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Bromoanisoles and methoxylated bromodiphenyl ethers in macroalgae from Nordic coastal regions†

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Marine macroalgae are used worldwide for human consumption, animal feed, cosmetics and agriculture. In addition to beneficial nutrients, macroalgae contain halogenated natural products (HNPs), some of which have toxic properties similar to those of well-known anthropogenic contaminants. Sixteen species of red, green and brown macroalgae were collected in 2017–2018 from coastal waters of the northern Baltic Sea, Sweden Atlantic and Norway Atlantic, and analyzed for bromoanisoles (BAs) and methoxylated bromodiphenyl ethers (MeO-BDEs). Target compounds were quantified by gas chromatography-low resolution mass spectrometry (GC-LRMS), with qualitative confirmation in selected species by GC-high resolution mass spectrometry (GC-HRMS). Quantified compounds were 2,4-diBA, 2,4,6-triBA, 2′-MeO-BDE68, 6-MeO-BDE47, and two tribromo-MeO-BDEs and one tetrabromo-MeO-BDE with unknown bromine substituent positions. Semiquantitative results for pentabromo-MeO-BDEs were also obtained for a few species by GC-HRMS. Three extraction methods were compared; soaking in methanol, soaking in methanol–dichloromethane, and blending with mixed solvents. Extraction yields of BAs did not differ significantly ($p > 0.05$) with the three methods and the two soaking methods gave equivalent yields of MeO-BDEs. Extraction efficiencies of MeO-BDEs were significantly lower using the blend method ($p < 0.05$). For reasons of simplicity and efficiency, the soaking methods are preferred. Concentrations varied by orders of magnitude among species: $\sum_2$BAs 57 to 57 700 and $\sum_5$MeO-BDEs < 10 to 476 pg g^{-1} wet weight (ww). Macroalgae standing out with $\sum_2$BAs >1000 pg g^{-1} ww were *Ascophyllum nodosum*, *Ceramium tenuicorne*, *Ceramium virgatum*, *Fucus radicans*, *Fucus serratus*, *Fucus vesiculosus*, *Saccharina latissima*, *Laminaria digitata*, and *Acrosiphonia/Spongomorpha* sp. Species *A. nodosum*, *C. tenuicorne*, *Chara virgata*, *F. radicans* and *F. vesiculosus* (Sweden Atlantic only) had $\sum_5$MeO-BDEs >100 pg g^{-1} ww. Profiles of individual compounds showed distinct differences among species and locations.

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Environmental significance

Marine macroalgae ("seaweeds") are used worldwide for human consumption and animal feed. In addition to beneficial nutrients, macroalgae contain brominated phenolic compounds, some of which have toxic properties similar to those of well-known anthropogenic contaminants. Knowledge of the bromophenolic content of macroalgae is needed to understand environmental pathways, including human exposure through consumption as well as bioaccumulation by grazers and transfer through the aquatic food web. Here we report bromoanisoles and methoxylated bromodiphenyl ethers in 16 species of macroalgae from the northern Baltic and Atlantic coasts of Sweden and Norway. This is the largest survey of these compounds in macroalgae from the Nordic region and the first for the Atlantic coasts.

Introduction

Macroalgae ("seaweeds", "sea vegetables") are used worldwide for human consumption (>80% in 2016), animal feed, cosmetics and agriculture. The global market is large and growing, $11.1 U.S. billion in 2016 and projected to reach $22.1 U.S. billion by 2024.[1] Macroalgae are a rich source of many nutrients, including vitamins, minerals, fiber, phytochemical antioxidants (fucoxanthin, polyphenols) and omega-3s.[2–5] Several health benefits are attributed to consumption of whole macroalgae and they exert a preservative effect when

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incorporated into foods, due to their antimicrobial activity.[3] On the other hand, caution for over-consumption has been advised due to the presence of accumulated heavy metals and other pollutants[2,3] and to excessive exposure to iodine, linked to hypothyroidism.[3]

Many halogenated natural products (HNPs) are found in marine macroalgae. Bromophenolic compounds are a prominent subset of HNPs, comprising bromophenols (BPs) and their transformation products bromoanisoles (BAs), hydroxylated and methoxylated bromodiphenyl ethers (OH-BDEs, MeO-BDEs) and polybrominated dibenzo-*p*-dioxins (PBDDs).[6–12] BPs add characteristic and desirable flavors to seafood,[13,14] and due to their high food quality macroalgae have been used to supplement the feed of aquacultured fish[15,16] and farm animals.[4] Several brominated polyphenols have antioxidant, antimicrobial, anticancer, antidiabetic and antithrombotic activities.[4,17] Bromophenolic compounds enter the human diet through macroalgae consumption and from compounds bioaccumulated in seafood, and profiles of MeO-BDE congeners in human serum reflect dietary exposure.[18] Toxic properties associated with bromophenolic compounds include disruption of hormone synthesis or activity (OH-BDEs and MeO-BDEs)[19] and oxidative phosphorylation (OH-BDEs),[20] and binding to the aryl hydrocarbon (Ah) receptor (PBDDs).[21,22]

Bromophenolic compounds have been identified in several macroalgae species from the Baltic Sea: *Dictyosiphon foeniculaceus*, *Ceramium tenuicorne*, *Polysiphonia fucoides* and *Pilayella littoralis*, as well as in cyanobacteria *Nodularia spumigena* and *Aphanizomenon flos-aquae*,[8–11] but quantitative data have only been reported for *C. tenuicorne*,[11,23–25] *D. foeniculaceus*[8] and *N. spumigena*.[8] Brominated polyphenols were isolated from the red alga *Vertebrata lanosa* collected on the coast of Norway.[26] Several simple BPs were identified in macroalgae species from the families Ceramiaceae, Delesseriaceae, Bonnemaisoniaceae, Rhodophyllaceae, Corallinaceae and Rhodomelaceae, collected on the Swedish west coast.[27] One of these compounds, lanosol (2,3-dibromo-4,5-dihydroxybenzyl alcohol) was also identified in seawater. To our knowledge, this is the only report of bromophenolic compounds in macroalgae from the west coast of Sweden.

