Identification of Hydroxylated and Methoxylated Polybrominated Diphenyl Ethers in Baltic Sea Salmon (*Salmo salar*) Blood

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Methoxylated and hydroxylated polybrominated diphenyl ethers (MeO-PBDEs and OH-PBDEs) have recently been reported to be present in wildlife from Northern Europe. The structures of a majority of these compounds have however been unknown. In the present study, nine OH-PBDEs and six MeO-PBDEs were identified in Baltic Sea salmon (*Salmo salar*) blood. All OH- and MeO-PBDEs identified were substituted with four or five bromines, and five of these had one chlorine substituent. Fourteen of the OH- and MeO-PBDEs have the methoxy or hydroxy group substituted in the ortho position to the diphenyl ether bond. Identification was done by comparison of relative retention times of authentic reference standards with compounds present in salmon plasma on two gas chromatographic columns of different polarities. The identification was supported by comparisons of full-scan mass spectrometric data: electron ionization (EI) and electron capture negative ionization (ECNI). Nine of the 15 OH- and MeO-PBDEs identified have not previously been reported to occur in the environment. The structures of several identified OH- and MeO-PBDEs support natural origin. However, at least one of the OH-PBDEs may be a hydroxylated metabolite of anthropogenic polybrominated diphenyl ether (PBDE).

Introduction

During the past decade, an increased interest has been focused on brominated flame retardants (BFRs) and particularly on polybrominated diphenyl ethers (PBDEs), which are known as ubiquitous environmental contaminants (*1*). PBDEs are synthetic anthropogenic compounds and have not to our knowledge been shown to occur as natural products. Hydroxylated PBDEs (OH-PBDEs) have been detected as metabolites in fish (*2*), mice, and rats (*3*) after exposure to 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47), the most abundant PBDE congener in wildlife (*1*). Two of the OH-PBDE metabolites found in fish have tentatively been identified as 6-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether (6-OH-BDE-47) and 2′-hydroxy-2,3′,4,4′-tetrabromodiphenyl ether (2′-OH-BDE-66) (*2*). Other environmentally relevant PBDE congeners, 2,2′,4,4′,5-pentabromodiphenyl ether (BDE-99) (*4*) and decaBDE (BDE-209) (*5*), have been shown to form OH-PBDE metabolites in rats. On the other hand, OH-PBDEs have been isolated, structurally identified, and determined as natural products in marine sponges (*6*, *7*) and ascidians (tunicates) (*8*, *9*). OH-PBDEs have also been detected in red alga (*10*). Methoxylated PBDEs (MeO-PBDEs) have been reported as natural products present in marine sponge (*11*, *12*) and green alga (*13*). The occurrence of these compounds in environmental samples can therefore originate either from natural or anthropogenic sources.

OH-PBDEs and MeO-PBDEs have been shown to be present in species at high trophic levels. Two MeO-tetraBDEs were reported from green turtle and marine mammals (whales, dolphins, dugong, and seals) from the Southern Hemisphere and at concentrations higher than the levels of PBDEs (*14*, *15*). The concentrations of PBDEs in Australian marine mammals were at least 2 orders of magnitude lower as compared to the MeO-PBDEs (*15*). One of these MeO-PBDEs, 2′-methoxy-2,3′,4,5′-tetrabromodiphenyl ether (2′-MeO-BDE-68), and a diMeO-PBDE, 2′,6-dimethoxy-2,3′,4,5′-tetrabromodiphenyl ether (2′,6-diMeO-BDE-68), were identified (*14*). The reference standards used for these identifications were based on compounds isolated from a marine sponge collected within the same geographic area as the majority of the investigated animals. Hence these results support a natural origin of these identified compounds. Three MeO-tetraBDEs with unknown structure were reported to be present in whales collected in the Atlantic (*16*), and a human milk sample from the Faeroe Islands was shown to contain 2′-MeO-BDE-68 and a MeO-tetraBDE with unknown structure (*17*). From Northern Europe, in the Baltic Sea area, MeO-PBDEs have been reported in marine fish (*18*, *19*), freshwater fish (*20*), birds (*21*), and seals (*18*). OH-PBDEs were reported in fish (*19*) and humans (*22*). 6-Methoxy-2,2′,4,4′-tetrabromodiphenyl ether (6-MeO-BDE-47) has been detected in fish (*19*, *20*) and birds (*21*). The corresponding phenolic compound, 6-OH-BDE-47, has been identified in salmon (*Salmo salar*) plasma (*19*) and in human plasma (*22*). Nevertheless, the majority of the OH- and MeO-PBDEs, all substituted with 4−5 bromine atoms, indicated in previous studies have not yet been identified (*18*, *19*). In the latter studies, it was not clarified whether the OH- and MeO-PBDEs originated from natural or anthropogenic sources.

The objective of this work was to identify the structures of OH-PBDEs and MeO-PBDEs present in Baltic Sea salmon plasma. This was done by comparison of synthesized MeO-PBDE standards with compounds extracted from the salmon plasma using relative retention times on a polar and nonpolar GC column and by comparing MS data. Coelution between PBDE and MeO-PBDE congeners and between MeO-PBDE congeners are reported, and the potential sources of these compounds are discussed.

Materials and Methods

Chemicals. The chemical name and abbreviation of the individual MeO-PBDE congeners used as authentic reference standards are given in Table 1 and were synthesized as described elsewhere (*23*, *24*). Thirty-three PBDE congeners (abbreviated according to Ballschmiter et al.) (*25*): BDE-30, -32, -17, -25, -28, -33, -35, -37, -75, -51, -49, -71, -47, -66, -77, -100, -119, -99, -116, -85, -155, -105, -154, -153, -139, -140, -138, -166, -128, -183, -181, -190, and BDE-203 (presented in

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TABLE 1. Relative Retention Times (RRTs) Given in Elution Order of All MeO-PBDEs Used as Reference Standards on CP-SIL 8 and SP-2331 GC Columns[a]

