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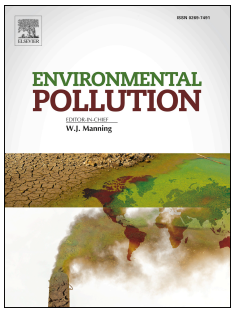

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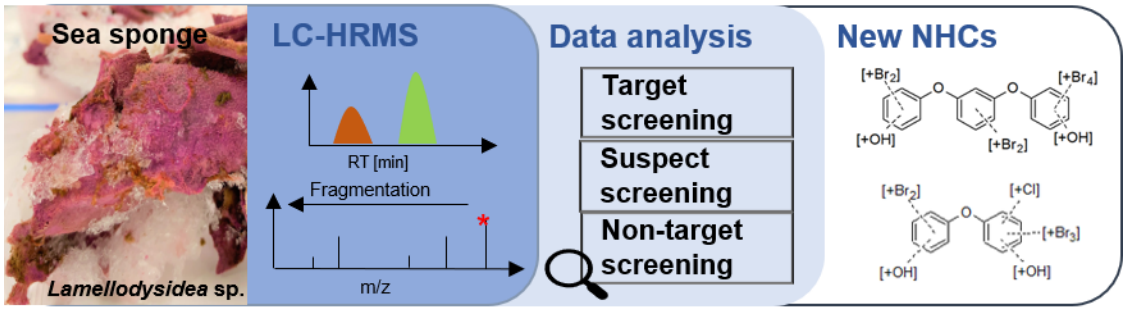
Adrian Covaci: Conceptualization, Methodology, Resources, Writing – review and editing, Supervision, Project Administration, Funding acquisition

Naturally occurring halogenated compounds (NHCs)



Identification of novel halogenated naturally occurring compounds in marine biota by high-resolution mass spectrometry and combined screening approaches

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Abstract:

Marine animals, plants or bacteria are a 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). This study applied a comprehensive screening approach using liquid chromatography high-resolution mass spectrometry and combining target, suspect and non-target screening with the aim to identify new hydroxylated NHCs which might be missed by commonly applied gas chromatographic methods. 24 alga samples, 4 sea sponge samples and 7 samples of other invertebrates were screened. Target screening was based on 19 available reference standards of BPs, (di)OH-BDEs and diOH-BBs and yielded seven unequivocally identified compounds. 6OH-BDE47 was the most frequently detected compound with a detection frequency of 31%. Suspect screening yielded two additional compounds identified in alga samples as well as 17 and 8 compounds identified in sea sponge samples of *Lamellodysidea* sp. and *Callyspongia* sp., respectively. The suspect screening results presented here

confirmed the findings of previous studies conducted on sea sponge samples of *Lamellodysidea* sp. and *Callyspongia* sp. Additionally, in *Lamellodysidea* sp. and *Callyspongia* sp. 13 and 4 newly identified NHCs are reported including heptabrominated diOH-BDE, monochlorinated pentabrominated diOH-BDE, hexabrominated OH-MeO-BDE and others. Non-target screening allowed the identification of 31 and 20 polyhalogenated compounds in *Lamellodysidea* sp. and *Callyspongia* sp. samples, respectively. Based on the obtained fragmentation spectra, polybrominated dihydroxylated diphenoxybenzenes (diOH-PBDPBs), such as hepta-, octa- and nonabrominated diOH-BDPBs, could be identified in both species. To our knowledge, this study is the first report on the environmental presence of OH-PBDPBs.

Keywords: naturally-occurring halogenated compounds, seafood, sea sponges, HO-BDE, polybrominated dihydroxylated diphenoxybenzenes, target screening, suspect and non-target screening

Introduction:

Natural halogenated compounds (NHCs) are a diverse class of compounds commonly detectable in the marine environment and produced by marine animals, plants or bacteria (Gribble, 2003). The most relevant NHC classes found in biota include bromophenols (BPs), bromoanisoles (BAs) and hydroxylated or methoxylated analogues of brominated biphenyls (HO-BBs and MeO-BBs), of polybrominated diphenyl ethers (HO-PBDEs and MeO-PBDEs), and of brominated dibenzo-p-dioxins (HO-BDDs and MeO-BDDs) (Bidleman et al., 2019a; Bidleman et al., 2019b). These brominated compounds may originate both from natural and anthropogenic sources. For example, several BPs are used as brominated flame retardants (BFRs) and precursors for the production of more complex BFRs (Thomsen et al., 2001), but also may occur in red, green and brown algae, as well as sea sponges (Haraguchi et al., 2010; Liu et al., 2011). HO-PBDEs are metabolites of anthropogenic PBDEs, but can also have a natural origin e.g., when produced by marine bacteria (Agarwal et al., 2014). MeO-PBDEs are presumed to be of natural origin only (Hamers et al., 2008; Wan et al., 2009). BDDs are produced as by-products in the industrial processing or thermal decomposition of BFRs, but have also been described to occur in red alga (Malmvarn et al., 2008).

The resistance to chemical and biological degradation of natural and anthropogenic halogenated compounds can lead to their bioaccumulation and biomagnification in the environment. The bioaccumulation and biomagnification potential of PBDEs and their analogies was studied in different aquatic food chains by determining the concentration of PBDEs, HO-PBDEs and MeO-PBDEs in mammals, fish and other invertebrates which represented different trophic levels (Dahlgren et al., 2016; Kim et al., 2015; Sun et al., 2020; Weijs et al., 2009). Recent studies showed that the bioaccumulative potential is highest for MeO-PBDEs, followed by PBDEs and HO-PBDEs, respectively (Losada et al., 2009; Sun et al., 2020). This in turn can lead to biomagnification of NHCs in higher trophic levels of the food chain (Choo et al., 2019). Due to this fact and the structural similarities of NHCs to man-made toxic environmental pollutants (e.g., BFRs), potential toxic effects of NHCs have been a topic of interest during

the last decade (Dingemans et al., 2008; Linares et al., 2015). For example, described toxic effects of OH-PBDEs included disruption of thyroid hormone homeostasis, oxidative phosphorylation disruption, neurotoxic effects and altered estradiol synthesis showing that the investigation of hydroxylated NHCs is of high relevance to access possible toxic effects on humans (Dingemans et al., 2008; Li et al., 2010; Su et al., 2014; Van Boxtel et al., 2007).

Most studies on characterization and quantification of NHCs are carried out applying gas chromatography (GC) coupled to tandem mass spectrometry (MS/MS) or high-resolution mass spectrometry (HRMS). Thereby, the separation of neutral and hydroxylated NHCs is carried out by fractionation within sample preparation followed by derivatization of hydroxylated NHCs to methoxylated derivates prior to analysis (Lacorte et al., 2010; Malmvarn et al., 2005). While this approach shows great selectivity for the detection and identification 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 (Kato et al., 2012).

To address this issue, several methods using liquid chromatography (LC) coupled HRMS or MS/MS for the analysis of hydroxylated NHCs have been introduced in the past (Kato et al., 2012; Lupton et al., 2010; Wang et al., 2011). Thereby, the application of targeted methods allows the tentative identification (and quantification) of known NHCs providing valuable information to access potential human exposure (Kato et al., 2009). 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 (Leon et al., 2019). Previous studies applying NTS for the identification of novel NHCs in biota samples have shown the potential of these techniques (Agarwal et al., 2015; Cariou et al., 2020). Nevertheless, a study combining the advantages of both targeted and non-target approaches using LC-HRMS for the detection and identification of HO-NHCs is still lacking.

Therefore, we aim to comprehensively screen a variety of biota samples using a combination of target, suspect and non-target screening approaches with the purpose to simultaneously detect known NHCs and potentially discover new.

