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Article

High Amounts of Halogenated Natural Products in Sperm Whales (*Physeter macrocephalus*) from Two Italian Regions in the Mediterranean Sea

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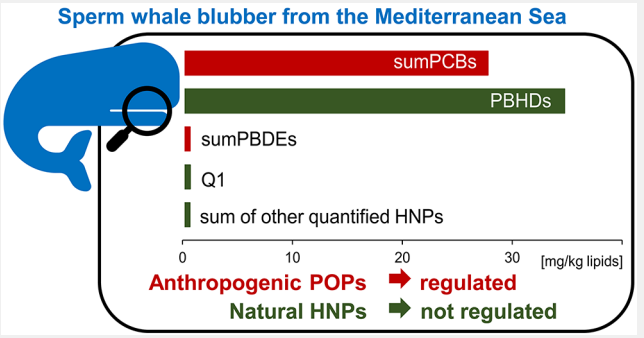
ABSTRACT: Halogenated natural products (HNPs) are considered to be emerging contaminants whose environmental distribution and fate are only incompletely known. Therefore, several persistent and bioaccumulative HNP groups, together with man-made polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs), were quantified in the blubber of nine sperm whales (*Physeter macrocephalus*) stranded on the coast of the Mediterranean Sea in Italy. The naturally occurring polybrominated hexahydroxanthene derivatives (PBHDs; sum of TetraBHD and TriBHD) were the most prominent substance class with up to 77,000 ng/g blubber. The mean PBHD content (35,800 ng/g blubber) even exceeded the one of PCBs (28,400 ng/g blubber), although the region is known to be highly contaminated with man-made contaminants. Based on mean values, Q1 ∼ PBDEs > MeO-BDEs ∼ 2,2′-diMeO-BB 80 and several other HNPs followed with decreasing amounts. All blubber samples contained an abundant compound whose molecular formula ($C_{16}H_{19}Br_3O_2$) was verified using high-resolution mass spectrometry. The only plausible matching isomer was (2*S*,4′*S*,9*R*,9′*S*)-2,7-dibromo-4′-bromomethyl-1,1-dimethyl-2,3,4,4′,9,9′-9,9′-hexahydro-1*H*-xanthen-9-ol (OH-TriBHD), a hydroxylated secondary metabolite previously detected together with TriBHD and TetraBHD in a sponge known to be a natural producer of PBHDs. The estimated mean amount of the presumed OH-TriBHD was 3000 ng/g blubber, which is unexpectedly high for hydroxylated compounds in the lipids of marine mammals.



KEYWORDS: *Halogenated natural product, naturally occurring polyhalogenated compound, persistent organic pollutant, sperm whale, Italy, polar metabolite*

1. INTRODUCTION

With currently >8000 known representatives, halogenated natural products (HNPs) represent a remarkable substance class of predominantly marine origins.[1] Individual HNPs differ considerably in molecular size, as well as the type and number of halogen substituents.[1−3] Interestingly, some HNPs have been detected at elevated amounts in higher organisms.[4−7] This implies that these HNPs are persistent and bioaccumulative, i.e. detrimental properties mainly known from man-made polyhalogenated compounds that have partly been classified as persistent organic pollutants (POPs) due to their reported toxicity or adverse effects on different ecosystems.[7−9] This subgroup of HNPs includes bromophenols such as 2,4,6-tribromophenol (TBP)[10] and bromoanisoles such as 2,4,6-tribromoanisole (TBA)[11] along with polybrominated hexahydroxanthene derivatives (PBHDs),[4] polyhalogenated 1′-methyl-1,2′-bipyrroles (PMBPs) including the heptachlorinated Q1,[9] polyhalogenated 1,1′-dimethyl-2,2′-bipyrroles (PDBPs) such as 5,5′-dichloro-1,1′-dimethyl-3,3′,4,4′-tetrabromo-2,2′-bipyrrole (5,5′-Cl_2-3,3′,4,4′-Br_4-DBP),[12] polyhalogenated methoxy diphenyl ethers (especially 2′-MeO-BDE 68 and 6-MeO-BDE 47), and the dimethoxy diphenyl ether 2′,6-diMeO-BDE 68.[13−16] Further frequently reported persistent and bioaccumulative HNPs are 2,2′-dimethoxy-3,3′,5,5′-tetrabromobiphenyl (2,2′-diMeO-BB 80),[17] 2,3,4,5-tetrabromo-*N*-methylpyrrole (TBMP),[8] and the mixed halogenated monoterpene (1*R*,2*S*,4*R*,5*R*,1′E)-2-bromo-1-bromomethyl-1,4-dichloro-5-(2′-chloroethenyl)-5-methylcyclohexane (MHC-1).[18]

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As a consequence, several persistent and bioaccumulative HNPs have been classified as emerging contaminants by the Arctic Monitoring and Assessment Programme,[19] although their occurrence on earth may cover multiple millions of years.[1] Still, comparably little is known about the global distribution of HNPs in the oceans and their bioactivity and toxicity.[7−9,20−23]

In this study, we aimed to determine HNPs in the blubber of nine sperm whales (*Physeter macrocephalus*) found stranded at two different regions of the Italian part of the Mediterranean Sea. This area is known for high levels of anthropogenic POPs.[24−26] However, marine mammals including sperm whales frequently also feature considerable amounts of HNPs which could even exceed those of POPs.[6,7,9,17,27−29] Accordingly, the availability of sperm whale blubber samples enabled us to quantify HNPs and selected man-made POPs by gas chromatography with electron capture negative ion mass spectrometry operated in the selected ion monitoring mode (GC/ECNI-MS-SIM). Nontargeted GC/ECNI-MS screening of the samples indicated the presence of an HNP hitherto unreported in marine mammals whose molecular formula was investigated with high resolution mass spectrometry.

