2′-OH-6-MeO-BDE68 and 2′,6-diOH-BDE68 isolated by preparative reversed-phase LC were >84 and >91 %, respectively. These compounds were used as external standards for quantification of each homolog group by LC/MS/MS.

Sponge collection

One specimen of *Callyspongia* sp. (phylum Porifera, order Haplosclerida, family Callyspongia) and two specimens of *Lamellodysidea* sp. (formerly known as *Dysidea*; phylum Ceractinomorpha, order Dictyoceratida, family Dysideidae) were collected while scuba diving at Nikko Bay Korrol, Palau, in August 2005. Freshly collected sponge materials were immediately frozen and stored at −20 °C prior to molecular and chemical analyses. The three specimens of sponges were phytogenetically identified by the method of Meixner et al. [30]. Voucher specimens have been deposited at the Department of Analytical Chemistry, Daiichi College of Pharmaceutical

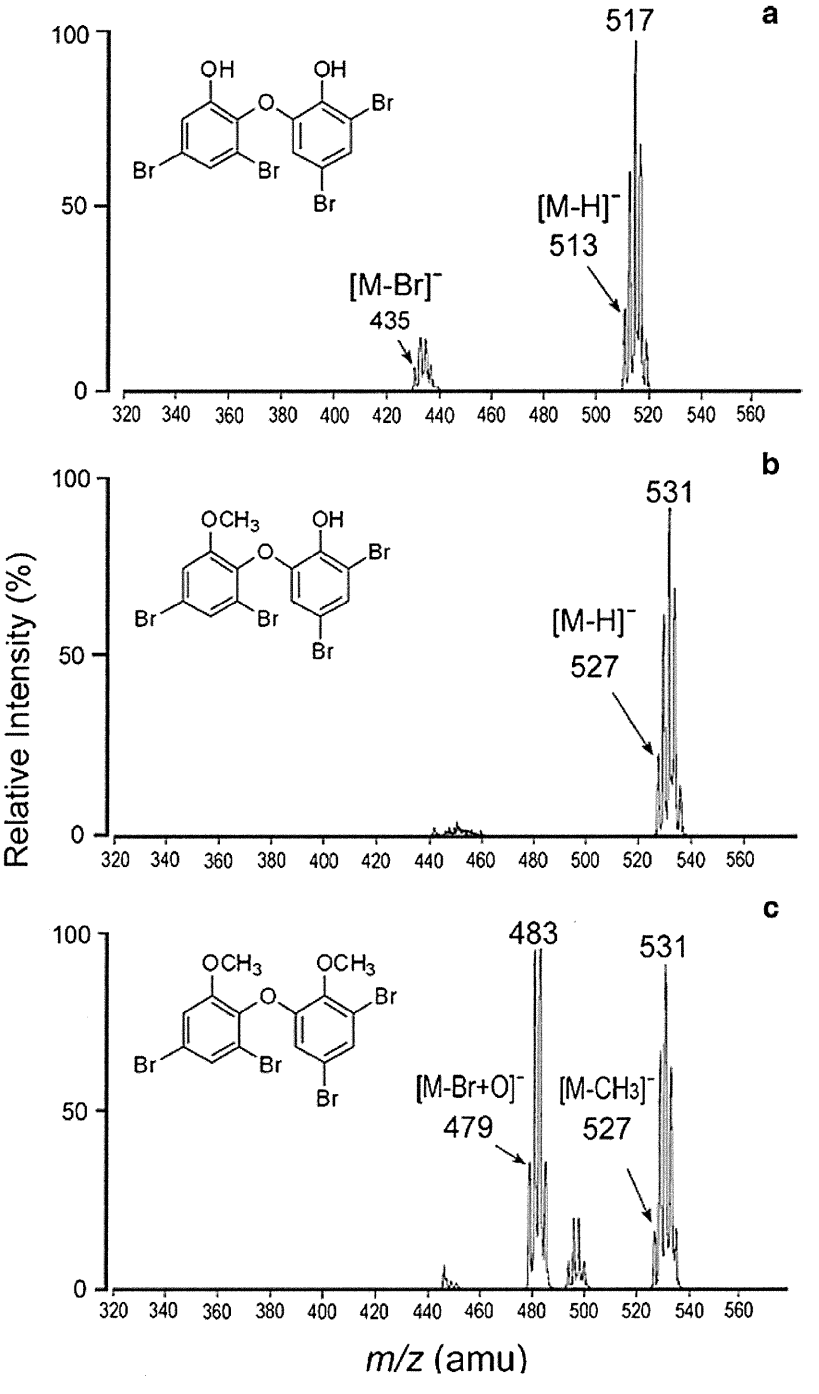

Fig. 1 Chemical structures and LC/MS spectra (precursor ion scan of Br^-) in negative APCI mode of authentic 2′,6-diOH-BDE68 (**a**), 2′-OH-6-MeO-BDE68 (**b**), and 2′,6-diMeO-BDE68 (**b**). The concentration of each standard was 2 μg mL^{-1}

Sciences, as numbers SP05-319 (*Callyspongia* sp.), SP05-321, and SP05-322 (*Lamellodysidea* sp.).

Sample preparation

Sponge tissues were cut into pieces and the homogenate was extracted with methanol/ethyl acetate (1:1, *v/v*) at room temperature. The crude extracts were filtered, and the organic matter was determined gravimetrically. The purification was performed as described previously [31]. Briefly, portions of the crude extracts were purified by gel-permeation chromatography (Bio-Beads, S-X3, Bio-Rad Laboratories, Hercules, CA, USA), with elution with dichloromethane/*n*-hexane (1:1, *v/v*). The eluate, which contained phenolic PBDEs, was concentrated to dryness and dissolved in *n*-hexane. The hexane extracts were partitioned in 2 M KOH/ethanol (7:3, *v/v*). The organic phase (neutral fraction) was purified by silica-gel column chromatography (0.2 g, Wako gel S-1, Wako Pure Chemical Industries Ltd., Osaka, Japan), eluted with dichloromethane/*n*-hexane (12:88, *v/v*). The aqueous layer was acidified with 2 M HCl and back-extracted three times with *n*-hexane/diethyl ether (8:2, *v/v*; phenolic fraction).

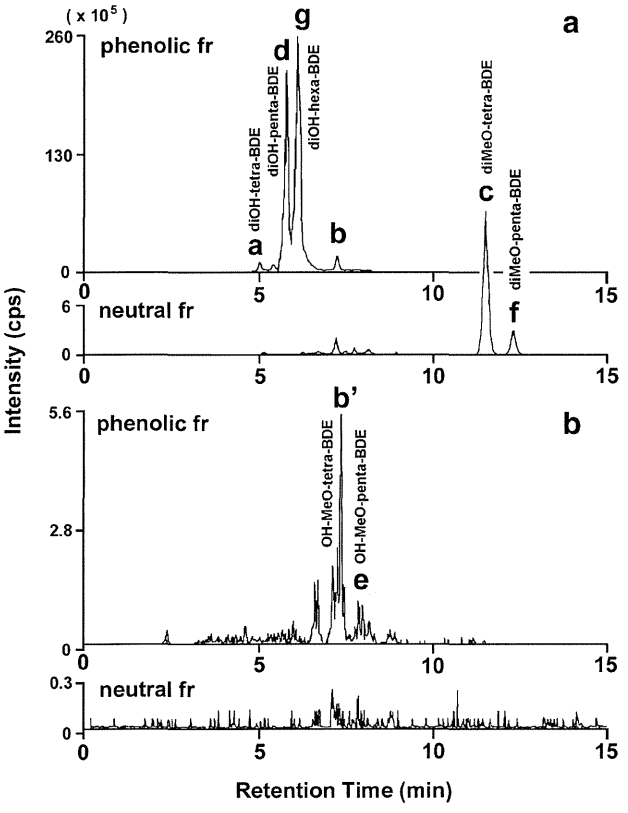

Fig. 2 Total ion chromatograms of PBDE analogs in the phenolic and neutral fractions of sponge extracts from *Lamellodysidea* sp. (*upper*) and *Callyspongia* sp. (*lower*)

Both the neutral and phenolic fractions were concentrated and dissolved in acetonitrile for LC/MS/MS analysis.

APCI-LC/MS/MS analysis

Analyses were carried out using a liquid chromatograph (Prominence 20A; Shimadzu Co., Kyoto, Japan) coupled to a tandem mass spectrometer (3200QTRAP-MS/MS system; AB SCIEX, Tokyo, Japan). The column conditions and MS/MS parameters were optimized as described previously [28]. A reversed-phase Shim-pack FC-ODS column (150× 4.6 mm, i.d., 3.0 μm particle size; Shimadzu Co., Kyoto, Japan) was used. The isocratic mobile phase composition was acetonitrile/water (9:1, *v/v*) at 0.5 mL min^{-1}. Full-scan data acquisition was performed by scanning from *m/z* 50 to 700 (Q1 scan range) in profile mode, using a scan time of 1 s with a step size of 0.1 amu and a pause between each scan of 5 ms. To select the fragmentation patterns of *m/z* (Q1)→*m/z* (Q3) ions for the multiple reaction monitoring (MRM) transitions, product ion scan mass spectra were recorded by collision-activated dissociation of selected precursor ions. The optimized parameters for each analyte are summarized in Table 1.

