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Naturally occurring organobromine compounds (OBCs) including polybrominated dibenzo-*p*-dioxins in the marine sponge *Hyrtios proteus* from The Bahamas

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ABSTRACT

Halogenated natural products (HNPs) were identified from organic extracts of the marine sponge *Hyrtios proteus* from The Bahamas using gas chromatography with electron capture negative ion mass spectrometry and non-targeted gas chromatography with electron ionization mass spectrometry. The HNPs found have similar properties to anthropogenic persistent organic pollutants (POPs). Two *ortho*-methoxy brominated diphenyl ethers (MeO-BDEs) 2′-MeO-BDE 68 and 6-MeO-BDE 47 were the most abundant compounds. Fourteen other MeO-BDEs were detected along with several polybrominated dibenzo-*p*-dioxins (PBDDs) (1,3,7-triBDD, 1,3,6,8-tetraBDD and 1,3,7,9-tetraBDD) and MeO-PBDDs. Further analysis of a higher trophic level octopus (*Octopus maya*) from the same FAO fishing area showed that the major HNPs detected in *Hyrtios proteus* were also predominant. Moreover, HNPs were more than 30-fold higher in abundance than the major POPs in the octopus, i.e., polychlorinated biphenyls. Hence, Caribbean marine organisms, including those potentially used for food, harbor relatively high concentrations of HNPs.

1. Introduction

During the last 20 years, several halogenated natural products (HNPs) have been detected in samples of marine origin including food (Gribble, 2010; Vetter, 2006). Their detection in the tissue of marine mammals and fish indicated that HNPs may share similar bioaccumulation potential as anthropogenic persistent organic pollutants (POPs) (Vetter, 2006). For this reason certain HNPs have been classified as environmental contaminants due to possible toxic effects on humans and wildlife (Bidleman et al., 2019b; Moore et al., 2002). Typically, HNPs are biosynthesized as secondary metabolites by lower trophic marine biota such as seaweed and sponges (Gribble, 2010; Teuten et al., 2006; Vetter et al., 2001a, 2002). Frequently, however, the natural producers are unknown, and this makes it difficult to predict or model their occurrence and distribution in different habitats. To elucidate HNP sources it is necessary to study their occurrence in their natural producers such as seaweed and sponges. Previous studies have focused on regions such as the Baltic and the Mediterranean Seas (Europe) (Bidleman et al., 2019a; Melcher et al., 2007), Western Indian Ocean (Wu et al., 2021) and especially the Great Barrier Reef (Australia) (Vetter et al., 2001b, 2002, 2009). Similarly, the Caribbean Sea including the Bahamas (Fig. S1) is a prime habitat for HNP-producing sponges that dominate Caribbean coral reef communities (McMurray et al., 2008; Pawlik, 2011). Several of the more than 10,000 sponge species worldwide were already identified as producers of environmentally relevant HNPs (Assmann et al., 2000; Gribble, 2010; Vetter et al., 2002; Vetter and Janussen, 2005). These HNPs may be used by sponges as chemical defenses against predators, competitors and pathogens (Assmann et al., 2000; Pawlik, 2011).

In this study, we screened the nonpolar fraction of several sponge species collected from the Bahamas Islands by gas chromatography coupled with mass spectrometry operated in the electron-capture negative ion mode (GC/ECNI-MS). This method allows the selective and sensitive detection of polyhalogenated compounds (Buser, 1986;

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Vetter, 2001). Among these samples, the marine sponge *Hyrtios proteus* stood out as it featured several abundant peaks originating from polyhalogenated compounds. Here, we present the details of our structural investigation of these HNPs. In addition, a higher trophic level marine organism *Octopus maya* was collected from the same FAO fishing area as sponges and analyzed to investigate the bioaccumulation of HNPs into seafood.

2. Materials and methods

2.1. Samples

Three sponge species were analyzed but only *H. proteus*, collected at Dog Rocks, near Exuma Cays, Bahamas Islands (Western Central Atlantic, FAO fishing area 31, Fig. S1) in July 2010, was studied in detail. Two additional species, *Smenospongia reticulatus* and *Stylissa caribica*, also collected from Dog Rocks in May and July 2010, respectively, were also analyzed from HNPs for comparison. Sponges collected by divers, transferred to (polyethylene) bags underwater, returned to the surface, stored at −20 °C for the remainder of the cruise and for some months thereafter until final transfer to a −80 °C freezer. Octopus (*Octopus maya*) was purchased in a local supermarket in Stuttgart, Germany, which was harvested in FAO fishing area 31 in September 2020.

2.2. Chemicals and reagents

Methanol (MeOH, ≥99.9%) was obtained from Fisher Scientific (Fairlawn, NJ, USA). Dichloromethane (DCM), *n*-hexane and isooctane (all suitable for trace analysis at below the part-per-billion level quality) were purchased from Burdick and Jackson (Muskegon, MI, USA). Toluene (HPLC grade) and diethyl ether (>97%) were obtained from Sigma-Aldrich (Steinheim, Germany). Anhydrous sodium sulfate and Florisil (0.150–0.250 mm, 60–100 mesh) were from Carl Roth (Karlsruhe, Germany). Sources of HNPs standards were reported in Wu et al. (2019). "ICES-7 PCBs" (PCB 28, PCB 52, PCB 101, PCB 118, PCB 138, PCB 153 and PCB 180) were obtained from Dr. Ehrenstorfer (Augsburg, Germany). Solutions of 2,4,4′-[$^{13}C_{12}$] tribromodiphenyl ether ($^{13}C_{12}$-BDE 28), 2,2′,4,4′-tetrabromo-6-methoxy-[$^{13}C_{12}$] diphenyl ether [$^{13}C_{12}$-MeO-BDE 47], and 2,2′,4,4′,5,5′-[$^{13}C_{12}$] hexabromodiphenyl ether ($^{13}C_{12}$-BDE 153) were from Wellington Laboratories (Guelph Ontario, Canada). Solutions of 2,2′,4,4′,6,6′-[$^{13}C_{12}$] hexachlorobiphenyl ($^{13}C_{12}$-PCB 155) and $^{13}C_{12}$-2,2′,4,4′,5,5′-[$^{13}C_{12}$] hexachlorobiphenyl ($^{13}C_{12}$-PCB 153) were from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Solutions of 4′-fluoro-2,3,3′,4,5,6-hexabromodiphenyl ether, 3′-fluoro-3,4-dibromodiphenyl ether, and 4′-fluoro-2,3′,4-tribromodiphenyl ether were from AccuStandard (New Haven, CT, USA). Mixtures and dilutions of these compounds used as internal and recovery standards were prepared gravimetrically. The internal standard solution in isooctane contained the following compounds: 3′-fluoro-3,4-dibromodiphenyl ether, 4′-fluoro-2,3′,4-tribromodiphenyl ether, $^{13}C_{12}$-PCB 153, $^{13}C_{12}$-BDE 153 and $^{13}C_{12}$-MeO-BDE 47. The mixture used as recovery standard contained $^{13}C_{12}$-PCB 155, $^{13}C_{12}$-BDE 28, and 4′-fluoro-2,3,3′,4,5,6-hexabromodiphenyl ether.