Macroalgae and cyanobacteria are important sources of bromophenolic compounds to the Baltic ecosystem and they are transferred through the food web from macroalgae to invertebrate grazers to fish.[24] Several studies have reported bromophenolic compounds in Baltic mussels, which filter-feed on cyanobacteria[8,10,21,28–30] and transferred from mussels to seaducks.[28] Macroalgae from the Nordic coastal region are being promoted as human food, with published recipes utilizing local species.[31] Consideration should be given to the bromophenolic compounds and other HNPs present in macroalgae used for human consumption, to place exposure in perspective with anthropogenic compounds.

In 2017–2018, we collected >30 species of macroalgae from the Bothnian Sea (northern Baltic), Sweden Atlantic coast (Skagerrak) and Norway Atlantic coast. Objectives are to establish analytical methods and determine the variability of

Table 1 Collection of Nordic macroalgae and concentrations of ∑BAs[a] and ∑MeO-BDEs[b]

						pg g^{-1} ww	
Abbreviation	Group	Species[c]	Latitude N	Longitude E	Collection date	$\sum_2$BAs	$\sum_5$MeO-BDEs
Bothnian Sea							
Cet	Red alga	*Ceramium tenuicorne*	60.769	17.349	2017-08-24	3360	199
Chv	Green alga[d]	*Chara virgata*	63.414	19.491	2017-08-26	57	103
Clg	Green alga	*Cladophora glomerata*	63.461	19.805	2017-07-08	591	56
Dif	Brown alga	*Dictyosiphon foeniculaceus*	63.462	19.803	2017-07-08	324	61
Fur	Brown alga	*Fucus radicans*	60.806	17.356	2017-08-24	6690	476
Stt	Brown alga	*Stictyosiphon tortilis*	60.791	17.381	2017-08-24	976	56
Uli	Green alga	*Ulva intestinalis*	63.461	19.805	2017-07-08	726	45
Skagerrak							
Asn	Brown alga	*Ascophyllum nodosum*	58.868	11.059	2017-10-06	41 000	396
Cev	Red alga	*Ceramium virgatum*	58.868	11.059	2017-10-06	1180	30
Ful	Red alga	*Furcellaria lumbricalis*	58.869	11.143	2017-10-06	854	<10
Fus	Brown alga	*Fucus serratus*	58.868	11.059	2017-10-06	1160	85
Fuv	Brown alga	*Fucus vesiculosus*	58.868	11.059	2017-10-06	5570	253
Rhc	Red alga	*Rhodomela confervoides*	58.869	11.143	2017-10-06	437	<10
Sal	Brown alga	*Saccharina latissima*	58.868	11.059	2017-10-06	1120	<10
Coastal Norway							
Ac/Sp	Green alga	*Acrosiphonia/Spongomorpha* sp.	65.200	11.933	2018-05-13	1420	<10
Asn	Brown alga	*Ascophyllum nodosum*	65.200	11.933	2018-05-13	57 700	34
Fuv	Brown alga	*Fucus vesiculosus*	65.200	11.933	2018-05-13	3970	52
Lad	Brown alga	*Laminaria digitata*	65.200	11.933	2018-05-13	12 400	<10

[a] ∑2,4-DiBA and 2,4,6-TriBA. [b] ∑2′-MeO-BDE68, 6-MeO-BDE47, one tetrabromo-MeO-BDE and two tribromo-MeO-BDEs with unknown bromine substituent positions. [c] Nomenclature follows Algae Base (www.algaebase.org). [d] Stonewort.

bromophenolic compounds among species and locations, as a prelude to estimating the role of macroalgae in supplying bromophenolic compounds to Nordic estuaries. Here we report concentrations of some neutral bromophenolic compounds (BAs and MeO-BDEs) in an initial survey of 16 species. A major task was to compare methods of extraction for these compounds, since different methods have been applied in previous studies. Compounds with free phenolic groups (BPs, OH-BDEs) were not included at this stage because of the additional steps required for their isolation and derivatization.

Materials and methods

Macroalgae sampling

Macroalgae were collected from coastal sites in the Bothnian Sea (northern Baltic), Skagerrak (Sweden Atlantic) and Norway Atlantic. Amounts collected exceeded 20 g wet weight (ww) in most cases, except when limited by availability (*Chara virgata* 3 g, *Ceramium tenuicorne* 10 g ww). After species identification, the macrophytes were rinsed with deionized water, blot dried with laboratory tissues, and frozen at −20 °C until analysis. A subset of 16 macroalgae species (18 specimens, some species from two locations), including one stonewort, was selected for the initial survey of BAs and MeO-BDEs. Collection details for these are given in Table 1 and locations are shown in Fig. 1. Species were chosen to represent different taxonomic groups (red, green, brown algae), locations, morphologies and anticipated content of BAs and MeO-BDEs.

Extraction and analytical methods

Materials. Solvents were gas chromatography quality (Suprasolv from Merck, Solna, Sweden). Florisil (100–200 mesh), granular anhydrous sodium sulfate and 99% sulfuric acid were obtained from Fisher Scientific (Gothenburg, Sweden), potassium chloride was reagent grade from Scharlau S.L. (Sentmenat, Spain). An analytical standard of mixed MeO-BDE congeners was kindly supplied by Prof. Lillemor Asplund, Department of Analytical Chemistry and Environmental Sciences (ACES), Stockholm University, Stockholm, Sweden. Standards of BAs and internal standard 2,2′,6,6′-tetrachlorobiphenyl (CB-54) were purchased from Accustandard (New Haven, CT, USA).

Extraction and cleanup. Frozen macroalgae were defrosted and blot dried with laboratory tissues. The mass of macroalgae (containing residual water) taken for analysis ranged from 1.1 to 3.6 g (mean 2.8 ± 0.7 g). Macroalgae were cut into small pieces with scissors and extracted using one or more of three methods:

SOAK 1.[7] Macroalgae pieces were placed in a glass jar with a polytetrafluoroethylene-lined cap and recovery surrogates were added: 15 ng 2,4,6-tribromoanisole-d_5 (2,4,6-triBA-d_5) and two PBDE congeners not found in commercial mixtures: 6.0 ng 3,3′,5-tribromodiphenyl ether (BDE-35) and 24 ng 2,3′,4,4′,6-pentabromodiphenyl ether (BDE-119) (Table 2). The macroalgae were covered with 15 mL methanol (MeOH) and allowed to stand in the refrigerator at 4 °C for one week.