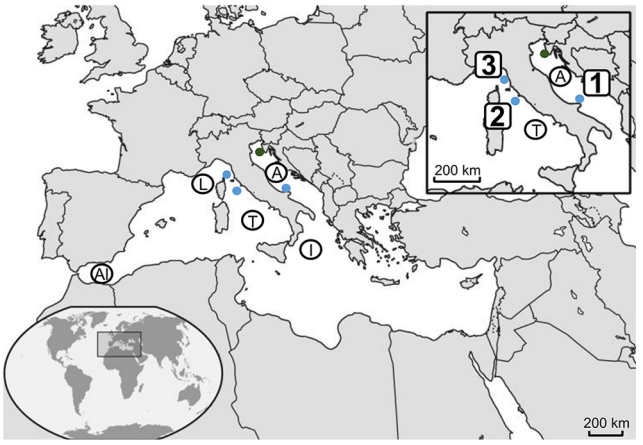

Figure 1. Map showing the different sites where the analyzed sperm whales stranded (blue dots) as well as where the beforehand analyzed *Scalarispongia scalaris* sponge[34,42] (green dot) was sampled including different parts of the Mediterranean Sea (Table S2). With A: Adriatic Sea, T: Tyrrhenian Sea, Al: Alboran Sea, I: Ionian Sea, and L: Ligurian Sea.

2. MATERIALS AND METHODS

2.1. Chemicals and Standards

"For pesticide residue analysis", grade *i*-octane and *n*-hexane were purchased from Honeywell Riedel-de Haen (Seelze, Germany) and Chemsolute Th. Geyer (Renningen, Germany). Cyclohexane (p.a. ≥ 99.8%), ethyl acetate (p.a. ≥ 99.5%), both distilled in mixture prior to use, and 2,3,6,7-tetrachloronaphthalene (TCN) were ordered from Sigma-Aldrich, (Steinheim, Germany). Silica gel 60 (for column chromatography grade) and sodium sulfate (>99%, water free, p.a.) were obtained from Carl Roth (Karlsruhe, Germany). The internal standards perdeuterated α-hexachlorocyclohexane (α-PDHCH) and 6′-MeO-BDE 66 (BCIS) were synthesized in our research group.[30,31] Standard mixtures used for quantification of polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) were purchased from Dr. Ehrenstorfer (Augsburg, Germany) and AccuStandard (New Haven, CT, USA), while the origins of the synthesized or isolated HNPs were described in Wu et al.[32] PBHD standards were isolated from a sponge (*Cacospongia* sp.) by Garson et al.,[33] and quantitative standard solutions were prepared by Melcher et al.[34]

2.2. Samples and Characteristics of *Physeter macrocephalus*

Blubber of male sperm whales stranded on the east (n = 7, A1-A7, Adriatic Sea (A)) and west (n = 2, T1, T2, Tyrrhenian Sea (T)) coasts of Italy were collected between 2009 and 2016 (Figure 1, Table S1). Information on the sampling of the sperm whales that were previously analyzed on POPs is shown in Bartalini et al.[26]

Sperm whales (*P. macrocephalus*) are found worldwide over a wide latitudinal range, with the Mediterranean subpopulation considered endangered.[35,36] According to genetic analyzes, Mediterranean sperm whales are presumed to be a semi-isolated subpopulation.[35] Adult females have mean lengths of about 11 m and weigh in at about 15 tons, while males are even larger (∼16 m long, ∼45 tons).[36] Sperm whales mainly feed on larger organisms such as squid and demersal fishes which they hunt on dives of up to more than 1000 m depth.[36] Ecological studies indicated a good habitat fidelity of this subpopulation (normal residence area with a diameter of 10−20 km, up to 90 km/day during preying).[36]

2.3. Sample Cleanup

Between 1.0 and 1.5 g of the blubber samples were analyzed in duplicates (Figure S1). Lipids were extracted with cyclohexane/ethyl acetate (46/54, w/w, Figure S1) in a Dionex 350 ASE system (Thermo Fisher Scientific, Waltham, MA, USA).[37] The samples were spiked with 107 ng α-PDHCH before extraction for the later determination of the recovery rate (Figure S1).[37] Extracts of samples T1 and T2 were cloudy, so they were centrifuged, the supernatant was decanted, and the residue was re-extracted twice with cyclohexane/ethyl acetate (46/54, w/w) (Figure S1). The (combined) solutions of all samples were condensed to adjust to exactly 5 mL of which 4.4 mL (88%) were subjected to gel permeation chromatography (GPC) (Figure S1).[37] Since several HNPs (and most brominated flame retardants) are not stable against sulfuric acid,[38] its use was omitted and GPC was used instead. From the remaining share, 500 μL was used for the determination of the lipid content (Figure S1). The volume of the GPC extract was reduced to 0.5 mL, and the solvent was changed to ∼1 mL of *n*-hexane (Figure S1). Following that, the sample solution was subjected to adsorption chromatography using 3 g of deactivated silica gel (30% water, w/w, glass column with 1 cm internal diameter) and eluted with 60 mL of *n*-hexane (Figure S1).[37] A further cleanup step ("group separation") was performed with 80% of the sample mainly to separate PCBs from HNPs by adsorption chromatography using 8 g of activated silica gel eluting the first fraction using 48 mL of *n*-hexane (Fraction 1) followed with 50 mL of *n*-hexane/ethyl acetate (9/1, v/v) for Fraction 2 (Figure S1).[39] The remaining 20% was used directly for measurement by GC/ECNI-MS. Since the internal standard α-PDHCH eluted into Fraction 2, sample extracts were supplemented with 107 ng of TCN to determinate the recovery rate in Fraction 1 (Figure S1). The nonpolar HNPs were quantified in fractions 1 and 2, respectively. Exemplarily, in the case of sample T1 an additional fraction was collected with 50 mL of ethyl acetate (Fraction 3), which targets among others the more polar bromophenols which were not detected during the initial screening of the samples before the group separation (for this reason only Fractions 1 and 2 of the group separation were collected in this study, Figure S1).[39] The fractions were concentrated to 800 μL (Fraction 1) and 80 μL (Fraction 2 and Fractions 1−3 of sample T1) (Figure S1). Either 190.6 or 19.06 ng of BCIS was added (Figure S1) to compensate for run-to-run variations in GC/ECNI-MS measurements (section 2.4).