Materials and methods

Chemicals

HPLC-grade methanol and acetonitrile were obtained from Biosolve Chimie SARL (Dieuze, France). A PURELAB Flexsystem was used to obtain ultrapure water (18.2 MΩ cm, Milli-Q, Millipore). Ammonium acetate was purchased from Sigma-Aldrich (eluent additive for LC-MS). Solvents used for sample preparation included ethyl acetate, methanol, isooctane, dichloromethane, diethyl ether, sodium sulphate (all from Merck KGaA, Darmstadt, Germany), hexane (Acros Organics, Geel, Belgium), 1M KOH, ethanol (EtOH) and 1M hydrogen chloride (HCl). 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. Standards available for targeted screening included 14 OH-PBDEs, 19 BPs, 2,2'-diOH-BB80 and 2,2'-diOH-BDE68. The exact compounds including their full name, molecular formula and commercial source are summarized in Table S1. Stock solutions were available at concentrations of 10 µg/mL or 50 µg/mL in acetonitrile. The same solvent was used to prepare working solutions at a concentration of 5 µg/mL and 0.5 µg/mL, which were stored at -20 °C prior to analysis.

Samples

A total of 35 samples were collected from the natural habitat of the species or purchased from the commercial market. For each sample, the sample identification, the sample type, the species, the edibility and the origin are shown in Table S2. Sample 31 was a stored extract which was prepared by the sample preparation method described in Kato et al. (2012). The samples included 24 samples of seaweed and 11 samples of various species such as sea sponge, sea cucumber, oyster, mussel, shrimp

and clams. All investigated seaweed species belong to the class of brown seaweed (*Phaeophyta*), except for sample 35 which was nori, a red seaweed. Among the samples of seaweed, ten samples of *Sargassum sp.*, five samples of *Laminaria sp.*, four samples of *Fucus sp.* and two samples of *Undaria sp.* were obtained. All sea sponge samples belong to the class of *Demospongiae*. Most of the purchased samples were dried at the point of purchase. Samples collected directly from the sea were dried at room temperature or freeze-dried after collection. All dried samples (MT 1 to 25, 31 to 35) were stored at room temperature, and all wet samples (MT 26 to 30) were stored at -20°C.

Sample preparation

For sample preparation a previously established method was used with modifications (Fujii et al., 2014). In brief, 300 to 400 mg of each dry sample and 1000 to 1500 mg of each wet sample were twice extracted with 6 mL of a ethyl acetate:MeOH:DCM (1:1:1, v/v/v) mixture. After centrifugation, the combined supernatants were evaporated to dryness and reconstituted in 5 mL of hexane. One milliliter 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. The hexane fraction was subject of another study and will not be discussed.

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. After vortexing and centrifugation, 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. Due to the complex matrices, samples were diluted with a ratio of 1 to 5 in methanol prior to injection.

A detailed description of the sample preparation procedure can be found in the supporting information. Procedural blank was prepared using the procedure described above.

Instrumentation and LC-QTOF-MS analysis

All measurements were conducted on an Agilent Infintity 1290 UPLC coupled to an Agilent 6530 quadrupole time-of-flight (QTOF) mass spectrometer equipped with an electrospray ionization (ESI) source (Agilent Technologies, Santa Clara, USA). The final chromatographic method included 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 final optimized chromatographic conditions and ion source parameters are summarized in Table S3. The QTOF was operated in negative ionization mode. For sample analysis using target and suspect screening, the Auto MS/MS acquisition mode was applied to allow an automatic selection of precursor ions (max. 4 per cycle). The quadrupole was operated in narrow selection mode (m/z ± 1.3) and fragments were recorded in a mass range of m/z 50 to 1500 applying collision energies of 10, 20 and 40 eV. The monoisotopic mass of the $[M-H]^-$ ion of each target compound (Table S1) was included as preferred for fragmentation. Based on the results of target and suspect screening, the sea sponge samples were selected for further investigation using non-target screening. Therefore, these samples were injected in full scan MS mode (mass range m/z 100-1700) to enhance cluster detection. Additionally, a selection of the most abundant highly brominated compounds detected within non-target screening were analyzed in target MS/MS mode to obtain further structural information from their MS/MS spectra. Therefore, the most abundant ion was chosen as the target m/z value, and the selection mode was set to narrow (m/z ± 1.3).

Data analysis

Targeted screening: The available standards (Table S1) were analyzed under the same conditions as the biota samples. The acquired retention times and fragmentation spectra were summarized in a compound database (using MassHunter PCDL Manager, B.08.00; Agilent Technologies) together with the name, molecular formula and monoisotopic mass of each standard. The data were matched against

all biota samples using MassHunter Qualitative Analysis Software (B.07.00). Matching criteria of ± 0.1 min for the retention time (RT) and ± 5 ppm for the mass error were applied.

Suspect screening: 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. Biphenyls, diphenyl ethers and dibenzodioxins were set with up to a total of 7 bromine and/or chlorine atoms, but only up to 3 chlorine atoms. Phenols were set with all possible combinations with maximum 5 bromine and/or chlorine atoms. The suspect list can be found in Table S4 (shortened overview) and Table S5 (full version). After chromatogram deconvolution and alignment, 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.

Non-target screening: HaloSeeker 1.0 was used to analyze samples acquired in full scan MS mode. This R based open source software allows the identification of halogenated (i.e. chlorinated and/or brominated) compounds based on the characteristic mass differences and relative intensities between $^{35}Cl/^{37}Cl$ and $^{79}Br/^{81}Br$ isotopes, respectively (Leon et al., 2019). After automatic raw data conversion into mzXML format by built in msConvert software version 3.0.9810 (Chambers et al., 2012), 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 and Cl atoms (RT tolerance = 5 s; *m*/*z* tolerance = 0.5 mDa) (Cariou et al., 2016). The results were displayed in MD plots representing the fractional part of an *m*/*z* value using a H/Cl scale instead of the common IUPAC scaling (Taguchi et al., 2010). This allows to distinguish between alkane and halogenated series as latter align on horizontal lines while alkane series show a diagonal alignment. To the obtained MD plots the F2+ filter was applied. This filter represents the fourth level of filtering available within the

HaloSeeker software. While the F0 filter would display all detected features and the F1 filter retain all paired features, the F2 filtering step applies additional rules retaining only paired features showing ion ratios corresponding to halogenated clusters. Lastly, the F2+ filter retains only polyhalogenated clusters. 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. Series of interest which were not fragmented in Auto MS/MS mode were analyzed in target MS/MS mode to obtain further structural MS/MS information.

Results and discussion

Method optimization and quality control

For the optimization of the chromatographic separation, different gradient slopes and four different columns were tested: Poroshell 120 EC-C18 columns with two different dimensions (i.e., 3.0 mm x 50 mm and 3.0 mm x 100 mm, both with 2.7 µm particle size; Agilent Technologies), a Kinetex Biphenyl column (2.1 mm x 50 mm, 1.7 µm particle size; Phenomenex, Utrecht, Netherlands) and a Kinetex Phenylhexyl column (2.1 mm x 50 mm, 1.7 µm particle size; Phenomenex, Utrecht, Netherlands). The aim of method optimization was to achieve a clear separation and satisfactory peak shapes for the two main compound classes covered within target screening (i.e., BPs and OH-PBDEs) rather than the separation of isomers as latter would require long run times as observed in previous studies (Chi et al., 2017). Ultimately, the Kinetex Biphenyl column (2.1 mm x 50 mm, 1.7 µm) gave the best results regarding peak shape and width and was chosen for further analyses. The retention times (RT) and observed fragments of all available standards are summarized in Table S6.