SOAK 2. As for SOAK 1, but using 10 mL MeOH + 5 mL dichloromethane (DCM).

Extracts from SOAK 1 or SOAK 2 were diluted with 60 mL deionized water (DIW), 3 mL saturated potassium chloride was added, and the mixture was partitioned three times with 20 mL DCM followed by 20 mL hexane. Combined organic layers were filtered through glass wool and concentrated by rotary evaporation and nitrogen blowdown into 5 mL hexane.

BLEND.[32,33] Macroalgae pieces were placed in a thick-walled glass jar and the above surrogates were added. A stainless steel

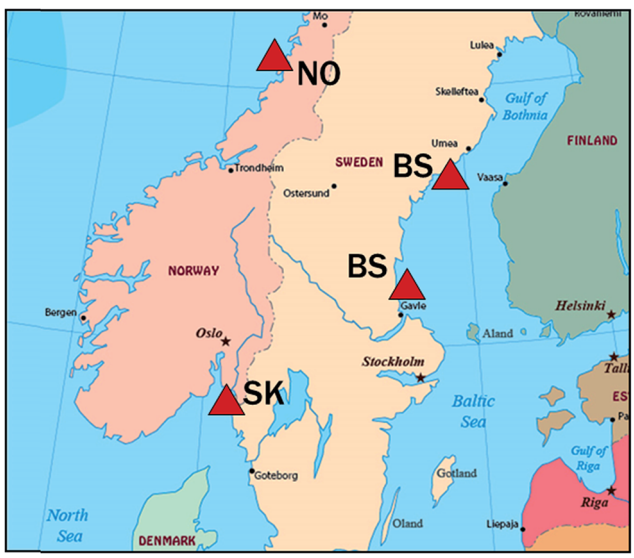

Fig. 1 Locations for sampling macroalgae in the Bothnian Sea (BS), Skagerrak (SK) and coastal Norway (NO). See Table 1 for coordinates and sampling dates.

Table 2 Percent recoveries of spiked surrogate compounds[a] using three extraction methods

		2,4,6-TriBA-d_5	BDE-35	BDE-119
SOAK 1[b]				
Mean		75.9	89.7	76.5
SD		10.4	19.3	16.6
N		18	17	17
SOAK 2[b]				
Mean		85.1	103	98.4
SD		10.7	13.3	14.0
N		28	28	28
BLEND[b]				
Mean		81.4	109	103
SD		9.4	10.3	8.9
N		17	17	17
Significance[c]				
SOAK 1	SOAK 2	Y	Y	Y
SOAK 1	BLEND	N	Y	Y
SOAK 2	BLEND	N	N	N

[a] 15 ng 2,4,6-triBA-d_5, 6.0 ng BDE-35 and 24 ng BDE-119. [b] SOAK 1: methanol; SOAK 2: methanol–dichloromethane; BLEND: mixed solvents. [c] One-way ANOVA, *post hoc* Tukey HSD, $p < 0.05$.

stick blender (Ultra Turrax T25, Janke & Kunkel GmbH, IKA Labortechnik, Staufen, Germany) was used to homogenize the macroalgae with 25 mL acetone + 10 mL hexane followed by two portions of 20 mL hexane + 3 mL diethyl ether, blending for one minute each time. The combined extracts were diluted with 60 mL DIW, 3 mL saturated potassium chloride was added and the mixture was partitioned. The organic layer was removed and the aqueous phase was extracted twice more with 20 mL hexane + 3 mL diethyl ether. Combined organic layers were filtered and concentrated as in the SOAK methods.

Of the 18 specimens, three were extracted in replicate (3–4) with each method, five were extracted once with each method and ten were extracted only with SOAK 2 (Tables 3, 4 and S1†).

Internal standard (59 ng 2,2′,6,6′-tetrachlorobiphenyl, CB-54) was added to the macroalgae extracts, which were then vortexed for 1 min with 2 mL 99% sulfuric acid and placed in the refrigerator overnight to allow the phases to separate. *Fucus serratus* required a second sulfuric acid treatment. The upper hexane layer was transferred with hexane rinsings to a Pasteur pipet containing 0.5 g Florisil topped with 0.5 cm granular anhydrous sodium sulfate and the column was eluted with

Table 3 Comparison of extraction methods, replicate analyses of three macroalgae, pg g^{-1} ww[a]

Sample	Method[c]	2,4-DiBA	2,4,6-TriBA	triU1[b]	triU2[b]	2′-68[b]	6-47[b]	tetraU3[b]
Cladophora glomerata								
Mean (n = 3–4)	SOAK 1	118	519	16.2			43.1	
s.d.		10.6	27.9	0.6			10.1	
RSD %		9.0	5.4	3.5			23.3	
Mean (n = 3–4)	SOAK 2	114	524	15.6			36.5	
s.d.		14.2	79.5	2.6			5.9	
RSD %		12.4	15.2	16.5			16.2	
Mean (n = 3–4)	BLEND	90.1	403	11.6			34.3	
s.d.		30.8	36.5	2.3			16.3	
RSD %		34.2	9.0	19.5			47.6	
Significance ($p < 0.05$)[d]								
SOAK 1	SOAK 2	N	N	N			N	
SOAK 1	BLEND	N	Y	Y			N	
SOAK 2	BLEND	N	Y	N			N	
Fucus radicans								
Mean (n = 3–4)	SOAK 1	829	6700	59.4	153	58.7	170	52.6
s.d.		88.7	828	10.1	32.0	3.1	15.3	20.9
RSD %		10.7	12.3	17.0	20.9	5.3	9.0	39.7
Mean (n = 3–4)	SOAK 2	711	6400	49.7	131	66.8	160	50.2
s.d.		95.3	928	7.1	46.7	11.8	23.4	16.2
RSD %		13.4	14.5	14.2	35.6	17.6	14.6	32.2
Mean (n = 3–4)	BLEND	611	4830	25.0	89.6	40.7	89.8	32.5
s.d.		37.3	940	3.4	22.2	3.6	11.9	13.3
RSD %		6.1	19.5	13.5	24.8	8.8	13.3	40.9
Significance ($p < 0.05$)[d]								
SOAK 1	SOAK 2	N	N	N	N	N	N	N
SOAK 1	BLEND	Y	Y	Y	N	Y	Y	N
SOAK 2	BLEND	N	N	Y	N	Y	Y	N
Fucus vesiculosus								
Mean (n = 4)	SOAK 1	967	5520		58.2	107	37.1	85.7
s.d.		178	1720		12.7	21.0	6.8	32.1
RSD %		18.4	31.1		21.7	19.6	18.5	37.4
Mean (n = 4)	SOAK 2	928	5260		58.3	84.7	25.3	49.3
s.d.		287	1920		20.9	25.6	5.2	9.8
RSD %		30.9	36.5		35.8	30.2	20.7	19.9
Mean (n = 4)	BLEND	593	3440		26.1	46.8	14.6	24.8
s.d.		123	923		6.3	10.0	3.3	7.2
RSD %		20.8	26.9		24.2	21.4	22.9	29.2
Significance ($p < 0.05$)[d]								
SOAK 1	SOAK 2	N	N		N	N	Y	N
SOAK 1	BLEND	N	N		Y	Y	Y	Y
SOAK 2	BLEND	N	N		Y	N	Y	N