	CP-SIL 8 CB (nonpolar)			SP-2331 (polar)		
MeO-PBDE	abbreviation	RRT	ID no.	abbreviation	RRT	ID no
6′-methoxy-2,2′,4-triBDE	6′-MeO-BDE-17	0.754		4′-MeO-BDE-30	0.495	
4′-methoxy-2,4,6-triBDE	4′-MeO-BDE-30	0.755		6′-MeO-BDE-17	0.544	
2′-methoxy-2,4,4′-triBDE	2′-MeO-BDE-28	0.768		2′-MeO-BDE-28	0.554	
3′-methoxy-2,4,4′-triBDE	3′-MeO-BDE-28	0.783		**6′-MeO-BDE-49**	**0.571**	**1**
4′-methoxy-2,2′,4-triBDE	4′-MeO-BDE-17	0.783		4′-MeO-BDE-17	0.582	
6′-methoxy-2,2′,4,5′-tetraBDE	**6′-MeO-BDE-49**	**0.813**	**1**	**2′-MeO-BDE-68**	**0.587**	**2**
2′-methoxy-2,3′,4,5′-tetraBDE	**2′-MeO-BDE-68**	**0.824**	**2**	3′-MeO-BDE-28	0.609	
6-methoxy-2,2′,4,4′-tetraBDE	**6-MeO-BDE-47**	**0.836**	**3**	**3′-Cl-6′-MeO-BDE-49**	**0.647**	**5**
4′-methoxy-2,3′,4,6-tetraBDE	4′-MeO-BDE-69	0.839		**6′-Cl-2′-MeO-BDE-68**	**0.658**	**6**
3-methoxy-2,2′,4,4′-tetraBDE	3-MeO-BDE-47	0.850		**5-Cl-6-MeO-BDE-47**	**0.662**	**7**
2′-methoxy-2,3′,4,4′-tetraBDE	2′-MeO-BDE-66	0.851		**6-MeO-BDE-47**	**0.669**	**3**
5-methoxy-2,2′,4,4′-tetraBDE	5-MeO-BDE-47	0.854		4′-MeO-BDE-69	0.671	
4′-methoxy-2,2′,4,5′-tetraBDE	**4′-MeO-BDE-49**	**0.856**	**4**	2′-MeO-BDE-66	0.683	
3′-chloro-6′-methoxy-2,2′,4,5′-tetraBDE	**3′-Cl-6′-MeO-BDE-49**	**0.864**	**5**	4′-MeO-BDE-121	0.689	
6′-chloro-2′-methoxy-2,3′,4,5′-tetraBDE	**6′-Cl-2′-MeO-BDE-68**	**0.869**	**6**	3-MeO-BDE-47	0.695	
5-chloro-6-methoxy-2,2′,4,4′-tetraBDE	**5-Cl-6-MeO-BDE-47**	**0.870**	**7**	**6-MeO-BDE-90**	**0.724**	**8**
4′-methoxy-2,3′,4,5′,6-pentaBDE	4′-MeO-BDE-121	0.886		**4′-MeO-BDE-49**	**0.731**	**4**
4-methoxy-2,2′,3,4′-tetraBDE	4-MeO-BDE-42	0.888		**6-MeO-BDE-99**	**0.731**	**9**
5′-chloro-2′-methoxy-2,3′,4,4′-tetraBDE	5′-Cl-2′-MeO-BDE-66	0.898		5-MeO-BDE-47	0.743	
6-methoxy-2,2′,3,4′,5-pentaBDE	**6-MeO-BDE-90**	**0.900**	**8**	5′-Cl-2′-MeO-BDE-66	0.760	
6-methoxy-2,2′,4,4′,5-pentaBDE	**6-MeO-BDE-99**	**0.903**	**9**	3-Cl-6-MeO-BDE-47	0.832	
3-chloro-6-methoxy-2,2′,4,4′-tetraBDE	3-Cl-6-MeO-BDE-47	0.905		2-MeO-BDE-123	0.836	
4-methoxy-2,2′,3,4′,5-pentaBDE	4-MeO-BDE-90	0.929		4-MeO-BDE-90	0.836	
2-methoxy-2′,3,4,4′,5-pentaBDE	2-MeO-BDE-123	0.935		4-MeO-BDE-42	0.853	
6-methoxy-2,2′,3,4,4′-pentaBDE	6-MeO-BDE-85	0.947		6-MeO-BDE-85	0.946	
2,2′,3,4,4′,5′-hexaBDE	BDE-138 (IS)	1.000		BDE-138 (IS)	1.000	
6-methoxy-2,2′,3,4,4′,5-hexaBDE	6-MeO-BDE-137	1.044		6-MeO-BDE-137	*b*	

[a] Recorded by ECNI *m/z* 79 and 81. Matching compounds from the salmon blood samples are marked in bold with identification numbers as in Figures 1 and 2 (chromatograms) and in Figure 3 (structures). [b] Did not elute within the GC program used.

elution order on a CP-Sil 8 CB GC column; BDE-17/BDE-25 and BDE-28/BDE-33 are coeluting pairs) were used to investigate any potential chromatographic coelution with MeO-PBDEs. The PBDE congeners have been synthesized as previously reported (*26*, *27*), but the preparation of BDE-105, -155, -139, and -183 are unpublished. The abbreviation system used for the OH-PBDE and MeO-PBDE congeners is the same as that used for polyhalogenated biphenylols and their methyl ether derivatives (*28*). The nomenclature used for OH- and MeO-PBDEs deviate from IUPAC rules (i.e., phenoxyphenols and methoxyphenoxybenzene), and these compounds are instead referred to as diphenyl ethers in the present work to simplify comparison with PBDE congeners. All solvents, acids, and other chemicals used in the synthesis and analysis were of pro analysis quality.

Synthesis of Reference Standards. Three partially chlorinated MeO-PBDE mixtures were prepared from 6-OH-BDE-47, 6′-hydroxy-2,2′,4,5′-tetrabromodiphenyl ether (6′-OH-BDE-49), and 2′-OH-BDE-66 and used as monochlorinated MeO-tetraBDE reference compounds. An attempt was also made to chlorinate 2′-hydroxy-2,3′,4,5′-tetrabromodiphenyl ether (2′-OH-BDE-68) in the same manner as described below, but no monochlorinated OH-tetraBDE product was formed. The mixtures were prepared as follows: Aqueous sodium chlorate (50 μL, 50 μmol) was added to a solution of the OH-tetraBDE congener (*23*) (35 mg, 70 μmol), mentioned above, in acetic acid (4 mL) and concentrated hydrochloric acid (0.25 mL) at room temperature. The reaction mixture was stirred at room temperature for 0.5 h, and water (5 mL) was added. The aqueous phase was removed from the precipitated product mixture. This mixture was washed with water (4 mL) and dissolved in dichloromethane (3 mL). The organic phase was washed with saturated aqueous sodium hydrogen carbonate (3 mL) followed by water (2 × 3 mL), and the products were concentrated in a rotary evaporator. The crude chlorinated OH-PBDE mixture was treated with sodium hydroxide (8.4 mg, 0.21 mmol) and tetrabutylammonium hydroxide (34 mg, 0.14 mmol) in water (2 mL) after which iodomethane (20 μL, 0.35 mmol) in dichloromethane (2 mL) was added and the reaction mixture was stirred at room temperature for 4 h. The layers were separated, and the water phase was extracted with dichloromethane (2 mL). The combined organic phases were dried with sodium sulfate, filtered, and concentrated. The crude product mixture was purified on a silica gel column (2 × 10 cm) from polar substances using dichloromethane/*n*-hexane (1:1) as the mobile phase.

