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

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## Credit author statement

**Lidia Belova:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft preparation, Visualization

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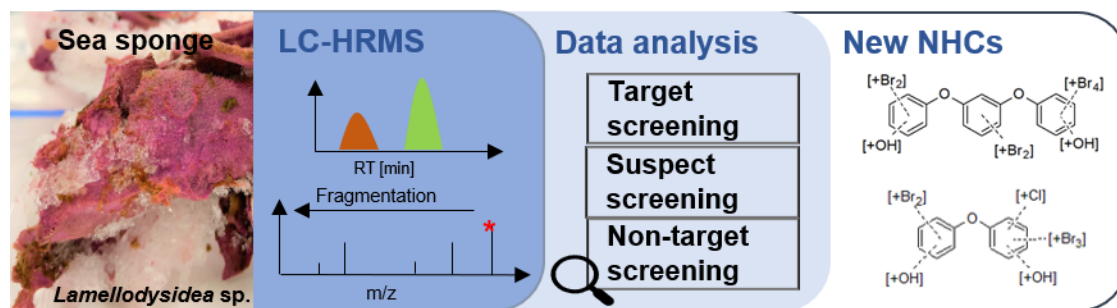
**Monika Śmielowska:** Methodology, Formal analysis, Investigation, Writing – review and editing

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Journal Pre-proof

## Naturally occurring halogenated compounds (NHCs)



Journal Pre-proof



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

3  
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14 **Abstract:**

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

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

36

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

39

**40 Introduction:**

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

55  
56 The resistance to chemical and biological degradation of natural and anthropogenic halogenated  
57 compounds can lead to their bioaccumulation and biomagnification in the environment. The  
58 bioaccumulation and biomagnification potential of PBDEs and their analogies was studied in different  
59 aquatic food chains by determining the concentration of PBDEs, HO-PBDEs and MeO-PBDEs in  
60 mammals, fish and other invertebrates which represented different trophic levels (Dahlgren et al., 2016;  
61 Kim et al., 2015; Sun et al., 2020; Weijs et al., 2009). Recent studies showed that the bioaccumulative  
2 potential is highest for MeO-PBDEs, followed by PBDEs and HO-PBDEs, respectively (Losada et al.,  
3 2009; Sun et al., 2020). This in turn can lead to biomagnification of NHCs in higher trophic levels of the  
4 food chain (Choo et al., 2019). Due to this fact and the structural similarities of NHCs to man-made toxic  
5 environmental pollutants (e.g., BFRs), potential toxic effects of NHCs have been a topic of interest during



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

71

72 Most studies on characterization and quantification of NHCs are carried out applying gas  
73 chromatography (GC) coupled to tandem mass spectrometry (MS/MS) or high-resolution mass  
74 spectrometry (HRMS). Thereby, the separation of neutral and hydroxylated NHCs is carried out by  
75 fractionation within sample preparation followed by derivatization of hydroxylated NHCs to methoxylated  
76 derivates prior to analysis (Lacorte et al., 2010; Malmvarn et al., 2005). While this approach shows great  
77 selectivity for the detection and identification of methoxylated NHCs, the identification of e.g. HO-MeO-  
78 PBDEs or HO-MeO-BBs can be hampered by the presence of corresponding dihydroxy analogues and  
79 vice versa (Kato et al., 2012).

80 To address this issue, several methods using liquid chromatography (LC) coupled HRMS or MS/MS for  
81 the analysis of hydroxylated NHCs have been introduced in the past (Kato et al., 2012; Lupton et al.,  
82 2010; Wang et al., 2011). Thereby, the application of targeted methods allows the tentative identification  
83 (and quantification) of known NHCs providing valuable information to access potential human exposure  
84 (Kato et al., 2009). However, this approach leaves many unknown potentially bioactive or toxic NHCs  
85 undetected. The use of HRMS for suspect or non-target screening (NTS) approaches allows the  
86 simultaneous analysis of a high number of compounds. Thereby, the application of novel bioinformatics  
87 tools, such as HaloSeeker, enables a selective detection of halogenated compounds based on their  
8 specific isotopic patterns (Leon et al., 2019). Previous studies applying NTS for the identification of novel  
9 NHCs in biota samples have shown the potential of these techniques (Agarwal et al., 2015; Cariou et  
0 al., 2020). Nevertheless, a study combining the advantages of both targeted and non-target approaches  
1 using LC-HRMS for the detection and identification of HO-NHCs is still lacking.



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

95

## 96 **Materials and methods**

### 97 **Chemicals**

98 HPLC-grade methanol and acetonitrile were obtained from Biosolve Chimie SARL (Dieuze, France). A  
99 PURELAB Flexsystem was used to obtain ultrapure water (18.2 M $\Omega$  cm, Milli-Q, Millipore). Ammonium  
100 acetate was purchased from Sigma-Aldrich (eluent additive for LC-MS). Solvents used for sample  
101 preparation included ethyl acetate, methanol, isooctane, dichloromethane, diethyl ether, sodium  
102 sulphate (all from Merck KGaA, Darmstadt, Germany), hexane (Acros Organics, Geel, Belgium), 1M  
103 KOH, ethanol (EtOH) and 1M hydrogen chloride (HCl). 4-hydroxy-polychlorinated biphenyl-159 (4-OH-  
104 PCB159) was used as internal standard and d18-gamma-hexabromocyclododecane (d18- $\gamma$ -HBCD) as  
105 recovery standard, respectively. Both standards were provided by Wellington Laboratories, Canada.  
106 Standards available for targeted screening included 14 OH-PBDEs, 19 BPs, 2,2'-diOH-BB80 and 2,2'-  
107 diOH-BDE68. The exact compounds including their full name, molecular formula and commercial source  
108 are summarized in Table S1. Stock solutions were available at concentrations of 10  $\mu$ g/mL or 50  $\mu$ g/mL  
109 in acetonitrile. The same solvent was used to prepare working solutions at a concentration of 5  $\mu$ g/mL  
110 and 0.5  $\mu$ g/mL, which were stored at -20 °C prior to analysis.

111

### 112 **Samples**

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





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

### 126 **Sample preparation**

127 For sample preparation a previously established method was used with modifications (Fujii et al., 2014).  
128 In brief, 300 to 400 mg of each dry sample and 1000 to 1500 mg of each wet sample were twice extracted  
129 with 6 mL of a ethyl acetate:MeOH:DCM (1:1:1, v/v/v) mixture. After centrifugation, the combined  
130 supernatants were evaporated to dryness and reconstituted in 5 mL of hexane. One milliliter of 1M  
131 KOH:EtOH (7:3, v/v) was added. After vortexing and centrifugation, the hexane fraction was transferred  
132 to another glass tube. The process was repeated with 5 mL of hexane. The hexane fraction was subject  
133 of another study and will not be discussed.