[a] No entry indicates compound < LOD in more than one replicate. See Table S1. [b] Tri-U1, tri-U2 and tetraU3 = MeO-BDEs with unknown tribromo- or tetrabromo-substituent positions. 2′-28 = 2′-MeO-BDE68, 6-47 = 6-MeO-BDE47. [c] SOAK 1: methanol, SOAK 2: methanol–dichloromethane, BLEND: mixed solvents. [d] One-way ANOVA, *post hoc* Tukey HSD test.

Table 4 Comparison of extraction methods, ratios of yields for eight macroalgae[a,b]

Sample	Method[c]	2,4-DiBA	2,4,6-TriBA	triU1	triU2	2′-68	6-47	tetraU3
Ascophyllum nodosum								
SOAK 1/SOAK 2		1.56	1.32		0.92	0.92	1.32	
BLEND/SOAK 2		0.91	0.91		0.47	0.55	0.76	
Cladophora glomerata[d]								
SOAK 1/SOAK 2		1.03	0.99	1.04			1.18	
BLEND/SOAK 2		0.79	0.77	0.67			0.94	
Fucus radicans[d]								
SOAK 1/SOAK 2		1.16	1.05	1.20	1.16	0.88	1.06	1.05
BLEND/SOAK 2		0.86	0.75	0.50	0.68	0.61	0.56	0.65
Fucus serratus								
SOAK 1/SOAK 2		0.49	0.61		1.04	1.32		
BLEND/SOAK 2		0.89	1.30		0.83	0.60		
Fucus vesiculosus[d]								
SOAK 1/SOAK 2		1.04	1.05		1.00	1.26	1.46	1.74
BLEND/SOAK 2		0.64	0.65		0.45	0.55	0.57	0.45
Rhodomela confervoides								
SOAK 1/SOAK 2		1.34	0.92					
BLEND/SOAK 2		0.86	0.93					
Saccharina latissima								
SOAK 1/SOAK 2		0.73	0.58					
BLEND/SOAK 2		1.02	0.78					
Ulva intestinalis								
SOAK 1/SOAK 2		1.14	0.89				0.56	
BLEND/SOAK 2		1.11	1.23				0.55	
Summary								
	Mean BAs	SD BAs	Significance[e]	Mean MeO-BDEs	SD MeO-BDEs	Significance[e]	Mean BAs	SD BAs
SOAK 1/SOAK 2	0.99	0.29	N	1.12	0.26	N	0.99	0.29
BLEND/SOAK 2	0.90	0.19	N	0.61	0.13	Y	0.90	0.19

[a] tri-U1, tri-U2 and tetraU3 = MeO-BDEs with unknown tribromo- or tetrabromo-substituent positions. 2′-28 = 2′-MeO-BDE68, 6-47 = 6-MeO-BDE47. [b] Concentration data in Tables 3 and S2. No entry indicates compound < LOD. [c] SOAK 1: methanol, SOAK 2: methanol–dichloromethane, BLEND: mixed solvents. [d] Ratios calculated from mean concentrations in Table 3. [e] Ratio significantly different from 1.00, *t*-test at $p < 0.05$.

12 mL 3 : 2 (v/v) DCM–hexane. The eluate was concentrated into 100 to 200 μL isooctane. Blanks were run by carrying solvents through the extraction and cleanup procedures.

Analysis. BAs, tribromo-MeO-BDEs and tetrabromo-MeO-BDEs were quantitatively determined by capillary gas chromatography-electron impact low-resolution mass spectrometry (GC-LRMS) using a J&W DB-5MS Ultra-inert column (30 m × 25 mm, 0.25 μm film), Agilent 6890N chromatograph-5975 mass selective detector (Agilent Technologies, Santa Clara, CA), monitoring two or more selected ions. Temperatures, oven programs and monitored ions were previously described.[34,35] Qualitative confirmation of tribromo- and tetrabromo-MeO-BDEs and identification of pentabromo-MeO-BDEs was done for nine macroalgae species by GC-high resolution mass spectrometry (GC-HRMS), as previously described.[34] Analytical standards included 2,4-, 2,5-, 2,6-, and 3,5-diBAs, 2,4,6-triBA, 2′-methoxy-2,4,4′-tribromodiphenyl ether (2′-MeO-BDE28), 2′-methoxy-2,3′,4,5′-tetrabromodiphenyl ether (2′-MeO-BDE68), 6-methoxy-2,2′,4,4′-tetrabromodiphenyl ether (6-MeO-BDE47), 6-methoxy-2,2′,3,4,4′-pentabromodiphenyl ether (6-MeO-BDE85), 6-methoxy-2,2′,3,4′,5-pentabromodiphenyl ether (6-MeO-BDE90), 6-methoxy-2,2′,4,4′,5′-pentabromodiphenyl ether (6-MeO-BDE99) and 2-methoxy-2′,3,4,4′,5-pentabromodiphenyl ether (2-MeO-BDE123), as well as recovery surrogates (2,4,6-triBA-d_5, BDE-35 and BDE-119) and the internal standard (CB-54). Response factors were derived from peak areas relative to the area of CB-54. Relative standard deviations (% RSD) of daily response factors averaged 7.5% for BAs and 8.5% for MeO-BDEs.

