IDENTIFICATION OF NOVEL HALOGENATED NATURALLY OCCURRING COMPOUNDS IN SEA SPONGE BY HIGH-RESOLUTION MASS SPECTROMETRY AND COMBINED SCREENING APPROACHES

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Introduction

Marine sponges are a rich source of bioactive naturally occurring halogenated compounds (NHCs), such as bromophenols (BPs), bromoanisoles (BAs) and hydroxylated or methoxylated analogues of polybrominated diphenyl ethers (HO-PBDEs, MeO-PBDEs) and bromobiphenyls (HO-BBs, MeO-BBs)[1,2]. These brominated compounds may originate both from natural and anthropogenic sources[3-5]. The resistance to chemical and biological degradation of natural and anthropogenic halogenated compounds can cause their bioaccumulation and biomagnification in higher trophic levels of the food chain[6,7]. Thereof, the identification of new NHCs potentially occurring in higher trophic levels has been a topic of interest during the last decade[8,9].

Most studies on the characterization and quantification of NHCs are carried out applying gas chromatography (GC) coupled to electron capture negative ion mass spectrometry (ECNI-MS) or high-resolution mass spectrometry (HRMS). This requires the derivatization of hydroxylated NHCs prior to analysis[10,11]. While this approach shows great selectivity for the detection of methoxylated NHCs, the identification of e.g., HO-MeO-PBDEs or HO-MeO-BBs can be hampered by the presence of corresponding dihydroxy analogues and vice versa[8]. To address this issue, several methods using liquid chromatography (LC) coupled to HRMS or tandem mass spectrometry (MS/MS) for the analysis of hydroxylated NHCs have been introduced by applying targeted methods for tentative identification of known NHCs[12,13]. However, this approach leaves many unknown potentially bioactive or toxic NHCs undetected. The use of HRMS for suspect or non-target screening (NTS) approaches allows the simultaneous analysis of a high number of compounds. Thereby, the application of novel bioinformatics tools, such as HaloSeeker, enables a selective detection of halogenated compounds based on their specific isotopic patterns[14]. Previous studies applying NTS for the identification of novel NHCs in biota samples have shown the potential of these techniques[15]. Nevertheless, a study applying these novel approaches using LC-HRMS for the detection and identification of (new) HO-NHCs is still lacking.

Therefore, this study aims to comprehensively screen sea sponge samples using a combination of suspect and non-target screening to identify new HO-NHCs.

Materials and methods

Chemicals: Solvents and chemicals used for sample preparation and LC-HRMS analysis were obtained from Biosolve Chimie SARL (Dieuze, France) and Merck KGaA (Darmstadt, Germany). A PURELAB Flexsystem was used to obtain ultrapure water (18.2 MΩ cm, Milli-Q, Millipore). All solvents used for LC-HRMS analysis were of HPLC-grade quality. 4-hydroxy-polychlorinated biphenyl-159 (4-OH-PCB159) was used as internal standard and d18-gamma-hexabromocyclododecane (d18-γ-HBCD) as recovery standard, respectively. Both standards were provided by Wellington Laboratories, Canada.

Samples: Two sea sponge samples from *Lamellodysidea* sp. (sample MT-11) and *Callyspongia* sp. (sample MT-31) were investigated. Sample MT-11 was collected from the natural habitat of the species and dried at room temperature. Sample MT-31 was a stored extract which was prepared by the sample preparation method described by Kato et al.[8]

Sample preparation: 1000 mg of sample MT-11 were twice extracted with 6 mL of a ethyl acetate:MeOH:DCM (1:1:1, *v/v/v*) mixture. The combined supernatants were evaporated to dryness and reconstituted in 5 mL of hexane. One millilitre of 1M KOH:EtOH (7:3, *v/v*) was added. After vortexing and centrifugation, the hexane fraction was transferred to another glass tube. The process was repeated with 5 mL of hexane.

Then, 1.3 mL of 1M HCl and 5 mL of hexane:diethylether (1:1, *v/v*) were added to the 1M KOH:EtOH (7:3, *v/v*) fraction. The hexane:diethylether (1:1, *v/v*) fraction was transferred to another glass tube and the procedure was repeated. The combined fractions were concentrated to nearly dryness and reconstituted in 50 µL of recovery standard (d18-γ-HBCD, 150 pg/µL) and 150 µL of MeOH. Samples were diluted with a ratio of 1:5 in methanol prior to injection.