GC/MS analysis for comparison

The phenolic fraction from the sponge extract was further methylated with diazomethane for GC/MS analysis. The methylated phenolic fraction was analyzed using a gas chromatograph (Agilent 6980N, Agilent, Santa Clara, CA, USA) equipped with a mass-selective detector (5973i) in electron-ionization and selected-ion-monitoring mode. The GC/MS conditions were the same as described elsewhere [31].

Quality assurance and quality control

An eight-point calibration curve for 2′,6-diOH-BDE68, 2′-OH-6-MeO-BDE68 and 2′,6-diMeO-BDE68 in the concentration range 2–2,000 ng mL^{-1} was used to determine linearity. The limits of quantification (LOQs) were determined using spiked samples (n=5) at 5 and 20 ng mL^{-1}. LOQs using a signal-to-noise ratio (*S/N*) of 10 were measured for 2′-OH-6-MeO-BDE68, 2′,6-diOH-BDE68, and 2′,6-diMeO-BDE68. Recoveries of the three compounds were assessed by spiking with 10 and 50 ng of each compound throughout the entire extraction procedure. The repeatability (percentage relative standard deviation (RSD%)) for all analytes was evaluated by five consecutive injections of a 20–50 ng mL^{-1} standard solution and by measuring the same standard solution on different days. All reported concentrations were calculated by comparing the MRM peak areas to the peak areas produced by external

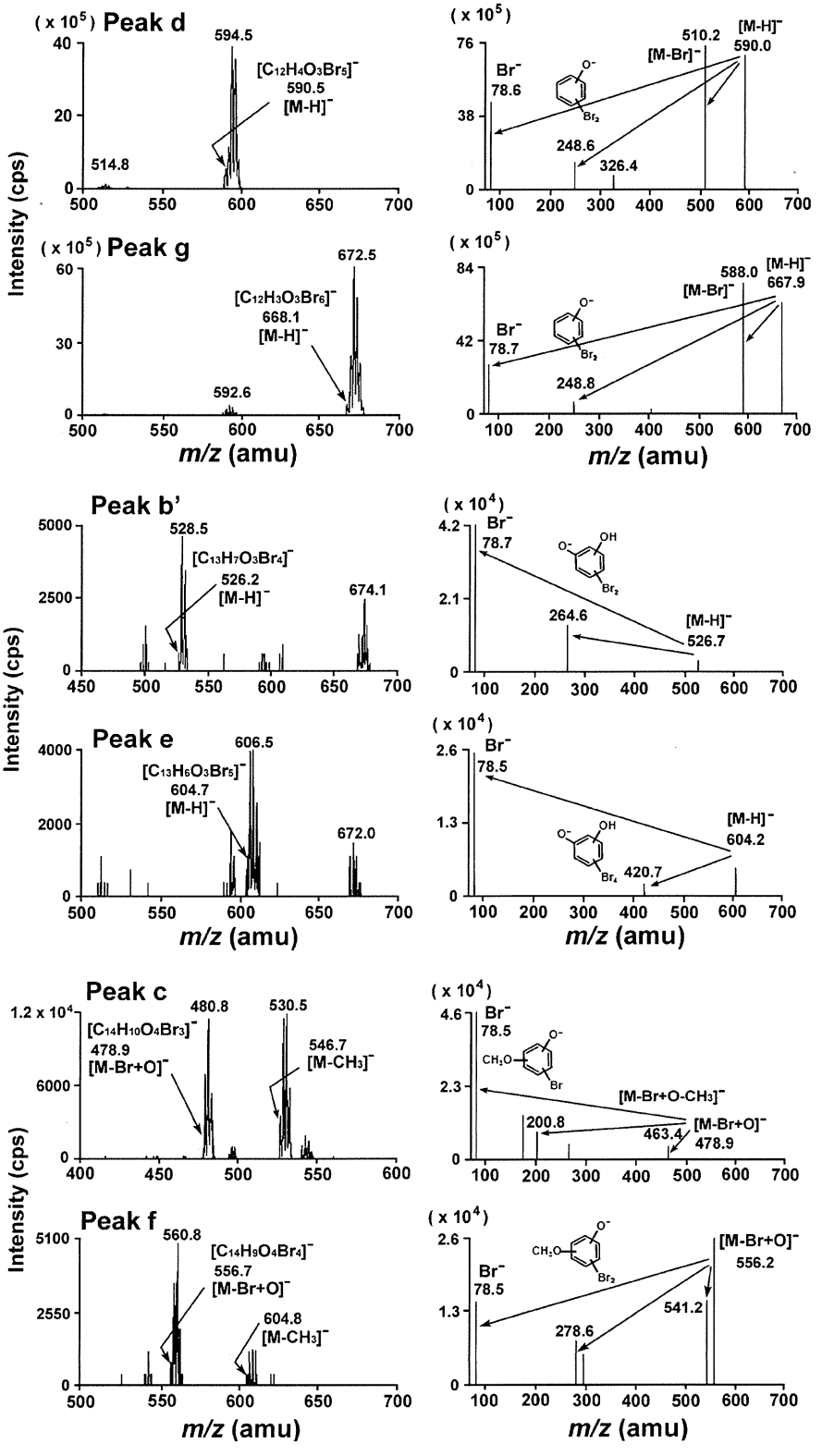

Fig. 3 LC/MS spectra (precursor ion Q1 and product ion Q3 scan) of peaks labeled according to Fig. 2; peaks d and g are diOH-penta-/hexa-BDEs in the phenolic fraction of *Lamellodysidea* sp., peaks b' and e are OH-MeO-tetra-/penta-BDEs in the phenolic fraction of *Callyspongia* sp., and peaks c and f are diMeO-tetra-/penta-BDEs in the neutral fraction of *Lamellodysidea* sp.

standards. Each group of compounds was quantified based on the assumption that the MRM responses of ($[M+4-H]^- \rightarrow Br^-$) for tetra-, penta-, and hexa-BDE analogs are the same as those of 2′-OH-6-MeO-BDE68, and that the MRM responses of ($[M+4-Br+O]^- \rightarrow Br^-$) are the same as those of 2′,6-diMeO-BDE68.

Results

The phenolic PBDEs (i.e., 2′,6-diOH-BDE68 and 2′-OH-6-MeO-BDE68) exhibited a base peak $[M+4-H]^-$ and a weak fragment peak $[M+4-Br]^-$, whereas the dimethoxylated PBDE (i.e., 2′,6-diMeO-BDE68) exhibited two characteristic ions, $[M+4-Br+O]^-$ and $[M+4-CH_3]^-$ (Fig. 1). The MRM parameters, LC retention times, LOQs, recoveries, and repeatabilities for the five authentic standards are shown in Table 1. Each congener was well separated on the reversed-phase column within 15 min, and none of the MRM peaks interfered with the others. These five standards were used for validation of the method and for quantification of each homolog as an external standard. The five-point calibration curve (20–1,000 ng mL^{-1}) of each congener was linear (R^2>0.999), and the procedural recoveries were 77–92 % (n=5) for 2′-OH-6-MeO-BDE68 in the phenolic fraction and 88–93 % (n=4) for 2′,6-diMeO-BDE68 in the neutral fraction. The LOQ of 2′-OH-6-MeO-BDE68 was 9.1 ng mL^{-1} (S/N=10), which was about 5-fold higher than that for 2′-OH-BDE68 (1.9 ng mL^{-1}) (Table 1). The repeatabilities (n=5, RSD%) for each standard were 15–22 % for inter-day variations.

Total ion chromatograms (TICs; precursor ion scan of Br^-) for brominated compounds in the phenolic and neutral fractions of the two sponge species are shown in Fig. 2. The Q1 scans of major peaks in the phenolic fraction (Fig. 3) showed intense molecular-related ions $[M+4-H]^-$ for diOH-penta-BDEs (*m/z* 595, peak d) and diOH-hexa-BDEs (*m/z* 673, peak g), OH-MeO-tetra-BDEs (*m/z* 531, peak b′), and OH-MeO-penta-BDEs (*m/z* 609, peak e), whereas the Q1 scans of the peaks in the neutral fraction gave characteristic ions, $[M+4-Br+O]^-$ and $[M+4-CH_3]^-$, for diMeO-tetra-BDEs (*m/z* 483, peak c), and diMeO-penta-BDEs (*m/z* 561, peak f), respectively. No mass spectra were acquired for OH-MeO-hexa-BDEs.