Results and discussion

Compound identification and quality control

Only 2,4-diBA and 2,4,6-triBA were identified and quantified in the macroalgae by GC-LRMS, while 2,6-diBA and BAs with *meta*-substitutions were absent. The tetrabromo-compounds 2′-MeO-

BDE68 and 6-MeO-BDE67 were identified using GC-LRMS and GC-HRMS by their retention times relative to authentic standards and ion ratios that fell within ±20% of those for standards, and quantified by GC-LRMS. Two tribromo-MeO-BDEs and one additional tetrabromo-MeO-BDE with unknown bromine substituent positions were also identified from their monitored ions by both LRMS and HRMS (Fig. 2). The unknown tribromo-MeO-BDEs were quantified by GC-LRMS *versus* the response factor (relative to CB-54) for a standard of 2′-MeO-BDE28 (which was not found in any of the macroalgae species) and the unknown tetrabromo-MeO-BDE was quantified by using the average of response factors for 2′-MeO-BDE68 and 6-MeO-BDE47. The two tribromo-MeO-BDEs appear to be the same ones previously identified in Baltic water and air samples.[34] Pentabromo-MeO-BDEs showed poor response by GC-LRMS, but three compounds were identified in some selected macroalgae species by HRMS using authentic standards: 6-MeO-BDE85, 6-MeO-BDE90 and 6-MeO-BDE99; 2-MeO-BDE123 was also sought but not found. Due to poor performance of the CB54 internal standard in GC-HRMS runs, semi-quantitative results for pentabromo-MeO-BDEs were estimated without use of an internal standard and assuming a 200 μL sample volume.

Four blanks were run by carrying solvents through the extraction and cleanup procedures. No target peaks were found, and blank quantities were estimated by integrating baseline noise in the vicinity of expected peaks. Blanks (mean ± SD) by compound class were: BAs 6.3 ± 2.6 pg, tribromo-MeO-BDEs 5.7 ± 3.4 pg, tetrabromo-MeO-BDEs 22 ± 9 pg. Some BAs and MeO-BDEs were absent from all macroalgae; 2,6-diBA and 2′-MeO-BDE28, and other MeO-BDEs were absent in some of the species. "Blanks" were also assessed using a larger data set from algae extractions and quantifying baseline signal in the absence of a noticeable target peak. The overall limits of detection, LOD = (mean blank + 3 × SD)/sample weight, from both types of blanks were: BAs 15 pg g^{-1}, tribromo-MeO-BDEs 10 pg g^{-1} and tetrabromo-MeO-BDEs 17 pg g^{-1}, based on a 2.5 g (ww) sample.

Percent recoveries of 2,4,6-triBA-d_5, BDE-35 and BDE-119 surrogate spikes varied among the three extraction methods, with means ranging from 76% to 109% and relative standard deviations (% RSD) ranging from 8.7% to 21.7% (Table 2). While mean recoveries of all surrogates using each method exceeded 75%, higher yields were generally obtained by SOAK 2 (MeOH–DCM) and BLEND methods than by SOAK 1 (MeOH). Differences in mean recoveries between SOAK 2 and SOAK 1 were significant for all three surrogates (single factor ANOVA, *post hoc* Tukey HSD, $p < 0.05$), between SOAK 1 and BLEND for the two BDEs and not significant between SOAK 2 and BLEND for any surrogate ($p > 0.05$). Concentrations of BAs and MeO-BDEs in macroalgae were corrected on a per-sample basis for surrogate yields (2,4,6-triBA-d_5 recovery for BAs, average of BDE-35 and BDE-119 recoveries for MeO-BDEs).

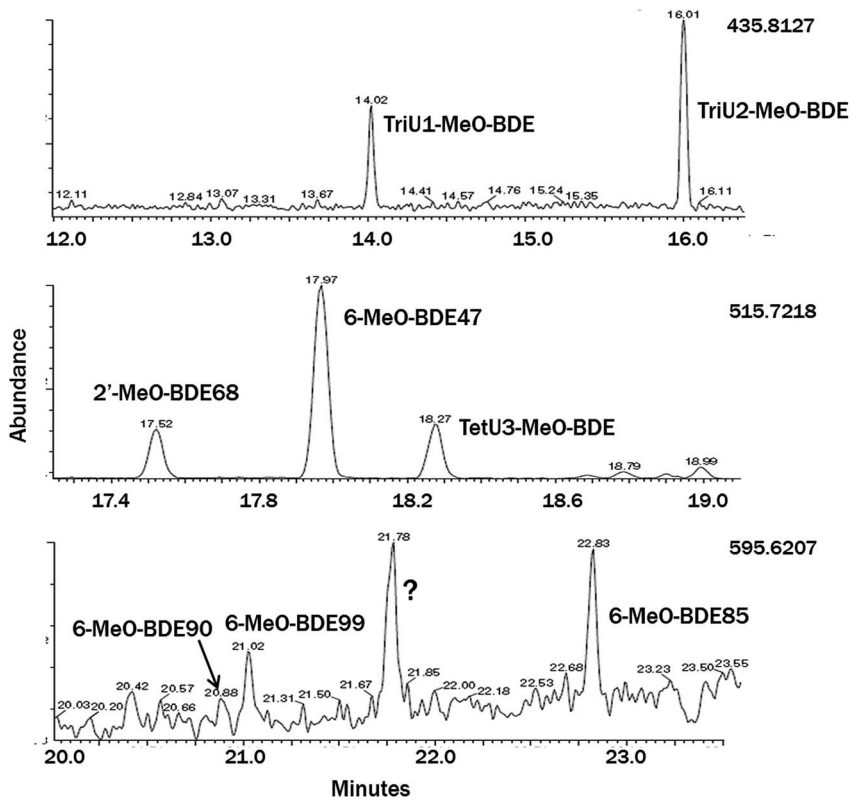

Fig. 2 GC-HRMS of tribromo-, tetrabromo- and pentabromo-MeO-BDEs (upper, middle and lower, respectively) in *Fucus radicans* from the Bothnian Sea. Monitored ions are given at the upper right of each panel. TriU1-MeO-BDE, TriU2-MeO-BDE and TetU3-MeO-BDE have unknown bromine substituent positions. The marked peak (?) at 21.78 min has not been identified, but does not have the correct ion ratios for a pentabromo-MeO-BDE.