Instrumentation and LC-QTOF-MS analysis: All measurements were conducted on an Agilent Infinity 1290 UPLC coupled to an Agilent 6530 quadrupole time of flight (QTOF) mass spectrometer equipped with an electrospray ionization (ESI) source in negative ionization mode (Agilent Technologies, Santa Clara, USA). Chromatographic separation was performed on a Kinetex Biphenyl column (2.1 mm x 50 mm, 1.7 µm particle size; Phenomenex, Utrecht, Netherlands) equipped with a SecurityGuard™ ULTRA guard column (i.d. 2.1 mm; Phenomenex, Utrecht, Netherlands) with the same stationary phase. The mobile phases consisted of water with 5 mM ammonium acetate (A) and methanol (B). The applied gradient was as follows: 0-0.5 min (40%B); 9 min (95%B); 9-12min

(95%B); 12.6-15min (40%B). Samples were analyzed both in Auto MS/MS and full scan MS (*m*/*z* 100-1700) modes. For Auto MS/MS, 4 precursors per cycle were selected and fragmented at collision energies of 10, 20 and 40 eV.

Data analysis: A suspect list was developed which contained 1) mono-hydroxylated, 2) di-hydroxylated, and 3) mono-hydroxylated and mono-methoxylated compounds of BPs, BBs, BDEs, and BDDs with up to a total of 7 bromine and/or chlorine atoms, but only up to 3 chlorine atoms. The suspect list was matched against the analyzed samples using the "targeted feature extraction" algorithm of the Profinder software (B.08.00; Agilent Technologies, USA). Thereby, matching criteria of ± 10 ppm for the mass error and ± 0.2 min for RT alignment were used. For non-target screening, HaloSeeker 1.0 was used. Peak picking was performed applying the xcms package (version 3.2.0; *m*/*z* tolerance = 3; peakwidth = 5-60; prefilter step = 3; prefilter level = 10 000; sntresh = 10). This provided a list of extracted signals (i.e., features) which was paired based on the specific mass differences and isotopic patterns of C, Br an Cl atoms (RT tolerance = 5 s; *m*/*z* tolerance = 0.5 mDa). The results were filtered based on the F2+ filter which only retains polyhalogenated features. For the obtained series, molecular formula (selected elements: H, C, O, Cl, Br, S; *m*/*z* tolerance = 10 mDa; relative abundance tolerance = 20%) were predicted. The MS/MS spectra of the most abundant series were investigated to obtain additional structural information.

Results and discussion

Suspect screening:

Suspect screening of sponge samples yielded 18 identified compounds. In this report a selection of the most relevant hydroxylated compounds will be discussed (Table 1). All these compounds were present in the *Lamellodysidea* sp. sample (MT-11), while seven compounds were identified in sample MT-31 (*Callyspongia* sp.). Tri-, penta-, hexa- and heptabrominated diOH-BDEs were identified in *Lamellodysidea* sp. (MT-11). Whilst there were no fragmentation spectra available for diOH tribrominated BDE resulting in confidence level (CL) 4 according to Schymanski et al.[16], the fragmentation spectra of penta- and hexabrominated diOH-BDE showed a characteristic fragment of $[C_6H_3Br_2O]^-$ (theoretical *m*/*z* 248.8556) indicating that both compounds carry a dibrominated phenolic moiety. Accordingly, fragments corresponding to a tri- and tetrabrominated phenolic moiety were detected in the penta- and hexabrominated diOH-BDE, respectively. The described fragments also confirm that the hydroxy groups are located on different aromatic rings.

For both sponge species, the fragmentation spectrum of diOH-hepta BDE showed fragments of tetra- and tribrominated phenolic moieties, again indicating the positioning of hydroxy groups on different aromatic rings. The fragmentation spectra of all diOH-BDEs showed fragments corresponding to [Br]- and a loss of HBr confirming the bromination of the parent compounds.

Table 1: Summary of suspect screening results of sea sponge samples. For each compound the retention time (RT), mass error and level of identification confidence are reported. n.d. = not detected.

			Lamellodysidea sp.			*Callyspongia* sp.		
Compound	**Formula**	**Monoisot. mass**	RT [min]	Mass error [ppm]	Conf. level	RT [min]	Mass error [ppm]	Conf. level
Br3-diOH-diphenyl ether	C12H7Br3O3	435.7945	5.52	-0.57	4	n.d.	n.d.	n.d.
Br5-diOH-diphenyl ether	C12H5Br5O3	591.6156	5.86 6.74	0.94 -3.44	3 3	6.29	-1.59	3
Br6-diOH-diphenyl ether	C12H4Br6O3	669.5261	6.09	1.81	3	6.55	0.79	3
Br7-diOH-diphenyl ether	C12H3Br7O3	747.4366	7.23	-3.06	3	7.27	1.40	4
Br4-OH-MeO-diphenyl ethers	C13H8Br4O3	527.7207	7.83	-0.85	3	7.89	-3.66	4
Br5-OH-MeO-diphenyl ethers	C13H7Br5O3	605.6312	8.10 8.44	-1.15 -5.72	3 4	8.11 8.45	-2.63 -4.68	4 4
Br6-OH-MeO-diphenyl ethers	C13H6Br6O3	683.5417	7.08 8.25 8.89	-0.95 -6.61 -7.86	4 4 4	7.12 8.90	-4.04 -3.28	4 4
Br3-OH-diphenyl ether/ Br5-diOH-biphenyl	C12H5Br5O2	575.6206	7.27	-4.58	3	n.d.	n.d.	n.d.
Cl1-Br5-diOH-diphenyl ethers	C12H4Br5ClO3	625.5766	6.36	-1.18	3	6.48	-3.42	3