2.4. GC/MS Analysis

HNPs and POPs were determined with an Agilent 7890/5975C system (Waldbronn, Germany) equipped with a 30 m × 0.25 mm inner diameter × 0.25 μm d_f Optima 5 MS column (Macherey-Nagel, Düren, Germany). GC/ECNI-MS operating parameters (temperatures of ion source and quadrupole: 150 °C, transfer line: 300 °C) and the oven temperature program (50 °C, held for 1 min, 10 °C/min

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to 300 °C, held for 14 min) were those described by Wu et al.[37] Runs in full scan mode covered m/z 30−600 (solvent delay: 8 min) while GC/ECNI-MS-SIM values for quantification and verification are compiled in Table S2.

Sample aliquots after adsorption chromatography with deactivated silica gel (30% water, w/w, Figure S1) were additionally analyzed by GC-Orbitrap-ECNI-HRMS using a GC-Q-Exactive Orbitrap instrument (Thermo Fisher, Waltham, MA, USA) equipped with a 15 m × 0.25 mm i.d. × 0.25 μm d_f HP-5MS UI column (Agilent, Waldbronn, Germany). The oven temperature program was started at 60 °C, held for 2 min, and then ramped up at 50 °C/min to 300 °C, held for 11 min. The temperature of the ion source was 180 °C. The covered mass range was m/z 30−630. The resolution of the Orbitrap was tuned to 120,000 (fwhm) and mass calibration was stable with a deviation < 1 ppm and not older than 48 h.

2.5. Quality Assurance

Glassware was precleaned with detergent, demineralized water, acetone, and distilled cyclohexane/ethyl acetate (46/54, w/w). Blanks (all chemicals, no sample) were cleaned-up in the same way as the samples. With the exception of sample T1, the recovery rate of α-PDHCH was >70%. Therefore, sample T1 will be discussed individually and it was not considered calculating the mean values. One determination of sample A5 had a recovery rate slightly below 70% and only the other one will be presented and discussed in the following. Exemplary evaluations of Q1, TetraBHD and PCB 153 resulted in variations of <20% for all duplicate samples. With a typical range of 0.1−1 pg, the lowest limit of detection (LOD) was 0.04 pg (TBMP) while the maximum was 14 pg (Cl_6-DBP). Silica Fraction 1 (second step, Figure S1) contained an artifact peak from sample cleanup which did not affect the quantification of the analytes.

2.6. Safety

No unexpected, new, and/or significant hazards or risks were associated with the reported work.

3. RESULTS AND DISCUSSION

3.1. Polybrominated Hexahydroxanthene Derivatives (PBHDs) in the Samples

PBHDs (Figure 2a,b) accounted for the majority of the HNPs, to which they contributed more than 90% (Table 1). Their pronounced dominance in GC/ECNI-MS chromatograms resembled previous reports in Mediterranean fish, deep sea fish, and, to a lesser degree, in delphinoids from the Alboran Sea on the west side of the Mediterranean Sea (Figure 1, Al; Figure 3a).[4,9,34,40]

TetraBHD amounts in individual samples varied only by a factor of 5 between 14,600 and 77,700 ng/g lipids, and six samples ranged between 23,000 and 32,500 ng/g lipids TetraBHD (Table 1). The mean amount of 34,300 ng/g lipids TetraBHD was among the highest levels of an HNP hitherto determined in marine mammals.[9] TriBHD amounts (940−2800 ng/g lipids, mean 1430 ng/g lipids) were considerably lower but the ratio of ∼23 ± 6 between TetraBHD and TriBHD was relatively constant in the samples (Table 1). Most of the few other samples from the Mediterranean Sea were richer in TriBHD,[4,9,34] with the exception of deep-sea fish samples caught along the Apulian coast on the east side of Italy, which was also the habitat of samples A1−A7 (site 1). In these deep-sea fish samples, TetraBHD accounted for up to 98% of PBHDs, while PBHDs represented 90−99% of all organobromine compounds.[40] Both the proximity of the sampling sites and the deep dives of sperm whales during preying are plausible arguments for the pronounced predominance of TetraBHD in the sperm whale blubber.[36]

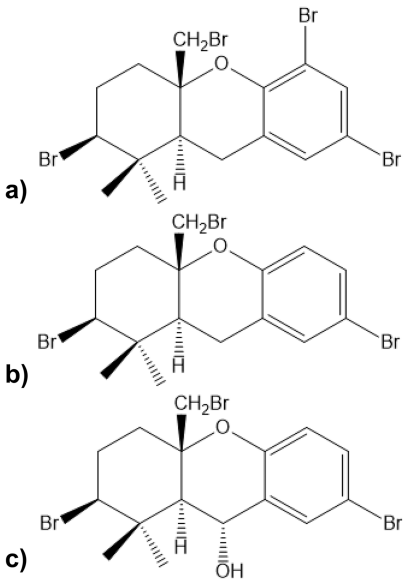

Figure 2. Structures of the PBHD derivatives (a) TetraBHD, (b) TriBHD, and (c) OH-TriBHD which was determined after extraction from a sponge of the *Cacospongia sp.* followed by structure elucidation by nuclear magnetic resonance spectroscopy (NMR).[41]