Comparison of extraction methods and reproducibility

Replicate extractions (3 to 4) of *C. glomerata* and *F. radicans* from the Bothnian Sea and *F. vesiculosus* from Skagerrak were done using SOAK 1 (MeOH), SOAK 2 (MeOH–DCM) and BLEND (mixed solvents) methods. Analytical results are summarized in Table 3 and elaborated in Table S1.† Percent relative standard deviations (% RSD) for replicate analyses of a particular species with a specified method ranged from 5% to 36% for BAs and 4% to 48% for MeO-BDEs, with overall average % RSDs of 18% and 23%, respectively (Table 3). Sources of this variability are in the precision of the analytical method itself (the average % RSD of all surrogate recoveries was 13%, Table 2) and probably also in the reproducibility of removing water by blot drying with laboratory tissues. In most cases no significant differences in extraction yields were found using SOAK 1 or SOAK 2 methods (one-way ANOVA, *post hoc* Tukey HSD test, $p > 0.05$). Differences between the SOAK and BLEND methods were sometimes significant ($p < 0.05$) and lower with BLEND, especially for MeO-BDEs. The difference appears to be due to lower extraction efficiency of MeO-BDEs from the algal matrix, since surrogate recoveries with BLEND were high and not different from SOAK 2.

Five other macroalgae species, *U. intestinalis* from the Bothnian Sea, and *A. nodosum*, *F. serratus*, *R. confervoides* and *S. latissima* from Skagerrak, were extracted once with each method. Here the extraction methods were compared by expressing yields relative to SOAK 2; *i.e.* SOAK 1/SOAK 2 and BLEND/SOAK 2. Results for these five macroalgae plus the three species used in methods replication (Table 3) are summarized in Table 4. Averaged over all eight species and compounds, SOAK 1/SOAK 2 yielded ratios of BAs (0.99 ± 0.29) and MeO-BDEs (1.12 ± 0.26) that were not significantly different from 1.00 (*t*-test, $p > 0.05$). BLEND/SOAK 2 ratios for BAs were slightly, but not significantly, lower (0.90 ± 0.19, $p > 0.05$), while BLEND/SOAK 2 ratios for MeO-BDEs were significantly lower (0.61 ± 0.13, $p < 0.001$). Thus, the extraction efficiency of BAs from these test species did not differ significantly by the three methods. Yields of MeO-BDEs were not significantly different by SOAK 1 and SOAK 2. The extraction efficiency for MeO-BDEs was significantly lower using BLEND, confirming results from replicate extractions of three species. For reasons of simplicity and efficiency, the soaking methods are preferred.

Six macroalgae species (*A. nodosum*, *C. glomerata*, *F. radicans*, *F. serratus*, *F. vesiculosus* and *R. confervoides*) that had been extracted by SOAK 1 or SOAK 2 received second week-long extractions with fresh solvents. Second extraction percentages were $6.6 \pm 3.5\%$ of first-extraction yields for BAs and $21 \pm 19\%$ for MeO-BDEs. The MeO-BDE percentages are likely inflated because second extraction quantities were often near or below the LOD (LOD/2 was entered).

Concentrations of BAs and MeO-BDEs in macroalgae

Table 1 gives concentrations of the $\sum_2$BAs (2,4-diBA + 2,4,6-triBA) and $\sum_5$MeO-BDEs (2′-MeO-BDE68, 6-MeO-BDE47, one tetrabromo-MeO-BDE and two tribromo-MeO-BDEs) in pg g^{-1} ww. For those macroalgae receiving extractions by different methods, yields by all three methods were averaged for BAs, while only SOAK 1 and SOAK 2 results were averaged for MeO-BDEs. Composition details are shown in Fig. 3 and Table S2.† BAs were found in all sixteen macroalgae species examined, while eleven species contained one or more tribromo- or tetrabromo-MeO-BDEs.

The bromophenolic content varied over several orders of magnitude; Σ_2BAs 57 to 57 700 pg g^{-1} ww, and Σ_5MeO-BDEs < 10 to 476 pg g^{-1} ww (Table 1). Macroalgae standing out with $\sum_2$BAs >1000 pg g^{-1} ww were *A. nodosum*, *C. tenuicorne*, C. *virgatum*, *F. radicans*, *F. serratus*, *F. vesiculosus*, *S. latissima*, *L. digitata* and *Acrosiphonia/Spongomorpha* sp. The species *A. nodosum*, *C. tenuicorne*, *C. virgata*, *F. radicans* and *F. vesiculosus* (Skagerrak only) had $\sum_5$MeO-BDEs >100 pg g^{-1} ww. The Bothnian Sea and Skagerrak species tended to have higher levels of $\sum_5$MeO-BDEs, while the $\sum_5$MeO-BDEs was low in all four species examined from coastal Norway. Considering all species and locations, there was no significant relationship between concentrations or logarithm transformed concentrations of $\sum_2$BAs and $\sum_5$MeO-BDEs ($p > 0.05$). The proportion of 2,4,6-triBA, expressed by the fraction 2,4,6-triBA/(2,4,6-triBA + 2,4-diBA), was significantly higher ($p < 0.05$) in Bothnian Sea macroalgae (0.883 ± 0.109), than in the combined set of Atlantic coast species (Skagerrak and Norway, 0.747 ± 0.154) (*t*-test of means, unequal variance).

Nine species were checked for pentabromo-MeO-BDEs by HRMS (Table S3†). Due to poor performance of the CB54 internal standard in GC-HRMS runs, semiquantitative results were estimated without use of an internal standard and assuming a 200 μL sample volume. Only *C. tenuicorne* and *F. radicans* from the Bothnian Sea, and *A. nodosum* and *F. vesiculosus* from Skagerrak contained one or more of 6-MeO-BDE85, 6-MeO-BDE90 and 6-MeO-BDE99 at concentrations ranging from 1.3 to 18 pg g^{-1} ww.