2.3. Sample preparation

2.3.1. Extraction

Freshly thawed sponges were cut into small pieces (approximately 0.5 × 0.5 cm) enough for 15 mL volume. Sponge sample volume was determined by displacement in water or solvent. Besides the volume, the wet weight of sponge tissue was also recorded. Then added 35 mL MeOH/DCM (1:1) solution to the chopped sponge sample. The procedural blank included 50 mL MeOH/DCM was prepared and analyzed using the same process as for samples. Samples were transferred into pressurized fluid extraction cells and were spiked gravimetrically with 400 μL of the internal standard solution, and then were placed on a shaker overnight. Resulting extracts were filtered over baked glass wool into medium sized Turbovap tubes (first extract). Remaining sponge tissue was re-extracted on the shaker with 40 mL MeOH overnight (second extract). First extracts were evaporated to 1 mL using a Turbovap (EC-1, VLM, Bielefeld, Germany) under nitrogen flow, transferred into a 1.5 mL amber vial and then capped, wrapped with Teflon tape, and placed in the freezer. Second extracts were filtered over glass wool (baked at about 600 °C, >1 h) and combined with the first extracts. Combined first and second extracts were evaporated to a volume of 1–2 mL and transferred to 15 mL test tubes after rinsing with 3–4 mL *n*-hexane.

The resulting sample extracts were separated into neutral and phenolic compounds according to Hovander et al. (2000). Briefly, 2 mL of KOH solution (0.5 mol/L dissolved in 50% ethanol) was added to each test tube. The test tubes were then capped, inverted, and shook for 3 min and then centrifuged for 4 min at 1500 rpm. After centrifugation, the organic (top) layer of each sample was transferred into a separate 125 mL Turbovap tube (first separation). 3 mL of *n*-hexane was added to the remaining, lower layer in the test tubes, manually shaken for 3 min and centrifuged for 4 min on 1500 rpm. The organic (top) layer was combined with the first separation, and isooctane was added as a keeper solvent before evaporating the samples down to 1 mL using nitrogen stream.

2.3.2. Sample purification

The neutral (nonpolar) fraction was additionally clean-up by absorption chromatography with deactivated silica gel according to Weichbrodt et al. (2000). Briefly, the method used 3 g of silica gel topped with 1 cm of Na_2SO_4 in 0.9 cm diameter columns. Sample extracts (1 mL) were added to the columns and then eluted with 60 mL *n*-hexane. The eluate was evaporated with nitrogen stream to 1 mL using the Turbovap. The samples were transferred into solvent rinsed 1.5 mL amber auto sampler vials. Each sample was amended gravimetrically with 400 μL (about 40 ng) of recovery standard solution. A separate solution was prepared in parallel containing 400 μL of each internal and recovery standard solution, combined gravimetrically.

Freeze-dried octopus samples were extracted by ASE using the mixed solvent of C/E (cyclohexane/ethyl acetate, 46:54, w/w) and lipids were removed by gel permeation chromatography (GPC) (Weichbrodt et al., 2000) with subsequent absorption chromatography with deactivated silica gel as described above.

2.3.3. Separation of polybrominated compounds according to planarity on Florisil

The separation of coplanar polybrominated compounds by Florisil was performed according to Klimm et al. (2019) with slight modifications. Briefly, a glass column (length 30 cm, inner diameter 1 cm) was sequentially filled with glass wool, sea sand and 5 g of Florisil (deactivated with 1% distilled water, w/w). The sample (1 mL in *n*-hexane) was then added into the column. Nonplanar polybrominated compounds were eluted with 100 mL of *n*-hexane (fraction 1), while coplanar polybrominated compounds were eluted with 50 mL of diethyl ether/toluene (1:9, v/v, fraction 2). To ensure that all compounds were eluted, an additional 50 mL of diethyl ether/toluene (1:9, v/v) was eluted and collected as fraction 3.

2.4. Instrumental analysis

Gas chromatography with electron capture negative ion mass spectrometry (GC/ECNI-MS) was performed on a 6890 GC coupled with HP 5973 MSD (Agilent Technologies, Waldbronn, Germany). An HP-5MS column (30 m length, 0.25 mm internal diameter, 0.25 μm film thickness) was installed in the GC oven. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. Sample solutions (1 μL) were injected splitless into the injection port maintained at 250 °C. Methane (purity

99.9995%) served as the reagent gas at a flow rate of 40 mL/min. Sensitive and selective detection of organohalogen compounds was performed in the low mass selected ion monitoring (SIM) mode according to Vetter (2001) with modifications: *m/z* 35 and *m/z* 37 ($[Cl]^-$), *m/z* 79 and *m/z* 81($[Br]^-$), *m/z* 114 and *m/z* 116 ($[ClBr]^-$), *m/z* 159 and *m/z* 161 ($[HBr_2]^-$) and *m/z* 370 and *m/z* 372 ($[M]^-$ of ^{13}C PCB 153) were recorded throughout the run. In the full scan mode, *m/z* 30–750 was recorded with a solvent delay of 7 min. Gas chromatography with electron ionization mass spectrometry (GC/EI-MS) was performed with the same instrument and the same settings. The non-targeted screening via GC/EI-MS-SIM was performed according to the method of Rosenfelder et al. (2010) (Table S1, Supplementary materials). Namely, mass ranges of 112 amu in three time windows were screened by eight non-target runs (NT1-NT8) using 15 subsequent masses, respectively.

2.5. Quality assurance and quality control

Internal standards added at the beginning of sample processing were used to monitor overall recoveries. Each set of samples included a blank sample (no sponge tissue, only solvent) spiked with the internal standard mixture, prepared in parallel. GC/MS instrumental blanks (injection of pure solvent) were included in each sequence of samples. The retention times (t_R) of the peaks in the samples should vary less than ±0.05 min relative to external reference compounds. Before the use of any glassware, tubes, caps, foil, metal ware, all materials were rinsed thoroughly with *n*-hexane as to avoid laboratory contamination during sample preparation. Compounds were considered brominated when (i) the ratio of *m/z* 79 to 81 was 1.03 ± 0.10, (ii) the integration start and end times of both ion traces were identical or nearly so (±0.5 s) and (iii) the signal-to-noise ratio was >10.