In agreement with previous studies,[4,9,34,40] isomers of TriBHD and TetraBHD were not detected in the samples. However, all GC/ECNI-MS chromatograms contained a peak that eluted between TriBHD and TetraBHD that did not match the GC retention times of other HNPs (and POPs) available to us (Figure 3a). The high abundance of Br^- (m/z 79/81) and Br_2^- (m/z 158/160/162) fragment ions confirmed the presence of (at least two) bromine substituents. In the high mass range, this compound showed a molecular ion (M^-) at m/z 480 with a tribromo isotope pattern. Accordingly, its M^- was 16 u higher than the one of (the also tribrominated) TriBHD (M^-, m/z 464, $C_{16}H_{19}Br_3O$). Given the high amounts of TriBHD and TetraBHD in the samples, it was reasonable to assume that this compound was a hexahydroxanthene derivative. More precisely, the 16 u higher mass compared to TriBHD pointed out the presence of an additional oxygen substituent and a molecular formula of $C_{16}H_{19}Br_3O_2$. The correctness of this hypothesis was confirmed by subsequent GC-Orbitrap-ECNI-HRMS analysis. Specifically, the Br_3 isotope pattern agreed by 99.6% with the theoretical one of the molecular formula $C_{16}H_{19}Br_3O_2$ and the exact masses of each of the isotope peaks of M^- deviated <1 ppm from the theoretical value (Figure 3b−d). Exact masses of other theoretically possible molecular formulas deviated unacceptably more from the measured values or were not reasonable to expect (Table S3). Interestingly, the sponge *Cacospongia sp.* from Australia from which TriBHD and TetraBHD were initially isolated, additionally featured a minor secondary metabolite which deviated from TriBHD only by an additional hydroxyl group on C-9.[41] This hydroxylated secondary metabolite (2*S*,4′*S*,9*R*,9′*S*)-2,7-dibromo-4′-bromomethyl-1,1-dimethyl-2,3,4,4′,9,9′-9,9′-hexahydro-1*H*-xanthen-9-ol (OH-TriBHD, Figure 2c) had been extracted from the wet sponge with ethanol.[41] OH-TriBHD (0.007% mass of the dry weight of the sponge) amounted to ∼1% TriBHD in this *Cacospongia sp.* sample.[33] Subsequent nontarget GC/ECNI-MS-SIM measurements of purified extracts of the Mediterranean sponge *Scalarispongia scalaris* had indicated a tribrominated compound

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Table 1. Contents of the Different HNPs [ng/g Lipids] Determined by GC/ECNI-MS-SIM

	A1	A2	A3	A4	A5[a]	A6	A7	T1[bc]	T2
TriBHD	2200	1100	1300	1200	1000	2800	940	1800	1000
TetraBHD	48,700	29,100	23,400	23,500	25,300	77,700	14,600	23,100	32,300
Q1	330	380	690	250	270	1360	250	350	740
2′-MeO-BDE 68	130	83	68	100	80	140	40	98	44
6-MeO-BDE 47	170	130	94	140	110	160	70	150	89
2,2′-diMeO-BB 80	160	96	81	100	100	150	43	68	67
MHC-1	3.2	7.6	4.3	4.7	12	16	2.4	n.d.	n.d.
5,5′-Cl_2-3,3′,4,4′-Br_4-DBP	19	19	20	14	19	15	11	23	24
Br_6-DBP	8.4	8.3	12	9.2	12	9.1	9.0	14	15
Br_5Cl-DBP	1.6	1.9	2.1	1.4	1.9	1.9	2.0	3.3	4.1
Cl_6-DBP	150	110	120	78	47	130	160	210	330
TBMP	n.d.[d]	n.d.	n.d.	n.d.	2	0.2	0.2	0.1	0.2

[a]Single determination due to low recovery of the second determination; all other values represent mean values of duplicate samples. [b]Recovery rate < 70% in this sample; the amounts may thus be slightly higher (no extrapolation to 100% recovery). This sample was not considered for the calculating of mean values. [c]All samples males and mature except sample T1 (male, juvenile). [d]n.d. compound was not detected.