Under the optimized conditions (Table 2), the MRM chromatograms gave peaks a, d, and g for diOH-tetra-/penta-/hexa-BDEs (t_R=4.5–6.5 min), peaks b, b′, e, and e′ for OH-MeO-tetra-/penta-BDEs (t_R=6.5–8.0 min), and peaks c and f for diMeO-tetra-/penta-BDEs (t_R=11–14 min) in the extracts from the two sponge species (Fig. 4). No peaks were observed in the MRMs for OH-MeO-/diMeO-hexa-BDEs. Each MRM peak for ($[M+4-H]^- \rightarrow Br^-$) was synchronized with those of the isocratic transition ($[M-H]^- \rightarrow Br^-$) at an isotope ratio of about 1:3 to 1:6 for $[M-H]^-/[M+4-H]^-$. In the MRMs for diOH-tetra-BDEs, peak a was identified as 2′,6-diOH-BDE68. The signal next to peak a (Fig. 4a) represents an interfering peak from fragment ions (e.g., $[M+4-Br]^-$) of diOH-penta-BDEs (peak d).

In the MRMs for OH-MeO-tetra-BDEs, peak b′ (t_R=7.35 min) was tentatively identified as 2′-MeO-6-OH-BDE68 in extracts of *Callyspongia* sp. (Fig. 4a), whereas the other peak b (t_R=6.60 min) was identified as the isomeric 2′-OH-6-MeO-BDE68 in the extracts of *Lamellodysidea* sp. Similarly, in the MRMs for OH-MeO-penta-BDE, peak e was observed in the *Lamellodysidea* sp. and an additional peak e′ was observed in the *Callyspongia* sp. (Fig. 4b); these were identified as the isomeric 2′-MeO-6-OH and/or 2′-OH-6-MeO analogs. In the MRMs for diMeO-PBDEs, two major components (peaks c and f) were observed in both species, of which peak **c** had the same t_R as 2′,6-diMeO-BDE68.

The identification of each homolog group in the phenolic fraction was confirmed by GC/MS in EI mode, following derivatization with diazomethane (Fig. 5); there was one tetra-BDE homolog, two penta-BDE homologs, and one hexa-BDE homolog in the methylated phenolic fraction.

Using three external standards, we quantified three homolog groups of diOH-, OH-MeO-, and diMeO-PBDEs in the extracts from three marine sponge species (two genus) using APCI-LC/MS/MS. Table 3 shows the concentrations (in micrograms per gram wet weight, (ww)) of the mono-oxygenated (OH- and MeO-) tetra-BDE, and di-oxygenated (diOH-, OH-MeO-, and

Table 2 MRM parameters used for quantitative determination of PBDE homologs

Compound	Chemical formula	Precursor ion	Product ion	MRM transition (*m/z*)	DP (V)	CE (V)
Tetra-BDE homolog						
diOH-tetra-BDE	$C_{12}H_6O_3Br_4$	$[M+4-H]^-$	Br^-	516.7→78.9	−30	−40
OH-MeO-tetra-BDE	$C_{13}H_8O_3Br_4$	$[M+4-H]^-$	Br^-	530.7→78.9	−30	−40
diMeO-tetra-BDE	$C_{14}H_{10}O_3Br_4$	$[M+4-Br+O]^-$	Br^-	482.8→78.9	−30	−40
Penta-BDE homolog						
diOH-penta-BDE	$C_{12}H_5O_3Br_5$	$[M+4-H]^-$	Br^-	594.6→78.9	−30	−40
OH-MeO-penta-BDE	$C_{13}H_7O_3Br_5$	$[M+4-H]^-$	Br^-	608.6→78.9	−30	−40
diMeO-penta-BDE	$C_{14}H_9O_3Br_5$	$[M+4-Br+O]^-$	Br^-	560.7→78.9	−30	−40
Hexa-BDE homolog						
diOH-hexa-BDE	$C_{12}H_4O_3Br_6$	$[M+4-H]^-$	Br^-	672.5→78.9	−30	−40
OH-MeO-hexa-BDE	$C_{13}H_6O_3Br_6$	$[M+4-H]^-$	Br^-	686.5→78.9	−30	−40
diMeO-hexa-BDE	$C_{14}H_8O_3Br_6$	$[M+4-Br+O]^-$	Br^-	638.7→78.9	−30	−40

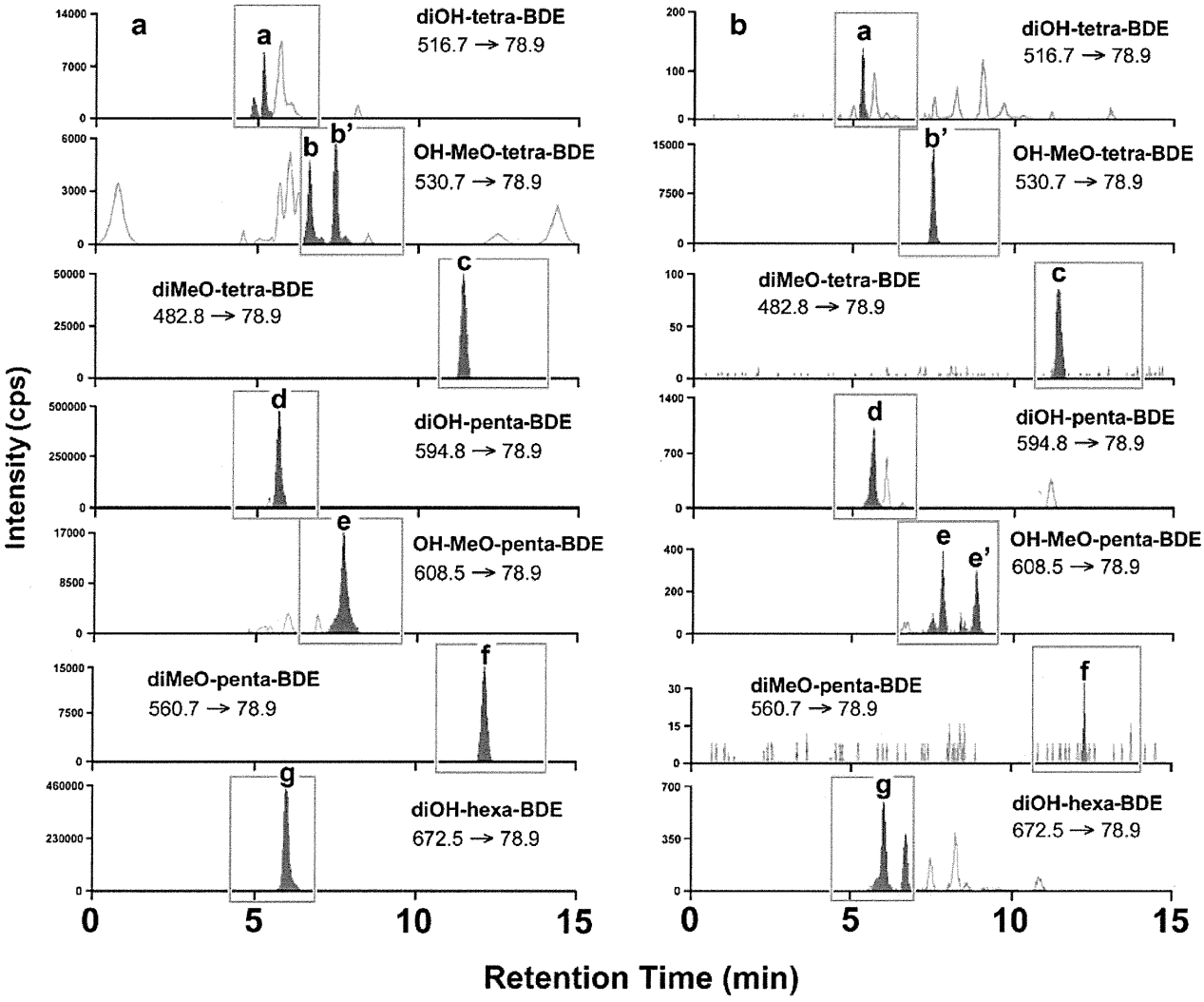

Fig. 4 MRM chromatograms of diOH-, OH-MeO-, and diMeO-substituted tetra-, penta-, and hexa-BDEs in marine sponges: *Lamellodysidea* sp. (**a**) and *Callyspongia* sp. (**b**). Peak labeling is according to Fig. 2. Stationary phase, 150×4.6 mm, i.d. Shim-pack FC-ODS column; mobile phase, acetonitrile:water (9:1, *v/v*) at a flow rate of 0.5 mL min^{-1}

diMeO-) tetra-, penta-, and hexa-BDEs in the two genus. For mono-oxy-substituted PBDEs, OH-tetra-BDEs (62 μg g^{-1} ww in the *Lamellodysidea* sp. (SP05-322) and 3.0 μg g^{-1} ww in the *Callyspongia* sp.) were detected at higher levels than the corresponding MeO-tetra-BDEs (10 and 1.8 μg g^{-1} ww, respectively). In the *Lamellodysidea* sp.(SP05-322), the levels of diOH-tetra-BDE (31 μg g^{-1} ww), diOH-penta-BDE (264 μg g^{-1} ww), diOH-hexa-BDE (196 μg g^{-1} ww) were higher than those of the corresponding OH-MeO analogs (less than 4.9 μg g^{-1} ww). In contrast, the phenolic fraction of the *Callyspongia* sp. contained higher amounts of OH-MeO-tetra-BDEs (6.0 μg g^{-1} ww) than of diOH-tetra-BDEs (0.2 μg g^{-1} ww) or diMeO-tetra-BDEs (0.4 μg g^{-1} ww). The percentage contributions (%) of diOH-, OH-MeO-, and diMeO-tetra-BDEs were 5:1:94 in the *Lamellodysidea* sp. and 3:92:5 in the *Callyspongia* sp. (Table 3). The total percentage contributions of monohydroxy- and dihydroxy-substituted homologs were 5.7 % (72 μg g^{-1} ww) and 93 % (1,174 μg g^{-1} ww), respectively, in the *Lamellodysidea* sp., and 40 % (4.8 μg g^{-1} ww) and 60 % (7.3 μg g^{-1} ww), respectively, in the *Callyspongia* sp.