3. Results and discussion

3.1. Natural OBCs in H. proteus

The GC/ECNI-MS chromatogram of *H. proteus* (neutral fraction) featured several abundant polyhalogenated compounds some of which could be identified by reference standards. Namely, 2′-MeO-BDE 68 (BC-2, Fig. 1a), 6-MeO-BDE 47 (BC-3, Fig. 1b) were the two predominant compounds (Fig. 2). In addition, 2,4,6-tribromoanisole (2,4,6-TBA, Fig. 1c), 2,4,6-tribromophenol (2,4,6-TBP, Fig. 1c), 2′,6-diMeO-BDE 68 (BC-11, Fig. 1d) and 2,2′-diMeO-BB 80 (BC-1, Fig. 1e) could be unequivocally assigned in the neutral fraction extract. *Hyrtios* sp. is a known source of various secondary metabolites, mainly in the form of sesterterpenes, sesquiterpene quinones and macrolides (Hooper and van Soest, 2002; Lee et al., 2009). Polybrominated compounds (bromoindole derivatives and monobrominated tetracyclic compounds, Fig. S2) have

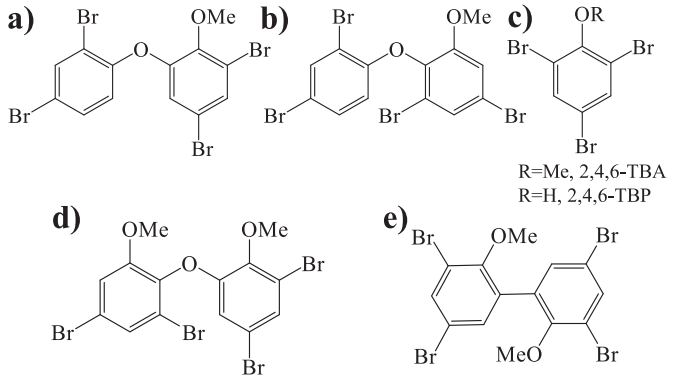

Fig. 1. Chemical structures of a) 2′-MeO-BDE 68 (BC-2), b) 6-MeO-BDE 47 (BC-3), c) 2,4,6-tribromoanisole (2,4,6-TBA) and 2,4,6-tribromophenol (2,4,6-TBP), d) 2′,6-diMeO-BDE 68 (BC-11) and e) 2,2′-diMeO-BB 80 (BC-1).

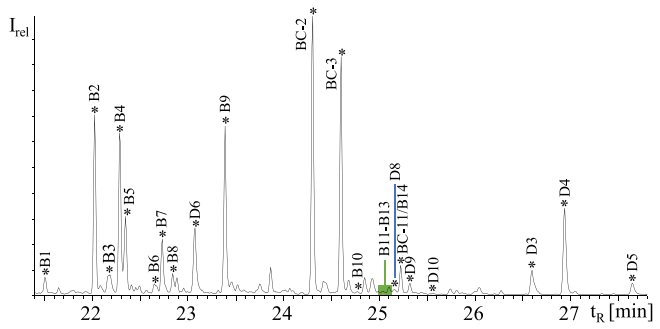

Fig. 2. GC/ECNI-MS-SIM chromatogram (extracted *m/z* 79) of *Hyrtios proteus* from The Bahamas (Caribbean Sea).

previously been identified in *Hyrtios* sp. from different locations in the Pacific Ocean (Solomon and Fiji islands) (Aoki et al., 2001; Longeon et al., 2011; Nasu et al., 1995; Utkina, 2009). That polybrominated compounds from *Hyrtios* sp. from the tropical Pacific differ from those found in the present study of a species from the tropical Atlantic (Fig. 2). Moreover, the high abundance of the major compounds excluded accumulation of the HNPs (and POPs) from the water phase which may also occur (Vetter, 2006; Vetter and Janussen, 2005; Wu et al., 2020a). Therefore, the two tetrabrominated MeO-BDEs (BC-2 and BC-3) must represent secondary metabolites of the sponge *H. proteus* (Fig. 2).

The search for additional polybrominated compounds was based on monitoring *m/z* 79/81 and low mass fragment ions in GC/ECNI-MS-SIM mode (Vetter, 2001). Additional presence of *m/z* 159/161 ($[HBr_2]^-$ (selectively formed by polybrominated compounds with a diphenyl ether backbone)) in compounds B1–B9 indicated that these earlier eluting compounds (Fig. 2) could be MeO-triBDEs (Melcher et al., 2005). Unfortunately, the molecular ions or abundant fragment ions in the higher mass range (>*m/z* 300) could not be detected by GC/ECNI-MS in full scan mode. Therefore, the sample was studied by non-target GC/EI-MS measurement in eight runs covering the whole array of potential molecular ions (see Experimental). Using this approach, 76 polybrominated compounds (~200 compounds in total) could be detected in the sample. As anticipated, B1–B9 were detected in window 2 of non-target run seven (NT-r7-w2, *m/z* 434–448, Table S1) by means of M^+ at *m/z* 434 featuring a tribromo pattern (Fig. 3a, Table 1). Melcher et al. showed that abiotic hydrodebromination of BC-2 and BC-3 resulted in the formation of eight tribrominated phenoxy anisoles, all of which were also detected in the common dolphin *D. delphis* and humpback dolphin *S. chinensis* from Australia (Melcher et al., 2005). Moreover, retention times of five MeO-triBDEs (B2, B3, B4, B6 and B7) relative to BC-2 in this study were almost consistent with the abiotic hydrodebromination products of BC-2 and BC-3 reported by Melcher et al. (2005) (Table S2). Therefore, B2, B4 and B7 were tentatively assigned to be 6-MeO-BDE 17, 6′-MeO-BDE 17 or 2-MeO-BDE 39 (co-elution) and 2′-MeO-BDE 28, respectively (Melcher et al., 2005) (Table 1). Four MeO-triBDEs were also detected in the marine sponge *Haliclona* sp. from Nikko Bay, Koror, Palau (Haraguchi et al., 2011).