134 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)  
135 fraction. After vortexing and centrifugation, the hexane:diethylether (1:1, v/v) fraction was transferred to  
136 another glass tube and the procedure was repeated. The combined fractions were concentrated to  
137 nearly dryness and reconstituted in 50 µL of recovery standard (d18-γ-HBCD, 150 pg/µL) and 150 µL  
138 of MeOH. Due to the complex matrices, samples were diluted with a ratio of 1 to 5 in methanol prior to  
139 injection.

0 A detailed description of the sample preparation procedure can be found in the supporting information.

1 Procedural blank was prepared using the procedure described above.

### 3 **Instrumentation and LC-QTOF-MS analysis**



144 All measurements were conducted on an Agilent Infinity 1290 UPLC coupled to an Agilent 6530  
145 quadrupole time-of-flight (QTOF) mass spectrometer equipped with an electrospray ionization (ESI)  
146 source (Agilent Technologies, Santa Clara, USA). The final chromatographic method included a Kinetex  
147 Biphenyl column (2.1 mm x 50 mm, 1.7  $\mu\text{m}$  particle size; Phenomenex, Utrecht, Netherlands) equipped  
148 with a SecurityGuard™ ULTRA guard column (i.d. 2.1 mm; Phenomenex, Utrecht, Netherlands) with the  
149 same stationary phase. The mobile phases consisted of water with 5 mM ammonium acetate (A) and  
150 methanol (B). The final optimized chromatographic conditions and ion source parameters are  
151 summarized in Table S3. The QTOF was operated in negative ionization mode. For sample analysis  
152 using target and suspect screening, the Auto MS/MS acquisition mode was applied to allow an automatic  
153 selection of precursor ions (max. 4 per cycle). The quadrupole was operated in narrow selection mode  
154 ( $m/z \pm 1.3$ ) and fragments were recorded in a mass range of  $m/z$  50 to 1500 applying collision energies  
155 of 10, 20 and 40 eV. The monoisotopic mass of the  $[\text{M-H}]^-$  ion of each target compound (Table S1) was  
156 included as preferred for fragmentation. Based on the results of target and suspect screening, the sea  
157 sponge samples were selected for further investigation using non-target screening. Therefore, these  
158 samples were injected in full scan MS mode (mass range  $m/z$  100-1700) to enhance cluster detection.  
159 Additionally, a selection of the most abundant highly brominated compounds detected within non-target  
160 screening were analyzed in target MS/MS mode to obtain further structural information from their MS/MS  
161 spectra. Therefore, the most abundant ion was chosen as the target  $m/z$  value, and the selection mode  
162 was set to narrow ( $m/z \pm 1.3$ ).

### 164 **Data analysis**

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



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

171

172 *Suspect screening:* A suspect list was developed which contained 1) mono-hydroxylated, 2) di-  
173 hydroxylated, and 3) mono-hydroxylated and mono-methoxylated compounds of BPs, BBs, BDEs, and  
174 BDDs. Biphenyls, diphenyl ethers and dibenzodioxins were set with up to a total of 7 bromine and/or  
175 chlorine atoms, but only up to 3 chlorine atoms. Phenols were set with all possible combinations with  
176 maximum 5 bromine and/or chlorine atoms. The suspect list can be found in Table S4 (shortened  
177 overview) and Table S5 (full version). After chromatogram deconvolution and alignment, the suspect list  
178 was matched against the analyzed samples using the “targeted feature extraction” algorithm of the  
179 Profinder software (B.08.00; Agilent Technologies, USA). Thereby, matching criteria of  $\pm 10$  ppm for the  
180 mass error and  $\pm 0.2$  min for RT alignment were used.

181

182 *Non-target screening:* HaloSeeker 1.0 was used to analyze samples acquired in full scan MS mode.  
183 This R based open source software allows the identification of halogenated (i.e. chlorinated and/or  
184 brominated) compounds based on the characteristic mass differences and relative intensities between  
185  $^{35}\text{Cl}/^{37}\text{Cl}$  and  $^{79}\text{Br}/^{81}\text{Br}$  isotopes, respectively (Leon et al., 2019). After automatic raw data conversion  
186 into mzXML format by built in msConvert software version 3.0.9810 (Chambers et al., 2012), peak  
187 picking was performed applying the xcms package (version 3.2.0;  $m/z$  tolerance = 3; peakwidth = 5-60;  
188 prefilter step = 3; prefilter level = 10 000; sntresh = 10). This provided a list of extracted signals (i.e.,  
189 features) which was paired based on the specific mass differences and isotopic patterns of C, Br and Cl  
190 atoms (RT tolerance = 5 s;  $m/z$  tolerance = 0.5 mDa) (Cariou et al., 2016). The results were displayed  
1 in MD plots representing the fractional part of an  $m/z$  value using a H/Cl scale instead of the common  
2 IUPAC scaling (Taguchi et al., 2010). This allows to distinguish between alkane and halogenated series  
3 as latter align on horizontal lines while alkane series show a diagonal alignment. To the obtained MD  
4 plots the F2+ filter was applied. This filter represents the fourth level of filtering available within the



195 HaloSeeker software. While the F0 filter would display all detected features and the F1 filter retain all  
196 paired features, the F2 filtering step applies additional rules retaining only paired features showing ion  
197 ratios corresponding to halogenated clusters. Lastly, the F2+ filter retains only polyhalogenated clusters.  
198 For the obtained series, molecular formula (selected elements: H, C, O, Cl, Br, S;  $m/z$  tolerance = 10  
199 mDa; relative abundance tolerance = 20%) were predicted. Series of interest which were not fragmented  
200 in Auto MS/MS mode were analyzed in target MS/MS mode to obtain further structural MS/MS  
201 information.

202

## 203 **Results and discussion**

### 204 **Method optimization and quality control**

205 For the optimization of the chromatographic separation, different gradient slopes and four different  
206 columns were tested: Poroshell 120 EC-C18 columns with two different dimensions (i.e., 3.0 mm x  
207 50 mm and 3.0 mm x 100 mm, both with 2.7  $\mu\text{m}$  particle size; Agilent Technologies), a Kinetex Biphenyl  
208 column (2.1 mm x 50 mm, 1.7  $\mu\text{m}$  particle size; Phenomenex, Utrecht, Netherlands) and a Kinetex  
209 Phenylhexyl column (2.1 mm x 50 mm, 1.7  $\mu\text{m}$  particle size; Phenomenex, Utrecht, Netherlands). The  
210 aim of method optimization was to achieve a clear separation and satisfactory peak shapes for the two  
211 main compound classes covered within target screening (i.e., BPs and OH-PBDEs) rather than the  
212 separation of isomers as latter would require long run times as observed in previous studies (Chi et al.,  
213 2017). Ultimately, the Kinetex Biphenyl column (2.1 mm x 50 mm, 1.7  $\mu\text{m}$ ) gave the best results  
214 regarding peak shape and width and was chosen for further analyses. The retention times (RT) and  
215 observed fragments of all available standards are summarized in Table S6.