Using the Kinetex Biphenyl column (Phenomenex, Utrecht, Netherlands) several gradients of different lengths and slopes were tested aiming to achieve separation of the partially coeluting bromophenols and isomers of tetrabrominated OH-BDEs (i.e., 2'OH-BDE68, 4'OH-BDE49, 6OH-BDE47, 5OH-BDE47). As flatter, longer gradients did not allow complete isomer separation while having a negative

influence on the peak shapes, the gradient shown in Table S3 was chosen as it allowed a clear separation between BPs and OH-PBDEs, satisfying peak shapes and a short run time.

The IS was detectable in all samples with an average retention time of 8.24 min ± 0.02 min. As the IS which was added prior to sample preparation to indicate potential analyte losses was detectable in all samples, the data of all samples was considered.

Target screening

The retention times and acquired fragmentation spectra (Table S6) of all available standards were summarized in a compound database. The database was matched against all analyzed samples. This allowed the identification of compounds with confidence level (CL) 1 according to Schymanski et al. (Schymanski et al., 2014). However, this assignment was only made if all identifiers (i.e., the retention time, exact mass, fragmentation spectra and isotopic pattern) unequivocally matched the reference data. Since bromine fragments (*m*/*z* 78.9189 and *m*/*z* 80.9168) were the most abundant in all fragmentation spectra and the detection of less abundant fragments in the samples was limited, the unequivocal identification of isomeric structures based on this information is challenging. This is additionally hampered by the limited chromatographic separation of isomers. Also, several samples showed overall low abundant fragmentation spectra and missing ions in the isotopic patterns of target compounds. Latter was observed for low abundant compounds and is especially problematic as the characteristic isotopic pattern of brominated compounds provides valuable information for their identification. These observations did not allow an unequivocal assignment of CL 1. In this case, level 3 was assigned.

To allow inclusion of information about the observed fragments and their abundances in the report of identification certainty, we propose a subdivision of CL 3 in levels 3A to 3D (Table 1). This subdivision was considered useful as it also allows to report differences in the observed isotopic patterns which is of high relevance for the identification of halogenated analytes. Also, the observed abundances of

fragmentation spectra were considered (3A/3B vs. 3C/3D). Thereby, level 3A or 3B was assigned if the most abundant fragment in the fragmentation spectra showed an intensity above 10^3 counts. This threshold was chosen based on empirical observations made within the analysis of samples for target screening. An intensity $<10^3$ counts in fragmentation spectra always correlated with the presence of only bromine fragments leading to limited information on the identity of the compounds and thus a lower identification confidence (i.e., CL 3C/3D).

Table 1: Subdivision of confidence level 3 (Schymanski et al., 2014) proposed for the identification of NHCs within target and suspect screening. For the subdivision, complete (✓) and incomplete (✕) matches of the isotopic pattern as well as the observed intensities of fragmentation spectra were considered. Within target screening a match of the retention time with the corresponding reference standard was necessary (✓).

Confidence level (CL)	Retention time	Fragmentation	Isotopic pattern
CL 3A	✓	High intensity (≥10^3 counts)	✓
CL 3B	✓	High intensity (≥10^3 counts)	✕
CL 3C	✓	Low intensity (≤10^3 counts)	✓
CL 3D	✓	Low intensity (≤10^3 counts)	✕

The results of target screening can be found in Tables S7 (OH-PBDEs in algae samples), S8 (OH-PBDEs in sea sponge samples) and S9 (BPs in all samples). None of the compounds reported there were present in the procedural blanks. From the group of OH-PBDEs and dihydroxylated compounds, 6OH-BDE47 and 2,2-diOH-BB80 were the most frequently detected compounds and were found in eleven and thirteen out of 35 samples, respectively. In invertebrates' samples (MT-25 to MT-30) no compounds were detected within target screening. Therefore, only the results of algae and sea sponge samples are discussed in the following sections.

Algae

6OH-BDE47 was detected in seven alga samples of *Sargassum* sp. and *Laminariaceae* sp. All these samples derived from the Asia/Pacific region. These results are in line with previous studies in which

6OH-BDE47 was one of the most frequently detected compounds in a set of 16 aquatic plant samples collected from Luzon Island, Philippines (Haraguchi et al., 2010).

2,2-diOH-BB80 was detected in ten alga samples (*Sargassum fusiforme*, *Laminariaceae* sp., *Undaria pinnatifida*, *Fucus vesiculosus, Fucus spiralis* and *Himanthalia elongata*). Thereby, *Sargassum fusiforme* and *Laminariaceae* sp. derived from the Asia/Pacific region whilst *Undaria pinnatifida*, *Fucus vesiculosus, Fucus spiralis* and *Himanthalia elongate* were collected in the Atlantic. The detection of 2,2-diOH-BB80 in samples from the Asia/Pacific region again confirms the findings of Haraguchi et al. who reported the presence of 2,2-diOH-BB80 and its dimethoxylated analogue 2,2-diMeO-BB80 in aquatic plant samples from the Philippines (Haraguchi et al., 2010). Additionally, another dihydroxylated compound (2,2-diOH-BDE68) was detected in two samples of edible kombu (*Laminariaceae* sp. and *Laminaria ochroleuca*). These findings can serve as a first indication of a more ubiquitous presence of dihydroxylated and dimethoxylated analogues of BB80 and BDE68 in marine biota from both the Atlantic and the Asia/Pacific.

From the group of BPs (Table S9), monobromophenol was detected in two alga samples (kelp kombu, *Laminaria digitata* and bladderwrack, *Fucus vesiculosus*) which both derived from the Atlantic. However, the identification of the position of bromine was not possible, as isomers of monobromophenol were not available as individual standards. 2,4-Dibromophenol (2,4-DBP) was detected in brown seaweed (Indonesia) and kelp kombu (*Laminaria digitata*, Ireland). 2,4,6-Tribromophenol (2,4,6-TBP) was the most frequently detected BP and was found in 9 alga samples, confirming previous findings. For example, within a study on BPs in a large set of marine algae samples (n = 87), 2,4,6-TBP was reported with a detection frequency (DF) of 100% (Whitfield et al., 1999). However, this study also reported a DF of 100% for 2,4-DBP which was not confirmed by our data. This might indicate a limited sensitivity of the applied sample preparation or instrumental method for the detection of BPs. Further optimizations of the chromatographic conditions and additional reference standards can further improve the results presented here.

294 Sea sponge

295 It must be noted that two of the sea sponge samples, namely MT-11 (*Lamellodysidea* sp.) and MT-31
296 (*Callyspongia* sp.), have previously been screened by Kato et al. for (di)OH-, (di)MeO- and OH-MeO-
297 PBDEs using tandem mass spectrometry (MS/MS) with atmospheric pressure chemical ionization (Kato
298 et al., 2012). Additionally, MT-31 was included in a study on OH- and MeO-PBDEs conducted by
299 Haraguchi et al. using GC-MS/MS (Haraguchi et al., 2011). Therefore, our study aimed to serve as a
300 complementary approach to identify new OH-NHCs through the application of LC-HRMS.
301 The results of target screening of sea sponge samples are summarized in table S8 (OH-PBDEs and
302 dihydroxylated compounds) and table S9 (BPs). From the group of BPs, only 2,4-DBP (CL1) and
303 tetrabromophenol (CL4) as well as 2,4,6-TBP (CL 3D) were detected in *Lamellodysidea* sp. (MT-11)
304 and *Demospongiae* sp. (MT-32), respectively.
305 6OH-BDE47 was detected in all investigated sea sponge samples. These observations are in
306 agreement with the results of target screening on algae samples (see above) as well as previous studies
307 which described 6OH-BDE47 as the predominant compound in sea sponges and species from higher
308 trophic levels (Haraguchi et al., 2011; Wan et al., 2009). Additionally, Haraguchi et al. had identified
309 6OH-BDE47 in *Callyspongia* sp. (MT-31) which was confirmed by our study (Haraguchi et al., 2011).
310 This also applies to the confirmed identification of 2,2'diOH-BDE68. Kato et al. identified a mono-HO
311 and di-HO-tetrabrominated-BDE in *Lamellodysidea* sp. (MT-11). However, due to the unavailability of a
312 reference standard, no unequivocal isomer identification was possible (Kato et al., 2012). Our study
313 therefore provides additional identification confidence by reporting 6OH-BDE47 and 2,2'diOH-BDE68 in
314 the *Lamellodysidea* sp. sample. Furthermore, 4OH-BDE17, 6OH-BDE85, 3OH-BDE154 and
315 2,2'diOH-BB80 were identified in *Lamellodysidea* sp. (MT-11; CL 1 for all compounds, except for
6 3OH-BDE154) which have not been described by Kato et al. Since the focus of their study of was mainly
7 placed on diOH- and diMeO-PBDEs, the mono-OH congeners were not reported (Kato et al., 2012).
8 Our study thus provides additional information on OH-PBDEs and a tetrabrominated di-OH-BB present