The partial chlorination and the subsequent methylation of 6-OH-BDE-47, 6′-OH-BDE-49, and 2′-OH-BDE-66 resulted in two isomeric monochlorinated 6-MeO-BDE-47; in one monochlorinated isomer product from each of 6′-MeO-BDE-49 and 2′-MeO-BDE-66, respectively; and in a mixture with the starting compound. The structures were tentatively assigned as follows: the monochlorinated MeO-PBDEs had (i) a loss of $BrCH_3$ as determined by MS in electron ionization (EI) mode, indicating the methoxy group in the ortho position of the diphenyl ether bond (*23*); (ii) the two fragment ions $[M - C_6HBr_2ClOCH_3]^-$ *m/z* 249 and $[M + H - OC_6H_3Br_2]^-$ and/or $[M - C_6H_3Br_2CH_3]^-$ *m/z* 298 that were shown by MS in electron capture negative ionization (ECNI) mode, indicating the numbers of halogen atoms in each ring (*18*, *29*); (iii) a molecular ion at *m/z* 546 determined by EI; and (iv) an ion isotopic halogen pattern that was in agreement with the expected pattern for all ions assigned. One of the methylated monochlorinated products from 6-OH-BDE-47 had identical MS spectra and RRT as the authentic 5-Cl-6-MeO-BDE-47 previously synthesized (*23*). Accordingly, the other isomer formed is most likely 3-Cl-6-MeO-BDE-47. Since there was only one monochlorinated product formed from both 6′-OH-BDE-49 and 2′-OH-BDE-66, the most likely substitution position is para to the hydroxy group (the ortho positions are occupied), which support the structures of 3′-

Cl-6′-MeO-BDE-49 and 5′-Cl-2′-MeO-BDE-66, respectively, after methylation. Furthermore, the authentic reference 6′-Cl-2′-MeO-BDE-68 (*23*) gives distinct loss of BrCl by MS in EI mode, while the chlorinated and methylated product of 2′-OH-BDE-66 only gave traces of this loss, indicating that the chlorine is substituted in the 5′-position and not in the 6′-position. As mentioned, 2′-OH-BDE-68 did not give any monochlorinated OH-tetraBDEs products at all under the preparation conditions used. This further supports the ortho/para directing properties of the aromatic hydroxy group as the conclusive factor during the chlorination, despite the fact that the aromatic ring is substituted with four functional groups. On the basis of the data and the reasoning above, the structures of the chlorinated MeO-PBDEs are suggested to be 3-Cl-6-MeO-BDE-47 and 5-Cl-6-MeO-BDE-47 from 6-OH-BDE-47, 3′-Cl-6′-MeO-BDE-49 from 6′-OH-BDE-49, and 5′-Cl-2′-MeO-BDE-66 from 2′-OH-BDE-66.

Instruments. Gas chromatography−mass spectrometry (GC−MS) analyses were performed on a Finnigan MAT SSQ 710 with a Varian 3400 gas chromatograph and a split/splitless injector operated in the splitless mode. Two fused-silica capillary columns with different polarities were used. A nonpolar column with a 5% phenyl, dimethylpolysiloxane phase, CP-Sil 8 CB (30 m × 0.25 mm i.d. and 0.25 μm film thickness) was purchased from Chrompack (EA Middelburg, The Netherlands). The column was programmed as follows: 80 °C (2 min), 10 °C min^{-1} to 300 °C (10 min). The injector and transfer line temperatures were 260 and 270 °C, respectively. The other GC column was a polar column with an 80% biscyanopropyl, 20% cyanopropylphenyl, siloxane phase, SP-2331 (30 m × 0.25 mm i.d. and 0.2 μm film thickness) obtained from Supelco (Bellefonte, USA). The column temperature program was 80 °C (1 min), 20 °C min^{-1} to 200 °C (1 min), 3 °C min^{-1} to 270 °C (12 min). The injector and transfer line temperature were both 260 °C. Helium was used as carrier gas.

Two different ionization techniques were used: electron ionization (EI) and electron-capture negative ionization (ECNI). For ECNI, methane (quality 4.5, <5 ppm O_2, AGA, Stockholm, Sweden) was used as the reagent gas. The electron energy was 70 eV, and the ion source temperature was 150 °C for both MS techniques. The spectra were scanned from 200 to 750 *m*/*z* in EI and from 32 to 750 *m*/*z*, in ECNI. The chromatographic data were recorded and processed with the ICIS data system (Finnigan MAT, USA).

Samples. Thirty female sea-run Baltic salmon from the Dalälven River population sampled in 1995 were used for the preparation of the pooled samples (for a more detailed description, see Asplund et al. (*19*)).

Cleanup and Analysis. The salmon blood plasma (5 g) were extracted and cleaned up as described elsewhere (*19*). In brief: the plasma samples (5 g) were denaturated by hydrochloric acid (6 M) and 2-propanol, followed by extraction of the organohalogens with *n*-hexane/methyl *tert*-butyl ether. Neutral and phenolic compounds in the extract were separated by partitioning between *n*-hexane and potassium hydroxide (0.5 M). After acidification of the aqueous phase, phenolic compounds were extracted into an organic phase and derivatized by diazomethane. Lipids present in the phenolic and neutral fractions were removed by HR-GPC and further cleaned up on a silica gel column (0.5 g) eluted with dichloromethane. The neutral phase was fractionated by HPLC on a nitrophenyl silica column, eluted with *n*-hexane. Two fractions were collected: fraction 1, where the PCBs eluted, and fraction 2 (back flush), where PBDE and MeO-PBDE eluted. The phenolic and neutral fractions were pooled separately. The solvent volume was reduced to approximately 0.5 mL and transferred to an activated (300 °C overnight) silica column (1 g in a Pasteur pipet). Three fractions were collected: fraction 1, *n*-hexane (6 mL); fraction 2, 20% dichloromethane in *n*-hexane (4 mL); and fraction 3, 50% dichloromethane in *n*-hexane (4 mL). Fractions 2 and 3 were finally pooled, and the solvent volume was reduced to 50 μL prior to GC−MS analysis.

The neutral fraction of muscle and egg samples from the same salmons, which were used for supporting ECNI information in the present work (of four MeO-PBDEs), were cleaned up as described earlier (*19*). Furthermore, they were similarly pooled and fractionated on silica as the salmon blood samples.

Results

The identification of the OH- and MeO-PBDEs in the salmon blood plasma was supported by similar RRTs versus BDE-138 on two columns compared to the synthetic references as shown in Table 1. The RRTs were determined from full-scan ECNI chromatograms by recording the ions *m*/*z* 79 and 81. In all cases, the difference in RRT between each reference standard and the corresponding MeO-PBDE in the salmon blood sample was less than ±0.001 on both columns used. Mass chromatograms of the phenolic and the neutral fractions on both nonpolar and polar GC columns are shown in Figures 1 and 2, respectively. The identified compounds, nine OH-PBDEs and six MeO-PBDEs, are labeled on the mass chromatograms, and their structures are shown in Figure 3.