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



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

222

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

226

### 227 **Target screening**

228 The retention times and acquired fragmentation spectra (Table S6) of all available standards were  
229 summarized in a compound database. The database was matched against all analyzed samples. This  
230 allowed the identification of compounds with confidence level (CL) 1 according to Schymanski et al.  
231 (Schymanski et al., 2014). However, this assignment was only made if all identifiers (i.e., the retention  
232 time, exact mass, fragmentation spectra and isotopic pattern) unequivocally matched the reference  
233 data. Since bromine fragments ( $m/z$  78.9189 and  $m/z$  80.9168) were the most abundant in all  
234 fragmentation spectra and the detection of less abundant fragments in the samples was limited, the  
235 unequivocal identification of isomeric structures based on this information is challenging. This is  
236 additionally hampered by the limited chromatographic separation of isomers. Also, several samples  
237 showed overall low abundant fragmentation spectra and missing ions in the isotopic patterns of target  
238 compounds. Latter was observed for low abundant compounds and is especially problematic as the  
239 characteristic isotopic pattern of brominated compounds provides valuable information for their  
240 identification. These observations did not allow an unequivocal assignment of CL 1. In this case, level  
241 3 was assigned.

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



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

252

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

Confidence level (CL)	Retention time	Fragmentation	Isotopic pattern
CL 3A	✓	High intensity ( $\geq 10^3$ counts)	✓
CL 3B	✓	High intensity ( $\geq 10^3$ counts)	✗
CL 3C	✓	Low intensity ( $\leq 10^3$ counts)	✓
CL 3D	✓	Low intensity ( $\leq 10^3$ counts)	✗

257

258 The results of target screening can be found in Tables S7 (OH-PBDEs in algae samples), S8 (OH-  
 259 PBDEs in sea sponge samples) and S9 (BPs in all samples). None of the compounds reported there  
 260 were present in the procedural blanks. From the group of OH-PBDEs and dihydroxylated compounds,  
 261 6OH-BDE47 and 2,2-diOH-BB80 were the most frequently detected compounds and were found in  
 262 eleven and thirteen out of 35 samples, respectively. In invertebrates' samples (MT-25 to MT-30) no  
 263 compounds were detected within target screening. Therefore, only the results of algae and sea sponge  
 4 samples are discussed in the following sections.

#### 5 Algae

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

268 6OH-BDE47 was one of the most frequently detected compounds in a set of 16 aquatic plant samples  
269 collected from Luzon Island, Philippines (Haraguchi et al., 2010).

270 2,2-diOH-BB80 was detected in ten alga samples (*Sargassum fusiforme*, *Laminariaceae* sp., *Undaria*  
271 *pinnatifida*, *Fucus vesiculosus*, *Fucus spiralis* and *Himanthalia elongata*). Thereby, *Sargassum*  
272 *fusiforme* and *Laminariaceae* sp. derived from the Asia/Pacific region whilst *Undaria pinnatifida*, *Fucus*  
273 *vesiculosus*, *Fucus spiralis* and *Himanthalia elongata* were collected in the Atlantic. The detection of  
274 2,2-diOH-BB80 in samples from the Asia/Pacific region again confirms the findings of Haraguchi et al.  
275 who reported the presence of 2,2-diOH-BB80 and its dimethoxylated analogue 2,2-diMeO-BB80 in  
276 aquatic plant samples from the Philippines (Haraguchi et al., 2010). Additionally, another dihydroxylated  
277 compound (2,2-diOH-BDE68) was detected in two samples of edible kombu (*Laminariaceae* sp. and  
278 *Laminaria ochroleuca*). These findings can serve as a first indication of a more ubiquitous presence of  
279 dihydroxylated and dimethoxylated analogues of BB80 and BDE68 in marine biota from both the Atlantic  
280 and the Asia/Pacific.

281

282 From the group of BPs (Table S9), monobromophenol was detected in two alga samples (kelp kombu,  
283 *Laminaria digitata* and bladderwrack, *Fucus vesiculosus*) which both derived from the Atlantic. However,  
284 the identification of the position of bromine was not possible, as isomers of monobromophenol were not  
285 available as individual standards. 2,4-Dibromophenol (2,4-DBP) was detected in brown seaweed  
286 (Indonesia) and kelp kombu (*Laminaria digitata*, Ireland). 2,4,6-Tribromophenol (2,4,6-TBP) was the  
287 most frequently detected BP and was found in 9 alga samples, confirming previous findings. For  
288 example, within a study on BPs in a large set of marine algae samples (n = 87), 2,4,6-TBP was reported  
289 with a detection frequency (DF) of 100% (Whitfield et al., 1999). However, this study also reported a DF  
0 of 100% for 2,4-DBP which was not confirmed by our data. This might indicate a limited sensitivity of  
1 the applied sample preparation or instrumental method for the detection of BPs. Further optimizations  
2 of the chromatographic conditions and additional reference standards can further improve the results  
3 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





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

321

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

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

334

### 335 **Suspect screening**

336 For suspect screening, the created suspect list (Table S5) was matched against all sample files acquired  
337 in Auto MS/MS mode. To further increase identification certainty, only matches with a matching score  
338  $\geq 75$  were considered. This matching score was calculated by the data analysis software (Profinder  
339 B.08.00; Agilent Technologies, USA) based on the accuracy of the experimental mass and isotopic  
340 pattern in comparison to theoretical values. As a matching score  $\geq 75$  always included a complete match  
1 of the isotopic pattern, confidence levels 3A or 3C were assigned depending on the intensities observed  
2 in fragmentation spectra. If no fragmentation spectra were acquired, CL 4 was assigned. As no reference  
3 MS or MS/MS library data was available, an assignment of CL 2 was not possible for the compounds  
4 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<sub>6</sub>-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<sub>6</sub>-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:  $[C_6H_3Br_2O]^-$ ;  $\Delta$ ppm -3.2 ppm) and  $m/z$   
354 406.6717 (proposed formula:  $[C_6HBr_4]^-$ ;  $\Delta$ ppm -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<sub>6</sub>-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<sub>6</sub>-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