NT-r5-w3 (*m/z* 506–520, Table S1) also indicated the additional presence of four, lower abundance isomers of BC-2 and BC-3 (B10 to B13, GC/ECNI-MS abundance 0.6–3.2% of BC-3) identified by examination of the tetrabromo isotope pattern at *m/z* 512 (Fig. 2b, Table 1). These isomers (B10-B13) eluted after BC-3 ($C_{13}H_8O_2Br_4$) from the GC column (Fig. 2b). Twenty-two isomers of BC-2 and BC-3 (eleven of which featured the methoxy group in *ortho*-position) have been described so far (https://scifinder.cas.org), and six of them (4-MeO-BDE 42, 3-MeO-BDE 47, 5-MeO-BDE 47, 4′-MeO-BDE 49, 6′-MeO-BDE 49 and 6′-MeO-BDE 66) were occasionally detected in biota (Table S3). Since *ortho*-substitution is a key attribute of natural MeO-BDEs, the *ortho*-substituted 6′-MeO-BDE 49 and 6′-MeO-BDE 66 were the most prominent candidates (Butryn et al., 2015; Cade et al., 2018; Geng et al., 2017;

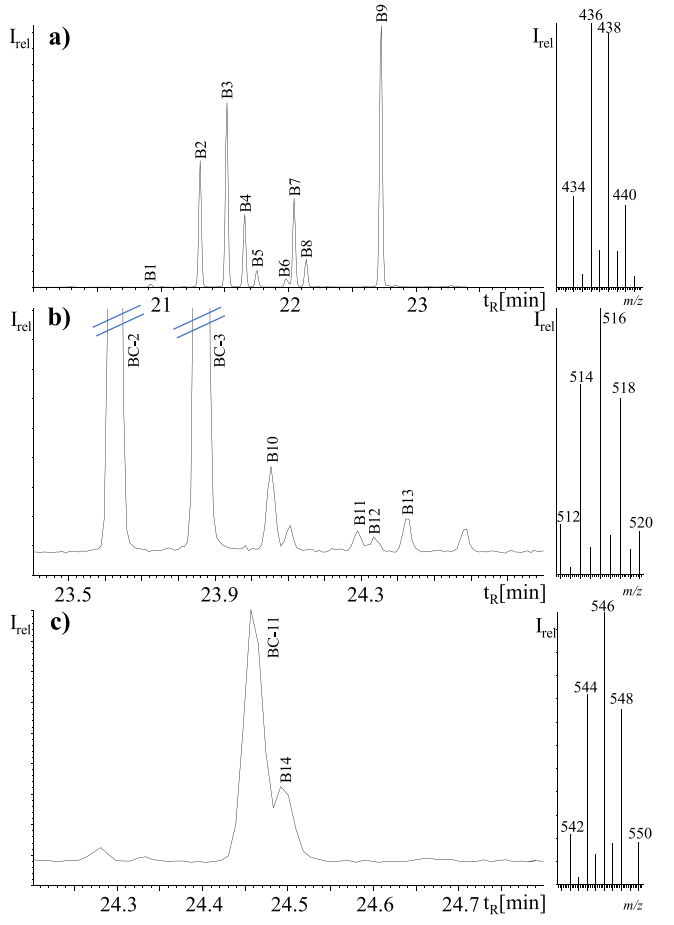

Fig. 3. GC/EI-MS-SIM chromatograms of (a) *m/z* 436 extracted from NT-r7-w2 and corresponding mass spectra of compounds B1–B9, (b) *m/z* 516 extracted from NT-r5-w3 and mass spectra of BC-2, BC-3 and B10-B13 and (c) *m/z* 546 in NT-r7- w3 and mass spectra of BC-11 and B14 of *Hyrtios proteus* from The Bahamas (Caribbean Sea).

Haraguchi et al., 2011; Kelly et al., 2008; Lacorte et al., 2010; Liu et al., 2014; Marsh et al., 2004; Sun et al., 2013; Verreault et al., 2005; Weijs et al., 2013, 2014).

Based on (relative) GC t_R data, B10 (1.018, Table 1) matched with 4′-MeO-BDE 69 (1.018) reported by Marsh et al. (2004). In addition, the partly co-eluting B11 and B12 (Fig. 1) were most likely a hitherto unknown 2-MeO-tetraBDE isomer and 6-MeO-BDE 42, respectively, based on relative t_R (1.028 and 1.030 in this study (Table 1) versus 1.031 and 1.032 by Vetter et al. (2011)). Similarly, B13 could by identified as 6′-MeO-BDE 66 (BC-IS) by means of a reference standard.

In addition, one potential dibrominated MeO-BDE congener was tentatively detected in NT-r1-w2 (*m/z* 350–364, Table S1) due to the agreement of measured abundance ratios of *m/z* 356: 358: 360 (57:100: 51) to theoretical values of 51:100: 49 (Table 1). Since GC/ECNI-MS responses of mono-and dibrominated compounds relative to more highly brominated compounds are low, the actual amount of this isomer may be underrepresented considering the low signal abundance. Unfortunately, in order to ensure the maximum sensitivity of the current NT scanning method, the high molecular ion of MeO-pentaBDEs (*m/z* 590) was not included in the NT scanning method of Rosenfelder et al. (2010) (Table S1). However, an additional GC/ECNI-MS-SIM run on MeO-pentaBDEs using ions *m/z* 590/592/594/596/598/600 verified that MeO-pentaBDEs were not present in the *H. proteus* sample.

The sample also featured 2′,6-diMeO-BDE 68 (BC-11, $C_{14}H_{10}O_3Br_4$, M^-, *m/z* 542) and a hitherto unreported isomer (B14) eluting slightly later and not fully resolved from BC-11 (Fig. 3c, Table 1). Only BC-11 was previously isolated from other sponges (e.g., *Dysidea* sp. (Cameron et al., 2000) and *Lamellodysidea herbacea* from Indonesia (Hanif et al., 2007)). However, the structurally related 2,6′-diOH-BDE 72 had been isolated from the marine sponge *Lamellodysidea herbacea* (Hanif et al., 2007). OH-BDEs are known precursors of corresponding MeO-BDEs, and possibly diOH-BDEs and diMeO-BDEs (Allard et al., 1987; Wan et al., 2009). Hence the detected isomer is potentially 2,6′-diMeO-BDE 72; however, the presence could not be verified due to the lack of an authentic reference standard.