Discussion

Until now, dihydroxylated or hydroxy-methoxy derivatives of PBDEs in the phenolic fractions from marine samples have been identified as diMeO-PBDEs by GC/MS,

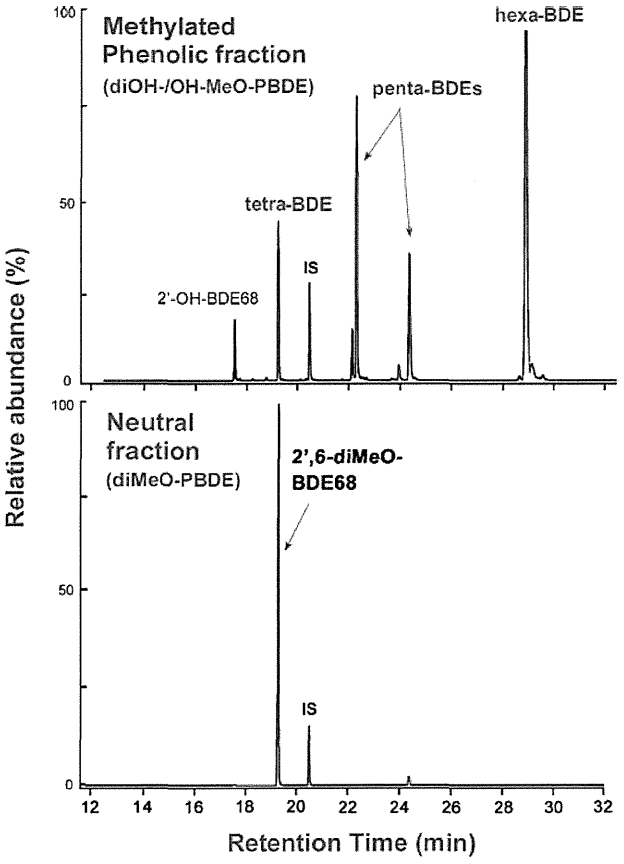

Fig. 5 GC/MS (EI) TIC of brominated products in the methylated phenolic fraction (*upper*) and neutral fraction (*lower*) from *Lamellodysidea* sp. extracts. The phenolic fraction (Fig. 2) was methylated with diazomethane. The peaks in this fraction are estimated as the sum of diOH and OH-MeO analogs (one peak for tetra-, two peaks for penta-, and one peak for hexabrominated homologs). IS internal standard, 4′-MeO-BDE121

following derivatization, and thus quantified as a mixture [31]. The present study demonstrates that dihydroxy, hydroxy-methoxy, and dimethoxy analogs of each PBDE could be separately determined by MRM of LC/MS/MS in negative APCI mode, so that the concentration ratios of the three analogs can be calculated in underivatized fractions.

The method was validated using 2′,6-diOH-BDE68, 2′-OH-6-MeO-BDE68, and 2′,6-diMeO-BDE68 (Fig. 2). The LOQs (9.1 ng mL^{-1}) of two phenolic standards by LC/MS/MS in the negative APCI mode are comparable to the LOQ of 2′-OH-6-MeO-BDE68 by GC/MS in EI mode after *O*-methylation. This allows the determination of diOH-PBDEs in human fluid matrices (e.g., blood). The present LOQ for diOH-BDE68 is slightly higher than the LOQs reported by Mas et al. [24] which were measured for eight OH-PBDEs (0.7–4.6 g mL^{-1}) in negative ion-spray ionization mode, and also those reported by Lai et al. [27] who reported LOQs of 0.2–2.8 ng mL^{-1} for nine OH-PBDEs, using the same mode. Higher ionization intensities may occur in higher brominated congeners such as penta-BDE or hexa-BDE [32]. The accuracies of spiked samples and repeatabilities for diOH-PBDEs (n=3, RSD<22 %) in this study are similar to those for OH-PBDEs obtained using other methods [24,25]. The present LC/MS/MS method with an isocratic mobile phase may give incomplete separation of OH-PBDE isomers, compared with the ultra-performance liquid chromatography method used by Lai et al. [27] who demonstrated efficient separation of nine OH-PBDEs using a gradient system. However, these could not be directly compared with our target analytes. In our study, the two isomeric 2′-OH-6-MeO- and 2′-MeO-6-OH-BDE68s were separated from each other under the HPLC conditions used. Furthermore, OH-MeO-tetra-BDE (peak b') can be distinguished by MRM from OH-tetra-BDE (2′-OH-BDE68), which it was close to in terms of LC retention times (t_Rs of 2′-OH-BDE68 and 2′-MeO-6-OH-BDE68 were 7.2 and 7.35 min, respectively). Mono- and dihydroxylated PBDE analogs from the marine food web can therefore be screened using the APCI-LC/MS/MS, without derivatization. We fractionated the phenolic and neutral compounds by partitioning with KOH and n-hexane. However, the fractionation following the GPC procedure may not be necessary, because the both products can be separated by HPLC (Fig. 2).

The developed method was applied to two marine sponges with different profiles of dihydroxylated analogs, from tetra- to hexabrominated. The Q1 scan (TIC) indicated that the diOH analogs of penta- and hexa-BDEs are predominant in the *Lamellodysidea* sp, whereas OH-MeO-tetra-BDEs are selectively present in the *Callyspongia* sp. The interfering peak which appeared in the MRMs of OH-MeO-BDEs may be caused by the fragment ion $[M-Br]^-$ of diOH-PBDEs. In fact, the *Lamellodysidea* sp. produced large amounts of diOH analogs (at levels two orders of magnitude higher than that for the OH-MeO analogs). As a result, the MRM ($[M+4-H]^- \rightarrow Br^-$) for OH-MeO-tetra-BDEs is interfered with by that for the diOH-penta-BDEs.

In the MRMs of OH-MeO-tetra-/penta-BDEs, the presence of two isomeric congeners is confirmed by GC/MS following derivatization of the phenolic fraction (Fig. 5). The methylated phenolic fraction gave one peak for the tetra-BDE homolog, two peaks for the penta-BDE homologs, and one peak for the hexa-BDE homolog, originating from the diOH and/or OH-MeO analogs. LC/MS/MS showed that a higher amount of diOH-tetra-BDE than of OH-MeO was observed in the *Lamellodysidea* sp., where diOH/OH-MeO was 10:1 for the tetra-BDE homologs, and 100:1for penta-BDE. In contrast, a higher amount of OH-MeO-tetra-BDE was observed in the *Callyspongia* sp. (the ratio, 1:30 for the tetra-BDE homologs). These findings suggest that the formation ratios of these phenolic PBDEs are species-dependent and support the

Table 3 Determination of dihydroxy-, hydroxy-methoxy-, and dimethoxy-PBDEs in marine sponges using APCI-LC/MS/MS

Homologs	Concentrations (μg g^{-1} wet weight)		
	Lamellodysidea sp.		*Callyspongia* sp.
	SP05-321	SP05-322	SP05-319
Mono-substituted			
OH-tetra-BDE	55.4	61.8	3.01
MeO-tetra-BDE	2.19	10.4	1.82
Total	57.6 (6.1 %)	72 (5.7 %)	4.8 (40 %)
Di-substituted			
Tetra-BDE homolog			
diOH-tetra-BDE	37.9	30.7	0.20
OH-MeO-tetra-BDE	3.79	4.93	5.98
diMeO-tetra-BDE	381	639	0.36
Total	423 (44.7 %)	675 (53.7 %)	6.5 (54.2 %)
Penta-BDE homolog			
diOH-penta-BDE	276	264	0.58
OH-MeO-penta-BDE	3.33	18.5	0.06
diMeO-penta-BDE	7.98	20	ND
Total	287 (30.3 %)	303 (24.1 %)	0.64 (5.3 %)
Hexa-BDE homolog			
diOH-hexa-BDE	170	196	0.06
OH-MeO-hexa-BDE	ND	ND	ND
diMeO-hexa-BDE	ND	ND	ND
Total	170 (18.0 %)	196 (15.6 %)	0.06 (0.5 %)
Total	947 (100 %)	1,256 (100 %)	12 (100 %)

ND not detected (less than 0.05 μg g^{-1} wet weight)

hypotheses that the bacterial *O*-methylation of diOH-PBDEs in the sponges may vary with the degree of bromination [33].