In *Lamellodysidea* sp., tetra-, penta- and hexabrominated OH-MeO-BDEs were detected. Thereby, penta- and hexabrominated OH-MeO-BDE showed two and three peaks, respectively, indicating the presence of various isomers. Hexabrominated OH-MeO-BDE and one of the isomers of pentabrominated OH-MeO-BDE were assigned with CL 4, as no fragmentation spectra could be acquired. The fragmentation spectra of both tetra- and

the first isomer of pentabrominated OH-MeO-BDE showed fragments which correspond to the loss of the methyl group and the loss of [-CH_3Br]. These observations confirm the methylation of the detected compounds. Additionally, both compounds showed a fragment corresponding to $[C_6H_2Br_2O_2]^-$ (theoretical *m/z* 263.8427) indicating that both compounds carry a dibrominated aromatic moiety. For pentabrominated OH-MeO-BDE, this was further confirmed by the detection of a fragment corresponding to a tribrominated phenolic moiety ($[C_6H_2Br_3O_2]^-$; theoretical *m/z* 341.7532). Interestingly, both described fragments still carried two oxygens while this was not observed for non-methylated diOH-BDEs, indicating different fragmentation pathways. Tetra-, penta- and hexabrominated OH-MeO-BDE were also detected in *Callyspongia* sp. However, due to low abundance and thereof no available fragmentation spectra, CL 4 had to be assigned.
It has to be noted that OH-MeO-tetra BDE, diOH-penta-BDE, OH-MeO-penta-BDE and diOH-hexa-BDE have previously been reported in both *Lamellodysidea* sp. and *Callyspongia* sp. by Kato et al.[8] Our study confirms these findings and provides further information on fragmentation spectra and on the proposed structures of detected compounds. Additionally, our study reports the detection of a OH-MeO-hexa-BDE and a diOH-hepta-BDE in both sponge species which has not been reported previously.
Furthermore, a pentabrominated monochlorinated diOH-BDE was detected in *Lamellodysidea* sp. and *Callyspongia* sp. Fragments which correspond to the loss of HBr and HCl demonstrated the mixed halogenation of the detected compound. The observed fragments with *m/z* 248.8500 ($[C_6H_3Br_2O]^-$) and *m/z* 360.7177 ($[C_6HBr_3ClO]^-$) indicate that the two phenolic moieties carry two and three bromines plus one chlorine, respectively. To our knowledge, this is the first study reporting a mixed halogenated diOH-BDE in sea sponge species.

Non-target screening:
For non-target screening, the sea sponge samples were analyzed using HaloSeeker 1.0 software, as a complementary approach to suspect screening. Molecular formulae were predicted for all detected features and their fragmentation spectra were analyzed aiming to gain additional structural information. In this report, only the compounds which were not reported previously, and which showed the highest CLs are included.
Based on the predicted molecular formulae, hepta-, octa- and nonabrominated dihydroxylated diphenoxybenzene (diOH-PBDPB) could be identified in *Lamellodysidea* sp. This was confirmed by the analysis of the corresponding fragmentation spectra shown in Figure 1. Based on this information, hepta- ($C_{18}H_7Br_7O_4$, Δppm -2.61 ppm), octa- ($C_{18}H_6Br_8O_4$, Δppm -1.19 ppm) and nonabrominated ($C_{18}H_5Br_9O_4$, Δppm -7.98 ppm) diOH-PBDPB were detected in *Lamellodysidea* sp. For heptabrominated diOH-PBDPB, the observed fragments with molecular formulae $[C_6H_3Br_2O_2]^-$ (theoretical *m/z* 266.8485) and $[C_6H_2Br_3O_2]^-$ (theoretical *m/z* 344.7590) gave evidence about the distribution of bromines between the three aromatic moieties. The same applies to octabrominated diOH-PBDPB, for which fragments with molecular formulae $[C_6H_3Br_2O_2]^-$ (theoretical *m/z* 266.8485) and $[C_6HBr_4O_2]^-$ (theoretical *m/z* 424.6675) were observed. This information provided additional confirmation of compound identification and was used to propose the structures given in Figure 1.