NT-r6-w3 (*m/z* 520–534, Table S1) featured three tetrabrominated isomers with M^+ at *m/z* 526 (compounds D3-D5 in Fig. 4a) which were also detected by GC/ECNI-MS-SIM (Fig. 2). An increase of M^+ by 14 amu relative to BC-2 and BC-3 ($C_{13}H_8Br_4O_2$, *m/z* 512) can be explained by M + O_2-2H ($C_{13}H_6Br_4O_3$) or by M + CH_4 ($C_{14}H_{10}Br_4O_2$). Corresponding tetrabrominated HNPs of both options (molecular ion at *m/z* 526) were previously detected in marine mammals, namely 6-MeO-5-Me-BDE 42 and 2,2′-dimethoxy-3,3′,5,5′-tetrabromobiphenyl (BC-1) and two tetrabrominated methoxy-dibenzo-*p*-dioxin (MeO-tetraBDDs) isomers, respectively (Marsh et al., 2005; Haglund et al., 2013; Unger et al.,

Table 1
OBCs detected by means of non-targeted GC/EI-MS-SIM run in *Hyrtios proteus* from The Bahamas (Caribbean Sea).

ID	Non-target run	rrt[a]	Isotopic pattern	Monoisotopic peak (*m/z*)	Possible structure
MeO-diBDEs ($C_{13}H_{10}O_2Br_2$)					
–	1	0.851	Br_2	356	Unknown
MeO-triBDEs ($C_{13}H_9O_2Br_3$)					
B1	7	0.885	Br_3	434	Unknown
B2	7	0.902	Br_3	434	6-MeO-BDE 17
B3	7	0.911	Br_3	434	Unknown
B4	7	0.917	Br_3	434	6′-MeO-BDE 17/2-MeO-BDE 39 (co-elution)
B5	7	0.921	Br_3	434	Unknown
B6	7	0.930	Br_3	434	Unknown
B7	7	0.933	Br_3	434	2′-MeO-BDE 28
B8	7	0.937	Br_3	434	Unknown
B9	7	0.962	Br_3	434	Unknown
MeO-tetraBDEs ($C_{13}H_8O_2Br_4$)					
–	5	1.000	Br_4	512	2′-MeO-BDE 68 (BC-2)
–	5	1.010	Br_4	512	6-MeO-BDE 47 (BC-3)
B10	5	1.018	Br_4	512	4′-MeO-BDE 69
B11	5	1.028	Br_4	512	2-MeO-tetraBDE
B12	5	1.030	Br_4	512	6-MeO-BDE 42
B13	5	1.034	Br_4	512	6′-MeO-BDE 66
DiMeO-tetraBDEs ($C_{14}H_{10}O_3Br_4$)					
–	7	1.035	Br_4	542	2′,6-diMeO-BDE 68 (BC-11)
B14	7	1.037	Br_4	542	2,6′-diMeO-BDE 72
MeO-triBDDs ($C_{13}H_7O_3Br_3$)					
D1	8	0.917	Br_3	448	Unknown
D2	8	0.944	Br_3	448	Unknown
MeO-tetraBDDs ($C_{13}H_6O_3Br_4$)					
D3	3&6	1.094	Br_4	526	Unknown
D4	3&6	1.112	Br_4	526	Unknown
D5	3&6	1.140	Br_4	526	Unknown
TriBDDs ($C_{12}H_5O_2Br_3$)					
D6	5&6	0.948	Br_3	418	1,3,7-triBDD
D7	5&6	0.987	Br_3	418	1,3,9-triBDD
TetraBDDs ($C_{12}H_4O_2Br_4$)					
D8	4	1.035	Br_4	496	1,3,6,8-tetraBDD
D9	4	1.041	Br_4	496	1,3,7,9-tetraBDD
D10	4	1.052	Br_4	496	Unknown

[a] rrt = relative retention time to BC-2.

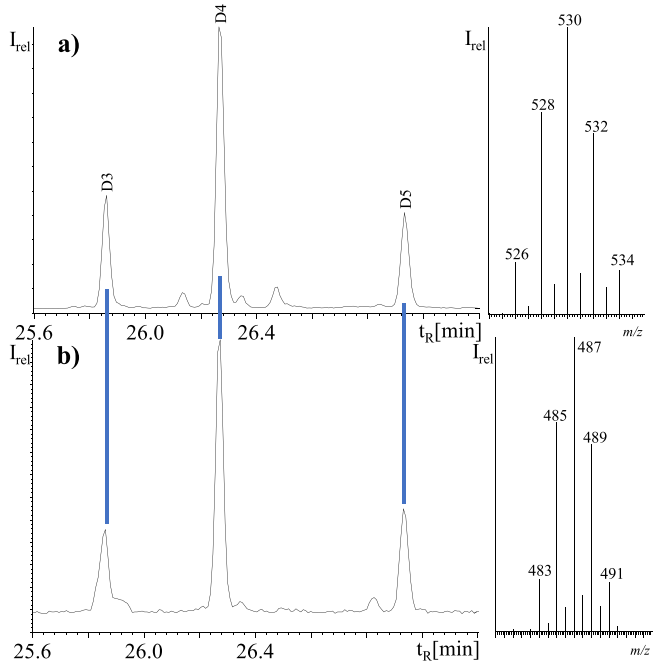

Fig. 4. GC/EI-MS-SIM chromatograms of (a) *m/z* 530 extracted from NT-r6-w3 and the corresponding mass spectra and (b) *m/z* 487 in NT-r3-w3 and corresponding mass spectra for compounds D3-D6 in *Hyrtios proteus* from The Bahamas (Caribbean Sea).