Mixed analogs of mono- and di-OH-tetra-BDEs have also been isolated from *Dysidea* spp. [5–8] and marine algae [10]. The present study also supported the suggestion that in most species the major component has the diphenyl ether structure oxygenated at the 2,6′-position. One of the methylated products, 2′,6-diMeO-BDE68, has been found to accumulate in fish [31], shark liver [19], and whale blubber [16,19,20]. The potential source may be the sponge brominated products in the phenolic fraction, but these phenolic products have not been detected, or were only present in trace amounts, in higher trophic organisms [28], probably because of the lower persistence and higher water solubility of phenolic PBDEs, and they accumulate as diMeO-tetra-BDEs following bacterial methylation of the hydroxyl group.

The simultaneous determination of OH-, OH-MeO-, and diOH-PBDEs in phenolic fractions are of great importance in monitoring studies of animals and humans, because these analogs exhibit a variety of bioactivities, depending on the structure (the presence of one or two hydroxyl groups in PBDE molecules) [7–9]. For example, diOH-PBDEs and OH-MeO-PBDEs are active against the Gram-positive bacterium *Bacillus subtilis*, whereas in their methylated analogs (diMeO-PBDEs), such activity is reduced [7]. In another report, OH-MeO-PBDEs were shown to have a greater biological effect than those of the corresponding diOH-PBDEs [34]. The proposed LC/MS/MS method should be an efficient tool for elucidating the *O*-methylation of diOH-PBDEs, or microsomal demethylation of diMeO-PBDEs.

Conclusions

This study demonstrated that negative APCI-LC/MS/MS is an efficient tool for the selective determination of diOH-PBDEs and the corresponding *O*-methylated analogs in marine biota. The method provides better selectivity for diOH-PBDEs and OH-MeO-PBDEs in the MRM transition ($[M-H]^- \rightarrow Br^-$), and HPLC could be used to resolve the homologs. The APCI-LC/MS/MS method was applied to two sponge extracts with different profiles of a set of diOH, OH-MeO, and diMeO analogs, within homologs and between species. The proposed approach will broaden monitoring studies dealing with the sources, metabolism, and fates of diOH-PBDEs in wildlife and humans.

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Levels of Mercury and Organohalogen Compounds in the Muscle and Liver of Kidako Moray Eels (*Gymnothorax kidako*) Caught off the Southern Region of Japan

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We analyzed the levels of total mercury (T-Hg), methylmercury (M-Hg) and Cd in the muscle and liver of kidako moray eels (*Gymnothorax kidako*) of different body lengths caught off Kochi Prefecture in southern Japan. Furthermore, we analyzed the levels of organohalogen compounds such as polychlorinated biphenyls (PCBs), *p,p'*-1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p'*-DDE), *trans*-nonachlor and 2,3,3',4,4',5,5'-heptachloro-1'-methyl-1-2'-bipyrrole (Q1) and stable isotope ratios of carbon (δ^{13}C) and nitrogen (δ^{15}N) in the muscle of eels. The concentrations of T-Hg and M-Hg in the muscle (edible part) were 0.31±0.08 μg/wet g and 0.25±0.06 μg/wet g (n=26), respectively, and those in large eels exceeded the Japanese legislated levels of T-Hg (0.4 μg/wet g) and M-Hg (0.3 μg/wet g) in fish and shellfish, respectively. The T-Hg and M-Hg concentrations in the liver were markedly higher than those in the muscle, respectively. The ratios of M-Hg to T-Hg in the muscle and liver were about 80 and 60%, respectively, and those ratios tended to decrease with increased body length. The Cd concentrations in the liver tended to increase proportionally with body length, while that in the muscle was trace (around or below 0.03 μg/wet g). The concentrations of PCBs, *p,p'*-DDE, *trans*-nonachlor in the muscle tended to increase proportionally with body length, while that of Q1 did not. The δ^{13}C and δ^{15}N values in the kidako moray eel were markedly higher than those in offshore habit predators reported elsewhere, which may reflect the inshore habitat of this eels.

Key words kidako moray eel (*Gymnothorax kidako*); mercury; cadmium; polychlorinated biphenyl; *p,p'*-1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene; stable isotope ratio

The kidako moray eel, *Gymnothorax kidako*, is a species of moray eel native to the Northwestern Pacific Ocean. The kidako moray eel is commonly found in the southern region of Japan, and small scale commercial fishing is undertaken along the regions bordering the Kuroshio current (a warm current), which include Kochi, Wakayama, Shizuoka and Chiba Prefectures. The kidako moray eels usually reside in crevices in shallow reefs, and primarily feed on fish, cephalopods, and crustaceans.[1] Although little is known about their body length (age) at maturation, the maximum body length of the mature animal and the life span of the kidako moray eel, the maximum body length appears to be about 120 cm as eels exceeding 115 cm have been sold in retail outlets in Kochi Prefecture. The cooked eel flesh, either lightly roasted (*Tataki*) or boiled down with soy sauce (*Tsukudani*), are famous and traditional dishes in Kochi Prefecture and Wakayama Prefecture, respectively.

Mercury (Hg) is a metal that typically accumulates in predators such as tuna, sharks and marine mammals *via* the marine food web in an age (body length)-dependent manner.[2–7] In our survey of Hg in fish and marine foods (unpublished data), the average concentration of total mercury (T-Hg) in roasted eels purchased in Kochi Prefecture between 2004 and 2005 was 0.42 μg/wet g (n=9), which is similar to the levels of T-Hg found in spiny dogfish[7] and yellowfin and albacore tuna.[8] As Hg toxicity due to fish consumption is estimated by the amount of ingested methylmercury (M-Hg),[9] further study of Hg in the kidako moray eel, including M-Hg determination, is necessary to estimate Hg toxicity due to their consumption more precisely. In contrast, cadmium (Cd) accumulation among marine biota is highest in mollusks, particularly cephalopods, and occurs *via* species-specific physiologic mechanisms and not *via* the marine food web.[10,11] Higher levels of Cd are reported to be accumulated in some predators which preferentially eat cephalopods.[4–6,10–12] A high level of Cd was expected to be accumulated in the kidako moray eel because of their preference for cephalopods. As far as we know, however, no data for Hg and Cd concentrations in the muscle of the kidako moray eel is available.

As with toxic heavy metals, predatory fish and marine mammals accumulate anthropogenic lipophilic compounds, such as polychlorinated biphenyls (PCBs), dichloro-diphenyl-trichloroethane (DDT) and its metabolites (DDTs; *p,p'*-DDT, *p,p'*-DDD and *p,p'*-1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p'*-DDE)), and chlordane-related compounds (CHLs; *trans*-chlordane, *cis*-chlordane, *trans*-nonachlor, *cis*-nonachlor and oxychlordane)[13–15] as well as naturally originating compounds of 2,3,3',4,4',5,5'-heptachloro-1'-methyl-1-2'-bipyrrole (referred to as Q1).[8,16] Q1 was first detected in fish and mammals from Australia and is known to be distributed throughout the world's oceans.[17] PCBs and DDTs are accumulated in predators in an age (body length)-dependent manner *via* the marine food web, but little is known about the trophic transfer and age-dependent accumulation of Q1.

Stable isotope analysis has been used as a tool by which to obtain information on the feeding ecology of marine biota. The δ^{15}N value shows stepwise increases in trophic level in the food chain,[18,19] whereas the δ^{13}C value is used to indicate

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the relative contribution of potential primary sources to the diet and can demonstrate differences between species taking coastal and offshore prey or between species taking pelagic and benthic prey.[18,19] Many studies on the trophic transfer of Hg and organochlorine compounds *via* the food web have utilized stable isotope ratios.[13–15,20,21] However, little is known about the relationships among Cd level, body length (age) and trophic level of marine biota evaluated by $\delta^{15}N$ value. Furthermore, no studies on the growth and accumulation of not only heavy metals but also organochlorine compounds have yet been undertaken for eel species.

Here, we report our analytical data for T-Hg, M-Hg, Cd, PCBs, *p,p'*-DDE (a major metabolite of DDTs), *trans*-nonachlor (a major chemical among CHLs) and Q1 levels and discuss the human health problems associated with M-Hg intake through the consumption of kidako moray eel. Furthermore, we present the relationships among the concentrations of those metals and organohalogen compounds, body length and stable isotope ratios in this eel species, and compare them with those of predatory fish such as spiny dogfish[7] and tuna species.[8]

MATERIALS AND METHODS

Sampling of Market Products Kidako moray eels caught off the coast of Kochi Prefecture were purchased from a retail outlet in Kochi (Kochi Prefecture) in December 2006 (n=14), January 2007 (n=6) and March 2007 (n=6). Total body length and total weight of the eels were measured, and muscle and liver samples were extracted and stored at −20°C until chemical analyses.