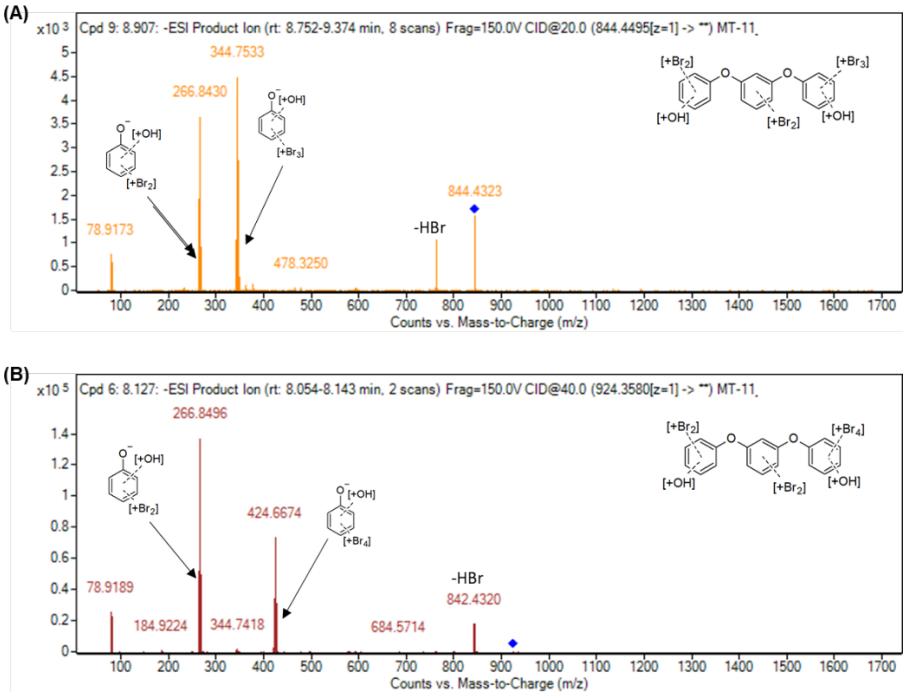

Figure 1: Experimental data of hepta- and octabrominated dihydroxylated diphenoxybenzenes (diOH-BDPBs) with the molecular formulae $C_{18}H_7Br_7O_4$ (A) and $C_{18}H_6Br_8O_4$ (B) detected in *Lamellodysidea* sp.

The analysis of *Callyspongia* sp. also yielded the detection of hepta-, octa- and nonabrominated diOH-PBDPB. Their fragmentation spectra showed fragments identical to the ones observed in *Lamellodysidea* sp. Therefore, the same assumptions regarding the molecular structures and distribution of bromine as described above can be made. To our knowledge, this is the first time that diOH-PBDPB are reported in the environment.
Additionally, a heptabrominated monochlorinated dihydroxylated diphenoxybenzene with the molecular formula $C_{18}H_6Br_7ClO_4$ was detected in *Callyspongia* sp. The given formula was proposed based on the grouping of the described compound on the same horizontal line in the H/Cl-plot as the non-chlorinated diOH-PBDPBs indicating the same degree of halogenation. This was additionally confirmed by the satisfying fit between experimental and theoretical isotopic patterns (Δppm -8.15 ppm). However, due to the low abundance of the compound, no fragmentation spectra were available to confirm the findings.

This study introduced a comprehensive combined screening approach applying suspect and non-target screening for the identification of new HO-NHCs in sea sponge samples. The use of LC-HRMS allowed the detection of a high variety of NHCs from different classes.
Suspect screening yielded a high number of compounds detected in sponge samples (17 and 8 compounds identified in sea sponge samples of *Lamellodysidea* sp. and *Callyspongia* sp., respectively) indicating a high variety of NHCs occurring in this species. Four of the identified compounds have been described in previous studies. Thus, the presented work introduces a high number of newly identified NHCs in sea sponge samples including heptabrominated diOH-BDE, monochlorinated pentabrominated diOH-BDE, hexabrominated OH-MeO-BDE and others.
Non-target screening allowed the identification of OH-PBDBPs, such as hepta-, octa- and nonabrominated diOH-BDBPs, in *Lamellodysidea* sp. and *Callyspongia* sp. samples. To our knowledge, this is the first study reporting these compounds in the environment. Non-target screening yielded 16 additional compounds in *Lamellodysidea* sp. and *Callyspongia* sp. samples, respectively, which could tentatively be identified through the assignment of predicted molecular formulae.
This study provides a comprehensive screening approach for polyhalogenated NHCs in biota samples which provided additional information on the occurrence and distribution of NHCs in alga and sea sponge species and can serve as a valuable tool for future screening studies.

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