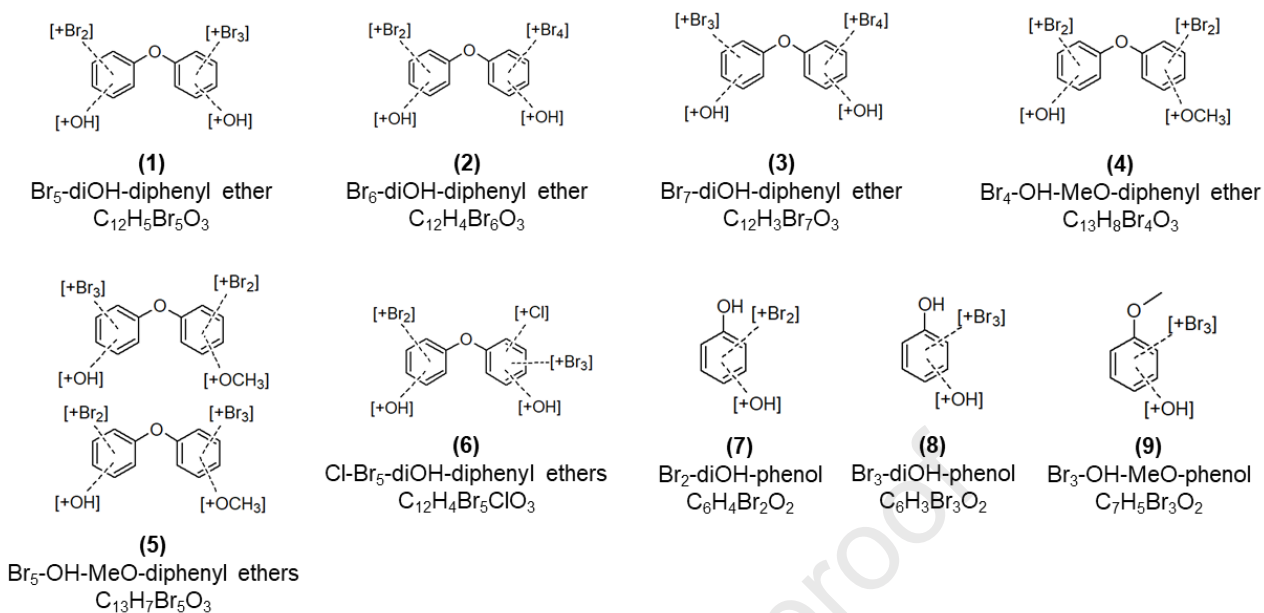
371 as predominant congeners in specific samples from low trophic levels (Dahlgren et al., 2016). To our  
372 knowledge, our study is the first report of a dihydroxylated hexabrominated BDE in alga samples. Further  
373 studies are necessary to give more insights on possible formation pathways and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

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

380

#### 381 Sea sponge

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



386

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

389 In addition to 2,2'-diOH-BDE68 (see target screening), tri-, penta-, hexa- and heptabrominated  
 390 diOH-BDEs were identified in *Lamellodysidea* sp. (MT-11). Whilst there were no fragmentation spectra  
 391 available for diOH-tribrominated-BDE resulting in CL 4, the fragmentation spectra of penta- **(1)** and  
 392 hexabrominated **(2)** diOH-BDE (Figure S3) showed a characteristic fragment of [C<sub>6</sub>H<sub>3</sub>Br<sub>2</sub>O]<sup>-</sup> (theoretical  
 393 *m/z* 248.8556) indicating that both compounds carry a dibrominated phenolic moiety. Accordingly,  
 394 fragments corresponding to a tri- and tetrabrominated phenolic moiety were detected in the penta- and  
 395 hexabrominated diOH-BDE, respectively. As discussed for the hexabrominated diOH-BDE (**OH-Br<sub>6</sub>-**  
 396 **BDE**) in the Algae section above, the characteristic fragment of [C<sub>6</sub>H<sub>3</sub>Br<sub>2</sub>O]<sup>-</sup> indicates an *ortho*- or *meta*-  
 397 position of the hydroxy group. The described fragments also confirm that the hydroxy groups are located  
 398 on different aromatic rings.

399 The fragmentation spectrum of diOH-hepta-BDE **(3)** (Figure S4) showed fragments of tetra- and  
 0 tribrominated phenolic moieties, again indicating the positioning of hydroxy groups on different aromatic  
 1 rings. The fragmentation spectra of all diOH-BDEs showed fragments corresponding to [Br]<sup>-</sup> and a loss  
 2 of HBr confirming the bromination of the parent compounds. The fragmentation spectra of penta- **(1)**  
 3 and hexabrominated **(2)** diOH-BDEs (Figure S5) detected in *Callyspongia* sp. (MT-31) showed similar

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

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409 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  
 410 are reported. n.d. = not detected.

Compound	Formula	Monoisot. mass	MT-11			MT-31			MT-33			MT-32		
			sea sponge			sea sponge			sea sponge			sea sponge		
			<i>Lamellodysidea</i> sp.			<i>Callyspongia</i> sp.			<i>Demospongiae</i>			<i>Demospongiae</i>		
			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 <sub>3</sub> -diOH-diphenyl ether	C <sub>12</sub> H <sub>7</sub> Br <sub>3</sub> O <sub>3</sub>	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 <sub>5</sub> -diOH-diphenyl ether	C <sub>12</sub> H <sub>5</sub> Br <sub>5</sub> O <sub>3</sub>	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 <sub>6</sub> -diOH-diphenyl ether	C <sub>12</sub> H <sub>4</sub> Br <sub>6</sub> O <sub>3</sub>	669.5261	6.69	1.81	3A	6.55	0.79	3A	6.69	-2.73	3A	6.72	-4.2	3A
Br <sub>7</sub> -diOH-diphenyl ether	C <sub>12</sub> H <sub>3</sub> Br <sub>7</sub> O <sub>3</sub>	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 <sub>4</sub> -OH-MeO-diphenyl ethers	C <sub>13</sub> H <sub>8</sub> Br <sub>4</sub> O <sub>3</sub>	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 <sub>5</sub> -OH-MeO-diphenyl ethers	C <sub>13</sub> H <sub>7</sub> Br <sub>5</sub> O <sub>3</sub>	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 <sub>6</sub> -OH-MeO-diphenyl ethers	C <sub>13</sub> H <sub>6</sub> Br <sub>6</sub> O <sub>3</sub>	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 <sub>3</sub> -OH-diphenyl ether/ Br <sub>5</sub> -diOH-biphenyl	C <sub>12</sub> H <sub>5</sub> Br <sub>5</sub> O <sub>2</sub>	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 <sub>5</sub> -diOH-diphenyl ethers	C <sub>12</sub> H <sub>4</sub> Br <sub>5</sub> ClO <sub>3</sub>	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 <sub>3</sub> -OH-MeO-biphenyl	C <sub>13</sub> H <sub>9</sub> Br <sub>3</sub> O <sub>2</sub>	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 <sub>4</sub> -OH-MeO-biphenyl	C <sub>13</sub> H <sub>8</sub> Br <sub>4</sub> O <sub>2</sub>	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 <sub>3</sub> -OH-MeO-biphenyl	C <sub>13</sub> H <sub>8</sub> Br <sub>3</sub> ClO <sub>2</sub>	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 <sub>2</sub> -Br <sub>2</sub> -OH-MeO-biphenyls	C <sub>13</sub> H <sub>8</sub> Br <sub>2</sub> Cl <sub>2</sub> O <sub>2</sub>	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 <sub>5</sub> -diOH-dibenzo-dioxins	C <sub>12</sub> H <sub>3</sub> Br <sub>5</sub> O <sub>4</sub>	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 <sub>2</sub> -diOH-phenol	C <sub>6</sub> H <sub>4</sub> Br <sub>2</sub> O <sub>2</sub>	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 <sub>3</sub> -diOH-phenol	C <sub>6</sub> H <sub>3</sub> Br <sub>3</sub> O <sub>2</sub>	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 <sub>3</sub> -OH-MeO-phenol	C <sub>7</sub> H <sub>5</sub> Br <sub>3</sub> O <sub>2</sub>	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.