Chemical Analyses The total mercury (T-Hg) concentrations in the muscle and liver samples were determined using a flameless atomic absorption spectrophotometer (Hiranuma Sangyo Co., Ltd., HG-1, Ibaraki, Japan) after digestion by a mixture of HNO_3, $HClO_4$ and H_2SO_4.[22] Methylmercury (M-Hg) concentrations in the samples were determined using a gas chromatograph (Shimadzu Co., Ltd., GC-14A, Kyoto, Japan) with a ^{63}Ni electron capture detector (ECD).[16] Cadmium (Cd) concentrations were analyzed using a Z-8100 Hitachi Polarized Zeeman flame atomic absorption spectrophotometer (Hitachi Ltd., Tokyo, Japan) after digestion by HNO_3 and $HClO_4$.[23] DOLT-2 (National Research Council of Canada) and CRB463 (BCR, European Commission) were used as analytical quality controls for T-Hg, M-Hg and Cd, as reported previously.[5,6,22,24] The mean recoveries of T-Hg, M-Hg and Cd from the quality controls were 95, 88 and 93% (n=5), respectively, and those of spiked metals from the muscle and liver samples were ranged from 85 to 102%. The M-Hg data were corrected against the recovery of the quality controls (88%).

Cleanup procedures of organohalogen compounds were performed according to a modification of our previous method.[16] Hexane extractable lipid (HEL; 10–100 mg) was spiked with an internal standard (IS) solution of CB205 (30 ng), and then removed by gel permeation chromatography (Bio-Beads, SX-3, Bio-Rad Laboratories), including elution with n-hexane–dichloromethane (1 : 1 v/v) for organohalogen residues. The eluate was purified by silica gel S-1 column (0.2 g, Wako Pure Chemical Industries, Ltd., Japan), including further elution with n-hexane (12 mL). Thirteen PCB congeners (CB99, CB101, CB118, CB138, CB146, CB149, CB153, CB170, CB183, CB187, CB194, CB199 and CB208), *p,p'*-DDE, *trans*-nonachlor, and Q1 were quantified using a gas chromatograph (Shimadzu Co., Ltd., GC-2014, Kyoto, Japan) equipped with an ECD. Procedure blanks were processed in parallel to control for any contamination. The recoveries of all analytes and IS in the spiking experiment ranged from 85 to 98%. The detection limits for all analytes were between 0.02 and 0.1 ng/wet g. Quality assurance for PCBs, *p,p'*-DDE and *trans*-nonachlor was confirmed by analyzing Standard Reference Materials (cod liver oil 1588b, NIST). Data from our laboratory were in good agreement with the certified value of the cod liver (relative standard deviation: 15%, n=5).

The stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) in the muscle samples after the removal of lipids were analyzed using a mass spectrometer (Delta S, Finnigan Co., Bremen, Germany) coupled with an elemental analyzer (EA1108, Fisons Co., Milan, Italy), as reported previously.[7,25] CERKU-1, -2 and -5, certified by the Center for Ecology Research, Kyoto University (Kyoto, Japan), were used as the $\delta^{13}C$ and $\delta^{15}N$ reference materials.[25]

The concentrations of T-Hg and M-Hg in the eel samples were expressed as Hg concentration per wet weight basis, and the organohalogen concentrations were expressed on a wet weight basis as well as lipid weight basis.

Statistical Analyses Student's t-test and Pearson's correlation coefficient test were used to analyze the data using the Statcell 2 program, and the level of significance was set at $p<0.05$. All data were expressed as the mean±S.D.

RESULTS

Analytical Results for Body Size, Stable Isotope Ratios, and Mercury, Cadmium and Organohalogen Compound Levels The analytical results for the kidako moray eel specimens are summarized in Table 1. The average body length and weight of the kidako moray eel specimens analyzed were 96±11 cm and 2.4±1.1 kg (n=26), respectively, and a significant correlation was found between body length and body weight of the eel specimens ($p<0.05$). The average $\delta^{13}C$ and $\delta^{15}N$ values found in the muscle samples were −14.6±0.4 and 14.0±0.6‰, respectively, and the average HEL was 0.27±0.14%. HEL did not correlate with the body length, body weight, $\delta^{13}C$ value or $\delta^{15}N$ value.

The average T-Hg and M-Hg concentrations in the muscle samples (edible part) were 0.35±0.11 μg/wet g and 0.27±0.07 μg/wet g (n=26), respectively. The highest T-Hg and M-Hg concentrations in the samples were 0.64 and 0.41 μg/wet g, respectively, and 7 and 8 samples, respectively, exceeded the Japanese limits for T-Hg (0.4 μg/wet g) and M-Hg (0.3 μg/wet g) in fish and shellfish. The average ratio of M-Hg to T-Hg in the muscle samples was 80±8%. No correlations were found between HEL level and T-Hg or M-Hg concentration.

The average concentrations of T-Hg and M-Hg in the liver samples were 1.50±1.13 μg/wet g and 0.84±0.54 μg/wet g (n=26), respectively, which were about 4 and 3 times higher than those in the muscle samples, respectively. The highest T-Hg and M-Hg concentrations found in the liver samples were 4.95 and 2.38 μg/wet g, respectively. The average ratio of M-Hg to T-Hg in the liver samples was 60±15%, which was significantly lower than that in the muscle sample (80±8%).

The average Cd concentration in the liver samples was

Table 1. Analytical Results of Kidako Moray Eels (n=26)

	Body		Liver							
	Length (cm)	Weight (kg)	T-Hg (μg/wet g)	M-Hg (μg/wet g)	Percent (M-Hg/T-Hg)	Cd (μg/wet g)				
Mean	96	2.4	1.50	0.84	60	1.37				
S.D.	11	1.1	1.13	0.54	15	1.08				
Range	76–115	1.6–4.5	0.42–4.95	0.30–2.38	30–85	0.3–3.88				
	Muscle									
	δ^{13}C (‰)	δ^{15}N (‰)	T-Hg (μg/wet g)	M-Hg (μg/wet g)	Percent (M-Hg/T-Hg)	HEL (%)	PCBs (ng/wet g)	*p,p'*-DDE (ng/wet g)	*trans*-Nonachlor (ng/wet g)	Q1 (ng/wet g)
Mean	−14.6	14.0	0.31	0.25	80	0.27	4.83 (1.84)	1.40 (0.56)	0.61 (0.22)	2.35 (0.88)
S.D.	0.4	0.6	0.08	0.06	8	0.14	4.49 (1.32)	0.88 (0.33)	0.52 (0.11)	1.56 (0.36)
Range	−15.1 to −13.8	13.2–15.2	0.18–0.64	0.14–0.41	66–89	0.11–0.59	1.32–20.41 (0.54–6.80)	0.32–3.59 (0.20–1.30)	0.21–2.01 (0.08–0.55)	0.74–6.31 (0.41–1.99)

Parentheses in mean, S.D. and range indicate the data of concentrations expressed by lipid basis (μg/g lipid).

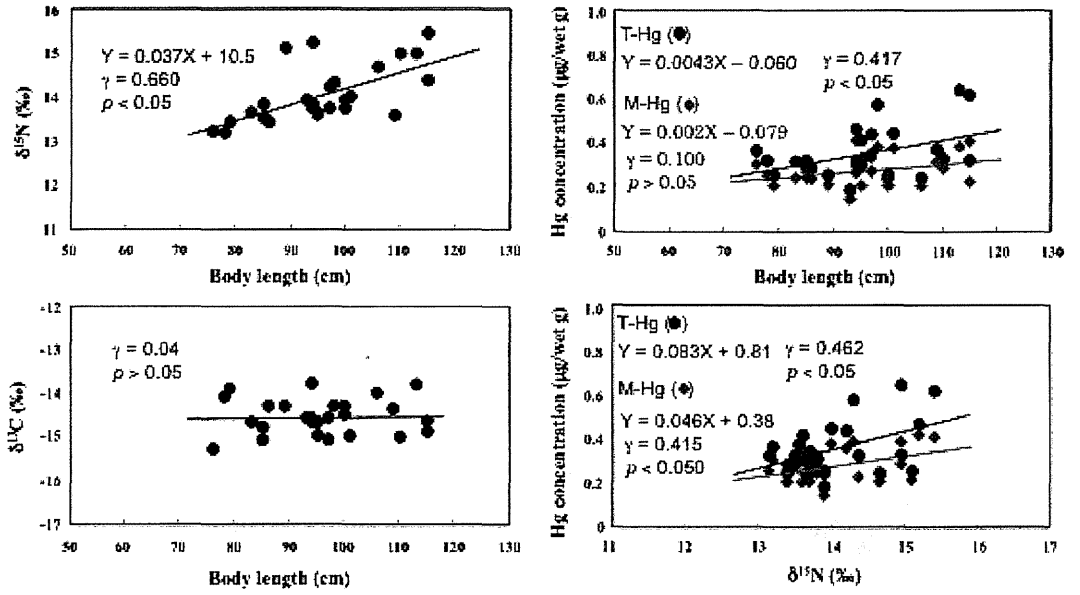

Fig. 1. Correlations among Body Length, $\delta^{15}N$ and $\delta^{13}C$ Values, and Hg Concentrations in the Muscle
See Table 1.