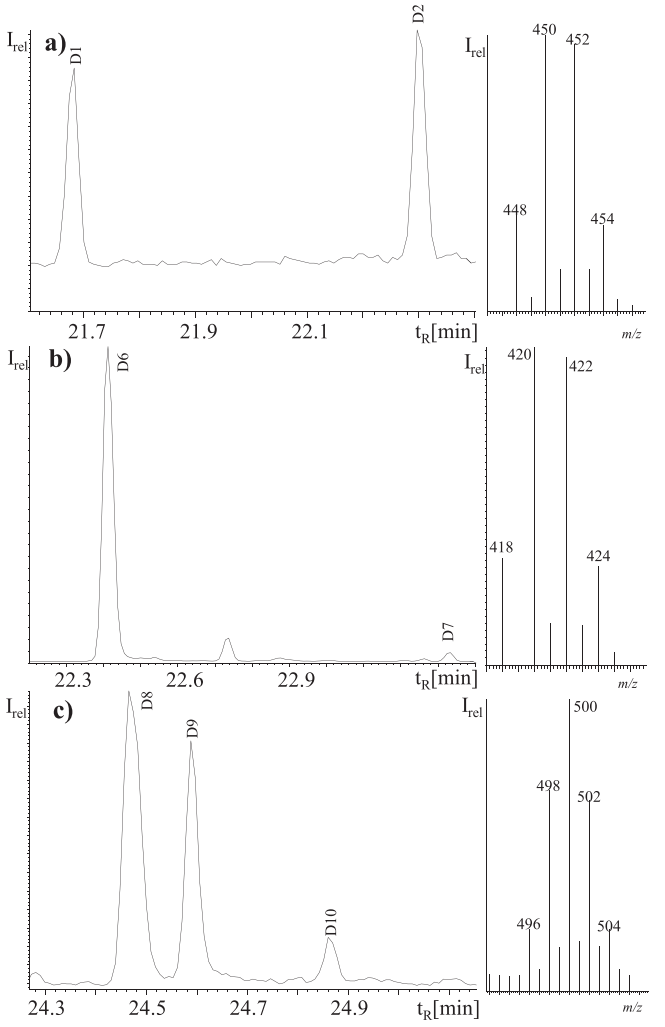

Fig. 5. GC/EI-MS-SIM chromatograms of (a) *m/z* 448 extracted from NT-r8-w2 and corresponding mass spectra of compounds D1 and D2, (b) *m/z* 420 extracted from NT-r5-w2 and corresponding mass spectra for compounds D6 and D7 and (c) *m/z* 500 extracted from NT-r4-w3 and corresponding mass spectra for compounds D8, D9 and D10 in *Hyrtios proteus* from The Bahamas (Caribbean Sea).

2010). Absence of $[HBr_2]^-$ (*m/z* 159/161) in the low mass GC/ECNI-MS-SIM run excluded the presence of a diphenyl ether backbone (Vetter, 2001) thus 6-MeO-5-Me-BDE 42. In addition, the later elution supported MeO-tetraBDDs for D3-D5 because coplanar molecules bind stronger to the stationary phase. Further verification for the presence of MeO-tetraBDDs could be derived from the diagnostic fragment ion at *m/z* 483 ($[M-43]^+$) detected in NT-r3-w3 (*m/z* 478–492) at the same t_R as *m/z* 526 in NT-r6-w3 (*m/z* 520–534, Fig. 4b, Table 1). This fragment ion was previously detected in the GC/EI-MS spectra of two MeO-tetraBDD isomers in the marine snail *Diloma subrostrata* from New Zealand (Haglund et al., 2013), which is likely formed by neutral loss of CH_3CO from M^+, ($[M-43]^+$), potentially via loss of CH_3 followed by CO (Haraguchi et al., 2011; Haglund et al., 2013). Also, one MeO-tetraBDD isomer (featuring M^+ at *m/z* 526 and *m/z* 483) was previously detected in the marine sponge *Callyspongia* sp. from the Nikko Bay, Koror, Palau (Haraguchi et al., 2011) while two MeO-tetraBDDs (2,3,6,8- and 3,4,6,8-tetrabromo-1-methoxy-dibenzo-*p*-dioxins) were isolated from the Australian marine sponge *Dysidea dendyi* (Utkina et al., 2002), and the latter two were only MeO-tetraBDDs with known structures. However, MeO-tetraBDDs have not been reported to occur in *H. proteus*. Co-occurrence of polybrominated phenoxyanisoles, phenoxyphenols and MeO-PBDDs has been reported before in other marine samples (Haraguchi et al., 2011; Haglund et al., 2013; Utkina et al., 2001, 2002). As well, two potential MeO-triBDDs (D1 and D2) were detected based on M^+ at *m/z* 448 in NT-r8-w2 (*m/z* 448–462) (Fig. 5a, Table 1), although *m/z* 405 ($[M-43]^+$, see above) could not be detected in this case (data not shown). Absence of *m/z* 405 may be due to very low abundance of D1 and D2 in the present sample; similarly, for MeO-triBDDs in *Haliclona* sp., *m/z* 405 ($[M-43]^+$) was not detected either (Haraguchi et al., 2011). Furthermore, the non-targeted GC/EI-MS-SIM method indicated the presence of two tribrominated isomers (D6 and D7) at *m/z* 418 (NT-r5&r6-w2, *m/z* 406–434) (Fig. 5b, Table 1) and three tetrabrominated isomers (D8-D10) at *m/z* 496 (NT-r4-w3, *m/z* 492–506) (Fig. 5c, Table 1). The late elution indicated the presence of PBDDs (Table 1). In agreement with that, D6 could be identified as 1,3,7-triBDD by means of the authentic reference standard (Fig. S3). According to Malmvärn et al. (2005), 1,3,7-triBDD is difficult to separate from 1,3,8-triBDD. Injection of both reference standards resulted in a slight resolution of both triBDD isomers (Fig. S3). The good match of the t_R of D6 and 1,3,7-triBDD verified its presence in our sample of *H. proteus* (Fig. S3). However, the presence of lower amounts of 1,3,8-triBDD could not be fully ruled out (Fig. S3). Presence of 1,3,7-triBDD and 1,3,8-triBDD is in agreement with their proposed formation from the phenoxyphenols of BC-3 (6-OH-BDE 47) and BC-2 (2′-OH-BDE 68) by elimination of HBr via bromoperoxidase mediated oxidative (BPO) cyclization or photochemical formation (Arnoldsson et al., 2012b; Haglund et al., 2007) (Fig. 6a, c; note that, due to the symmetric nature of the dioxin backbone, the carbon numbering is changed between both molecules). 1,3,7- and 1,3,8-triBDD were frequently detected as the most abundant triBDD isomers in environmental samples (sediment) and marine organisms (perch and blue mussels) from the Baltic Sea (Haglund, 2010). The second triBDD, isomer D7 in our sample, eluted 0.92 min later than the 1,3,7-triBDDs (D6) (Fig. 5b). GC t_R data of Malmvärn et al. (2005) indicated that D7 could be 1,3,9-triBDDs although this isomer was not detected in the Baltic Sea samples.