in sea sponge samples of *Lamellodysidea* sp. indicating the advantages of the applied comprehensive screening approach.

Within the analysis of the *Lamellodysidea* sp. sample (MT-11), strong matrix effects were observed which were assumed to hamper the detection and identification of lower abundant NHCs. After an additional dilution step (final dilution factor of 25), an additional tribrominated OH-PBDE (3OH-BDE28; CL 3B) was identified which had not been described by Kato et al. (Kato et al., 2012).

The detection of 6OH-BDE47 in samples of *Demospongiae* sp. (MT-32 and MT-33; CL 3B and 1, respectively) confirmed the ubiquitous occurrence of this compound. 6OH-BDE85, 2,2'-diOH-BDE68, and 2,2'-diOH-BB80 were detected in both *Demospongiae* sp. samples with CL 3D, 4 and 3D, respectively. Additionally, 3OH-BDE154 was identified in one of the *Demospongiae* sp. samples (MT-32; CL 3D). Even though these results have to be interpreted carefully since the identified compounds all showed low levels of identification confidence, to our knowledge this is the first report of mono-OH and di-OH BDEs and BBs in sea sponges of *Demospongiae* sp. Further studies are needed to provide higher identification levels as well as quantitative data on the described compounds.

Suspect screening

For suspect screening, the created suspect list (Table S5) was matched against all sample files acquired in Auto MS/MS mode. To further increase identification certainty, only matches with a matching score ≥75 were considered. This matching score was calculated by the data analysis software (Profinder B.08.00; Agilent Technologies, USA) based on the accuracy of the experimental mass and isotopic pattern in comparison to theoretical values. As a matching score ≥75 always included a complete match of the isotopic pattern, confidence levels 3A or 3C were assigned depending on the intensities observed in fragmentation spectra. If no fragmentation spectra were acquired, CL 4 was assigned. As no reference MS or MS/MS library data was available, an assignment of CL 2 was not possible for the compounds identified through suspect screening.

345

346 Algae

347 The compounds detected in algae samples and one invertebrate sample (MT-34; sea cucumber) are
348 summarized in Table S10. The $[M-H]^-$ ion of a hexabrominated diOH-BDE with the molecular formula
349 $C_{12}H_4Br_6O_3$ (denoted as **OH-Br$_6$-BDE**) was detected in three alga samples. The observed isotopic
350 pattern (in comparison with theoretical data), the proposed structure and the fragmentation spectrum of
351 **OH-Br$_6$-BDE** in sample MT-13 (kombu; *Laminaria ochroleuca*) are found in Figure S1. The high match
352 of the isotopic pattern between experimental and theoretical data confirms the number of bromines. The
353 observed fragments with *m*/*z* 248.8548 (proposed formula: $[C_6H_3Br_2O]^-$; Δppm -3.2 ppm) and m/z
354 406.6717 (proposed formula: $[C_6HBr_4]^-$; Δppm -7.1 ppm) indicate two phenolic moieties with two and
355 four bromines, respectively. Additionally, within target screening, the fragment with theoretical *m*/*z*
356 248.8556 ($[C_6H_3Br_2O]^-$) was only observed for standards which showed a hydroxy group in *ortho*- (2,2'-
357 diOH-BDE68) or *meta*-position (e.g., 5OH-BDE47, 3'OH-BDE28). For standards with a hydroxy group
358 in *para*-position, the mentioned fragment was not observed even though a dibrominated phenolic moiety
359 was present (e.g., 4'OH-BDE49). Therefore, the hydroxy group is assumed to be in the *ortho*- or *meta*-
360 position. In sample MT-35, **OH-Br$_6$-BDE** was the most abundant compound whose chromatographic
361 peak showed significant tailing indicating column overload. A further dilution of MT-35 was needed (final
362 dilution factor 25) to achieve satisfying chromatographic results. The observed isotopic pattern and
363 fragmentation spectra were similar to sample MT-13 (data not shown) resulting in the same assigned
364 level of identification confidence (Table S10). In sample MT-12, no fragmentation spectra of
365 **OH-Br$_6$-BDE** could be acquired resulting in an assignment of CL 4.
366 Dihydroxylated PBDEs, such as 2',6-diOH-BDE68, have been reported in sea sponge samples
7 (Haraguchi et al., 2011). A dihydroxylated hexabrominated BDE whose structure corresponds to the
8 mass spectral data observed in this study has been described as a major constituent in the *Dysideidae*
9 sponge family (Hanif et al., 2007). However, dihydroxylated hexabrominated BDEs have not been
0 reported in alga samples yet, even though penta- and hexabrominated OH-PBDEs have been described

as predominant congeners in specific samples from low trophic levels (Dahlgren et al., 2016). To our knowledge, our study is the first report of a dihydroxylated hexabrominated BDE in alga samples. Further studies are necessary to give more insights on possible formation pathways and to increase the identification certainty of some of the reported compounds.

Furthermore, a chlorinated phenol (denoted as **CP-1**) with the molecular formula C_6H_5ClO was detected in samples MT-8 (brown seaweed) and MT-34 (sea cucumber, *Holothuria atra*). The observed RT and mass error can be found in Table S10. Figure S2 shows the match between theoretical and observed isotopic patterns confirming the presence of chlorine. However, due to low abundance of the observed signals no fragmentation spectra were obtained leading to an identification with CL 4.

Sea sponge

Suspect screening of sponge samples yielded 18 identified compounds (Table 2). All these compounds were present in the *Lamellodysidea* sp. sample (MT-11), while eight compounds were identified in sample MT-31 (*Callyspongia* sp.). Compounds assigned with CL 3A which allowed to propose a possible chemical structure based on the observed fragmentation spectra are summarized in Figure 1.