1.37±1.08 μg/wet g (n=26), while that in the muscle samples was around or below the detection limit (0.03 μg/wet g, data not shown in Table 1).

The average levels of PCBs, *p,p'*-DDE, *trans*-nonachlor and Q1 in the muscle samples were 4.83±4.49, 1.40±0.88, 0.61±0.52 and 2.35±1.56 ng/wet g (n=26), respectively, and the concentrations normalized by HEL were 1.84±1.32, 0.56±0.33, 0.22±0.11 and 0.88±0.36 μg/g lipid, respectively. Due to the limited weight of the liver samples, the levels of organochlorine compounds in the liver could not be determined.

Relationships among Body Length, $\delta^{13}C$ and $\delta^{15}N$ Values and Hg Concentration in the Muscle The relationships among body length, $\delta^{13}C$ and $\delta^{15}N$ values and Hg concentration in the muscle samples were investigated (Fig. 1). The $\delta^{15}N$ value increased with increases in body length (p<0.05), whereas the $\delta^{13}C$ value did not. As data not shown in figure, no correlation was found between the $\delta^{13}C$ and $\delta^{15}N$ values. The T-Hg concentration in the muscle samples increased with increases in body length and $\delta^{15}N$ value (p<0.05). The M-Hg concentration increased with increases in $\delta^{15}N$ value (p<0.05), but not with increases in body length. The ratio of M-Hg to T-Hg in the muscle samples tended to decrease slightly with increases in body length and $\delta^{15}N$ value. Higher correlation coefficients (r) between body length or $\delta^{15}N$ value and Hg concentration were found in the normal plot of Hg concentration (Fig. 1) than in the logarithmic plot of Hg concentration (data not shown).

Relationships between Body Length, $\delta^{15}N$ Value and Hg or Cd Concentration in the Liver The relationships among body length, $\delta^{15}N$ value and Hg or Cd concentration in the liver samples are shown in Fig. 2. The T-Hg and M-Hg concentrations increased with increases in body length and $\delta^{15}N$ value (p<0.05), with the increases in T-Hg more prominent than those in M-Hg. The difference in the T-Hg and M-Hg concentrations and the ratio of M-Hg to T-Hg tended to increase and decrease with increases in body length and $\delta^{15}N$ value, respectively, and these tendencies were more prominent in the liver samples than in the muscle samples (Fig. 1). Similarly, the Cd concentration increased with increases in body length and $\delta^{15}N$ value (p<0.05). Higher correlation coefficients (γ) between body length or $\delta^{15}N$ value and Hg or Cd concentration in the liver were found in the normal plot of metal concentration (Fig. 2) than in the logarithmic plot (data not shown).

Relationships among Body Length, $\delta^{15}N$ Value and Organohalogen Concentration in the Muscle Figure 3 shows the relationship between the body length and organochlorine concentration expressed on a lipid weight basis. The concentrations of the anthropogenic compounds of PCBs and *p,p'*-DDE in the muscle samples significantly increased with increases in body length (p<0.05) and that of *trans*-nonachlor tended to increase (p>0.05), while the concentration of Q1, a naturally originating compound, did not increase (p>0.05). This tendency for the concentrations of PCBs, *p,p'*-DDE and *trans*-nonachlor to increase with increases in body length was less prominent when expressed on a wet weight basis (data not shown) than when expressed on a lipid weight basis (Fig. 3). The correlation coefficients (γ) between body length or $\delta^{15}N$ value and organohalogen concentrations were again higher in the normal plot of concentration (Fig. 3) than in logarithmic plot (data not shown). As data not shown in figure, the concentrations of PCBs, *p,p'*-DDE and *trans*-nonachlor, but not that of Q1, in the muscle samples showed weak tendencies to increase with increases in $\delta^{15}N$ value (p>0.05).

DISCUSSION

The average levels of T-Hg (0.35±0.11 μg/wet g) and M-Hg (0.27±0.07 μg/wet g) found in the muscle of kidako moray eels are similar to those in predatory fish such as spiny dogfish,[7] yellowfin and albacore tuna,[8] and skipjack tuna[26] caught off Japan. The contamination levels of T-Hg and M-Hg in the

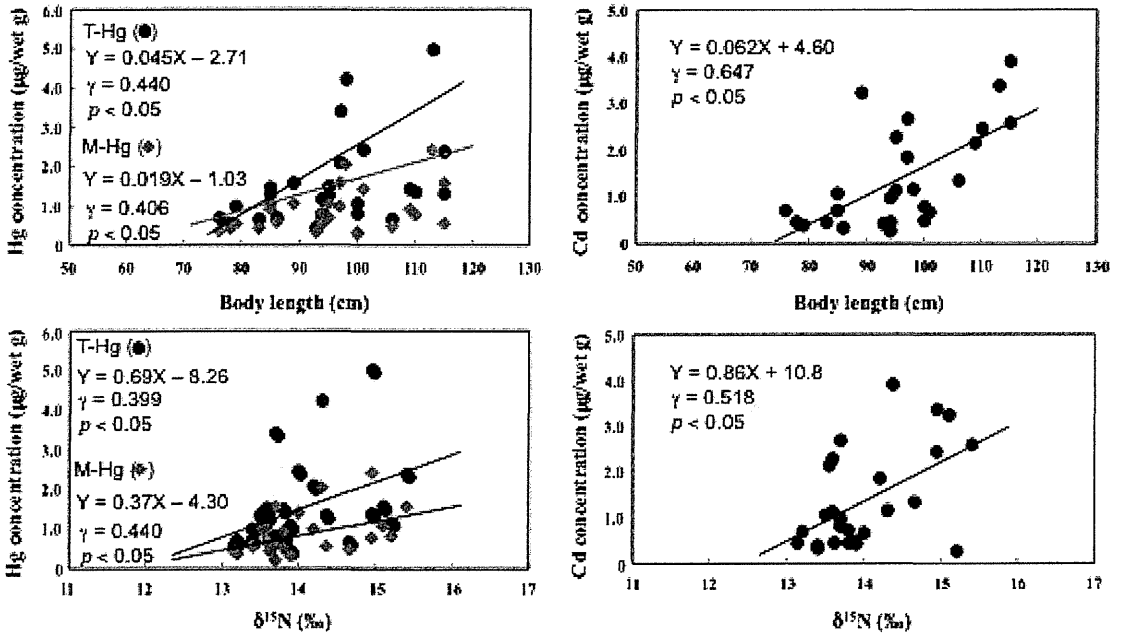

Fig. 2. Correlations among Body Length, $\delta^{15}N$ Value, and Hg and Cd Concentrations in the Liver
See Table 1.

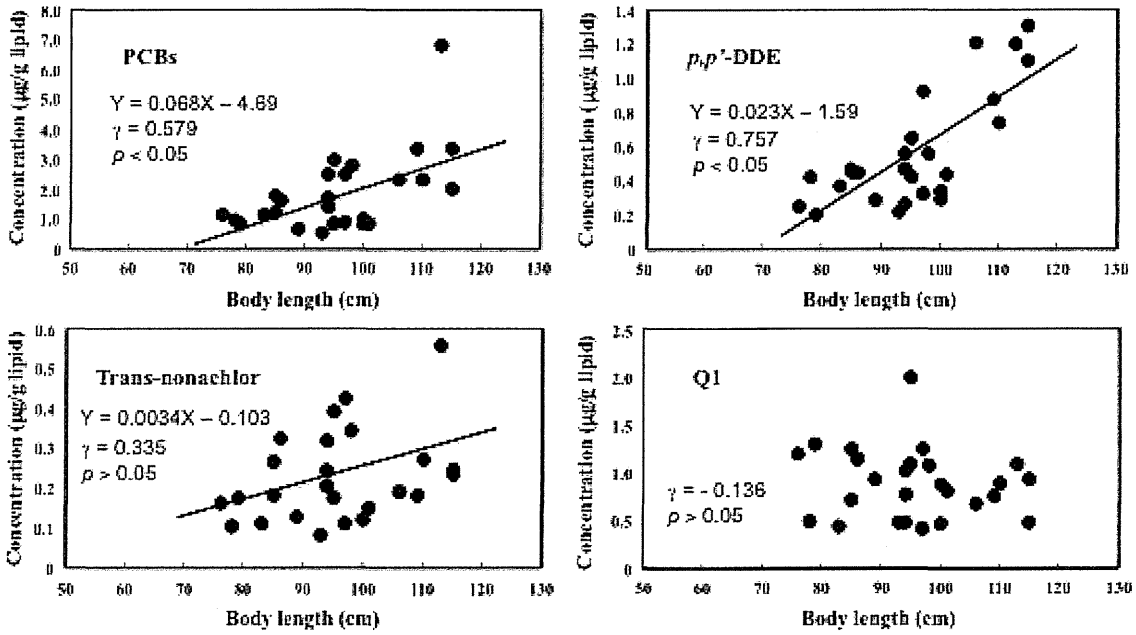

Fig. 3. Correlations among Organohalogen Compounds in the Muscle and Body Length
See Table 1.