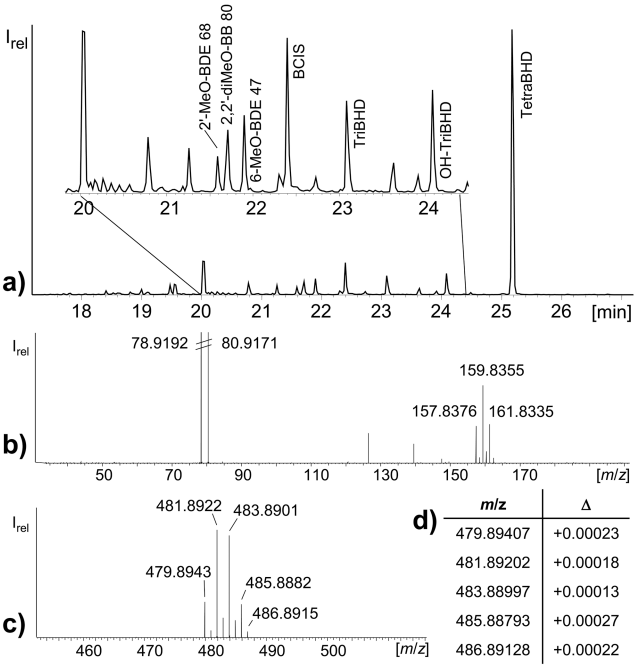

Figure 3. GC/ECNI-MS-SIM (method one, Table S2) total ion chromatogram of fraction two obtained by (a) group separation of sample A1 as well as (b) the mass spectrum of the unknown compound with a molecular ion at m/z 480 measured by GC/ECNI-HRMS from m/z 30−200 and (c) the isotope pattern of the molecular ion and (d) the m/z values of the isotope peaks of M^- of the unknown compound and their deviations (Δ) from the theoretical values of $C_{16}H_{19}Br_3O_2$.

with the corresponding M^- at m/z 480.[34,42] This potential natural PBHD producer was collected about 400 km away (Figure 1, green dot) from sampling site 1 (both sites located in the Adriatic Sea (Figure 1, A).[34,42] Therefore, it seemed plausible for the isomer with a molecular formula of $C_{16}H_{19}Br_3O_2$ detected here to be OH-TriBHD. However, OH-TriBHD had not been reported in fish or marine mammals so far.[34,40]

The GC/ECNI-MS abundance ratio of the presumed OH-TriBHD and TriBHD in the blubber sample extracts was 0.9 to 1.8 (mean value 1.5) in the low share of extract unused for the final fractionation into two groups (section 2.3, Figure S1). In this context it is important to note that the high amounts of a hydroxylated polyhalogenated compound in the blubber of marine mammals were surprising. For instance, OH-PBDEs were found to bioaccumulate less likely than PBDEs or MeO-BDEs.[43] Likewise, OH-PCBs were only detected in the plasma but not the blubber of harbor seals (*Phoca vitulina*).[44] Also, the present sample cleanup method (and assumedly others as well) does not cover hydroxylated compounds such as bromophenols, which require a more polar solvent for their elution (section 2.3). However, the ratio of the presumed OH-TriBHD and TriBHD in Fraction 2 of the group separation (1.0 to 1.8; mean value 1.5) was virtually identical with the one determined in the remaining share of sample extracts unused for the final group separation. A final test was carried out with the remaining share of the qualitative sample T1 (section 2.5) not used for the group separation (section 2.3) by introducing an additional more polar Fraction 3 which targets bromophenols and OH-POPs.[39] According to expectations, PCBs eluted into Fraction 1 while Fraction 2 contained all other brominated compounds/HNPs quantified in the sample. Also, the bulk of presumed OH-TriBHD (78%) was detected in Fraction 2 compared to that in Fraction 3. Apparently, the polarity of the unknown compound was lower than initially expected, which may explain why OH-TriBHD could possibly be detected (and accumulated) in the blubber of the sperm whale samples. Unfortunately, sample material was not left, and the sample preparation could not be repeated. Therefore, the partial loss may not be restricted to the last step so that amounts could not be determined accurately. Still, assuming a complete elution of the unknown compound into Fraction 2 (no loss during the sample cleanup) and using the response factor of TriBHD, the mean amount was about 3,000 ng/g lipids (i.e., more than TriBHD and 9% of the mean value of TetraBHD). Accordingly, the presence of a hydroxylated polyhalogenated compound at parts per million levels would be both remarkable and unusual for marine mammals.

Additional PBHD related compounds but not OH-TriBHD were recently detected in bivalves from Mediterranean and Atlantic Ocean coasts of France.[45] Their semiquantified concentrations ranged from undetectable to ∼40 ng/g dry weight in the samples from the Mediterranean coast.[45] PBHDs and the PBHD related compounds were almost exclusively found in the samples from the Mediterranean Sea, which supports that PBHD derivatives producing sponges are found

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throughout the Mediterranean Sea as well as in Oceania.[4,33,41,45]

Given the rather narrow PBHD contamination range (see above), no deviation could be made between the different sampling sites. Also, the highest and the lowest PBHD contamination was observed in two adult individuals (samples A6 and A7) from the same site (1, Figure 1). This indicated that the natural producer of PBHDs was likely found with similar abundance on both coasts of Italy assuming the sperm whales were staying within their normal residence area of 10−20 km.[36]

3.2. Further HNPs in the Sperm Whales

Q1 (Figure 4a) was the next most abundant HNP with a mean value of 530 ng/g lipids (range: 250 to 1360 ng/g lipids) in the samples (Table 1). As with PBHDs, the highest and lowest Q1 levels were determined in samples A6 and A7, respectively (Table 1). These Q1 levels were within the range of different dolphin species from the Mediterranean Sea (Q1:130−3580 ng/g lipids).[9] These dolphin samples were from the Alboran Sea (Figure 1, Al) which is >1000 km away from the present sampling sites that include two distinct marine areas (Tyrrhenian and Adriatic Seas, Figure 1, T and A). Irrespective of species-specific differences, these results produce strong evidence that Q1 is also uniformly present and evenly distributed in the Mediterranean Sea. Hitherto, Q1 amounts have scarcely been reported in sperm whales (*Physeter* sp.). However, one sperm whale (*Physeter catadon*; ∼12−18t) stranded in 2016 at the German North Sea coast contained 150 ng/g lipids Q1, which is lower than the amount in the present samples.[38] Eight further peaks of PMBPs (two $BrCl_6$-MBPs, two Br_2Cl_5-MBPs, three Br_3Cl_4-MBPs, and traces of Br_7-MBP) were detected in the samples, but their quantities could not be determined due to the lack of standards. According to the GC/ECNI-MS responses with the present setup, the predominant Q1 was followed by $BrCl_6$-MBPs (∼12% of Q1) while all other PMBPs were present in only trace amounts. High amounts of PMBPs including usually high shares of Q1 had already been detected in marine mammals.[9] Cetaceans (melon-headed whale, pygmy sperm whale, common dolphin and bottlenose dolphin) from Australia contained 7−27.5% $BrCl_6$-MBPs compared to Q1, while the higher brominated PMBPs were present with similarly low abundance ratios (Figure S2).[46] This indicated that the PMBPs were bioaccumulated at the same extent in the different cetaceans.