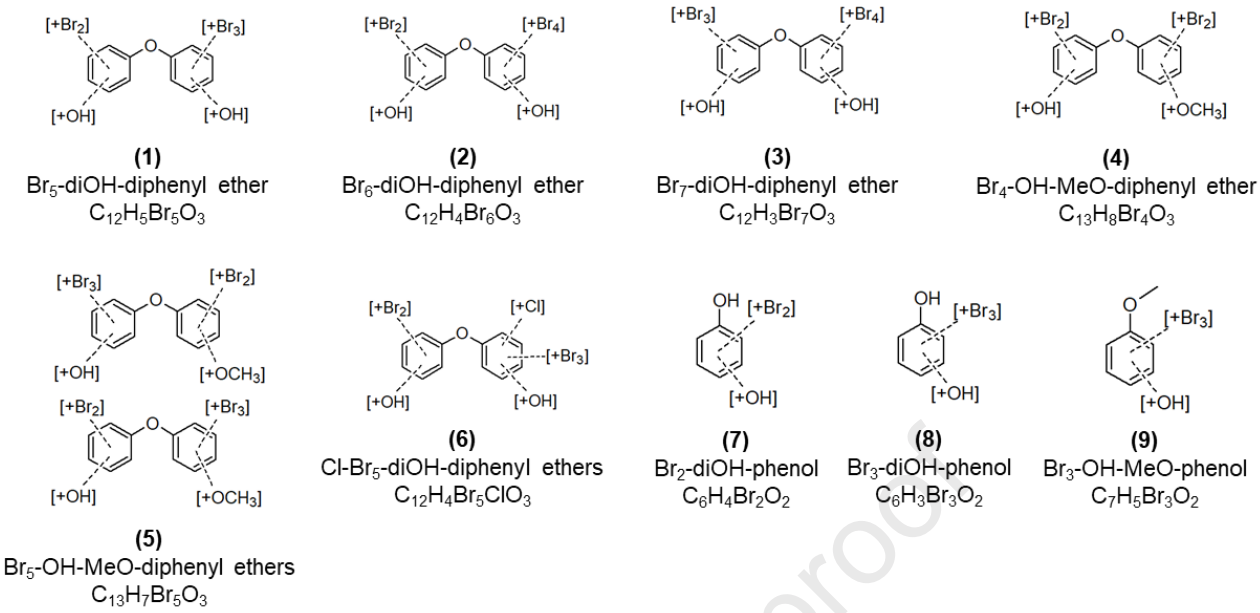

Figure 1: Proposed chemical structures of compounds detected in sea sponge samples through suspect screening and assigned with CL 3A.

In addition to 2.2'-diOH-BDE68 (see target screening), 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 CL 4, the fragmentation spectra of penta- **(1)** and hexabrominated **(2)** diOH-BDE (Figure S3) 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. As discussed for the hexabrominated diOH-BDE (**OH-Br_6-BDE**) in the Algae section above, the characteristic fragment of $[C_6H_3Br_2O]^-$ indicates an *ortho*- or *meta*-position of the hydroxy group. The described fragments also confirm that the hydroxy groups are located on different aromatic rings.

The fragmentation spectrum of diOH-hepta-BDE **(3)** (Figure S4) 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. The fragmentation spectra of penta- **(1)** and hexabrominated **(2)** diOH-BDEs (Figure S5) detected in *Callyspongia* sp. (MT-31) showed similar

fragments as in sample MT-11 allowing the same conclusions as described above. The same applies to the fragmentation spectra of diOH-pentabrominated-BDE in sample MT-33 and diOH-hexabrominated-BDE in samples MT-32 and MT-33, respectively (both *Demospongiae*). There, similar fragments as described above were observed, whereof similar conclusions about the structures of the detected compounds can be drawn.

Table 2: 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.

			MT-11			**MT-31**			**MT-33**			**MT-32**		
			sea sponge			sea sponge			sea sponge			sea sponge		
			Lamellodysidea sp.			*Callyspongia* sp.			*Demospongiae*			*Demospongiae*		
Compound	**Formula**	**Monoisot. mass**	RT [min]	Mass error [ppm]	Conf. level	RT [min]	Mass error [ppm]	Conf. level	RT [min]	Mass error [ppm]	Conf. level	RT [min]	Mass error [ppm]	Conf. level
Br_3-diOH-diphenyl ether	$C_{12}H_7Br_3O_3$	435.7945	5.52	-0.57	4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Br_5-diOH-diphenyl ether	$C_{12}H_5Br_5O_3$	591.6156	5.86 6.74	0.94 -3.44	3A 3A	6.29	-1.59	3A	6.32 6.81	-0.94 -3.22	4 4	6.33	-0.48	3A
Br_6-diOH-diphenyl ether	$C_{12}H_4Br_6O_3$	669.5261	6.69	1.81	3A	6.55	0.79	3A	6.69	-2.73	3A	6.72	-4.2	3A
Br_7-diOH-diphenyl ether	$C_{12}H_3Br_7O_3$	747.4366	7.23	-3.06	3A	7.27	1.4	4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Br_4-OH-MeO-diphenyl ethers	$C_{13}H_8Br_4O_3$	527.7207	7.83	-0.85	3A	7.89	-3.66	4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Br_5-OH-MeO-diphenyl ethers	$C_{13}H_7Br_5O_3$	605.6312	8.10 8.44	-1.15 -5.72	3A 4	8.11 8.45	-2.63 -4.68	4 4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Br_6-OH-MeO-diphenyl ethers	$C_{13}H_6Br_6O_3$	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	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Br_3-OH-diphenyl ether/ Br_5-diOH-biphenyl	$C_{12}H_5Br_5O_2$	575.6206	7.27	-4.58	3C	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cl-Br_5-diOH-diphenyl ethers	$C_{12}H_4Br_5ClO_3$	625.5766	6.36	-1.18	3A	6.48	-3.42	3A	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Br_3-OH-MeO-biphenyl	$C_{13}H_9Br_3O_2$	433.8153	7.87	0.49	4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Br_4-OH-MeO-biphenyl	$C_{13}H_8Br_4O_2$	511.7258	8.48	-3.3	4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cl-Br_3-OH-MeO-biphenyl	$C_{13}H_8Br_3ClO_2$	467.7763	8.29	-4.32	4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cl_2-Br_2-OH-MeO-biphenyls	$C_{13}H_8Br_2Cl_2O_2$	423.8268	8.10	-2.36	4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Br_5-diOH-dibenzo-dioxins	$C_{12}H_3Br_5O_4$	605.5948	5.41	4.04	4	5.45	-2.22	4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Br_2-diOH-phenol	$C_6H_4Br_2O_2$	265.8578	2.87	1.66	3A	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Br_3-diOH-phenol	$C_6H_3Br_3O_2$	343.7683	4.88	-1.31	3A	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Br_3-OH-MeO-phenol	$C_7H_5Br_3O_2$	357.7840	5.37	-5.62	3A	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

In *Lamellodysidea* sp. (MT-11), 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- **(4)** and the first isomer of pentabrominated **(5)** OH-MeO-BDE (Figure S6) 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 **(5)**, 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. (MT-31). However, due to low abundance and thereof no available fragmentation spectra, CL 4 was assigned, not allowing any assumption about the structure of the compounds.

In *Lamellodysidea* sp. (MT-11), 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- **(4)** and the first isomer of pentabrominated **(5)** OH-MeO-BDE (Figure S6) 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 **(5)**, 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. (MT-31). However, due to low abundance and thereof no available fragmentation spectra, CL 4 was assigned, not allowing any assumption about the structure of the compounds.

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 in both *Lamellodysidea* sp. (MT-11) and *Callyspongia* sp. (MT-31) (Kato et al., 2012). Our study has confirmed these findings and provided further information on fragmentation spectra and on the proposed structures of detected compounds. Additionally, our study reports the detection of a OH-MeO-hexabrominated-BDE and a diOH-heptabrominated-BDE in both sponge species. This compound was not detected by the study of Kato et al. (Kato et al., 2012). However, this finding has to be interpreted carefully as the reported compound could only be assigned with CL4.

In addition to the report of 6OH-BDE85 (RT 8.17 min) within target screening, a second compound with the molecular formula $C_{12}H_5Br_5O_2$ (RT 7.27 min) was detected in *Lamellodysidea* sp. (MT-11). The

fragmentation spectra only yielded fragments corresponding to $[Br]^-$. These findings do not allow to unequivocally distinguish whether the observed compound is an additional isomer of a pentabrominated OH-BDE or a diOH-BB.