Comparison with other macroalgae investigations

Relative to the plethora of reports on free BPs, OH-BDEs and polyphenols in macroalgae,[4–13,17,23–27,36] the database for BAs and MeO-BDEs in marine algae is surprisingly small and summarized in Table 5. BAs and/or MeO-BDEs have been reported in *C. tenuicorne* from the Baltic Proper[11,24] at pg g^{-1} ww concentrations of 2,4-diBA 4 to 70, 2,4,6-triBA 47 to 390, 2′-MeO-BDE68 3 to 100 and 6-MeO-BDE47 5 to 210. Reported average concentrations of these four compounds in *D. foeniculaceus*, also from the Baltic Proper, were 33, 230, 120 and 150 pg g^{-1}, ww, respectively.[8] These appear to be the only Baltic macroalgae species in which BAs and MeO-BDEs have been quantified. For comparison, our concentrations of 2,4-diBA, 2,4,6-triBA, 2′-MeO-BDE68 and 6-MeO-BDE47 were 71, 3290, 80 and 56 pg g^{-1}, ww in *C. tenuicorne*, and 106, 217, 20 and < 17 pg g^{-1}, ww in *D. foeniculaceus* (Table S2†).

Wide ranges of BAs and MeO-BDEs have been reported in 15 genera of red, green and brown macroalgae from Philippine waters, all collected in the same month: 2,4,6-triBA < 20 to 2200 pg g^{-1} ww, 2′-MeO-BDE68 < 20 to 229 000 pg g^{-1} ww and 6-MeO-BDE47 < 20 to 27600 pg g^{-1} ww.[7] Ranges of 2,4-diBA and 2,4,6-triBA in the red alga *Polysiphonia sphaerocarpa* from the littoral

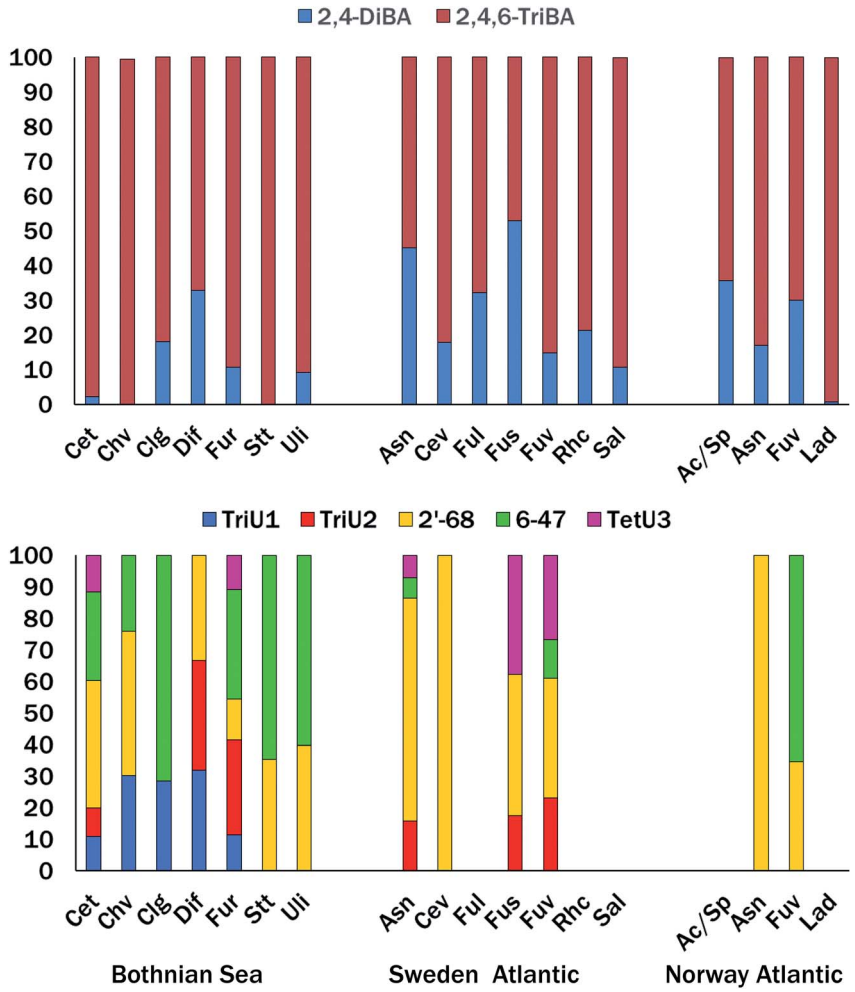

Fig. 3 Percent contribution of BAs (top) tribromo- and tetrabromo-MeO-BDEs (bottom) to $\sum_2$BAs and $\sum_5$MeO-BDEs in Nordic macroalgae. See Table 1 for species abbreviations. No bar indicates all compounds < LOD.

zone near Sydney, Australia were 100 to 300 and 200 to 700 pg g^{-1} ww.[36] Concentrations of BAs in our set of macroalgae are generally higher than those found in other investigations (Table 5). While BAs were highest in *Fucus* spp. and *A. nodosum*, which have not been analyzed by others, we also found higher levels in *C. tenuicorne* than previously reported. On the other hand, our levels of BAs in the brown alga *D. foeniculaceus* were comparable to those found by Löfstrand *et al.*,[8] while their concentrations of tetrabromo-MeO-BDEs were higher than ours. The semi-quantitative estimate of 17 pg g^{-1} ww 6-MeO-BDE85 in *C. tenuicorne* (Table S3†) compares favorably with 35 pg g^{-1} ww reported in the same species.[11]

In addition to variation among macroalgae species,[7] concentrations and proportions of bromophenolic compounds fluctuate with the season.[6,11,23,24] The collection months of Nordic macroalgae specimens was July–August in the Bothnian Sea, October in Skagerrak and May in coastal Norway (Table 1), which may account for observed differences. Concentrations of 2,4-diBA and 2,4,6-triBA in *C. tenuicorne*, collected from the Baltic Proper between June and August, ranged from 4 to 70 and 47 to 390 pg g^{-1} ww, respectively, while ranges of 2′-MeO-BDE68 and 6-MeO-BDE47 were 3 to 29 and 5 to 71 pg g^{-1} ww (Table 5).[24] Each of these bromophenolic compounds peaked in August. Ratios of compounds in *C. tenuicorne* varied over the spring-summer; 2,4,6-triBA/2,4-diBA from 5.5 to 17 and 6-MeO-BDE47/2′-MeO-BDE28 from 1.1 to 2.7.[24] Ratios of precursor 2,4,6-triBP/2,4-diBP concentrations also showed monthly fluctuations.[23] In comparison, 2,4,6-triBA/2,4-diBA was 46, while 6-MeO-BDE47/2′-MeO-BDE28 was 0.70 in our specimen of *C. tenuicorne*, collected in August. Stresses from salinity variations, light intensity and grazing by predators also cause changes in concentrations of 2,4,6-triBP.[23] These factors hinder comparisons among species, locations and sampling times.