Within the group of PDBPs, not only the most commonly known 5,5′-Cl_2-3,3′,4,4′-Br_4-DBP (Figure 4b) was detected and quantified but also Cl_6-DBP, Br_5Cl-DBP, and Br_6-DBP.[29,47,48] Interestingly, Cl_6-DBP (47−330 ng/g lipids) was about 1 order of magnitude higher in abundance than 5,5′-Cl_2-3,3′,4,4′-Br_4-DBP (11−24 ng/g lipids) (Table 1). Due to the synthesis of Martin et al., it could be shown that Cl_6-DBP has a very low GC/ECNI-MS response factor and was only quantified in a few studies.[49] For instance, Cl_6-DBP was not reported in any of the many studies of Tittlemier et al.,[12,29,47,48] which brought this substance class of PDBPs to the attention of scientists. In the Mediterranean Sea, striped

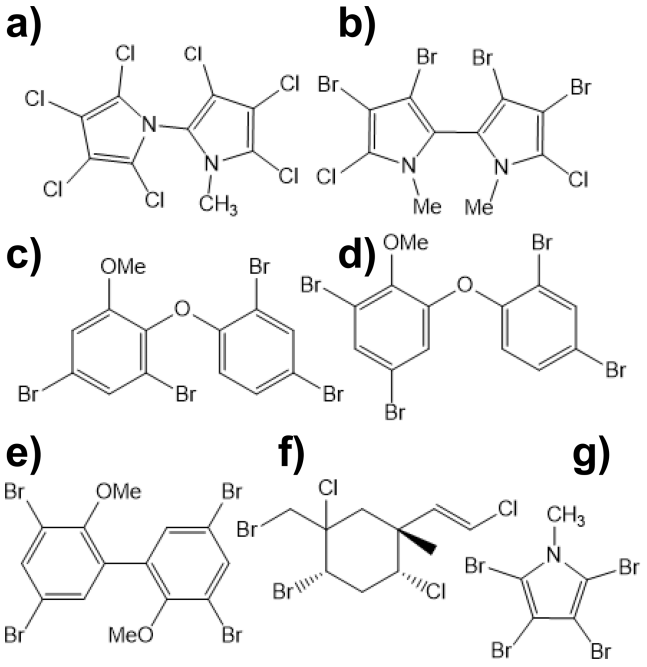

Figure 4. Structures of the analyzed HNPs (a) Q1, (b) 5,5′-Cl_2-3,3′,4,4′-Br_4-DBP, (c) 6-MeO-BDE 47, (d) 2′-MeO-BDE 68, (e) 2,2′-diMeO-BB 80, (f) MHC-1, and (g) TBMP.

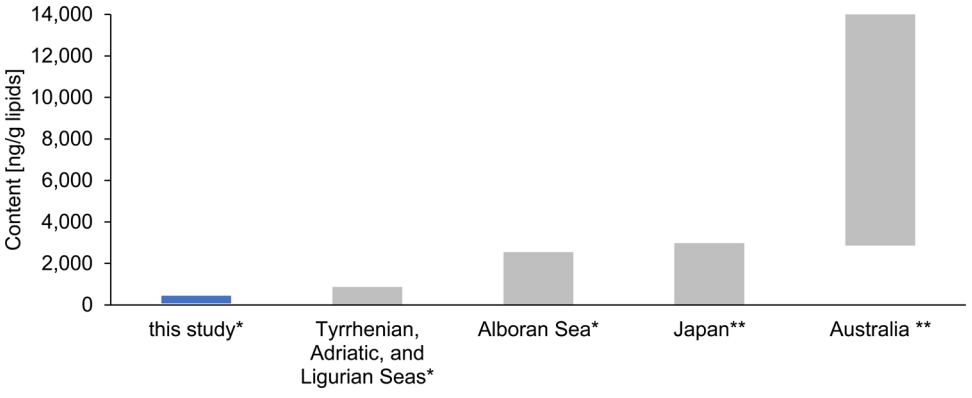

Figure 5. Content [ng/g lipids] of 2′-MeO-BDE 68 and 6-MeO-BDE 47 in the present sperm whale samples (blue) and literature data (gray) from different cetaceans from the Mediterranean Sea (Tyrrhenian, Adriatic, and Ligurian Seas[15] and Alboran Sea,[9] marked with *) and the Pacific Ocean (Japan[66] and Australia,[52] marked with **). In contrast to all other studies, liver was analyzed instead of blubber in the study of the Tyrrhenian, Adriatic, and Ligurian Seas.[15]

