652-2657.
9. Bali DKL, Liokas V, Garson MJ, Faulkner DJ (1990) Austr. J. Chem. 43, 2009-2020.
10. Pettersson A, van Bavel B, Engwall M, Jiménez B (2004) Arch. Environ. Contam. Toxicol. 47, 542-550.
11. Barón E, Hauler C, Gallistl C, Giménez J, Gauffier P, Castillo J, Fernández C, de Stephanis R, Vetter W, Eljarrat E, Barcelo D (2015) Environ. Sci. Technol. 49, 9073-9083.
12. Marsh G, Athanasiadou M, Athanassiadis I, Bergman Å, Endo T, Haraguchi K (2005) Environ. Sci. Technol. 39, 8684-8690.
13. Tittlemier S, Borrell A, Duffe J, Duignan PJ, Fair P, Hall A, Hoekstra P, Kovacs KM, Krahn MM, Lebeuf M, Lydersen C, Muir D, O'Hara T, Olsson M, Pranschke J, Ross P, Siebert U, Stern G, Tanabe S, Norstrom R (2002) Arch Environ Contam Toxicol. 43, 244-255
14. Hauler C, Martin R, Knölker H-J, Gaus C, Mueller JF, Vetter W (2013) Environ. Pollut. 178, 329-335.
15. Bartalini A, Muñoz-Arnanz J, Marsili L, Mazzariol S, Fossi MC, Jiménez B (2019) Sci. Total Environ. 653, 1417-1425