MS of Major Compounds. 6′-MeO-BDE-49, 2′-MeO-BDE-68, 6-MeO-BDE-47, and 6-MeO-BDE-90 (**1**−**3** and **8**) from the neutral fraction and the methylated corresponding OH-PBDEs as well as 6′-Cl-2′-OH-BDE-68 from the phenolic fraction (**1**−**3**, **6**, and **8**) all had matching EI mass spectra with comparison to the authentic references. A full-scan EI spectrum is shown in Figure 4a for 6′-Cl-2′-MeO-BDE-68 (**6**) from the methylated phenolic fraction. In EI mode, the presence of a $[M - CH_3]^+$ fragment ion is characteristic for MeO-PBDEs with the methoxy group in the para position to the diphenyl ether bond, the $[M - BrCH_3]^+$ fragment ion is characteristic for the methoxy group in the ortho position, and the absence of these two fragment ions indicates that the methoxy group is substituted in the meta position, as recently reported (*23*). This fragmentation is also in agreement with previous observations of chlorinated analogues (i.e., ortho-, meta-, and para-substituted methoxylated polychlorinated diphenyl ethers (MeO-PCDEs)) (*30*, *31*). A specific loss of $BrCH_3$ was observed for all compounds mentioned above, indicating that the methoxy group is substituted in the ortho position to the diphenyl ether bond, which is in agreement with the authentic references. 6′-Cl-2′-MeO-BDE-68 (**6**) had a distinct loss of BrCl giving the fragment ion $[M - BrCl]^+$ (Figure 4a), as observed for the authentic reference standard, but this fragment ion was absent or only present in trace amounts for all other investigated monochlorinated MeO-tetraBDEs used as reference standards. The identities of these compounds were also supported by ECNI full-scan spectra. An ECNI full-scan spectrum of the methylated 6′-Cl-2′-OH-BDE-68 (**6**) from the phenolic fraction is shown in Figure 4b. Besides the bromine ions *m*/*z* 79 and 81 $[Br]^-$ and 159, 161, and 163 $[HBr_2]^-$ and also the mixed bromine/chlorine ions 115, 117, and 119 $[HBrCl]^-$ for the chlorine-containing compounds, fragment ions above *m*/*z* 163 were also detected but with low abundance as compared with $[Br]^-$. For the compounds 6′-MeO-BDE-49, 2′-MeO-BDE-68, 6-MeO-BDE-47, and 6-MeO-BDE-90 (**1**−**3** and **8**), the higher *m*/*z* ions were more clearly detected in muscle and egg samples from the same salmon as compared to the phenolic and neutral fractions in the salmon blood sample. Also in ECNI mode, the major compounds identified formed specific fragment ions, which give important structural information. Due to cleavage at the diphenyl ether oxygen bond, the nonmethoxylated phenyl ring forms the fragment ion $[M - C_6H_{1-2}Br_{2-3}Cl_{0-1}OCH_3]^-$ and the methoxylated

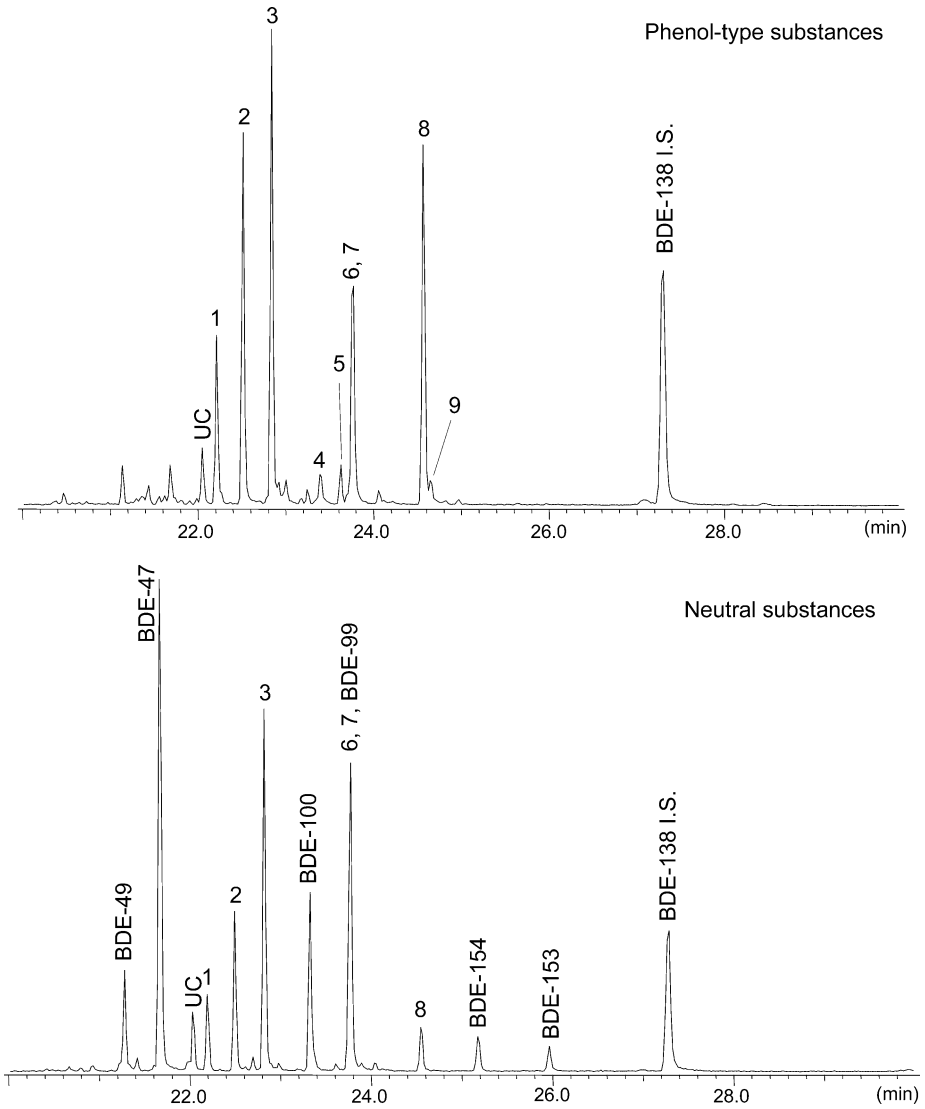


FIGURE 1. GC−MS (ECNI) chromatograms of bromide ions (*m/z*: 79, 81) of phenol-type substances (upper chromatogram) and neutral substances (lower chromatogram) in salmon blood analyzed on a CP-Sil 8 GC column. The phenolic compounds were methylated prior to GC−MS analysis. The peak identity numbers in the chromatograms refer to the chemical name and abbreviations given in Table 1, and the structures of these compounds are shown in Figure 3. A schematic chemical structure of the unidentified compound UC is also shown in Figure 3.

phenyl ring forms $[M - C_6H_3Br_2CH_3]^-$ and/or $[M + H - OC_6H_3Br_2]^-$ as shown for 6′-Cl-2′-MeO-BDE-68 (**6**) in Figure 4b. This fragmentation has previously been reported for MeO-PCDEs with the methoxy group in the ortho position to the diphenyl ether bond (*29*). The fragment ion $[M + H - OC_6H_3Br_2]^-$ has previously been discussed for a MeO-tetraBDE with a unknown bromine substitution pattern but with the methoxy group determined to be in the ortho position, according to EI (*18*). In each case the fragment ion $[M - C_6H_{1-2}Br_{2-3}Cl_{0-1}OCH_3]^-$ obtained from the MeO-PBDEs in the salmon samples was a dibromophenolate ion since this 2,4-dibromo-substituted (ortho and para to the diphenyl ether bond) nonmethoxylated diphenyl ether ring was the same for all compounds. Thus, these two specific ECNI fragment ions provide information about the number of halogen atoms in each phenyl ring, and this was also the case for the major MeO-PBDEs in the salmon samples, which was similar for the synthesized reference compounds. However, for 6-MeO-BDE-47 (**3**), this fragmentation was poor and shown in some MS runs but not in others, and this was the case both for the salmon samples and the reference standard. 6′-Cl-2′-MeO-BDE-68 (**6**) and 2′-MeO-BDE-68 (**2**) were the only identified compounds with an abundant clear $[M]^-$ in ECNI mode.