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Table 2. Comparison of the HNP Levels (ΣHNPs) with That of the Quantified Anthropogenic Pollutants (ΣPOPs)[a] [ng/g lipids] Determined by GC/ECNI-MS-SIM

sample	ΣPCBs	ΣBDEs	ΣPOPs	ΣHNPs	PBHDs	Q1	ratio ΣHNPs/ΣPOPs
A1	16,100	410	16,500	51,900	50,900	330	3.1
A2	20,700	310	21,000	31,000	30,200	380	1.5
A3	26,500	510	27,000	25,800	24,700	690	1.0
A4	18,300	460	18,800	25,400	24,700	250	1.4
A5[b]	9830	260	10,100	27,000	26,300	270	2.7
A6	58,700	900	59,600	82,500	80,500	1360	1.4
A7	12,900	270	13,200	16,100	15,500	250	1.2
T1[cd]	21,500	610	22,100	25,800	24,900	350	1.2
T2	63,900	770	64,700	34,600	33,300	740	0.53

[a]Sum contents of the quantified anthropogenic pollutants PCBs and PBDEs (sumPCBs, sumPBDEs) are listed as well (all determined values can be found in Table S4). [b]Single determination due to low recovery of the second determination; all other values represent mean values of duplicate samples. [c]Recovery rate < 70% in this sample; the amounts may thus be slightly higher (no extrapolation to 100% recovery). This sample was not considered for the calculating of mean values. [d]All samples males and mature except sample T1 (male, juvenile).

dolphins from the Catalonian Coast showed higher PDBP sum concentrations of 310−1480 ng/g lipids (Cl_6-DBP not included).[47] Therefore, PDBP contents seem to be dependent on the habitat of the mammals as suggested by Tittlemier et al., although species-specific factors could play a role as well.[47] In support of this assumption, a dolphin from the Great Barrier Reef was contaminated with 1100 ng/g lipids PDBPs (including the predominant Cl_6-DBP), which exceeds the PDBP amounts of 80−370 ng/g lipids in the present samples.[7] Other studies with data on PDBPs (excluding Cl_6-DBP) in Beluga from the North Pacific and Arctic showed levels of 0.5−99 ng/g lipids PDBPs with predominance of 5,5′-Cl_2-3,3′,4,4′-Br_4-DBP, whose amount was slightly lower than that in the present study.[47] Melon headed whales (2900−42,300 ng/g lipids 5,5′-Cl_2-3,3′,4,4′-Br_4-DBP) and killer whales (6000−24,000 ng/g lipids 5,5′-Cl_2-3,3′,4,4′-Br_4-DBP, 6500−26,000 ng/g lipids sum of PDBPs) from the North Pacific showed higher levels of PDBPs even without the determination of Cl_6-DBP.[50,51] In addition to the four quantified congeners, the present samples also contained three Br_2Cl_4-DBPs, one Br_3Cl_3-DBP, and one additional isomer each of 5,5′-Cl_2-3,3′,4,4′-Br_4-DBP as well as Br_5Cl-DBP. Compared to that, three isomers of 5,5′-Cl_2-3,3′,4,4′-Br_4-DBP were detected in the dolphins from the Great Barrier Reef along with two Br_5Cl-DBPs, four Br_2Cl_4-DBPs, and six Br_3Cl_3-DBPs.[7] Br_5Cl-DBPs, Br_6-DBPs, and Br_3Cl_3-DBPs were additionally already reported in marine mammals from the North and South Pacific.[47,51] For unknown reasons, PDBP patterns appear to be more varied than those of the PMBPs (see above).

MeO-PBDEs were detected in all samples with 6-MeO-BDE 47 (Figure 4c) being generally higher abundant than 2′-MeO-BDE 68 (Figure 4d) (mean levels 86 vs 120 ng/g lipids, Table 1). Semiquantitative investigation of liver samples of Mediterranean cetaceans from the Tyrrhenian, Adriatic, and Ligurian Seas (Figure 1, T, A, and L) also showed higher levels of 6-MeO-BDE 47 (7−630 ng/g lipids) compared to 2′-MeO-BDE 68.[15] Partly originating from the same areas of the Mediterranean Sea as the samples analyzed here, they were on a similar or lower level (Figure 5). Dolphins from the Alboran Sea (Figure 1, Al) showed on average higher MeO-PBDE levels than those in the present study with values ranging from undetectable to 2510 ng/g lipids (Figure 5).[9] Whales and dolphins from the Pacific Ocean (Japan and especially Australia) accumulated higher sum-levels of 6-MeO-BDE 47 and 2′-MeO-BDE 68 of up to ∼3000 ng/g lipids and ∼13,000 ng/g lipids (Figure 5).[52]

2,2′-diMeO-BB 80 (Figure 4e) amounted to 43−160 ng/g of lipids (mean 100 ng/g of lipids), with individual levels typically ranging between those of 2′-MeO-BDE 68 and 6-MeO-BDE 47 (Table 1). Slightly lower 2,2′-diMeO-BB 80 amounts were present in the two blubber samples from the Italian west coast (samples T1 and T2) with levels of ∼2/3 of the mean value of 100 ng/g lipids of all samples (Table 1, Figure 1). In comparison, marine mammals from the Pacific Ocean possessed 2,2′-diMeO-BB 80 levels of 12−800 ng/g lipids.[17] Accordingly, the 2,2′-diMeO-BB 80/MeO-BDE ratio was higher in the Mediterranean Sea than in the Pacific Ocean samples.