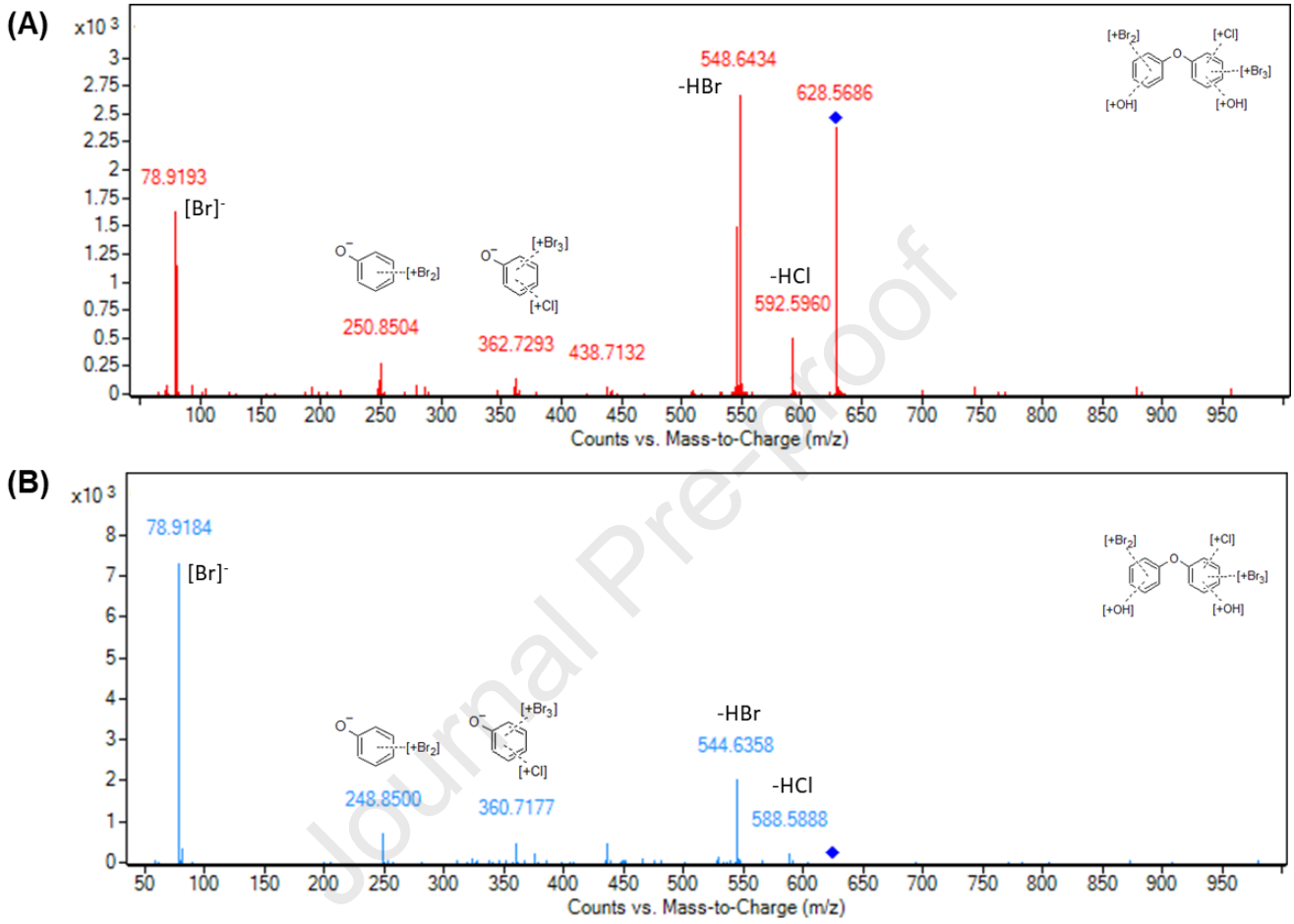

Figure 2: Experimental data of a pentabrominated monochlorinated diOH-BDE with the molecular formula $C_{12}H_4Br_5ClO_3$ detected in sample MT-11, *Lamellodysidea* sp. (A) and MT-31, *Callyspongia* sp. (B). The proposed structure and observed fragmentation spectrum at a collision energy of 40 eV are displayed. The most abundant fragments are assigned with the corresponding neutral losses or proposed structures. The most abundant fragments are assigned with the corresponding neutral losses or proposed structures.

A pentabrominated monochlorinated diOH-BDE **(6)** whose fragmentation spectra are shown in Figure 2 was detected in *Lamellodysidea* sp. (MT-11) and *Callyspongia* sp. (MT-31). 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. Again, the

characteristic fragment of $[C_6H_3Br_2O]^-$ indicates an *ortho*- or *meta*-position of the hydroxy group. To our knowledge, this is the first study reporting a mixed halogenated diOH-BDE in sea sponge species.

Four OH-MeO-BBs with the molecular formulae $C_{13}H_9Br_3O_2$, $C_{13}H_8Br_4O_2$, $C_{13}H_8Br_3ClO_2$ and $C_{13}H_8Br_2Cl_2O_2$ were detected in *Lamellodysidea* sp. (MT-11). Even though no fragmentation spectra were acquired, and CL 4 had to be assigned, it was assumed that the predicted molecular formulae correspond to OH-MeO-BBs and not MeO-BDEs as latter would not be detectable as $[M-H]^-$ ions with ESI due to the lack of a free hydroxy group. The observed limitations of the acquisition of fragmentation spectra for these compounds are assumed to be caused by their limited ionization or low abundance in the sample. Further optimization of the sample preparation and instrumental methods is needed to confirm the postulated findings. The same applies to the detection of a diOH-hexabromo-dibenzo-dioxin ($C_{12}HBr_5O_4$) which was detected in both *Lamellodysidea* sp. (MT-11) and *Callyspongia* sp. (MT-31). As mentioned above, the level of bromination was confirmed by the high match of the isotopic pattern. However, a more confident confirmation of the structure is not possible due to limited mass spectral information.

Last, diOH-dibrominated-BP **(7)**, diOH-tribrominated-BP **(8)** and OH-MeO-tribrominated-BP **(9)** were identified in *Lamellodysidea* sp. (MT-11; all CL 3A). The fragmentation spectra of the dihydroxylated compounds showed fragments corresponding to the loss of HBr, while the fragmentation spectrum of OH-MeO-tribrominated-BP confirmed its methylation by showing a characteristic loss of the methyl group. These findings give insights on new groups of compounds (i.e., diOH-BPs and OH-MeO-BPs) which have not been included in the studies conducted by Kato et al. (2012) and Haraguchi et al. (2011). The high number and variety of confirmed and newly identified compounds in sea sponge samples indicates the high added value of suspect screening analysis by HRMS. Additionally, sea sponge samples of *Lamellodysidea* sp. (MT-11) and *Callyspongia* sp. (MT-31) were analysed by non-target screening with the aim to identify further NHCs which were not included in the suspect list.