One OH-PBDE and its corresponding MeO-PBDE were verified in the salmon blood and marked with UC in Figures 1−3. The methylated derivative of this OH-PBDE was thus identical to the MeO-PBDE and had a molecule ion at *m/z* 468 with a chlorotribromo isotope pattern, as detected both with EI and ECNI. These compounds were substituated with a hydroxy/methoxy group in the ortho position to the diphenyl ether bond and with two bromine atoms in the nonhydroxylated/nonmethoxylated ring as well as one bromine and one chlorine atom in the hydroxylated/methoxylated ring, according to EI and ECNI. Unfortunately, no methoxylated chlorotriBDEs standards were available to confirm the identity of these compounds.

MS of Minor Compounds. EI full-scan spectra, present in relatively low abundance as compared with the major MeO-PBDEs, were recorded for three of the methylated OH-PBDEs from the phenolic fraction (i.e., 4′-MeO-BDE-49, 3′-Cl-6′-MeO-BDE-49, and 6-MeO-BDE-99 (**4, 5**, and **9**)) but could nevertheless give some structural information. 3′-Cl-6′-MeO-BDE-49 and 6-MeO-BDE-99 (**5** and **9**) also had a

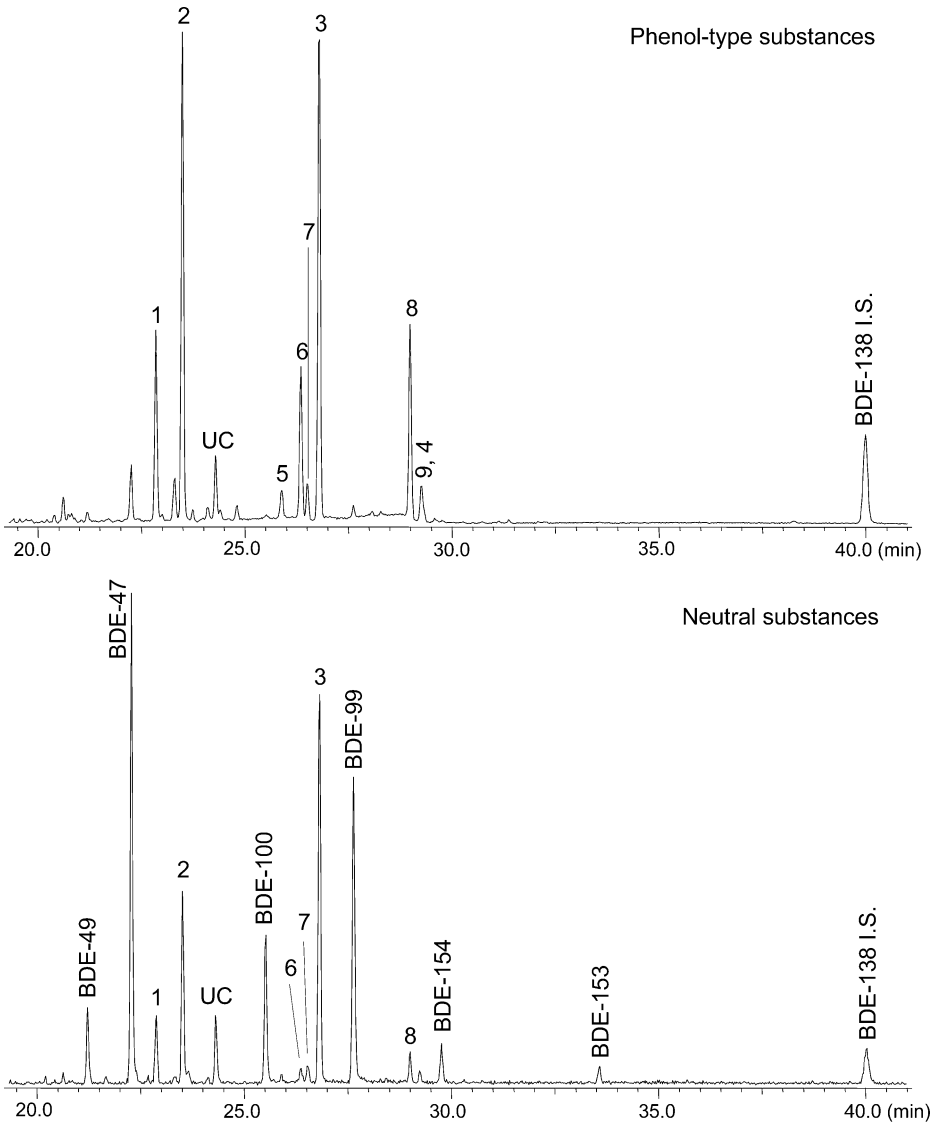


FIGURE 2. GC−MS (ECNI) chromatogram of bromide ions (*m/z*: 79, 81) of phenol-type substances (upper chromatogram) and neutral substances (lower chromatogram) in salmon blood analyzed on a polar SP 2331 column. The phenolic compounds were methylated prior to GC−MS analysis. The peak identity numbers in the chromatograms refer to the chemical name and abbreviations given in Table 1, and the structures of these compounds are shown in Figure 3. A schematic chemical structure of the unidentified compound UC is also shown in Figure 3.

loss of $BrCH_3$ whereas 4′-MeO-BDE-49 (**4**) did not. However, 4′-MeO-BDE-49 (**4**) had a loss of CH_3 and indicates the methoxy group in the para position to the diphenyl ether bond. This fragment ion was absent or only present in trace amounts for all other MeO-PBDEs detected, including the major once. All other compounds (i.e., the methylated 5-Cl-6-OH-BDE-47 (**7**) from the phenolic fraction and 5-Cl-6-MeO-BDE-47 (**7**) and 6′-Cl-2′-MeO-BDE-68 (**6**) from the neutral fraction) did not give any supporting EI information. No clear ECNI full-scan mass spectra could be obtained from these compounds that were present in the salmon blood plasma in low concentrations. However, these compounds all gave the bromine ions *m*/*z* 79 and 81 $[Br]^-$ and 159, 161, and 163 $[HBr_2]^-$, and the chlorine-containing compounds gave the mixed bromine/chlorine ions 115, 117, and 119 $[HBrCl]^-$. It should be emphasized that the 4′-MeO-BDE-49 (**4**) gave very poor fragmentation above *m*/*z* 163 for the authentic standard; consequently, it was difficult to find fragmental ions matching with the corresponding sample compound.