411 In *Lamellodysidea* sp. (MT-11), tetra-, penta- and hexabrominated OH-MeO-BDEs were detected. Thereby, penta- and hexabrominated  
 412 OH-MeO-BDE showed two and three peaks, respectively, indicating the presence of various isomers. Hexabrominated OH-MeO-BDE  
 413 and one of the isomers of pentabrominated OH-MeO-BDE were assigned with CL 4, as no fragmentation spectra could be acquired.  
 414 The fragmentation spectra of both tetra- (**4**) and the first isomer of pentabrominated (**5**) OH-MeO-BDE (Figure S6) showed fragments  
 415 which correspond to the loss of the methyl group and the loss of [-CH<sub>3</sub>Br]. These observations confirm the methylation of the detected

416 compounds. Additionally, both compounds showed a fragment corresponding to  $[C_6H_2Br_2O_2]^+$   
417 (theoretical  $m/z$  263.8427) indicating that both compounds carry a dibrominated aromatic moiety. For  
418 pentabrominated OH-MeO-BDE (**5**), this was further confirmed by the detection of a fragment  
419 corresponding to a tribrominated phenolic moiety ( $[C_6H_2Br_3O_2]^+$ ; theoretical  $m/z$  341.7532). Interestingly,  
420 both described fragments still carried two oxygens while this was not observed for non-methylated  
421 diOH-BDEs, indicating different fragmentation pathways. Tetra-, penta- and hexabrominated OH-  
422 MeO-BDE were also detected in *Callyspongia* sp. (MT-31). However, due to low abundance and thereof  
423 no available fragmentation spectra, CL 4 was assigned, not allowing any assumption about the structure  
424 of the compounds.

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425 In *Lamellodysidea* sp. (MT-11), tetra-, penta- and hexabrominated OH-MeO-BDEs were detected.  
426 Thereby, penta- and hexabrominated OH-MeO-BDE showed two and three peaks, respectively,  
427 indicating the presence of various isomers. Hexabrominated OH-MeO-BDE and one of the isomers of  
428 pentabrominated OH-MeO-BDE were assigned with CL 4, as no fragmentation spectra could be  
429 acquired. The fragmentation spectra of both tetra- (**4**) and the first isomer of pentabrominated (**5**) OH-  
430 MeO-BDE (Figure S6) showed fragments which correspond to the loss of the methyl group and the loss  
431 of  $[-\text{CH}_3\text{Br}]$ . These observations confirm the methylation of the detected compounds. Additionally, both  
432 compounds showed a fragment corresponding to  $[\text{C}_6\text{H}_2\text{Br}_2\text{O}_2]^-$  (theoretical  $m/z$  263.8427) indicating that  
433 both compounds carry a dibrominated aromatic moiety. For pentabrominated OH-MeO-BDE (**5**), this  
434 was further confirmed by the detection of a fragment corresponding to a tribrominated phenolic moiety  
435 ( $[\text{C}_6\text{H}_2\text{Br}_3\text{O}_2]^-$ ; theoretical  $m/z$  341.7532). Interestingly, both described fragments still carried two  
436 oxygens while this was not observed for non-methylated diOH-BDEs, indicating different fragmentation  
437 pathways. Tetra-, penta- and hexabrominated OH-MeO-BDE were also detected in *Callyspongia* sp.  
438 (MT-31). However, due to low abundance and thereof no available fragmentation spectra, CL 4 was  
439 assigned, not allowing any assumption about the structure of the compounds.