eel muscle increased proportionally with increases in body length (Fig. 1), and those of T-Hg and M-Hg found in large eels exceeded the Japanese limits of 0.4 μg/wet g for T-Hg and 0.3 μg/wet g for M-Hg, respectively. The highest concentration of M-Hg found in this study was 0.64 μg/wet g. Consumption of 150 g of this muscle would exceed the provisional tolerable weekly intake (PTWI) for M-Hg set by the FAO/WHO Joint Expert Committee on Food Additives (JECFA) in 2003 for someone with a body weight of 60 kg (1.6 μg/kg b-w/week). Frequent consumption of moray eel muscle may pose health problems for high risk populations such as pregnant women and children. In our unpublished data, the average T-Hg concentration in the cooked muscle of moray eels (*Tataki*) purchased in Kochi Prefecture was 0.42±0.16 μg/wet g (0.28–0.80 μg/wet g, n=9), which was slightly higher than the average T-Hg concentration in the uncooked muscle of moray

eels purchased in that prefecture (0.35±0.11 μg/wet g; Table 1). It is thought that cooking may reduce the moisture content in the muscle, thereby elevating the T-Hg concentration expressed on a wet weight basis.

The T-Hg and M-Hg concentrations in the liver samples were markedly higher than those in the muscle samples (Table 1), respectively. The differences in the T-Hg and M-Hg concentrations and the ratio of M-Hg to T-Hg in the liver samples were more and less than those in the muscle samples, respectively. Higher T-Hg concentrations in the liver than in the muscle have been reported in marine mammals[23,27] and mature tiger sharks[5] and those increases are explained by the formation of HgSe and the binding of inorganic mercury (I-Hg) to metallothineins (MTs) after the demethylation of M-Hg.[5,23,27,28] The determination of Se and MTs is necessary to elucidate the formation of HgSe and the binding of I-Hg to MTs.

Cd is preferentially accumulated in the liver of some predatory species, and high levels of Cd are ascribed to a diet in which cephalopods predominate.[10–12] As a reflection of their preferential feeding on cephalopods, the hepatic Cd concentration found in the kidako moray eel (1.37±1.08 μg/wet g, n=26, Table 1) was markedly higher than that in the tiger shark (0.15±0.24 μg/wet g, n=24),[5] but lower than that in toothed whales, such as the killer whale (7.84±2.90 μg/wet g, n=6),[4] melon-headed whale (7.24±2.08 μg/wet g, n=15)[6] and some dolphin species.[24] In contrast, the δ^{15}N value in the muscle of moray eels was higher than that in whales and dolphins caught off the central and southern regions of Japan[29] as well as that in tiger sharks (our unpublished data). A significant positive correlation, similar to those in other marine predators,[5,12] was observed between hepatic Cd concentration and body length (Fig. 2). Cd may be accumulated in the liver of cephalopod-feeding animals in a body length (age)-dependent manner, irrespective of trophic level as estimated by δ^{15}N value. As mentioned below, the higher δ^{15}N value in the moray eel may reflect its habitat within the crevices of shallow reefs (an inshore habitat). According to the literature,[23,27,28] most of the Cd in the liver of kidako moray eels is speculated to bind to MTs. Further study of not only Hg but also the MT-binding of Cd in the liver is necessary to confirm this speculation.

To our knowledge, no data for heavy metal concentrations in the kidako moray eel is available. Miao *et al.*[30] reported that T-Hg concentrations in the whole body of the yellow-edged moray eel (*Gymnothorax flavimarginatus*) and undulated moray eel (*Gymnothorax undulatus*) from the French Frigate Shoals were 0.34 and 0.42 μg/dry g, respectively, which correspond to approximately 0.08 and 0.11 μg/wet g, assuming a moisture content of 75% in the whole bodies. Miao *et al.*[30] also reported a Cd concentration of 1.0–15 μg/dry g in the whole bodies of those eels species, which corresponds to about 0.25–3.75 μg/wet g. The contamination levels of T-Hg found in the muscle and liver of the kidako moray eel were higher than that in the whole bodies of moray eel species from the French Frigate Shoals, while the contamination level of Cd in the kidako moray eel was lower. These differences in metal accumulation may reflect differences in feeding preference.

The concentrations of PCBs and *p,p'*-DDE in the muscle samples increased with increases in body length (p<0.05), while the increase in *trans*-nonachlor concentration was insignificant (Fig. 3). The lower correlation between body length and *trans*-nonachlor concentration may be related to the lower trophic transfer of *trans*-nonachlor reported in the Northwater Polynya[13] and Arctic marine[14] food webs. Provisional regulatory limitations of PCBs in fish and shellfish (edible parts) have been set by the Japanese government at 0.5 μg/wet g for the oceans and open sea, and at 3.0 μg/wet g for inland seas and bays, including inland waters. The level of PCBs found in the eel muscle was markedly lower than this legislated level. The levels of PCBs, *p,p'*-DDE and *trans*-nonachlor in the moray eels, expressed on a lipid weight basis as well as on a wet weight basis, were higher than those in the offshore-dwelling yellowfin and albacore tuna, respectively,[8] probably reflecting higher contamination levels of those compounds in the inshore area of Japan. It is worthy of note that the levels of Q1 (a naturally originating compound), expressed on both a wet weight basis and lipid weight basis, in the eel samples were markedly higher than those in tuna species,[8,16] which may be indicative of a link to the inshore food web. Furthermore, the accumulation of Q1 did not increase with increases in body length, which is in disagreement with the accumulation of anthropogenic compounds (Fig. 3). Differences in habitat, feeding preference, chemical properties and origin may explain these differences between Q1 and anthropogenic compounds, and further study of Q1 accumulation in marine biota is necessary to clarify these differences.

A significant positive correlation was found between the δ^{15}N value and body length in the kidako moray eel samples (Fig. 1), but the positive correlations between the δ^{13}C value and body length and between the δ^{13}C and δ^{15}N values were statistically insignificant. The wide variety of prey consumed by the kidako moray eel may be a possible reason for these weak correlations. The average δ^{13}C and δ^{15}N values in the moray eel samples (Table 1) were markedly higher than those in yellowfin (−16.5±0.5 and 10.3±1.2‰, n=53) and albacore tuna (−17.0±0.8 and 10.5±1.1‰, n=61) caught off the central and southern regions of Japan,[8] and spiny dogfish (−17.2± 0.4 and 12.9±0.9‰, n=75) caught off the northern region of Japan,[7] although the contamination level of T-Hg in the eel samples was similar to those in the tuna species[8] and spiny dogfish.[7] Furthermore, the average δ^{13}C and δ^{15}N values in the eel samples were higher than those in whale and dolphin samples caught off the central and southern regions of Japan, although the contamination level of T-Hg was markedly lower.[29] Kidako moray eels usually inhabit crevices in shallow reefs,[1] which is in contrast to the offshore habitat of tuna, spiny dogfish, whale and dolphin species. Higher δ^{13}C and δ^{15}N values have been reported in predators not only at higher trophic levels but also in those with inshore rather than offshore habits.[18,19] Higher values of δ^{13}C and δ^{15}N found in the kidako moray eel may reflect the inshore habit of this species rather than the trophic position or region in which they were taken.

In conclusion, the concentrations of T-Hg and M-Hg in the muscle (edible part) of kidako moray eels tended to increase with body length: the averages of T-Hg and M-Hg were 0.31± 0.08 μg/wet g and 0.25±0.06 μg/wet g, respectively, and those in large eels exceeded the Japanese legislation levels of T-Hg (0.4 μg/wet g) and M-Hg (0.3 μg/wet g) in fish and shellfish, respectively. The concentrations of T-Hg (1.50±1.13 μg/wet g) and M-Hg (0.84±0.54 μg/wet g) in the liver were markedly higher than those in the muscle, respectively, and the ratio of M-Hg to T-Hg in the liver (about 60%) was lower than that in

the muscle (80%). The Cd concentration in the liver tended to increase proportionally with body length, while that in the muscle was around or below the detection limit (0.03 μg/wet g). The concentrations of anthropogenic compounds of PCBs, *p,p'*-DDE and *trans*-nonachlor in the muscle tended to increase proportionally with body length, while the concentration of Q1, a naturally originating compound, did not. The $\delta^{13}C$ and $\delta^{15}N$ values in the kidako moray eel were markedly higher than those in predators with offshore habits, such as yellowfin and albacore tuna, spiny dogfish and marine mammals, reported elsewhere, which may reflect the inshore habitat of this species of moray eel.

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