Both the neutral fraction and the phenolic fraction from the salmon blood contained a number of other compounds that interfered with the MeO-PBDEs and methylated OH-PBDEs. An additional number of both MeO-PBDEs and OH-PBDEs were indicated in the salmon plasma but not possible to confirm since their concentrations were too low. All coelutions found from all reference compounds (PBDEs and MeO-PBDEs) on both polar and nonpolar GC columns are summarized in Table 2.

Discussion

PBDEs and Coelutions. The six PBDE congeners detected in the plasma correspond to the most dominating ones reported in biota (*1*, *32*, *33*). Of the identified compounds in the present study, BDE-99, 6′-Cl-2′-MeO-BDE-68 (**6**), and 5-Cl-6-MeO-BDE-47 (**7**) coeluted on the nonpolar CP-Sil-8 column whereas 4′-MeO-BDE-49 (**4**) and 6-MeO-BDE-99 (**9**) coeluted on the polar SP 2331 column. The coelution between BDE-99 and 6′-Cl-2′-MeO-BDE-68 (**6**) as well as 5-Cl-6-MeO-BDE-47 (**7**) on the common nonpolar DB-5 type CP-Sil-8 column must be considered to avoid errors in the quantification of BDE-99. BDE-99 has previously been reported to coelute with 2′,6-diMeO-BDE-68 in environmental samples of marine mammals from the Southern Hemisphere (*14*, *15*) and in a human milk from the Faeroe Islands (*17*).

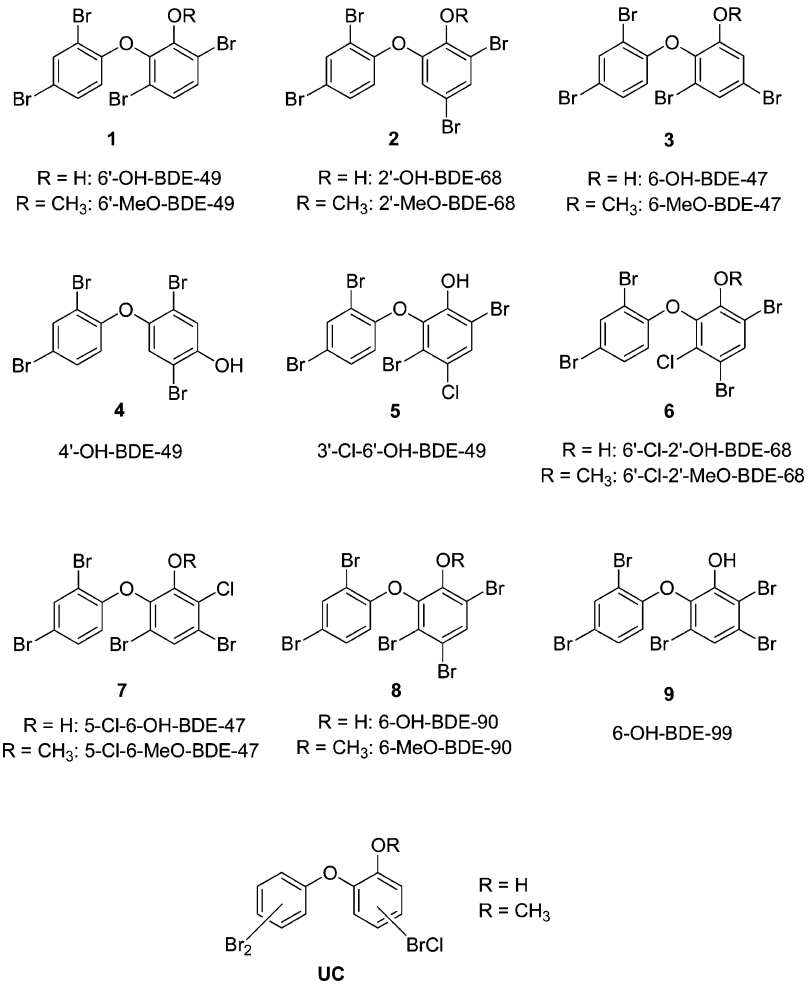


FIGURE 3. Structures of the compounds identified in the salmon blood. The identification numbers of corresponding OH-/MeO-PBDEs are the same (Figures 1 and 2) since the OH-PBDEs are methylated prior to GC−MS analysis.

Structural Information of the Identified OH- and MeO-PBDEs. MS data that gave characteristic structural information of the MeO-PBDEs was valuable for two purposes. As pointed out in the results, the major MeO-PBDEs detected in the salmon samples and partly also three of the minor compounds gave MS structural information, which strengthen the identity besides the matching RRTs. MS structural information was also used for the selection of potential MeO-PBDEs candidates, which were synthesized with respect to the present work. The MS information could together with an assumption (see below) reduce the relative large number of theoretical MeO-PBDE isomers to only a few, as illustrated in Table 3. The majority of the MeO-PBDEs used as reference compounds (*23*) were prepared according to this guidance. Hence, the major MeO-PBDEs in the salmon samples gave a loss of $BrCH_3$ indicating the methoxy group in the ortho position to the diphenyl ether bond, and the fragment ions $[M - C_6H_{1-2}Br_{2-3}Cl_{0-1}OCH_3]^-$ and $[M - C_6H_3Br_2CH_3]^-$ and/or $[M + H - OC_6H_3Br_2]^-$ in ECNI mode gave the number of halogens in each phenyl ring, which in each case were two bromine atoms in the nonmethoxylated phenyl ring and, consequently, the resulting halogens in the methoxylated phenyl ring. Furthermore, we assumed that the nonmethoxylated ring is 2,4-bromo-substituted since all OH- and MeO-PBDEs with natural origin reported in the literature, with two bromine atoms in the nonmethoxylated ring, are all substituted in this way. The over all dominating substitution of technical PBDEs (*34*) as well as PBDEs found in biota (*1*, *32*, *33*) with two bromine atoms in one of the phenyl rings is similarly, 2,4-bromo-substituted. Having this in mind, the potential candidates of MeO-PBDE isomers can be reduced to 6 MeO-tetraBDEs, 12 MeO-chlorotetraBDEs, and 4 MeO-pentaBDEs for the major compounds detected (Table 3). Of these compounds, 4 of the 6 MeO-tetraBDEs, 5 of the 12 MeO-chlorotetraBDEs, and all 4 MeO-pentaBDEs were made available as reference standards in the present study.

RRTs of certain synthesized MeO-PBDEs, with the methoxy group in the ortho position, gave also structural information since 2,3,4- and 3,4,5-trihalogenation in the methoxylated phenyl ring of these compounds seems to have longer RRTs as compared to equivalent isomers with 2,3,5- and 2,4,5-trihalogen pattern on the nonpolar column (Table 1). This latter information was especially useful for the preparation of MeO-chlorotetraBDEs (5-Cl-6-MeO-BDE-47 (**7**) and 6′-Cl-2′-MeO-BDE-68 (**6**)), which were the most recently synthesized compounds. Additional structural information about the major MeO-chlorotetraBDE investigated, originating from the phenolic fraction (identified as 6′-Cl-2′-OH-BDE-68 (**6**)), was obtained from the loss of BrCl in EI mode that indicated the chlorine substituent to be in the ortho position to the diphenyl ether bond.