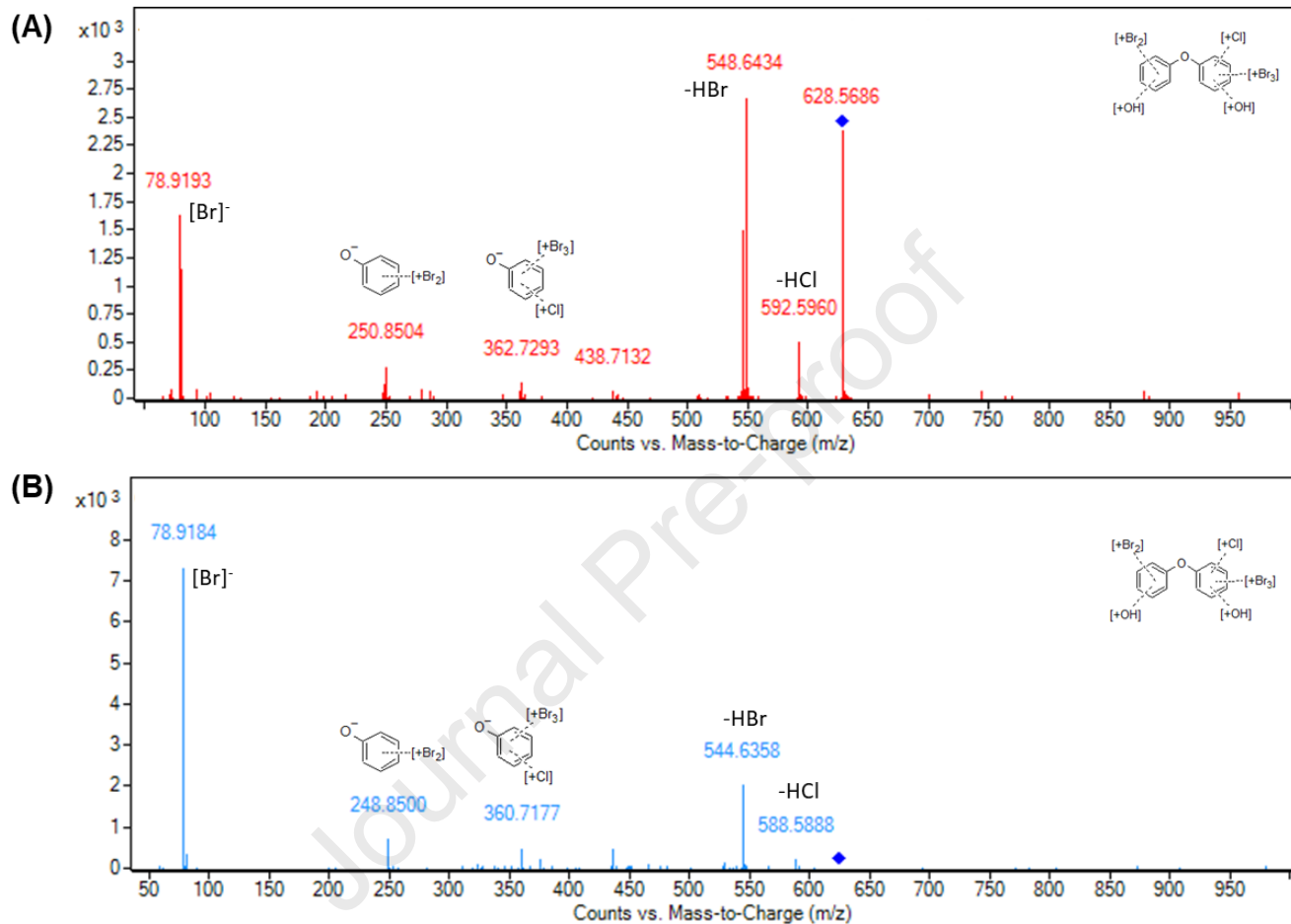
440  
441 It has to be noted that OH-MeO-tetra-BDE, diOH-penta-BDE, OH-MeO-penta-BDE and diOH-hexa-BDE  
442 have previously been reported in in both *Lamellodysidea* sp. (MT-11) and *Callyspongia* sp. (MT-31)  
443 (Kato et al., 2012). Our study has confirmed these findings and provided further information on  
444 fragmentation spectra and on the proposed structures of detected compounds. Additionally, our study  
445 reports the detection of a OH-MeO-hexabrominated-BDE and a diOH-heptabrominated-BDE in both  
446 sponge species. This compound was not detected by the study of Kato et al. (Kato et al., 2012).  
7 However, this finding has to be interpreted carefully as the reported compound could only be assigned  
8 with CL4.

9 In addition to the report of 6OH-BDE85 (RT 8.17 min) within target screening, a second compound with  
0 the molecular formula  $\text{C}_{12}\text{H}_5\text{Br}_5\text{O}_2$  (RT 7.27 min) was detected in *Lamellodysidea* sp. (MT-11). The





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



454 Figure 2: Experimental data of a pentabrominated monochlorinated diOH-BDE with the molecular formula  $\text{C}_{12}\text{H}_4\text{Br}_5\text{ClO}_3$   
 455 detected in sample MT-11, *Lamellodysidea* sp. (A) and MT-31, *Callyspongia* sp. (B). The proposed structure and observed  
 456 fragmentation spectrum at a collision energy of 40 eV are displayed. The most abundant fragments are assigned with the  
 457 corresponding neutral losses or proposed structures. The most abundant fragments are assigned with the corresponding  
 458 neutral losses or proposed structures.  
 459

460  
 461 A pentabrominated monochlorinated diOH-BDE (**6**) whose fragmentation spectra are shown in Figure 2  
 462 was detected in *Lamellodysidea* sp. (MT-11) and *Callyspongia* sp. (MT-31). Fragments which  
 463 correspond to the loss of HBr and HCl demonstrated the mixed halogenation of the detected compound.  
 464 The observed fragments with  $m/z$  248.8500 ( $[\text{C}_6\text{H}_3\text{Br}_2\text{O}]^-$ ) and  $m/z$  360.7177 ( $[\text{C}_6\text{H}_3\text{Br}_3\text{ClO}]^-$ ) indicate that  
 465 the two phenolic moieties carry two and three bromines plus one chlorine, respectively. Again, the

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

468

469 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  
470  $C_{13}H_8Br_2Cl_2O_2$  were detected in *Lamellodysidea* sp. (MT-11). Even though no fragmentation spectra  
471 were acquired, and CL 4 had to be assigned, it was assumed that the predicted molecular formulae  
472 correspond to OH-MeO-BBs and not MeO-BDEs as latter would not be detectable as  $[M-H]^-$  ions with  
473 ESI due to the lack of a free hydroxy group. The observed limitations of the acquisition of fragmentation  
474 spectra for these compounds are assumed to be caused by their limited ionization or low abundance in  
475 the sample. Further optimization of the sample preparation and instrumental methods is needed to  
476 confirm the postulated findings. The same applies to the detection of a diOH-hexabromo-dibenzo-dioxin  
477 ( $C_{12}HBr_5O_4$ ) which was detected in both *Lamellodysidea* sp. (MT-11) and *Callyspongia* sp. (MT-31). As  
478 mentioned above, the level of bromination was confirmed by the high match of the isotopic pattern.  
479 However, a more confident confirmation of the structure is not possible due to limited mass spectral  
480 information.