Similarly, 1,3,6,8-tetraBDD and 1,3,7,9-tetraBDD were the most prevalent tetraBDD isomers described so far in marine fish (perch, eel)

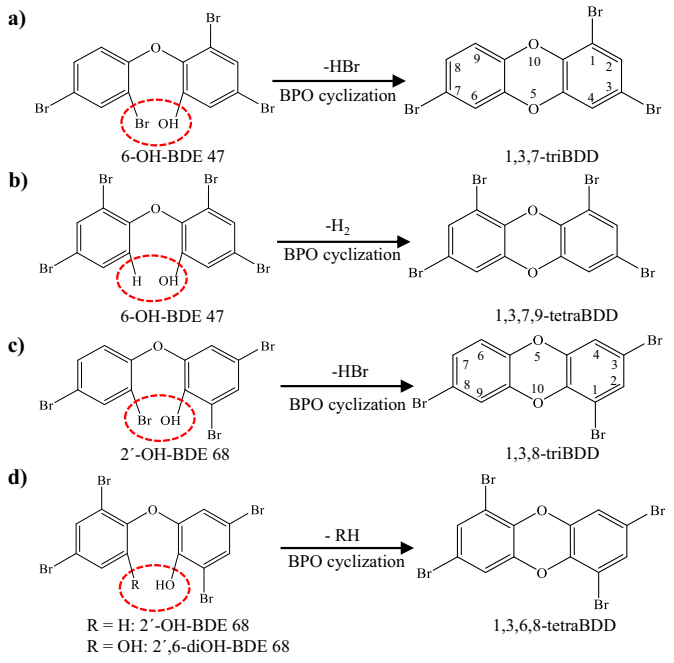

Fig. 6. Predicted formation pathways of (a) 1,3,7-triBDD from 6-OH-BDE 47, (b) 1,3,7,9-tetraBDD from 6-OH-BDE 47, (c) 1,3,8-triBDD from 2′-OH-BDE 68 and (d) 1,3,6,8-tetraBDD from 2′-OH-BDE 68 or 2′,6-diOH-BDE 68 in *Hyrtios proteus* from The Bahamas (Caribbean Sea).

and blue mussels from the Baltic Sea (Arnoldsson et al., 2012a; Haglund et al., 2007). Both could be related to the corresponding abundant potential precursors (OH-BDEs, structurally related to the (dimethylated) phenoxyanisoles BC-2 and BC-3) (Haglund, 2010). These tetraBDDs could be formed from *ortho*-OH-BDEs by elimination of H_2 via BPO cyclization (Wan et al., 2009). For instance, 2-OH-BDE 68 (desmethyl-BC-2) can be converted into 1,3,6,8-tetraBDD (Fig. 6d), and 6-OH-BDE 47 (desmethyl-BC-3) into 1,3,7,9-tetraBDD (Fig. 6b). In addition, 1,3,6,8-tetraBDD can also be formed by removal of H_2O from 2′,6-diOH-BDE 68 (desmethyl-BC-11) (Fig. 6d) (Allard et al., 1987; Wan et al., 2009). Moreover, 1,3,6,8-tetraBDD and 1,3,7,9-tetraBDD can be formed by bromoperoxidase-catalyzed enzymatic coupling of 2,4,6-TBP (Arnoldsson et al., 2012a). In any case, the possible precursors (BC-2, BC-3, BC-11 and 2,4,6-TBP) of 1,3,6,8-tetraBDD and 1,3,7,9-tetraBDD were detected in the present sample which strongly suggested that *H. proteus* has the ability to biosynthesize PBDDs. Similarly, 1,3,6,8-tetraBDD (and 1,3,6,8-tetraCDD) was found to elute prior to 1,3,7,9-tetraBDD (and 1,3,7,9-tetraCDD, respectively) from GC columns of different polarity (Table S4) (Arnoldsson et al., 2012a; Haglund et al., 2007; Haglund, 2010; Kishi et al., 2009; Liang et al., 2000; Ryan et al., 1991; Unger et al., 2009; van Bavel et al., 2015). On DB-5 (or ZB-5) columns, the GC t_R of 1,3,7,9-tetraCDD relative to 1,3,6,8-tetraCDD was always around 1.01 (Kishi et al., 2009; Ryan et al., 1991; van Bavel et al., 2015), which is consistent with the t_R of D9 relative to D8 in this study (an HP-5MS column was used, see Section 2.4) (Tables 1, S4). Therefore, all data is in support of 1,3,6,8-tetraBDD and 1,3,7,9-tetraBDD most likely being the structures of D8 and D9 (Table 1).

Co-existence of MeO-PBDDs and PBDDs was scarcely reported before in the literature. Namely, triBDDs and MeO-triBDDs were detected in the marine sponge *Haliclona* sp. (Haraguchi et al., 2011) while triBDDs, tetraBDDs and MeO-tetraBDDs were detected in the marine snail *Diloma subrostrata* (Haglund et al., 2013). As in the present study, MeO-BDEs were between one and two orders of magnitude more abundant than PBDDs.

3.2. Initial screening of HNPs in other samples from FAO fishing area 31

Release of HNPs produced by their primary producers (here: *H. proteus*) could contaminate seafood samples living in the same habitat (Wu et al., 2020a). The high abundance of several HNPs in the sponge *H. proteus* indicated that seafood from The Bahamas could also potentially be contaminated with these compounds. Unfortunately, higher organisms were not collected at the same time as the sponges. However, one octopus sample from the same catchment area (FAO fishing area 31) was bought through a commercial source (see Materials and methods). Around 100 OBCs were detected in the octopus sample, and 40% of them were also present in *H. proteus*. In agreement with *H. proteus* (Section 3.1), BC-2 and BC-3 were also predominant in the octopus sample (210 ng/g lw and 570 ng/g lw, respectively; abundance ratio ~ 1:3), but the abundance ratio of BC-2/BC-3 was different from *H. proteus* (~1:0.8). The concentration of the sum of PCBs (sum of PCB 153, PCB 138 and PCB 180: 12 + 9.5 + 2.7 = 24 ng/g lw, respectively) was <3% of the predominant HNPs, indicating that HNPs dominate the organohalogen burden in the sample. The octopus also featured 10 ng/g lw 2,3,3′,4,4′,5,5′-heptachloro-1′-methyl-1,2′-bipyrrole (Q1), while this HNP and PCBs were not detected in the sponge. Prevalent MeO-triBDEs (B2, B3, B4, B6, B7, B8 and B9) and three MeO-tetraBDEs (B11-B13) detected in *H. proteus* were also present in octopus (Fig. 7). Enrichment of coplanar polybrominated compounds (PBDDs and MeO-PBDEs) by Florisil fractionation also enabled the detection of 1,3,7-triBDD (D6), 1,3,6,8-tetraBDD and 1,3,7,9-tetraBDD (D8 and D9) in the octopus (Fig. 7). This indicated that both MeO-PBDEs and PBDDs produced by *H. proteus* (and probably other organisms) may be present in other biota in the Caribbean food web. For instance, MeO-BDEs (mainly BC-2 and BC-3) were already detected in the marine fish from Florida (close to The Bahamas, Fig. S1) (Weijs et al., 2015). In addition, BC-2 and BC-3 were also detected in the sponge-eating hawksbill turtles from the Caribbean region (Diez and van Dam, 2002; Dyc et al., 2015).

Also, the sponge *Stylissa caribica*, sampled in the same area, featured around 20 HNPs detected in *H. proteus* but at a much lower level (exception compound 3, Fig. S4). For instance, BC-2 was about two orders of magnitude lower in abundance in *Stylissa caribica* (Fig. S4). With a probability bordering on certainty, BC-2 and other HNPs were not naturally produced by *Stylissa caribica* but accumulated from the water phase, similarly to recent observations made with the HNP (1*R*,2*S*,4*R*,5*R*,1′*E*)-2-bromo-1-bromomethyl-1,4-dichloro-5-(2′-chloroethenyl)-5-methylcyclohexane (abbreviated as MHC-1) (Wu et al., 2020a). Furthermore, the consistent abundance ratio of shared compounds with similar molecular weight (retention time) indicated that *H. proteus* could be the HNP releasing organism (Fig. S4). Next to MeO-tetraBDEs (BC-2 and BC-3), the prevalent MeO-triBDEs (B4 and B9), and 1,3,7-triBDD (D6) were also detected in *Stylissa caribica*.

Compared to that, *Smenospongia reticulatus*, another sponge from the same habitat featured only BC-2 and five other HNPs detected in *H. proteus* (Fig. S4) while BC-3 and 1,3,7-triBDD (two highly abundant OBCs in *H. proteus*) were not detected in *Smenospongia reticulatus*. This discrepancy in the HNP pattern indicated species-specific differences, as previously also observed in chokka squid and sardine from the coasts of South Africa in the case of MHC-1 and Q1 (Wu et al., 2019, 2020b). Clearly, there are many gaps in knowledge of both the release of HNPs from their natural producers and their subsequent uptake from the water phase by other organisms.

4. Conclusion

The presence of high amounts of MeO-BDEs (BC-2 and BC-3) along with coplanar and potentially more toxic PBDDs and MeO-PBDDs in *H. proteus* and detection in the octopus indicate that potentially toxic HNPs occur in the Caribbean food web and points to the need for further research to define the presence in other species and biomagnification in higher trophic levels. This is also important because The Bahamas are

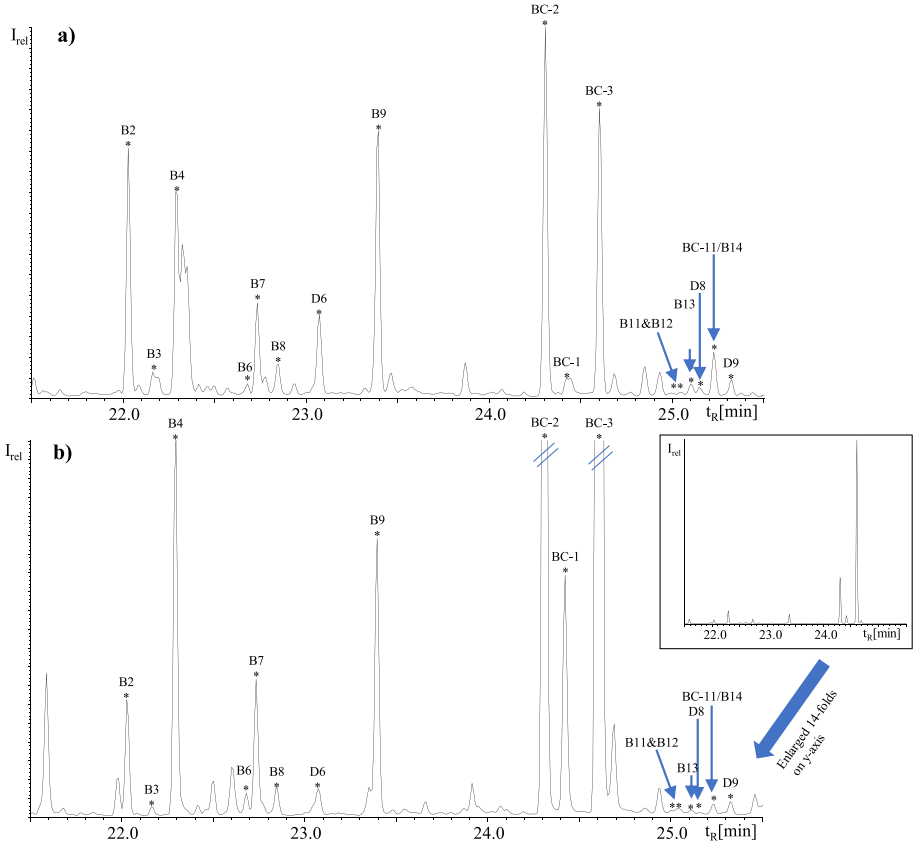

Fig. 7. GC/ECNI-MS-SIM chromatograms (extracted *m/z* 79) of (a) *Hyrtios proteus* and (b) *Octopus maya* of fraction 2 obtained from Florisil separation.

one of the world's important fishing grounds with the main seafood (spiny lobster, stone crabs and various fish species) being sold all over the world (http://www.fao.org). Up to now, only anthropogenic POPs were widely investigated in the Caribbean region (Hemming et al., 2003; Rauert et al., 2018; Vasquez et al., 2004), which played a minor role compared to the HNPs. In octopus, however, major POPs were more than one order of magnitude lower in abundance than HNPs. This may indicate that HNPs are the predominant organohalogen contaminants of other marine food products from the Caribbean Sea. Unfortunately, the toxicity of individual HNPs is largely unknown. Hence, there is an urgent need to screen marine samples from the Caribbean Sea on HNPs and study their ecotoxicological role.

CRediT authorship contribution statement

Qiong Wu: Investigation, Formal analysis, Visualization, Writing – Original draft.

Natalie Eisenhardt: Conceptualization, Investigation, Visualization, Writing – Review & Editing.

Stephanie Shaw Holbert: Investigation, Reading.

Joseph R. Pawlik: Conceptualization, Sample collection, Writing – Reading.

John R. Kucklick: Conceptualization, Writing – Review & Editing.

Walter Vetter: Conceptualization, Writing – Review & Editing, Supervision.

Declaration of competing interest

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.

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Disclaimer: Certain commercial equipment, instruments, or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2021.112872.

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