MHC-1 (Figure 4f) levels in the Adriatic Sea (2.4−16 ng/g lipids, (Figure 2, A) were lower than those of all other HNPs discussed so far, while MHC-1 was not detected (<1 ng/g lipids) in the west coast samples T1 and T2 (Tyrrhenian Sea, Figure 1, T) (Table 1). The occurrence of MHC-1 has been linked with the presence of its producer *Plocamium cartilagineum*.[18] This red seaweed is known to occur throughout the Mediterranean Sea including the Adriatic, the Tyrrhenian and the Ionian Sea (Figure 1, A, T, and I).[53−57] Assumedly, *Plocamium cartilagineum* was less prevalent on the east coast of Italy; however, the number of samples was small.[58,59] In addition, MHC-1 was found to be unevenly distributed in the marine environment.[8] In agreement with that, fish from the Mediterranean Sea (Italy and Greece) contained between not detectable and 52 ng/g lipid MHC-1 compared to slightly more (1.1−130 ng/g lipids MHC-1) in dolphins from the Alboran Sea (Figure 1, Al).[4,9] Apparently, MHC-1 was unevenly distributed in the Mediterranean Sea like in other marine regions.

TBMP (Figure 4g) was detected in only four samples at 0.2 (A6, A7, T2) or 2.0 (A5) ng/g lipids. Further polyhalogenated methyl bipyrroles (PMPs) as those described in blue mussels from the North Sea and Baltic Sea were not detected in the present samples.[8] Previous findings of TBMP in mussels, chokka squid and salmon from the Pacific and South Atlantic Ocean indicated regional and seasonal variations in the TBMP content.[32,60] Given the small activity range of this sperm whale population, this could explain why only part of the samples analyzed here contained TBMP (LOD 0.006−0.032 ng/g lipids).

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3.3. Comparison of HNP with Anthropogenic POP Levels

The present sperm whale samples were previously analyzed in terms of dioxin-like PCBs, PBDEs and polychlorodibenzo-*p*-dioxins (PCDDs) and -furans (PCDFs).[26] According to expectations, amounts of dioxin-like PCBs were reported by Bartalini et al. (mean 6410 ng/g lipids[26]) were lower than ΣPCBs in the present study (~10,000 to 64,000 ng/g lipids) which included the most prevalent congeners PCB 153, 138, and 180 (Tables 2 and S4, Figure S3). Still, PBHDs were on the same level with its mean value being even higher than that of PCBs confirming the relevance of PBHDs in the Mediterranean Sea (Table 2, Figure S3).[4,9,34] Levels of PBDEs (270−770 ng/g lipids) were comparable with those reported by Bartalini et al. in some of the samples (mean value 612 ng/g lipids).[26] PBDEs were ~2% of PCBs and PBHDs, and therefore on a similar level with the HNP Q1 (Table 2, Figure S3). MeO-PBDEs and PDBPs were detected in lower concentrations than PBDEs but were at a comparable level (Figure S3). Overall, the total amount of HNPs (ΣHNPs) was higher than that of ΣPCBs and ΣPBDEs (Table 2). However, the ratio ΣHNPs/ΣPOPs varied between 0.53 and 3.1, with only two samples being richer in POPs (Table 2). Higher amounts of HNPs compared to man-made pollutants were also determined in dolphins from the Mediterranean Sea.[9]

Bartalini et al. found that dioxin-like PCBs, PBDEs and PCDD/Fs in the present sperm whale samples were higher than in sperm whales from other geographical regions.[26] This illustrates that HNPs are relevant environmental contaminants, and they should be of general interest in a more thorough investigation of their toxic impact and environmental fate.

3.4. Conclusions

Several compounds and compound classes of persistent and bioaccumulative HNPs (PBHDs, PMBPs, MeO-PBDEs, PDBPs, 2,2′-diMeO-BB 80, MHC-1, and TBMP) were detected, and most of them could be quantified in the blubber of nine Mediterranean sperm whale (*P. macrocephalus*) samples. The mean amounts of the individual HNP classes spanned over more than 4 orders of magnitude with PBHDs being most and TBMP being least abundant. Especially the very high mean amounts of PBHDs, which were higher than the mean PCB level in this region highly polluted with man-made contaminants, are remarkable. The highest PBHD level of 77,000 ng/g lipids exceeds the 50−70 ppm boundary at which PCBs were considered to cause distortion of reproductive functions in seals and minks.[61] The reported PBHD level did not even include the presumed PBHD derivative with a molecular formula of $C_{16}H_{19}Br_3O_2$ that was reported for the first time in marine mammals. GC-Orbitrap-ECNI-HRMS proved to be important in the virtual identification of the molecular formula due to the high precision of the exact mass determination. In the future, the presence of hydroxyl groups may also be verified by derivatization similar to the approach used for the verification of tetrabrominated diphenols as transformation products of the flame retardant hexabromobenzene.[62]

On the one hand, OH-TriBHD (possessing the molecular formula $C_{16}H_{19}Br_3O_2$) is a known HNP which was detected together with TriBHD and TetraBHD in its natural producer.[33,41] On the other hand, a hydroxylated TriBHD could also be a metabolite that could be generated by the sperm whales, e.g., in the liver. The much higher content of TetraBHD and the lack of a hydroxylated TetraBHD in the samples speak more for the HNP hypothesis. Apart from that, in the case of PBDEs, hydroxylated OH-PBDEs were shown to be more toxic than the corresponding PBDEs.[63,64] The results of the present study clearly indicate the necessity of further research on the occurrence, the distribution, and, last but not least, the toxicity of PBHDs including OH-TriBHD. Also, there is an urgent need for pharmacokinetic data of HNPs, similar to the cytochrome oxidase-mediated biotransformation of PCBs into OH-PCBs.[65]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/envhealth.3c00182.

Flowchart of the sample cleanup, further information on the sperm whales (*Physeter macrocephalus*), GC/ECNI-MS-SIM methods used for the quantification, agreement of possible molecular formulas with the unknown compound, comparison of PMBP homologue patterns in this study with literature, quantified amounts of selected PCBs as well as PBDEs and comparison of the quantified amounts of HNPs and POPs (PDF)

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Notes

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