Non-target screening results of sea sponge samples

For non-target screening, samples of *Lamellodysidea* sp. (MT-11) and *Callyspongia* sp. (MT-31) were analysed in full scan MS mode to enhance feature detection using HaloSeeker 1.0 software, as a complementary approach to suspect screening. Since the results of non-target screening in the mass range below *m*/*z* 800 mainly yielded compounds which were already reported through target and suspect screening, the analysis was focused on a mass range between *m*/*z* 800 and 1500. Additionally, only polyhalogenated compounds which remained after applying the F2+ filtering within HaloSeeker 1.0 were considered. Molecular formulae were predicted for all detected features. If fragmentation spectra of sufficient quality were available for a feature and fragments could unequivocally be assigned, the most probably candidate was selected from the proposed formulae based on the assigned fragments. For the remaining features, molecular formulae which showed the highest matching score are reported. For *Lamellodysidea* sp. (MT-11), the non-target screening approach yielded 31 compounds (**L1** to **L31**) (Table S11). The corresponding H/Cl-scale can be found in Figure S8. Additionally, fragmentation spectra with satisfying intensities could be acquired for 15 compounds using the target MS/MS mode. Based on the predicted molecular formulae, compounds **L1** to **L4** were identified as hepta-, octa- and nonabrominated dihydroxylated diphenoxybenzene. This was confirmed by analysis of the corresponding masses in target MS/MS mode which yielded fragmentation spectra shown in Figure 3. Based on this information, hepta- ($C_{18}H_7Br_7O_4$, Δppm -2.61 ppm and -3.08 ppm), octa- ($C_{18}H_6Br_8O_4$, Δppm -1.19 ppm) and nonabrominated ($C_{18}H_5Br_9O_4$, Δppm -7.98 ppm) dihydroxylated diphenoxybenzenes were detected in *Lamellodysidea* sp. (MT-11). For heptabrominated dihydroxylated diphenoxybenzene, 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 dihydroxylated diphenoxybenzene, 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 3. To our knowledge, this is the first time that OH-PBDPB are reported in the environment.

For compounds **L5** to **L7**, the observed fragmentation spectra showed low abundances not providing additional structural information. The mass difference between compounds **L6** and **L7** and their diagonal orientation in the H/Cl-plot indicated that their molecular formulae differed by a methylene group. Molecular formulae with the highest matching score and which were in line with the described mass differences were selected and are reported in Table S11.

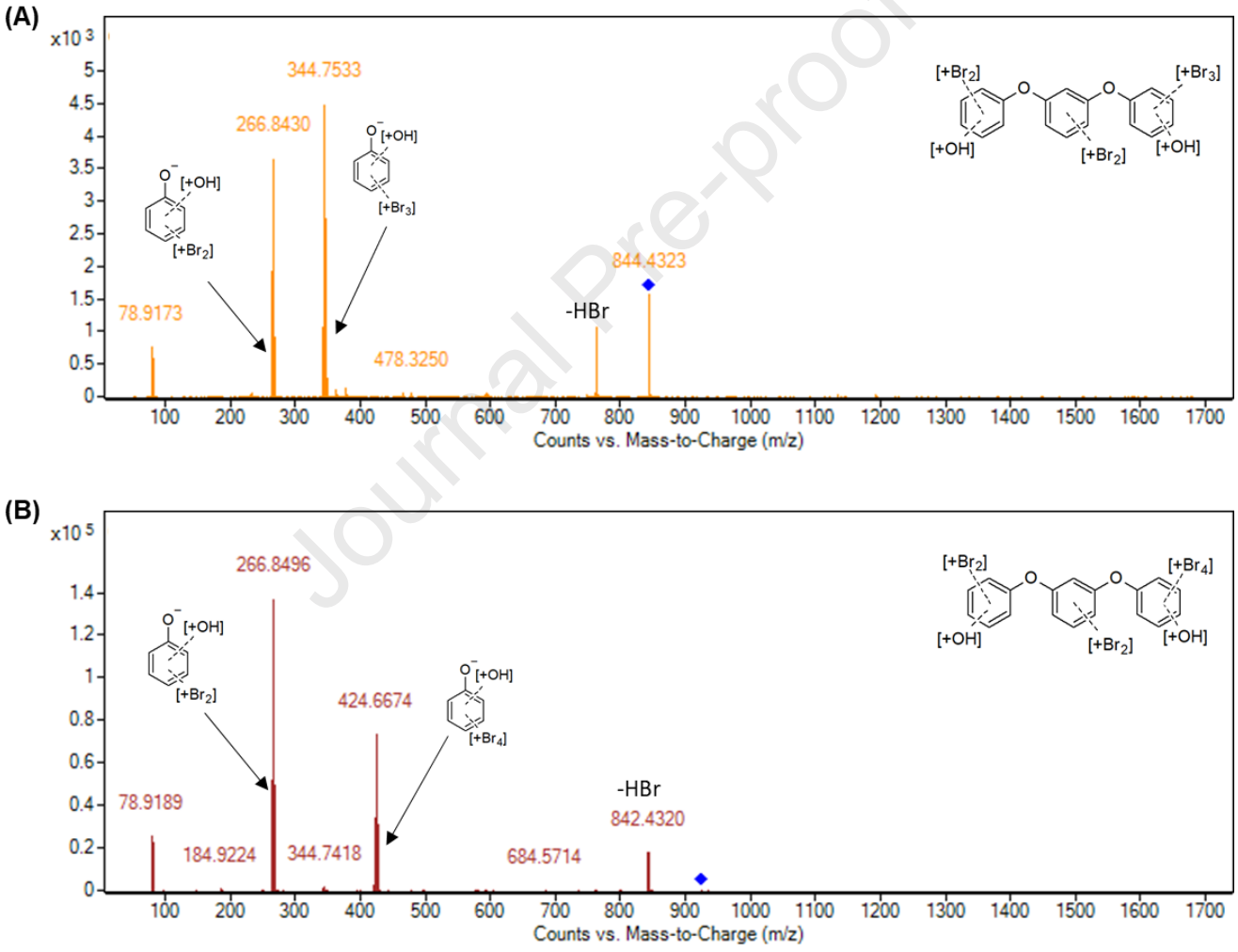

Figure 3: Experimental data and proposed chemical structures of hepta- and octabrominated dihydroxylated diphenoxybenzenes (diOH-BDPBs) with the molecular formulae $C_{18}H_7Br_7O_4$ (A) (**L1**/**L2**) and $C_{18}H_6Br_8O_4$ (B) (**L3**) detected in *Lamellodysidea* sp. (sample MT-11). The most abundant fragments are assigned with the corresponding neutral losses or proposed structures.

The fragmentation spectra of compounds **L8** to **L11** are found in Figures S8 and S9, respectively. All fragmentation spectra showed a fragment corresponding to $[HSO4]^-$ (theoretical *m*/*z* 96.9601) indicating that all four compounds carry at least one sulfate group. Additionally, the fragmentation spectra showed a fragment which could be assigned with the formula $[C_{12}H_4Br_5O_3]^-$. This formula and *m*/*z* corresponded to pentabrominated diOH-BDE. These findings were in line with the predicted formulae of compounds **L8** to **L11** which all included five bromines. The second highly abundant fragment observed in the fragmentation spectra was assumed to represent the non-brominated part of the molecule and differed by a methylene group when comparing compounds **L10** and **L11**. However, the acquired data did not allow a further characterization of the structure and the proposal of a structural formula. Compound **L12** showed similar fragmentation as described for compounds **L8** to **L11** (data not shown) with a fragment which was assigned with the formula $[C_{12}H_3Br_6O_3]^-$ (theoretical m/z 674.5127) indicating that compound **L12** carries an additional bromine in comparison to compound **L11**. This was confirmed by the fact that both compounds clustered on the same horizontal line in the H/Cl-plot.

Based on the horizontal clustering of compounds **L13** to **L16** in the H/Cl-plot, they were assumed to have similar molecular formulae with an increasing number of bromines. Compounds **L14**, **L15** and **L16** showed only two fragments within their fragmentation spectra, of which one corresponded to $[Br]^-$. The second fragment was *m*/*z* 528.6638 for **L14** and *m*/*z* 608.5827 for **L15** and **L16**, respectively. This confirmed that the compounds differed by a bromine but did not allow further assumptions about the structures. Therefore, molecular formula which showed the highest matching score and corresponded to the observed increasing degree of bromination were assigned to compounds **L13** and **L16** (see Table S11).