Both BAs and MeO-BDEs have been determined in macroalgae from Nordic ecosystems and the Philippines, with strikingly different results (Table 5). Even considering the spread of concentrations within each compound class and among species, the general trend is BAs > MeO-BDEs in Nordic ecosystems *versus* MeO-BDEs > BAs in the Philippines. Reasons are not apparent, and it would be interesting to conduct more comparisons between tropical and cold ecosystems.

Table 5 Comparison of BAs and MeO-BDEs in macroalgae, pg g^{-1} ww

Group	Genera	Location	2,4-DiBA	2,4,6-TriBA	2′-MeO-BDE68	6-MeO-BDE47	Reference
Brown algae	4	Nordic	<15–18 500	217–48 000	18–280	<17–165	This study
Red algae	3	Nordic	71–275	344–3290	<17–80	<17–56	This study
Green algae	3[a]	Nordic	<15–507	57–910	<17–47	<17–40	This study
Brown algae	3	Philippines		<20–700	300–229 000	100–27 600	7
Red algae	7	Philippines		<20–1300	100–78 000	50–29 000	7
Green algae	5[a]	Philippines		100–2200	<20–5900	<20–1700	7
Group	**Species**						
Brown alga	*Dictyosiphon foeniculaceus*	Bothnian Sea	106	217	20	<17	This study
Brown alga	*Dictyosiphon foeniculaceus*	Baltic Proper	33	230	120	150	8
Red alga	*Ceramium tenuicorne*	Bothnian Sea	71	3290	80	56	This study
Red alga	*Ceramium tenuicorne*	Baltic Proper[b]			75–100	160–210	11
Red alga	*Ceramium tenuicorne*	Baltic Proper[c]	4–70	47–390	3–29	5–71	24
Red alga	*Ceramium tenuicorne*	Baltic Proper[d]			85	40	23
Red alga	*Ceramium virgatum*	Skagerrak	210	970	30	<17	This study
Green alga	*Chara virgata*[f]	Bothnian Sea	<15	57	47	25	This study
Green alga	*Chara* sp.[f]	Philippines		300	<20	100	7
Green alga	*Cladophora glomerata*	Bothnian Sea	107	484	<17	40	This study
Green alga	*Cladophora* sp.	Philippines		1300	<20	<20	7
Red alga	*Polysiphonia sphaerocarpa*	Australia[e]	100–300	200–700			36

[a] Including stoneworts. [b] Ranges in a single collection period. [c] Ranges from June to August. [d] Peak concentrations, July. [e] East coast, near Sydney. [f] Stonewort.

Methods of extraction varied in the other studies. SOAK 1 as used here was employed to extract macroalgae from the Philippines.[7] Variations of the BLEND method were used for Baltic specimens.[8,11,24] BPs in macroalgae from Hong Kong[6] and Australia[12,36] were isolated by combined steam distillation-solvent extraction. Free bromophenols (BPs) were also determined in the studies listed in Table 5, with levels similar or higher than those of BAs.

The more polar BPs and OH-BDEs were not considered in this initial survey. These are also abundant in macroalgae.[6–12,36] BPs and OH-BDEs are partially ionized at seawater pH,[37,38] but are nevertheless subject to bioaccumulation.[24,28,39] Diverse toxic effects of 2,4,6-triBP[40] and OH-BDEs[18–20] have been reported, and beneficial antimicrobial activity has been attributed to 6-MeO-BDE47.[41] Many studies have demonstrated interconversion of BPs/BAs and OH-BDEs/MeO-BDEs through O-methylation/demethylation reactions,[18,19,40] and it has been suggested that wildlife acquire OH-BDEs through O-demethylation of accumulated MeO-BDEs.[42] Thus, it is important to include both free phenolic and O-methylated forms in subsequent surveys, along with additional HNPs that have been reported in marine macroalgae; *e.g.* PBDDs,[8,9,11,21] BDEs substituted with multiple OH- or MeO-groups, hydroxylated and methoxylated bromobiphenyls[7] and brominated methylpyrroles.[43] The SOAK 1 (methanol) method has been used to extract compounds with free phenolic groups from macroalgae.[7] Non-target screening by tandem GC-time of flight mass spectrometry (GCxGC-ToF-MS) is very effective for identifying HNPs.[44] GCxGC-ToF-MS[45–47] and advanced data processing techniques based on GC-MS[48] and LC-MS[49,50] methods have identified hundreds to thousands of natural and anthropogenic halogenated compounds in marine mammals and sediments but so far these have not been applied to identification of HNPs in macroalgae.

Conclusions

The data set for neutral bromophenolic compounds (BAs and MeO-BDEs) obtained in this survey is the largest for macroalgae in Nordic waters. A simple extraction procedure based on soaking the macroalgae in methanol (SOAK 1) or methanol-dichloromethane (SOAK 2) gave equivalent yields of both compound classes and was significantly more efficient for MeO-BDEs than blending with mixed solvents (BLEND) In retrospect, the sample sizes used in this survey are adequate for BAs, but somewhat low for MeO-BDEs, which were occasionally below or on the border of detection. Expanding the SOAK methods to accommodate larger samples is recommended.

Concentrations of BAs and MeO-BDEs in macroalgae were highly variable, spanning orders of magnitude, and compound profiles differed according to the species. The concentrations and compound profiles observed here represent only single sampling events, and it is likely that a more extensive survey would reveal seasonal and spatial variability. Other considerations for future research are expanding the list of target compounds to include the more polar BPs and OH-BDEs, search for other HNPs by nontarget screening, and examining the link between macroalgae and bioaccumulation of bromophenolic compounds and other HNPs in Nordic coastal waters.

Conflicts of interest

There are no conflicts of interest to declare.

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