Environmental Occurrence of the Identified OH- and MeO-PBDEs. Nine compounds (5-Cl-6-OH-BDE-47, 5-Cl-6-MeO-BDE-47, 6′-OH-BDE-49, 6′-MeO-BDE-49, 4′-OH-BDE-49, 3′-Cl-6′-OH-BDE-49, 6′-Cl-2′-OH-BDE-68, 6′-Cl-2′-MeO-BDE-68, and 6-MeO-BDE-90) are here reported for the first time to occur in the environment. The following OH- and MeO-PBDEs, reported herein, have previously been isolated, structurally identified, and confirmed as natural products: 6-OH-BDE-47 has been detected in marine sponges (*7*, *35*−*37*) and in ascidians (tunicates) (*8*, *9*); 2′-MeO-BDE-68 has been identified in marine sponge (*38*) as

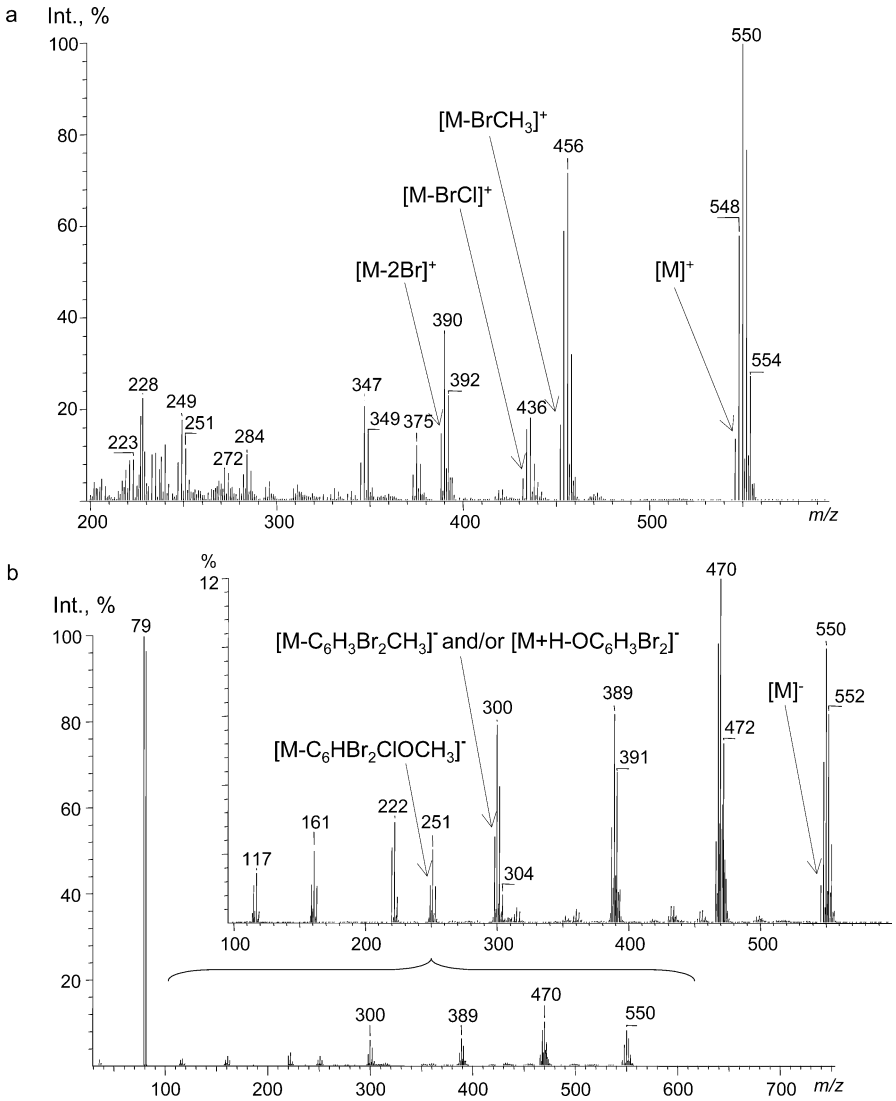


FIGURE 4. EI (a) and ECNI (b) mass spectra of 6′-chloro-2′-methoxy-2,3′,4,5′-tetraBDE (6′-Cl-2′-MeO-BDE-68), originating from the phenolic fraction in the salmon blood, as 6′-chloro-2′-hydroxy-2,3′,4,5′-tetraBDE (6′-Cl-2′-OH-BDE-68).

well as in green alga (*13*); 6-MeO-BDE-47 (*11*), 2′-OH-BDE-68 (*7*, *12*, *36*, *37*), and 6-OH-BDE-99 (*6*) have been identified in a marine sponge. In addition, 6-OH-BDE-47, 2′-OH-BDE-68, 6-OH-BDE-90, and 6-OH-BDE-99 have been detected in red alga by comparison of RRTs and ECNI full-scan mass spectra with authentic reference compounds (*10*). As pointed out in the Introduction, 6-OH-BDE-47, 6-MeO-BDE-47, and 2′-MeO-BDE-68 have previously been reported in biota of a high trophic level.

Sources of the Identified OH- and MeO-PBDEs. To our knowledge, OH- and MeO-PBDEs are not industrially produced and have not been reported as impurities in any brominated technical products. However, various technical chlorophenol products have been reported to contain byproducts of which OH-PCDEs were found as the major polychlorophenol dimer byproduct (*39*−*42*). MeO-PCDEs have also been determined in commercial polychlorophenol products (*43*, *44*). Contradictory, two technical polybromophenol mixtures (i.e., 2,4,6-tribromophenol and pentabromophenol) were found to contain no OH-PBDEs or other dimeric byproducts, such as MeO-PBDEs (*45*). According to the present knowledge about OH- and MeO-PBDEs, their appearance in environmental samples is most likely either as natural products and/or metabolites of anthropogenic PBDEs. OH-PBDEs that originate from PBDEs have so far only been observed in laboratory experiments (in mice, rat, and fish), and no MeO-PBDEs have hitherto been reported with that origin (*2*−*4*). However, it cannot be excluded that MeO-PBDEs are formed metabolically from PBDEs but then only as a minor pathway. Methoxyhydroxy-PBDE metabolites containing 6−7 bromine atoms have been shown in rats exposed to BDE-209 (*5*). OH-PCDEs have been reported to be microbially methylated (*46*), and this seems to be one possible pathway also for the methylation of OH-PBDEs.

The structure of a OH-PBDE (i.e., the position of the hydroxy group and the bromine atoms) may indicate its origin. Environmental samples and technical pentaBDE products contain the dominating tetraBDE congener BDE-47 as well as the minor tetraBDEs (BDE-49 and BDE-66), and the dominating pentaBDEs are BDE-85, BDE-99, and BDE-100 (*32*−*34*). According to what is known about metabolism of halogenated aromatic compounds via cytochrome P450-mediated transformations (*47*), via direct hydroxylation, or via hydroxylation with possible 1,2-shift resulting in a bromine or hydrogen migration, it is not possible to obtain more than four of the OH-PBDE discussed in the present paper (i.e., 6-OH-BDE-47, 6′-OH-BDE-49, 6-OH-BDE-99, and 4′-OH-BDE-49) from environmental relevant PBDEs. The presence of a chlorine atom in five of the identified compounds in the salmon blood excludes PBDEs as their precursors. On the other hand, one hydroxylated chlorotetrabromodiphenyl ether has been structurally identified and

TABLE 2. Coelutions Encountered for PBDE and MeO-PBDE Congeners on CP-Sil 8 and SP 2331 GC Columns, Presented in Elution Order

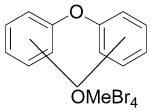
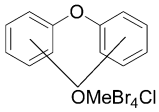
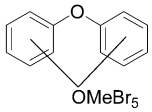
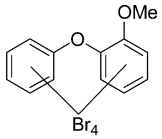
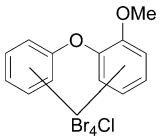
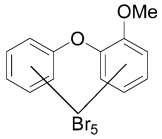
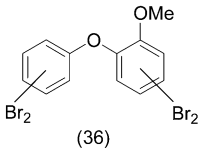
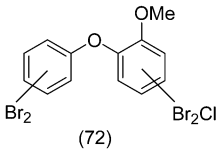
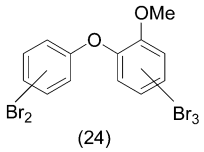
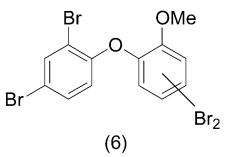
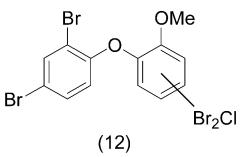
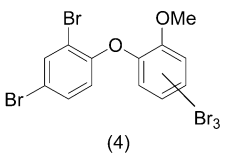
CP-Sil 8 (nonpolar)		
PBDEs	**vs**	**MeO-PBDEs**
BDE-71		3′-MeO-BDE-28
		4′-MeO-BDE-17
BDE-77		2′-MeO-BDE-68
BDE-100		5-MeO-BDE-47
		4′-MeO-BDE-49
BDE-85		6-MeO-BDE-99
BDE-99		5-Cl-6-MeO-BDE-47
		6′-Cl-2′-MeO-BDE-68
MeO-PBDEs	**vs**	**MeO-PBDEs**
6′-MeO-BDE-17		4′-MeO-BDE-30
3′-MeO-BDE-28		4′-MeO-BDE-17
3-MeO-BDE-47		2′-MeO-BDE-66
6′-Cl-2′-MeO-BDE-68		5-Cl-6-MeO-BDE-47
4′-MeO-BDE-121		4′-MeO-BDE-42
6-MeO-BDE-90		5′-Cl-2′-MeO-BDE-66
SP 2331 (polar)		
PBDEs	**vs**	**MeO-PBDEs**
BDE-66		2′-MeO-BDE-68
BDE-155		6-MeO-BDE-47
		4'-MeO-BDE-69
BDE-99		4′-MeO-BDE-121
BDE-154		5-MeO-BDE-47
BDE-153		2-MeO-BDE-123
		4-MeO-BDE-90
MeO-PBDEs	**vs**	**MeO-PBDEs**
6-MeO-BDE-99		4′-MeO-BDE-49
6-MeO-BDE-47		4′-MeO-BDE-69
2-MeO-BDE-123		4-MeO-BDE-90

determined as natural product from a marine sponge (i.e., 3-chloro-6-methoxy-2,2′,4,4′-tetrabromodiphenyl ether (3-Cl-6-MeO-BDE-47)) (*35*), and additionally one hydroxylated chlorotetrabromodiphenyl ether has been detected in red alga present in the Baltic Sea (*10*). 6-OH-BDE-47, 6′-OH-BDE-49, and 6-OH-BDE-99 may be metabolites of BDE-47, BDE-49, and BDE-99, respectively, and 6-MeO-BDE-47 and 6′-MeO-BDE-49 could possibly be formed through a metabolic methylation process. On the other hand, both 6-OH-BDE-47 and 6′-OH-BDE-49 have their corresponding MeO-PBDEs present in the salmon blood, similar to five other OH-PBDEs that also form MeO-PBDEs, but none of these are likely metabolites of PBDEs. Forming such a OH-/MeO-PBDE pair at the actual concentrations is thus suggested as evidence of natural formation of at least the major part of 6-OH/MeO-BDE-47 and 6′-OH/MeO-BDE-49. In the salmon blood, the concentrations of these MeO-PBDEs are approximately in the same range as the PBDEs, and the corresponding OH-PBDEs were estimated to be 20−30% of the MeO-PBDEs (*19*). In addition, 6-OH-BDE-47 and 6-OH-BDE-99 have been reported in red alga from the Baltic Sea (*10*), which supports natural origin.

4′-OH-BDE-49, however, may be a metabolite of BDE-47 and possibly also of BDE-49 because the hydroxy group is attached to the para position to the diphenyl ether bond. All natural occurring OH-PBDEs so far reported in the literature have the hydroxy group in the ortho position whereas mice and rats exposed to BDE-47 have the hydroxy group also in the meta and para positions (*3*). 4′-OH-BDE-49 is most probably one of the two metabolites with the hydroxy group in the para position as determined with EIMS formed in rat and mice exposed to BDE-47 (*3*), and most likely also in fish (exposed to BDE-47) (*2*) when comparing the characteristic chromatographic peak patterns in these two works. Findings of OH-PBDEs with the hydroxy group in a meta or para positions may indicate PBDE metabolism.

To summarize, the present knowledge about OH- and MeO-PBDEs as well as the structures of the identified OH- and MeO-PBDEs in the salmon blood indicate that all

TABLE 3. Step-by-Step Reduction of Possible MeO-PBDE and Methylated OH-PBDE Isomers Detected as Major Components of These Classes of Compounds in Baltic Sea Salmon Blood[a]

	MeO-tetraBDE	MeO-chlorotetraBDE	MeO-pentaBDE
Theoretical number of isomers	 (198)	 (894)	 (198)
MeO-group in the ortho position according to EI	 (76)	 (350)	 (76)
Known number of halogens in the phenyl rings according to ECNI	 (36)	 (72)	 (24)
Assumption: A 2,4-dibromo pattern in the non-MeO phenyl ring	 (6)	 (12)	 (4)

[a] The number of MeO-PBDE isomers are given in parentheses.

compounds except one (4′-OH-BDE-49) originate from natural sources. However, parts of 6-OH/MeO-BDE-47, 6′-OH/MeO-BDE-49, and 6-OH-BDE-99 present in the salmon blood might be metabolites of the corresponding PBDE congeners. 4′-OH-BDE-49 is most likely a metabolite of BDE-47 and possibly also one of BDE-49. Recently, Reddy at al. (*48*) reported that ^{14}C analysis is a useful tool to determine whether a compound is of natural or synthetic origin or both. PBDEs that are manufactured from petrochemicals are "free" of ^{14}C whereas natural products are not. Thus, proof of the origin of the OH- and MeO-PBDEs can be obtained if sufficient amount of the actual compounds can be isolated for a ^{14}C measurement.

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