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

1

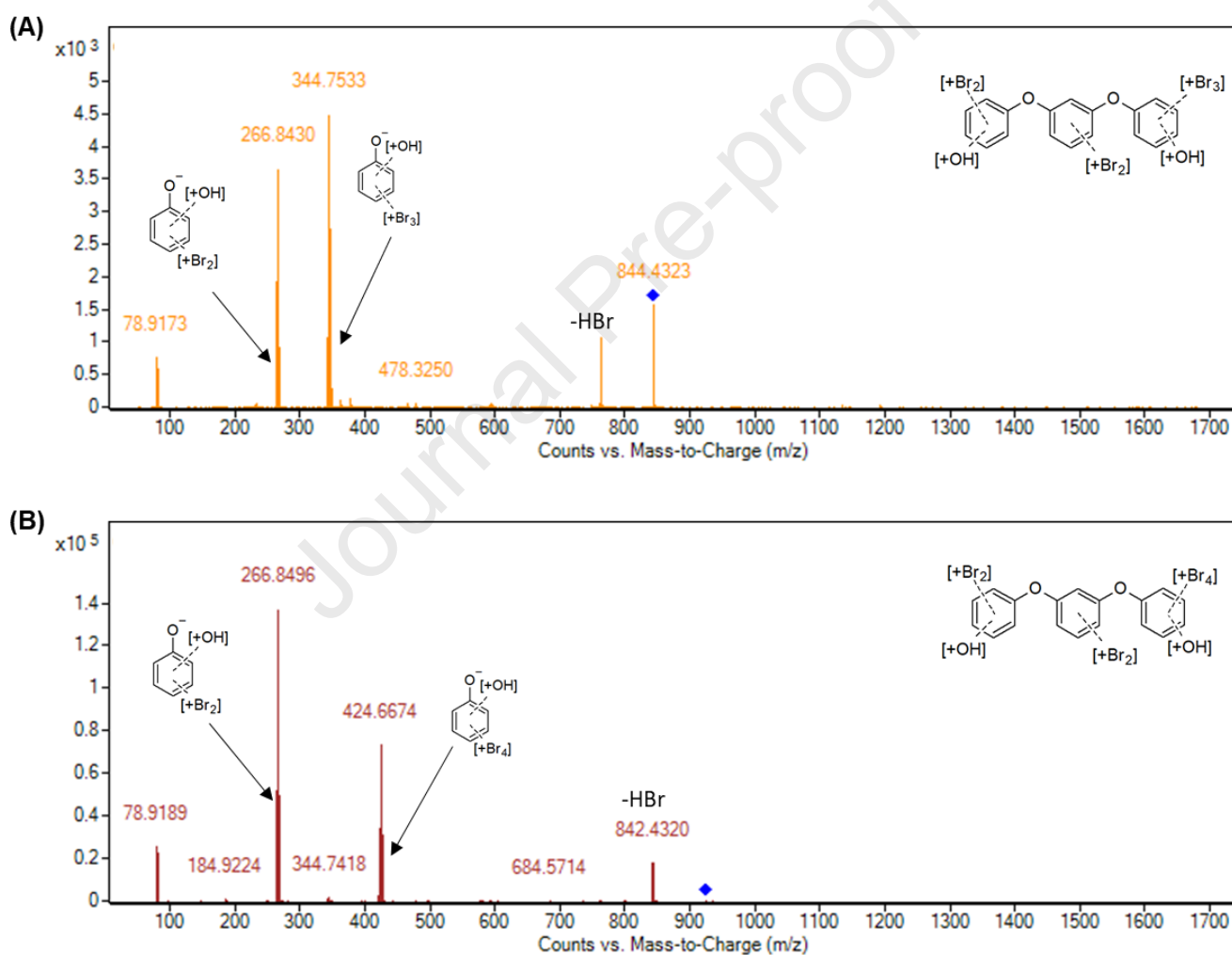


492 **Non-target screening results of sea sponge samples**

493 For non-target screening, samples of *Lamellodysidea* sp. (MT-11) and *Callyspongia* sp. (MT-31) were  
494 analysed in full scan MS mode to enhance feature detection using HaloSeeker 1.0 software, as a  
495 complementary approach to suspect screening. Since the results of non-target screening in the mass  
496 range below  $m/z$  800 mainly yielded compounds which were already reported through target and  
497 suspect screening, the analysis was focused on a mass range between  $m/z$  800 and 1500. Additionally,  
498 only polyhalogenated compounds which remained after applying the F2+ filtering within HaloSeeker 1.0  
499 were considered. Molecular formulae were predicted for all detected features. If fragmentation spectra  
500 of sufficient quality were available for a feature and fragments could unequivocally be assigned, the  
501 most probably candidate was selected from the proposed formulae based on the assigned fragments.  
502 For the remaining features, molecular formulae which showed the highest matching score are reported.  
503 For *Lamellodysidea* sp. (MT-11), the non-target screening approach yielded 31 compounds (**L1** to **L31**)  
504 (Table S11). The corresponding H/Cl-scale can be found in Figure S8. Additionally, fragmentation  
505 spectra with satisfying intensities could be acquired for 15 compounds using the target MS/MS mode.  
506 Based on the predicted molecular formulae, compounds **L1** to **L4** were identified as hepta-, octa- and  
507 nonabrominated dihydroxylated diphenoxybenzene. This was confirmed by analysis of the  
508 corresponding masses in target MS/MS mode which yielded fragmentation spectra shown in Figure 3.  
509 Based on this information, hepta- ( $C_{18}H_7Br_7O_4$ ,  $\Delta ppm$  -2.61 ppm and -3.08 ppm), octa- ( $C_{18}H_6Br_8O_4$ ,  
510  $\Delta ppm$  -1.19 ppm) and nonabrominated ( $C_{18}H_5Br_9O_4$ ,  $\Delta ppm$  -7.98 ppm) dihydroxylated  
511 diphenoxybenzenes were detected in *Lamellodysidea* sp. (MT-11). For heptabrominated dihydroxylated  
512 diphenoxybenzene, the observed fragments with molecular formulae  $[C_6H_3Br_2O_2]^-$  (theoretical  $m/z$   
513 266.8485) and  $[C_6H_2Br_3O_2]^-$  (theoretical  $m/z$  344.7590) gave evidence about the distribution of bromines  
4 between the three aromatic moieties. The same applies to octabrominated dihydroxylated  
5 diphenoxybenzene, for which fragments with molecular formulae  $[C_6H_3Br_2O_2]^-$  (theoretical  $m/z$   
6 266.8485) and  $[C_6HBr_4O_2]^-$  (theoretical  $m/z$  424.6675) were observed. This information provided



517 additional confirmation of compound identification and was used to propose the structures given in  
 518 Figure 3. To our knowledge, this is the first time that OH-PBDPB are reported in the environment.  
 519 For compounds **L5** to **L7**, the observed fragmentation spectra showed low abundances not providing  
 520 additional structural information. The mass difference between compounds **L6** and **L7** and their diagonal  
 521 orientation in the H/Cl-plot indicated that their molecular formulae differed by a methylene group.  
 522 Molecular formulae with the highest matching score and which were in line with the described mass  
 523 differences were selected and are reported in Table S11.



4  
 5 Figure 3: Experimental data and proposed chemical structures of hepta- and octabrominated dihydroxylated  
 6 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**)  
 7 detected in *Lamellodysidea* sp. (sample MT-11). The most abundant fragments are assigned with the  
 8 corresponding neutral losses or proposed structures.

530 The fragmentation spectra of compounds **L8** to **L11** are found in Figures S8 and S9, respectively. All  
531 fragmentation spectra showed a fragment corresponding to  $[\text{HSO}_4]^-$  (theoretical  $m/z$  96.9601) indicating  
532 that all four compounds carry at least one sulfate group. Additionally, the fragmentation spectra showed  
533 a fragment which could be assigned with the formula  $[\text{C}_{12}\text{H}_4\text{Br}_5\text{O}_3]^-$ . This formula and  $m/z$  corresponded  
534 to pentabrominated diOH-BDE. These findings were in line with the predicted formulae of compounds  
535 **L8** to **L11** which all included five bromines. The second highly abundant fragment observed in the  
536 fragmentation spectra was assumed to represent the non-brominated part of the molecule and differed  
537 by a methylene group when comparing compounds **L10** and **L11**. However, the acquired data did not  
538 allow a further characterization of the structure and the proposal of a structural formula. Compound **L12**  
539 showed similar fragmentation as described for compounds **L8** to **L11** (data not shown) with a fragment  
540 which was assigned with the formula  $[\text{C}_{12}\text{H}_3\text{Br}_6\text{O}_3]^-$  (theoretical  $m/z$  674.5127) indicating that compound  
541 **L12** carries an additional bromine in comparison to compound **L11**. This was confirmed by the fact that  
542 both compounds clustered on the same horizontal line in the H/Cl-plot.

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

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



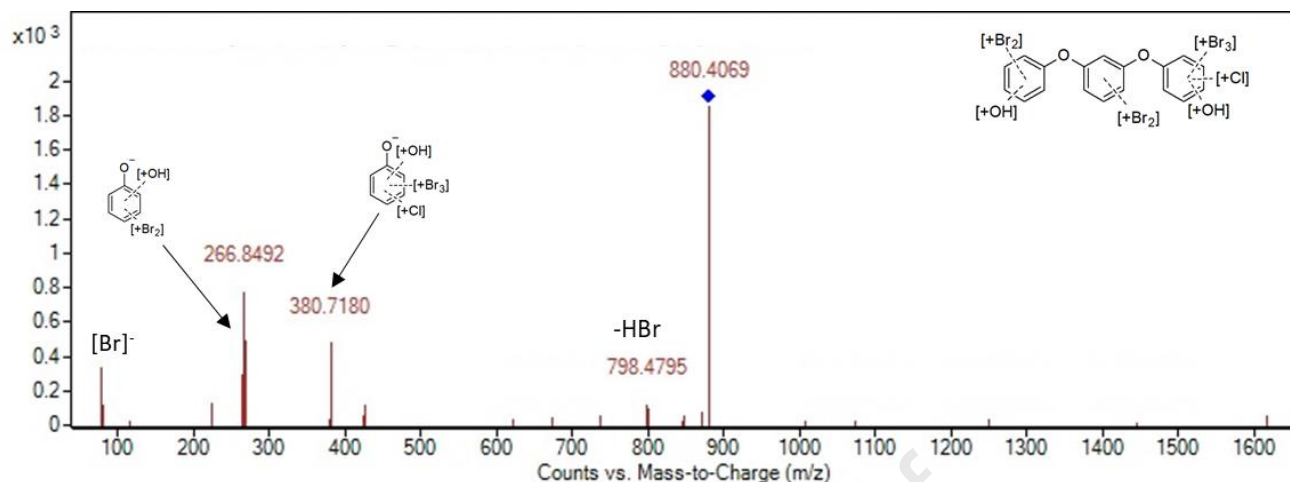
556 For the remaining compounds (i.e., **L17**, **L18**, **L21 - L31**) the information received from the predicted  
557 formulae or fragmentation spectra were not sufficient to allow a proposal of a molecular structure.  
558 Therefore, for these compounds the molecular formulae which showed the highest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

561

562 Non-target analysis of *Callyspongia* sp. (MT-31) yielded 20 compounds (**C1** to **C20**) which are  
563 summarized in Table S12. The corresponding H/Cl-plot can be found in Figure S10. Fragmentation  
564 spectra with satisfying intensities could be acquired for 9 compounds using the target MS/MS mode.

565 Similar to the results observed for *Lamellodysidea* sp. (MT-11), in *Callyspongia* sp. (MT-31) compounds  
566 **C1** to **C4** were identified as hepta-, octa- and nonabrominated dihydroxylated diphenoxybenzene. The  
567 fragmentation spectra obtained for hepta- and octabrominated dihydroxylated diphenoxybenzenes  
568 (Figure S11) showed fragments identical to the ones observed for sample MT-11. Therefore, the same  
569 assumptions regarding the molecular structures and distribution of bromine as described above can be  
570 made. Additionally, a heptabrominated monochlorinated dihydroxylated diphenoxybenzene (compound  
571 **C5**) with the molecular formula  $C_{18}H_6Br_7ClO_4$  was detected. The given formula was proposed based on  
572 the grouping of the described compound on the same horizontal line in the H/Cl-plot as the non-  
573 chlorinated diOH-diphenoxybenzenes indicating the same degree of halogenation. Additionally, the  
574 experimental and theoretical isotopic patterns ( $\Delta$ ppm -8.15 ppm; Figure S12) showed a satisfying fit.  
575 The obtained fragmentation spectrum of compound **C5** is shown in Figure 4. Similar to the results  
576 obtained for hepta- and octabrominated diOH-BDPBs, a fragment with the molecular formulae  
577  $[C_6H_3Br_2O_2]^-$  (theoretical  $m/z$  266.8485) was observed. A fragment which was assigned with the formula  
8  $[C_6HBr_3ClO_2]^-$  (theoretical  $m/z$  380.7178) confirmed that one of the aromatic moieties is carrying one  
9 chlorine atom.





580

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

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

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

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

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





## 601 **Conclusions**

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

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

612 Suspect screening yielded two compounds detected in alga samples (hexabrominated diOH-diphenyl  
613 ether and monochlorinated phenol). A high number of compounds was detected in sponge samples (17  
614 and 8 compounds identified in sea sponge samples of *Lamellodysidea* sp. and *Callyspongia* sp.,  
615 respectively) indicating a high variety of NHCs occurring in this species. Four of the identified  
616 compounds have been described in previous studies. Thus, the presented work introduces a high  
617 number of newly identified NHCs in sea sponge samples including heptabrominated diOH-BDE,  
618 monochlorinated pentabrominated diOH-BDE, hexabrominated OH-MeO-BDE and others.

619 Non-target screening allowed the identification of OH-PBDBPs, such as hepta-, octa- and  
620 nonabrominated diOH-BDBPs, in *Lamellodysidea* sp. and *Callyspongia* sp. samples. To our knowledge,  
621 this is the first study reporting these compounds in the environment. Non-target screening yielded  
622 additional and 16 compounds in *Lamellodysidea* sp. and *Callyspongia* sp. samples, respectively, which  
3 could tentatively be identified through the assignment of predicted molecular formulae.

4 This study provides a comprehensive screening approach for polyhalogenated NHCs in biota samples  
5 which provided additional information on the occurrence and distribution of NHCs in alga and sea  
6 sponge species and can serve as a valuable tool for future screening studies.





627

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638

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Journal Pre-proof



**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)

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**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:

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