Compounds **L19** and **L20** differed by two bromine atoms. While no satisfying fragmentation spectrum was obtained for compound **L19**, the fragmentation spectrum of compound **L20** showed two fragments of which one corresponded to $[Br]^-$. The second fragment with *m*/*z* 530.7074 could not unequivocally be assigned with a formula or structure. Therefore, the same approach as described previously was applied for the selection of predicted molecular formulae.

For the remaining compounds (i.e., **L17**, **L18**, **L21 - L31**) the information received from the predicted formulae or fragmentation spectra were not sufficient to allow a proposal of a molecular structure. Therefore, for these compounds the molecular formulae which showed the highest matching scores are reported (Table S11). Further analyses are needed to provide additional information on these compounds.

Non-target analysis of *Callyspongia sp.* (MT-31) yielded 20 compounds (**C1** to **C20**) which are summarized in Table S12. The corresponding H/Cl-plot can be found in Figure S10. Fragmentation spectra with satisfying intensities could be acquired for 9 compounds using the target MS/MS mode. Similar to the results observed for *Lamellodysidea* sp. (MT-11), in *Callyspongia* sp. (MT-31) compounds **C1** to **C4** were identified as hepta-, octa- and nonabrominated dihydroxylated diphenoxybenzene. The fragmentation spectra obtained for hepta- and octabrominated dihydroxylated diphenoxybenzenes (Figure S11) showed fragments identical to the ones observed for sample MT-11. Therefore, the same assumptions regarding the molecular structures and distribution of bromine as described above can be made. Additionally, a heptabrominated monochlorinated dihydroxylated diphenoxybenzene (compound **C5**) with the molecular formula $C_{18}H_6Br_7ClO_4$ was detected. 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-diphenoxybenzenes indicating the same degree of halogenation. Additionally, the experimental and theoretical isotopic patterns (Δppm -8.15 ppm; Figure S12) showed a satisfying fit. The obtained fragmentation spectrum of compound **C5** is shown in Figure 4. Similar to the results obtained for hepta- and octabrominated diOH-BDPBs, a fragment with the molecular formulae $[C_6H_3Br_2O_2]^-$ (theoretical *m*/*z* 266.8485) was observed. A fragment which was assigned with the formula $[C_6HBr_3ClO_2]^-$ (theoretical *m*/*z* 380.7178) confirmed that one of the aromatic moieties is carrying one chlorine atom.

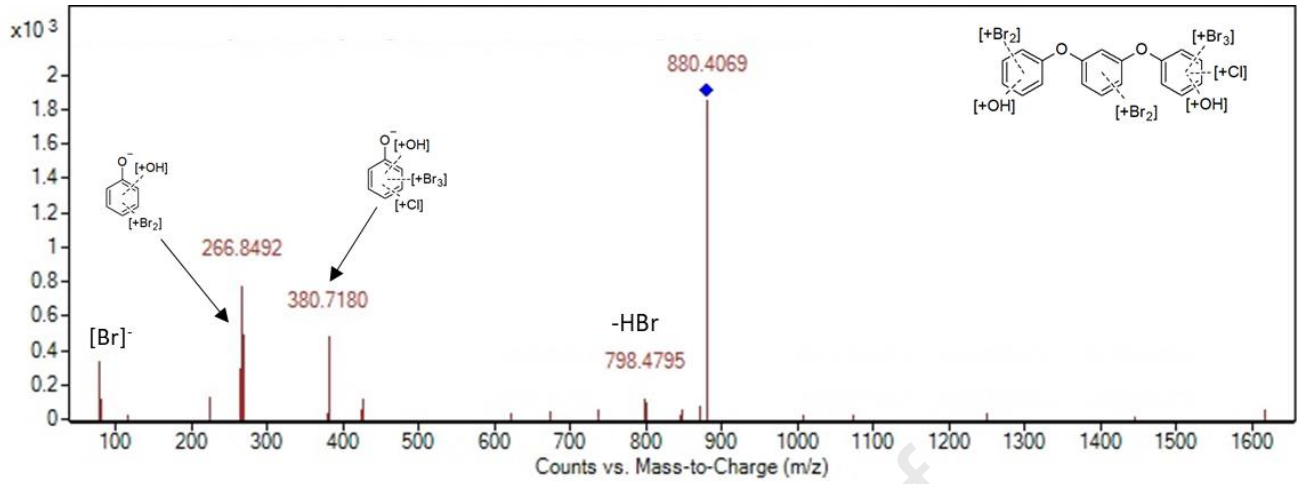

Figure 4: Experimental data and proposed chemical structure of a heptabrominated monochlorinated dihydroxylated diphenoxybenzene (diOH-BDPBs) with the molecular formula $C_{18}H_6Br_7ClO_4$ (C5) *Callyspongia sp.* (MT-31). The most abundant fragments are assigned with the corresponding neutral losses or proposed structures.

Based on the grouping in the H/Cl-plot and the observed mass differences, compounds **C9** and **C10** were assumed to differ by one bromine atom. As described for compounds **L14** to **L16** in *Lamellodysidea* sp. (MT-11), the fragmentation spectra obtained for compounds **C9** and **C10** in *Callyspongia* sp. (MT-31) again showed only two fragments (namely, bromine and *m*/*z* 528.6691 for compound **C9** and *m*/*z* for compound **C10**) not providing further structural information.

Compound **C8** was assigned with molecular formula $C_{24}H_8Br_{10}O_5$ which showed a satisfying match between theoretical and experimental isotopic patterns (Δppm -4.92 ppm; Figure S13). This formula was assumed to correspond to a decabrominated compound consisting of four aromatic rings which are linked by ether bonds and of which two carry a hydroxy group. This indicates another novel compound consisting of several aromatic rings detected in *Callyspongia* sp. However, there was no fragmentation spectra available to confirm the proposed structure.

For the remaining compounds available fragmentation spectra did not provide sufficient information for the assignment of probable structural formulae. Therefore, all remaining compounds (i.e., **C5** - **C8**, **C11** - **C20**) were assigned with molecular formulae which showed the highest matching scores.

In conclusion, nine compounds were detected in both sponge samples showing similar isotopic patterns and retention times. Accordingly, 22 compounds which were unique to *Lamellodysidea* sp. (sample MT-11) and 11 compounds which were unique to *Callyspongia* sp. (MT 31) are reported.

Conclusions

This study introduced a comprehensive combined screening approach applying target, suspect and non-target screening for the identification of new hydroxylated NHCs in biota samples. The use of liquid chromatography high-resolution mass spectrometry allowed the detection of a high variety of NHCs from different classes.

Target screening allowed the identification of seven compounds with 6OH-BDE47 being the most frequently detected compound. Additionally, the reported results showed the identification of two dihydroxylated compounds (2,2-diOH-BB80 and 2,2-diOH-BDE68) in matrices in which they have not been reported previously. The confirmation of previous results from quantitative studies showed that the approach of target screening gives a reliable overview of NHCs in biota samples with high confidence levels of identification.

Suspect screening yielded two compounds detected in alga samples (hexabrominated diOH-diphenyl ether and monochlorinated phenol). A high number of compounds was 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 additional and 16 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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Highlights:

- HRMS and suspect screening used to identify naturally occurring halogenated compounds
- First report of 2,2-diOH-BB80 in alga samples derived from the Atlantic
- Newly identified hydroxylated BDEs in sea sponge
- First detection of polybrominated dihydroxylated diphenoxybenzenes (diOH-PBDPBs)

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: