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Effects of temperature on the production of hydrogen peroxide and volatile halocarbons by brackish-water algae

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Abstract

Marine algae produce volatile halocarbons, which have an ozone-depleting potential. The formation of these compounds is thought to be related to oxidative stress, involving H_2O_2 and algal peroxidases. In our study we found strong correlations between the releases of H_2O_2 and brominated and some iodinated compounds to the seawater medium, but no such correlation was found for $CHCl_3$, suggesting the involvement of other formation mechanisms as well. Little is known about the effects of environmental factors on the production of volatile halocarbons by algae and in the present study we focused on the influence of temperature. Algae were sampled in an area of the brackish Baltic Sea that receives thermal discharge, allowing us to collect specimens of the same species that were adapted to different field temperature regimes. We exposed six algal species (the diatom *Pleurosira laevis*, the brown alga *Fucus vesiculosus* and four filamentous green algae, *Cladophora glomerata*, *Enteromorpha ahlneriana*, *E. flexuosa* and *E. intestinalis*) to temperature changes of 0–11 °C under high irradiation to invoke oxidative stress. The production rates, as well as the quantitative composition of 16 volatile halocarbons, were strongly species-dependent and different types of responses to temperature were recorded. However, no response patterns to temperature change were found that were consistent for all species or for all halocarbons. We conclude that the production of certain halocarbons may increase with temperature in certain algal species, but that the amount and composition of the volatile halocarbons released by algal communities are probably more affected by temperature-associated species shifts. These results may have implications for climatic change scenarios.

Keywords: Baltic Sea; Bromoform; Chloroform; Thermal discharge; Oxidative stress; Temperature adaptation

1. Introduction

The natural production of volatile low-molecular-weight halocarbon compounds (volatile halocarbons) has been widely investigated during the last decades. Much of this production is biogenic, notably by marine micro- and macroalgae (Gribble, 1992), although oxidation processes during chemical degradation of organic matter have been found to be significant as well (Keppler et al., 2000). It has been estimated that marine algae produce about 70% of global bromoform (Carpenter and Liss, 2000). Besides brominated and iodinated volatile halocarbons, also chlorinated compounds such as trichloroethylene (previously thought to have solely anthropogenic origin) and chloroform can be naturally produced (Lovelock, 1975; Collén et al., 1994; Abrahamsson et al., 1995a; Nightingale et al., 1995; Abrahamsson and Pedersén, 2000; Laturnus et al., 2002). Volatile halocarbons participate in a number of atmospheric reactions and change the oxidizing capacity of the atmosphere (Montzka et al., 1999). Thereby they influence the atmospheric lifetime of other greenhouse gases such as methane and chlorofluorohydrocarbons. It has been shown that the formation of volatile halocarbons by algae involves haloperoxidases and stress-induced H_2O_2 (Wever et al., 1991; Collén et al., 1994; Sundström et al., 1996; Pedersén et al., 1996a), which in turn is enzymatically reduced to H_2O with subsequent oxidation of bromide ions to HOBr (Wever et al., 1991) or chloride ions to HOCl (Pedersén et al., 1996b).

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However, the formation mechanisms are not yet fully understood, and different mechanisms may be involved in the formation of different halocarbon species (Collén et al., 1994).

Production rates of volatile halocarbons in marine macroalgae can be as high as 55 nmol per gram algal dry weight (DW) per day for the dominant halocarbon $CHBr_3$ (Gschwend et al., 1985) and 1–2 nmol (g DW)$^{-1}$ d^{-1} for $CHCl_3$ (Nightingale et al., 1995) and CH_2I_2 (Laturnus et al., 1996). The production of halocarbons by macroalgae has been investigated for around 70 species. Most previous studies have dealt with algae from temperate areas (Gschwend et al., 1985; Manley and Dastoor, 1987, 1988; Manley et al., 1992; Abrahamsson et al., 1995b; Ekdahl, 1997), but also from polar (Laturnus, 1996; Laturnus et al., 1996), and (sub)-tropical regions (Collén et al., 1994; Mtolera et al., 1996). Generally, algae from warmer regions have higher production rates compared those from colder regions (Ekdahl, 1997), and we speculated that this could be a result of differences in irradiation and temperature. Little is known about the effects of environmental factors on the production of volatile halocarbons. In the present study we investigated (1) if and how temperature affects the net production rates of H_2O_2 and volatile halocarbons in brackish-water macroalgae, (2) if temperature influences the quantitative composition of 16 halocarbons produced, and (3) if the pre-adaptation of algae (genetic and/or by acclimatization) to a certain temperature regime in the field will affect the production of H_2O_2 and volatile halocarbons. The production of H_2O_2 and volatile halocarbons is here defined as net production measured as increased concentrations in the seawater medium during incubations.

2. Results and discussion

2.1. Normalization of measurements

In this study we normalized all production rates of H_2O_2 and volatile halocarbons to the algal ash-free dry weight (ADW), which is the organic fraction of the dry weight (DW), to achieve a fair comparison between the six algal species we studied. When comparing the brown leathery species *Fucus vesiculosus* with four filamentous green algae and a diatom, its production rates on the basis of fresh weight (FW) would be overestimated because its DW/FW ratio is about twice that of the other five species (Table 1). When comparing the colonial diatom *Pleurosira laevis* with *F. vesiculosus* and four filamentous green algae (*Cladophora glomerata*, *Enteromorpha ahlneriana*, *E. flexuosa* and *E. intestinalis*), its production rates on the basis of FW or DW would be underestimated because its ADW/DW ratio is about half that of the others due to the diatom's heavy silica frustules (Table 1). These differences show that algal morphology is an important, but often neglected, factor to recognize when comparing different species.

2.2. The production of volatile halocarbons

Sixteen volatile halocarbons were detected in seawater after incubations of 66 algal samples in two experiments. These were: trichloromethane ($CHCl_3$ = chloroform), trichloroethene (C_2HCl_3 = trichloroethylene), tetrachloroethane (C_2Cl_4 = perchloroethylene), tribromomethane ($CHBr_3$ = bromoform), dibromomethane (CH_2Br_2), chlorodibromomethane ($CHClBr_2$), bromochloromethane (CH_2BrCl), bromodichloromethane ($CHCl_2Br$), diiodomethane (CH_2I_2), iodomethane (CH_3I), iodoethane (C_2H_5I), chloroiodomethane (CH_2ClI), 1-iodopropane (C_3H_7I), 2-iodopropane ($^{iso}C_3H_7I$), 1-iodobutane (C_4H_9I) and 2-iodobutane ($^{sec}C_4H_9I$). All 16 compounds occurred in 70–100% of the 66 samples, except for C_2HCl_3 (20%) which was only produced by two out of six species (*E. ahlneriana* and *F. vesiculosus*) and CH_2ClI (55%). C_2HCl_3 seems to be produced very irregularly among algal species, its highest producers known being the (sub)tropical red algae *Falkenbergia hillebrandii*, *Asparagopsis taxiformis* and *Meristiella gelidium* (Abrahamsson et al., 1995a; Abrahamsson and Pedersén, 2000). In the present study the overall highest production rates were found for $CHCl_3$, $CHBr_3$, $CHBr_2Cl$, CH_2Br_2 and CH_2I_2, whereas CH_2BrCl, CH_2ClI, CCl_4, $^{iso}C_3H_7I$ and $^{sec}C_4H_9I$ always were produced at very low rates. This relative halocarbon composition is in agreement with those reported from previous surveys of macroalgal species (Gschwend et al., 1985; Nightingale et al., 1995; Laturnus et al., 1996). Iodine was used about four times less in halocarbon formation by algae (mean and maximum production rates: 46 and 410 pmol (g ADW)$^{-1}$ h^{-1}) than chlorine (161 and 1781) and bromine (192 and 1264). A lower incorporation of iodine in volatile halocarbons was also observed for marine microorganisms in the Greenland Sea by Ekdahl (1997). The comparison of production rates with literature data is tricky because the rates depend on incubation times and conditions as well as which part of the algal thallus is used. No literature records of halocarbon production rates for our six species were found. The maximum production rate of $CHBr_3$ in our study was 74 pmol (g DW)$^{-1}$ h^{-1} by *E. intestinalis*, which is similar to the maximum rate reported for temperate green algae (Nightingale et al., 1995: 82 pmol (g DW)$^{-1}$ h^{-1} for *Enteromorpha* sp.), but low compared to the maximum rates reported for some brown algae from temperate and polar areas [ca. 5000 pmol (g DW)$^{-1}$ h^{-1}: Nightingale et al., 1995 for *Laminaria saccharina*; Laturnus et al., 1996 for *Desmarestia anceps*]. Our maximum production rates of $CHCl_3$ [405 pmol (g DW)$^{-1}$ h^{-1} in

Table 1
Production rates of H_2O_2 and three abundant volatile halocarbons, chloroform ($CHCl_3$), bromoform ($CHBr_3$) and diiodomethane (CH_2I_2), by six algal species, sampled from different temperatures in the field and incubated in the laboratory at 23 °C and 600 μmol photons PAR m^{-2} s^{-1} for 6 h. The other two abundant halocarbons, CH_2Br_2 and $CHBr_2Cl$, co-varied with $CHBr_3$ (r_P 0.84 and 0.87, respectively)

Species	Algal group	DW/FW Mean±S.D.	ADW/DW Mean±S.D.	Field temperature (°C)	H_2O_2 Mean±S.D. μmol (g ADW)$^{-1}$ h^{-1}	$CHCl_3$ Mean±S.D. pmol (g ADW)$^{-1}$ h^{-1}	$CHBr_3$ Mean±S.D. pmol (g ADW)$^{-1}$ h^{-1}	CH_2I_2 Mean±SD pmol (g ADW)$^{-1}$ h^{-1}
Pleurosira laevis	Diatom	0.08±0.01	0.36±0.02	17 (n=3)	1.5±0.4	57±34	2.6±4.4	29±50
n=6				23 (n=3)	No data	128±47	1.8±3.1	4.4±7.6
Fucus vesiculosus	Brown alga	0.17±0.01	0.84±0.01	12 (n=6)	1.8±0.6	46±98	27±5	5.8±5.2
n=6								
Cladophora glomerata	Green alga	0.06±0.02	0.72±0.02	12 (n=2)	No data	11±14	0.0±0.0	8.6±1.7
n=6				17 (n=2)	3.0±0.3	3.4±4.9	3.3±4.7	0.0±0.0
				23 (n=2)	2.8±0.9	2.2±0.6	1.1±1.5	0.0±0.0
Enteromorpha ahlneriana	Green alga	0.07±0.03	0.76±0.03	12 (n=3)	No data	156±265	4.5±7.5	67±109
n=9				17 (n=3)	22±18	17±23	7.7±6.2	3.4±5.8
				23 (n=3)	64±60	34±52	32±26	5.8±5.9
Enteromorpha flexuosa	Green alga	0.09±0.01	0.74±0.03	17 (n=3)	No data	13±22	16±28	23±9
n=6				23 (n=3)	5.6±3.1	0.9±0.9	31±27	10±4
Enteromorpha intestinalis	Green alga	0.09±0.03	0.77±0.02	12 (n=3)	No data	18±5	86±9	15±22
n=9				15 (n=3)	10±5	177±303	49±32	0.0±0.0
				17 (n=3)	26±1	8±12	55±12	29±19

E. intestinalis] and CH_2I_2 [147 pmol (g DW)$^{-1}$ h^{-1} in *E. ahlneriana*] were higher than those reported by Nightingale et al. (1995), Laturnus (1996) and Laturnus et al. (1996).

2.3. Production of halocarbons by different algal species in high temperature

Principal components analysis (PCA) summarized the distribution of 16 volatile halocarbons in 42 samples of six macroalgal species incubated at 23 °C, but sampled in the field at 12–23 °C. PCA is an ordination technique in which a reciprocal regression relation holds true between, in this case, halocarbon scores and halocarbon-derived sample scores of algal species/temperature combinations (Jongman et al., 1987). The halocarbon and sample scores together form a biplot that displays two-dimensional approximations (Fig. 1). Eigenvalues indicate the relative importance of each of the first four PCA ordination axes (0.43, 0.19, 0.14 and 0.08, respectively). Axis 1 explained most of the variation in the halocarbon composition of the algal samples as shown by its high eigenvalue compared to those of subsequent axes. Axis 1 was correlated to algal species (Fig. 1a), which suggests that the quantitative composition of the halocarbons produced was strongly species-dependent. The sample scores showed clear distribution patterns in the ordination: *F. vesiculosus* and *E. intestinalis* were situated to the left in the ordination, those of *E. flexuosa* in the center, and those of *C. glomerata*, *E. ahlneriana* and *P. laevis* to the right. These distributions were irrespective of the temperature at which the algae were sampled in the field as no patterns related to temperature were found within the species clusters (Fig. 1a). This indicates that although different production rates seemingly related to temperature stress were found for single halocarbons, no patterns consistent for all species were discovered in the data set, e.g. $CHBr_3$ production increased with temperature in *E. ahlneriana* and *E. flexuosa*, but decreased in *E. intestinalis* (Table 1). These results suggest little or no adaptation of halocarbon formation to the temperature regime in the field. However, field temperature was a significant factor when tested by multiple regression analysis on the ordination (arrow pointing to the right; Fig. 1b). This can be explained by the fact that neither *E. intestinalis* nor *F. vesiculosus* occurred at 23 °C in the field, whereas *E. flexuosa* and *P. laevis* did not occur at 12 °C. Thus, in this case field temperature was correlated with algal species and therefore it yielded a significant effect in the ordination, which is an indirect effect of temperature (temperature affecting species occurrences). We conclude that the production of certain halocarbons may increase with temperature in certain algal species, but that the amount and composition of the volatile halocarbons produced by algal communities are probably more affected by temperature-associated species shifts. In the northern Baltic Sea, increased temperature promotes algal communities with higher abundances of fast-growing ephemeral algae, especially *Enteromorpha* species (Snoeijs and Prentice, 1989; Snoeijs, 1992). Also eutrophication is known to promote the growth of *Enteromorpha* species, especially in shallow coastal bays (Snoeijs, 1999). Thus, both climatic changes (increased temperature) and eutrophication (increased nutrient availability) may lead to higher production of volatile halocarbons in coastal areas. As volatile halocarbons (especially chlorinated and brominated compounds) can

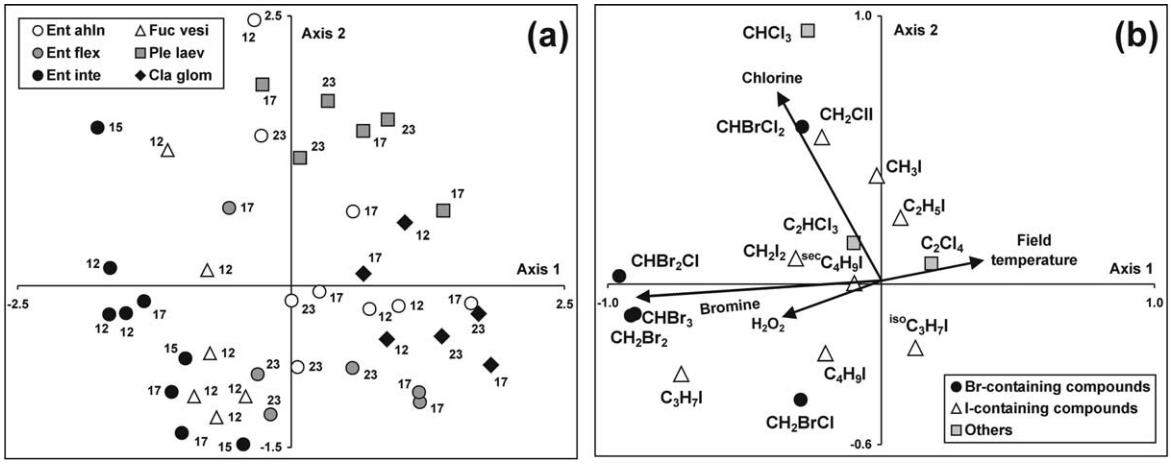

Fig. 1. Ordination plot of the PCA analysis of six different algal species, showing (a) sample scores labelled with algal species (symbols) and water temperature (in °C) during sampling, and (b) halocarbon scores for the 16 volatile halocarbons used in the analysis with arrows for the factors tested significant by multiple regression analysis on the results of the PCA (H_2O_2 production, bromine in halocarbons, chlorine in halocarbons and field water temperature during sampling). Algal species abbreviations: Cla glom = *Cladophora glomerata*, Ent ahln = *Enteromorpha ahlneriana*, Ent flex = *Enteromorpha flexuosa*, Ent inte = *Enteromorpha intestinalis*, Fuc vesi = *Fucus vesiculosus*, Ple laev = *Pleurosira laevis*.

reach tropospheric and stratospheric layers where the reactive halogen atoms can destruct ozone (Montzka et al., 1999; Sturges et al., 2000), climatic change and eutrophication could indirectly damage the earth's protecting ozone layer.

The production of H_2O_2 and the amounts of bromine and chlorine (but not iodine) incorporated in volatile halocarbons were significant factors when tested by multiple regression analysis on the results of the ordination (Fig. 1b). Such passively tested factors have explanatory power, but they were not used to construct the ordination itself. In the ordination plot, each of these factors is shown as an arrow, which indicates its direction and rate (arrow length) of change. The arrows for H_2O_2 and bromine point in the direction of the brominated compounds $CHBr_3$, CH_2Br_2, $CHBrCl_2$ and CH_2BrCl, as well as C_3H_7I and C_4H_9I, suggesting an involvement of H_2O_2 in the formation of these compounds. Table 1 confirms that *F. vesiculosus*, *E. intestinalis* and *E. flexuosa* were large producers of $CHBr_3$ compared to the other three species (with the exception of *E. ahlneriana* from 23 °C). *E. intestinalis* was the algal species with the highest overall production of brominated compounds with mean rates of 750 pmol bromine (g ADW)$^{-1}$ h^{-1}, followed by *F. vesiculosus* with mean rates of 332 pmol bromine (g ADW)$^{-1}$ h^{-1} (Fig. 2). *C. glomerata* produced least $CHBr_3$, which may be linked to its low production of H_2O_2 resulting from high activities of the H_2O_2 scavenging enzymes catalase and ascorbate peroxidase.

Along axis 2 of the ordination, there was a separation mainly according to high production of $CHCl_3$, which was the major compound determining the direction of the arrow for chlorine (Fig. 1b). The diatom *P. laevis* was found to be a stable large producer of this compound (Table 1). In the other species, high production

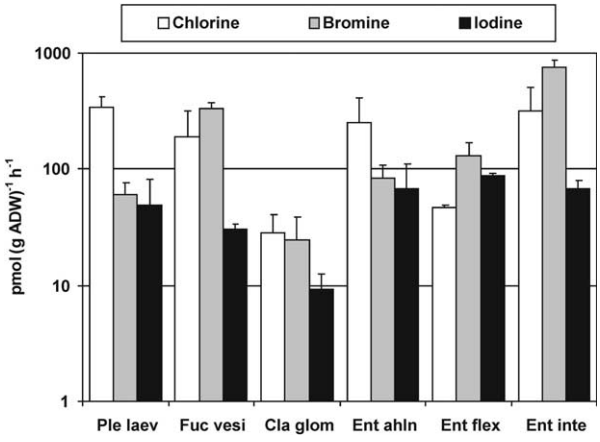

Fig. 2. Production rates of organo-chlorine, organo-bromine and organo-iodine by six algal species during 6 h of incubation. Error bars represent 1 S.E. of the mean. Algal species abbreviations cf. Fig. 1.

of $CHCl_3$ was found only in single samples, as shown by the high standard deviations for $CHCl_3$ in *F. vesiculosus* (from 12 °C), *E. ahlneriana* (from 12 °C) and *E. intestinalis* (from 15 °C) in Table 1. *E. ahlneriana*, *E. intestinalis*, *F. vesiculosus* and *P. laevis* were all large producers of chlorinated compounds with mean rates varying between 187 and 335 pmol chlorine (g ADW)$^{-1}$ h^{-1}. The compounds found in the center of the PCA ordination influenced the model to a minor extent, i.e. the production rates did not vary much among species. The three *Enteromorpha* species tended to have higher iodine values, 66–88 pmol I (g ADW)$^{-1}$ h^{-1} as compared to 9–49 pmol I (g ADW)$^{-1}$ h^{-1} for the other three species. Our results show no response pattern to temperature change that was consistent for all species or for all halocarbons.

The production of halocarbons may also depend on which part of the thallus is used in the experiments, as

shown by large differences in brominating activity between the stipe and the blade of the brown alga *Laminaria saccharina* (Mehrtens and Laturnus, 1997). Such differences cannot have influenced our results for the diatom and the filamentous green algae because their thalli are small and uniform, but within the thallus of *F. vesiculosus* differences may occur and therefore we always used the youngest upper 5 cm of the thalli where the cells are most actively growing.

2.4. Cross-incubation experiment

To examine if changes in temperature could induce H_2O_2 and halocarbon production, a 10-h cross-incubation experiment was performed with the two filamentous green algae *C. glomerata* and *E. ahlneriana* (Table 2). In this way different situations of temperature-dependent oxidative stress were created, e.g. low temperature and high radiation stimulates oxidative stress in algae. *C. glomerata* and *E. ahlneriana* dominate the upper littoral in the northern Baltic Sea in summer in single-species or mixed stands (Snoeijs, 1992, 1999). In Forsmark, they were growing in the field at all water temperatures between 12 and 23 °C. Different responses associated with temperature were found. Temperature change from 23 to 12 °C or from 12 to 23 °C increased H_2O_2 production six- to eightfold and CH_2I_2 production two- to fivefold in *C. glomerata* (Table 2). No correlation of this type was found for other common halocarbons such as $CHCl_3$ or $CHBr_3$. Instead, incubation of *C. glomerata* in 23 °C, irrespective of the field temperature during sampling, seemed to yield higher production of $CHCl_3$ and $CHBr_3$ (Table 2). However, this response of $CHCl_3$ to temperature was not stable because it did not occur in all replicates (see high SD in Table 2). An indication of adaptation to field temperature conditions was found in *E. ahlneriana* from 23 °C, which produced by far most H_2O_2 and $CHBr_3$ (Table 2). Laturnus et al. (2000) studied the influence of temperature on cultures of the red macroalga *Gymnogongus antarcticus* and recorded increased production of $CHBr_3$ as a result of increased temperature. Our results show that this can be the case, but that it is not a universal response to temperature in algae.

The variation in the composition of volatile halocarbons during the cross-incubation experiment was summarized by a PCA ordination (Fig. 3). The eigenvalues for the first four PCA ordination axes were 0.45, 0.29, 0.06 and 0.05, respectively. Axes 1 and 2 together explained most of the variation in the halocarbon composition of the algal samples as can be concluded from their high eigenvalues compared to axes 3 and 4. The sample scores (Fig. 3a) showed patterns related to both temperature and algal species. Along axis 1, three groups of sample scores were separated. To the far right of the ordination, all six *E. ahlneriana* sampled from 23 °C were situated (without a separation according to incubation temperature). This indicates that the halocarbon composition in this alga is quite different from all other samples, which may be the result of 17 years of adaptation (possibly genetically defined) to the heated discharge conditions. The sample scores of *E. ahlneriana* sampled from 12 °C were situated in the center of the ordination. The halocarbon composition of these samples appeared to be more like that of *C. glomerata* than that of *E. ahlneriana* sampled from 23 °C. The sample scores of *C. glomerata* showed a separation along axis 2: all six samples incubated in 12 °C (irrespective of the field temperature during sampling) were close together in the lower-left quadrant of the ordination plot, whereas those incubated in 23 °C occur all along axis 2. This designates a consistent halocarbon composition at 12 °C, but a variable composition at 23 °C mainly caused by high $CHCl_3$ production in some samples.

The scores of the different halocarbons were divided over the ordination plot according to the pattern shown by the two algal species in relation to temperature (Fig. 3b). The positions of the halocarbon scores and

Table 2
Production rates of H_2O_2 and three abundant volatile halocarbons, chloroform ($CHCl_3$), bromoform ($CHBr_3$) and diiodomethane (CH_2I_2), in the cross-incubation experiment with *Cladophora glomerata* and *Enteromorpha ahlneriana* sampled from different temperatures in the field (12 or 23 °C), during laboratory incubations at 12 or 23 °C and 600 μmol photons PAR m^{-2} s^{-1} for 10 h. The other two abundant halocarbons, CH_2Br_2 and $CHBr_2Cl$, co-varied with $CHBr_3$ (r_P 0.94 and 0.80, respectively)

Species	Field temperature (°C)	Incubation temperature (°C)	Final pH Mean±S.D. (after 10 h)	Number of replicates	H_2O_2 Mean±S.D. μmol (g ADW)$^{-1}$ h^{-1}	$CHCl_3$ Mean±S.D. pmol (g ADW)$^{-1}$ h^{-1}	$CHBr_3$ Mean±S.D. pmol (g ADW)$^{-1}$ h^{-1}	CH_2I_2 Mean±S.D. pmol (g ADW)$^{-1}$ h^{-1}
Cladophora glomerata	12	12	10.6±0.1	$n=3$	0.1±0.0	0.1±0.0	1.8±0.5	10±4
	12	23	10.6±0.1	$n=3$	0.8±0.4	102±159	3.3±1.5	18±18
	23	12	10.7±0.2	$n=3$	0.6±0.3	0.9±0.5	2.2±0.8	27±38
	23	23	10.6±0.1	$n=2$	0.1±0.1	109±151	3.3±0.3	4.8±3.3
Enteromorpha ahlneriana	12	12	10.7±0.1	$n=2$	11±5	7±10	3.7±5.3	6.9±0.7
	12	23	10.5±0.1	$n=3$	0.6±0.9	1.9±1.1	7.1±1.5	10±5
	23	12	10.7±0.2	$n=3$	23±17	1.3±1.2	53±10	11±5
	23	23	10.4±0.5	$n=3$	44±12	9±14	40±10	10±6

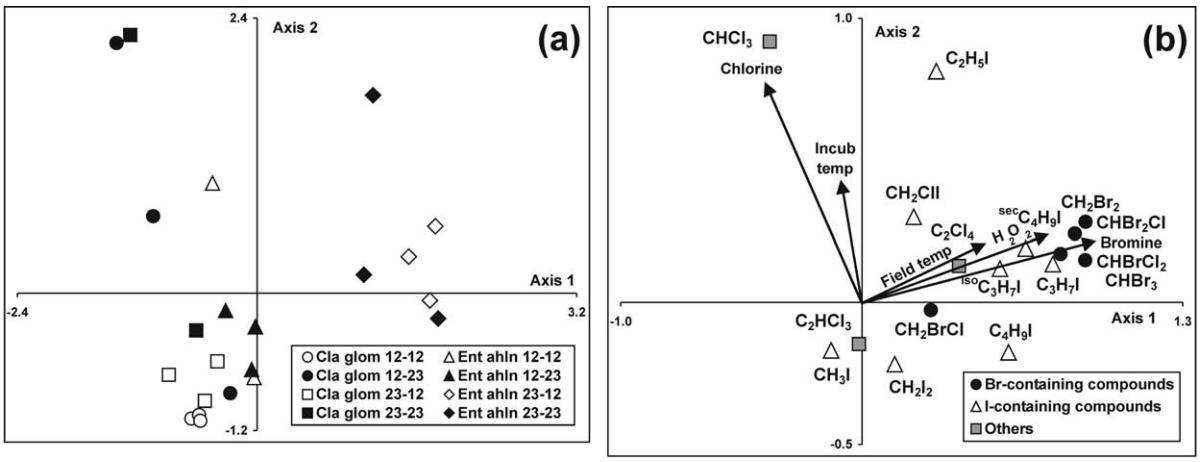

Fig. 3. Ordination plot of the PCA analysis of the cross-incubation experiment with *Cladophora glomerata* (Cla glom) and *Enteromorpha ahlneriana* (Ent ahln), showing (a) sample scores labelled with algal species (symbols)/temperature (first number = water temperature (in °C) during sampling, second number = water temperature during laboratory incubations) combinations, and (b) halocarbon scores for the 16 volatile halocarbons used in the analysis with arrows for the factors tested significant by multiple regression analysis on the results of the PCA (H_2O_2 production, bromine in halocarbons, chlorine in halocarbons, field water temperature during sampling and water temperature during incubation).

the arrows for bromine and field temperature indicated that *E. ahlneriana* sampled from 23 °C produced most brominated compounds, whereas the production of these compounds was lowest in all *C. glomerata* samples (Table 2), which were found at the opposite side of the ordination. *E. ahlneriana* sampled from 23 °C produced brominated compounds with mean rates of 169–213 pmol bromine (g ADW)$^{-1}$ h^{-1}, while rates in *C. glomerata* were only 7–12 pmol bromine (g ADW)$^{-1}$ h^{-1} (Fig. 4). When *C. glomerata* was incubated at 23 °C, a high production of $CHCl_3$ was induced in three out of five samples, irrespective of field temperature (Figs. 3a and b and 4). The arrows for chlorine and incubation temperature point in the direction of $CHCl_3$ as a result of the high production of this compound in the *C. glomerata* samples incubated at 23 °C. In concert with the increases in net H_2O_2 production in *C. glomerata* when cross-incubated, the incorporation of iodine in halocarbons more than doubled, from 13–23 to 43–56 pmol I (g ADW)$^{-1}$ h^{-1} (Fig. 4). Thus, three different types of responses to temperature were found in the cross-incubation experiment: adaptation (perhaps genetic) manifested by high production of brominated compounds in algae sampled from high temperature in the field, a cross-incubation effect manifested by high production of CH_2I_2 by algae incubated at a temperature higher or lower than the field temperature, and a high temperature effect manifested by high (but irregular) production of $CHCl_3$ in algae incubated at high temperature irrespective of the field temperature. Thus, also in the cross-incubation experiment, no response pattern to temperature change was found that was consistent for both species or for all halocarbons.

The production of H_2O_2 was a significant factor when tested by multiple regression analysis on the ordination (Fig. 3b). Like in the comparison of the six species, the arrow for H_2O_2 points in the direction of the brominated compounds $CHBr_3$, CH_2Br_2, $CHClBr_2$ and CH_2BrCl_2, as well as a group of iodinated halocarbons (C_3H_7I, $^{iso}C_3H_7I$, C_4H_9I, $^{sec}C_4H_9I$), suggesting involvement of H_2O_2 in the formation of these compounds. Significant correlation coefficients were found between the production rates of H_2O_2 and those of six halocarbons [$CHBr_3$ ($r_P = 0.69$), CH_2Br_2 ($r_P = 0.61$), $CHClBr_2$ ($r_P = 0.84$), CH_2BrCl_2 ($r_P = 0.76$), C_3H_7I ($r_P = 0.54$) and C_4H_9I ($r_P = 0.43$)], but no significant relationships were found between H_2O_2 and the other 10 halocarbons.

2.5. The formation mechanisms of halocarbons

As the production of some volatile halocarbons were clearly correlated with high algal H_2O_2 production and others were not, the formation mechanisms probably differ for different (groups of) halocarbon species. Marine algae possess bromoperoxidases (which can oxidize both I^- and Br^- but not Cl^-) and iodoperoxidases (which can oxidize only I^-) (Manley, 2002). These haloperoxidases can halogenate larger organic compounds with polyhalomethanes as by-products. Haloperoxidases and halogenated compounds occur in algae intracellularly (chloroplasts, and electron-dense vesicles in the cytosol, e.g. physodes in brown algae), but also at the middle lamella and the outer cell surface (Pedersén et al., 1980, 1996a; Manley, 2002). The haloperoxidases may protect the algal tissue from internally produced H_2O_2 (in photosynthesis, photorespiration, respiration and other metabolic processes), but also from H_2O_2 present in seawater due to leakage from the alga itself, leakage from other algae or abiotic photochemical reactions (Pedersén et al., 1996a). Also, iodine uptake in kelps may involve an extracellular haloperoxidase

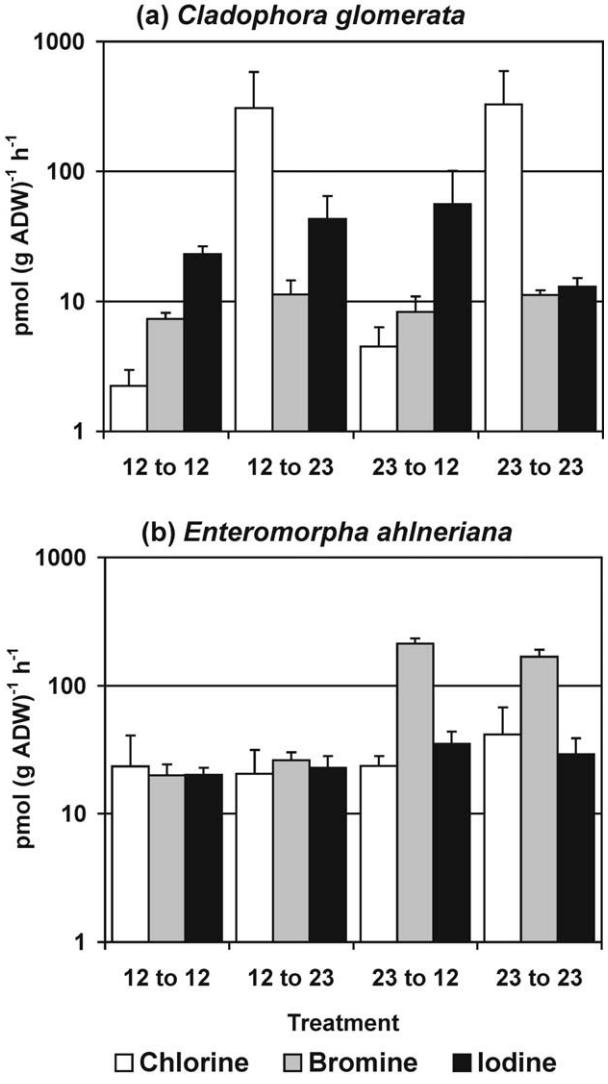

Fig. 4. Production rates of organo-chlorine, organo-bromine and organo-iodine by (a) *Cladophora glomerata* and (b) *Enteromorpha ahlneriana* during 10 h of incubation in the cross-incubation experiment. Error bars represent 1 S.E. of the mean.

(Küpper et al., 1998). The differences in the production of volatile halocarbons between species discovered in the present study are probably related to the types and activities of the haloperoxidases in the different species, as well as the algae's different production levels of H_2O_2. The latter is regulated by the degree of oxidative stress in the algal cells in conjunction with their defense mechanisms against oxidative stress (Pedersén et al., 1996a). In our study, the formation of brominated compounds was strongly correlated with the occurrence of H_2O_2 as previously found by Collén et al. (1994) and Manley and Barbero (2001). Also, the formation of iodinated propane and butane seemed to be correlated with H_2O_2. These results are not entirely consistent with the findings of Collén et al. (1994) who tested the production of halocarbons with addition of H_2O_2 in the tropical red alga *Meristiella gelidium* and found that the formation of brominated compounds, $CHCl_3$ and C_2HCl_3 increased, but not the formation of iodinated compounds. In both our data sets, no correlation between $CHCl_3$ and H_2O_2 was found and the lowest producer of H_2O_2, the diatom *P. laevis*, had the highest stable production of $CHCl_3$ (Table 1). Diatoms have also in other studies been shown to be large producers of $CHCl_3$, e.g. *Cyclotella* sp. (Plummer and Edzwald, 2001). Most probably, the algae used in our study did not possess haloperoxidases with chlorinating ability as $CHCl_3$ seemed to be formed by a mechanism independent of the occurrence of H_2O_2. Presently known H_2O_2-independent mechanisms for the phytogenesis of halomethanes include methyltransferase activity in which monohalomethanes appear to be by-products or 'accidents' of normal metabolism (Manley, 2002). Possible other formation mechanisms of volatile halocarbons independent of H_2O_2 in algae need to be investigated further.

Speculations about the evolutionary justification of the formation of volatile halocarbons include their possible function as allelochemicals. They have frequently been suggested to participate in chemical defense mechanisms against herbivores and epiphytes (Manley, 2002). Among our experimental algae the *Enteromorpha* species (especially *E. ahlneriana*) are usually markedly devoid of epiphytes, also in mixed stands with *C. glomerata* which is usually heavily overgrown by diatoms and cyanobacteria. Ohsawa et al. (2001) demonstrated that $CHBr_3$ produced by the red macroalga *Corallina pilulifera* could eliminate epiphytic organisms, especially diatoms from the macroalgal surface. Similarly, absence of epiphytes may be attributed to the higher release of H_2O_2 and halocarbons by *Enteromorpha* species in the Baltic Sea.

3. Experimental

3.1. Study area

The study area at Forsmark (60° 25.80″ N, 18° 11.14″ E) is situated on the Swedish east coast at the southern end of the Gulf of Bothnia (Baltic Sea), approximately 130 km north of Stockholm. Brackish (5 PSU = practical salinity units) cooling water from the Forsmark nuclear power plant (3200 MW) is led through an artificial enclosure, the Forsmark Biotest basin, before it is returned to the Baltic Sea. The basin was constructed especially for environmental impact studies (Snoeijs, 1994), and has received thermal discharge water since 1980. The Biotest basin has an area of ca. 1 km^2 and provides a range of environments, from stagnant water to fast flow. The water is heated by 10–12 °C in the main flow of the cooling water, but less in mixing areas, so that sites with temperature anomalies of 0–12 °C

above normal are found in the area. Thus, the study area offers a unique opportunity to sample algae that have adapted to different temperature regimes for longer periods of time in the field.

3.2. The algal material

The algae were sampled from sites in and around the Biotest basin on 8–12 September 1997, and immediately used in the experiments. The six algae used in the present study were the colony-forming diatom *Pleurosira laevis* Ehr. fo. *polymorpha* (Kütz.) Compère, the perennial brown alga *Fucus vesiculosus* L., and four species of ephemeral filamentous green algae, *Cladophora glomerata* (L.) Kütz., *Enteromorpha ahlneriana* Bliding, *Enteromorpha flexuosa* (Wulfen) J. Agardh and *Enteromorpha intestinalis* (L.) Nees. The brown and green algal species are all common in the brackish Baltic Sea, which has a year-round stable surface salinity of 5–7 PSU in its major basins (Snoeijs, 1999). The diatom is a non-indigenous 'warm water' species, which probably has been introduced from southern latitudes (Snoeijs, 1998). In the study area, *P. laevis* only occurs in flowing heated water in autumn in up to 0.5 m high colonies. In contrast, *F. vesiculosus*, which normally is belt-forming in the Baltic Sea, is completely absent from the heated water in Forsmark. In the northern Baltic Sea, the natural seasonal occurrence for *E. flexuosa* is restricted to summer and that of *E. ahlneriana* to summer-autumn, while *E. intestinalis* and *C. glomerata* occur from early spring to late autumn. In the cooling water discharge area these occurrence patterns are disrupted, allowing year-round occurrence, except for *E. flexuosa*, which remains restricted to the summer season (Snoeijs and Prentice, 1989; Snoeijs, 1992). Our study was carried out in late summer–early autumn (September) to assure access to healthy, growing algal material for all species.

3.3. Incubations for production measurements

Altogether, 42 samples of the six algal species (Table 1) were collected from 15 different sampling sites, which varied in water temperature from 12 °C (unheated sites) to 23 °C (maximally heated sites). Of each sample, 0.6 g of algal fresh weight (FW) was placed in a 70-ml gas-tight flask in natural seawater (NSW) without a headspace. The same (unfiltered) NSW stock of 5 PSU (25 l sampled on one occasion and kept in the dark) was used for all algal incubations and the controls without algae. The 42 flasks were incubated at 23 °C and 600 μmol photons PAR m^{-2} s^{-1} (obtained from daylight fluorescent tubes) for 6 h. As controls, 6 flasks with NSW without algae were incubated in the same conditions. After the incubations, H_2O_2 and halocarbon concentrations in the NSW were measured. The pH was measured at the end of the incubations as a measure of photosynthetic activity. For all algal samples dry weight (DW, after 24 h at 80 °C) and ash-free dry weight (ADW, after 24 h at 550 °C) were determined. All results were normalized to ADW.

3.4. Cross-incubation experiment

A cross-incubation experiment was carried out with the two species that had the widest temperature range in the field (12–23 °C), *C. glomerata* and *E. ahlneriana* (Tables 1 and 2). The algae were sampled from two sites where they grew together, one site had a field temperature of 12 °C and the other site had 23 °C. Of each of the four samples, six replicate 70-ml gas-tight flasks were prepared with 0.6 g of algal FW in NSW without a headspace. For each sample, three replicate flasks were incubated at 23 °C, and the other three at 12 °C, for 10 h at 600 μmol photons PAR m^{-2} s^{-1}. As controls, three flasks with NSW without algae were incubated at 23 °C, and three others at 12 °C. Altogether, the cross-incubation experiment included 24 flasks with algae and 6 controls without algae. Immediately after the incubations, H_2O_2 and halocarbon concentrations in the NSW were measured. The pH was measured at the end of the incubations as a measure of photosynthetic activity. For all algal samples DW and ADW were determined.

3.5. Determination of H_2O_2 in seawater

Luminol-dependent chemiluminescence (LDC) was measured using an LKB 1250 luminometer and a flatbed recorder. A method described by Glazener et al. (1991) and later adapted by Collén and Pedersén (1994) was followed with some minor modifications. Immediately before each single measurement, 48 μl Horseradish peroxidase (Sigma™) stock solution (11 μkat ml^{-1} in 0.1 M phosphate buffer, pH = 7.0) and 16 μl luminol (5-amino-2,3-dihydro-1,4-phtalazinedone, Sigma™) stock solution (8.8 mg luminol + 1 ml NaOH in 9 ml 0.4 M MOPS buffer solution, pH = 7.6) were mixed, and a sub-sample of 1 ml NSW was injected into this mixture. The mean recordings of three injections were used to calculate the H_2O_2 concentration of the NSW in each flask. A standard curve of H_2O_2 (p.a. Merck™) was prepared by diluting 30% H_2O_2.

3.6. Determination of halocarbons in seawater

Liquid halocarbon standard solutions were made in acetone (p.a. Merck™) using standards purchased from Fluka™. The halocarbons were pre-concentrated with a purge-and-trap system, which was connected to a Varian 3400 gas chromatograph equipped with an electron capture detector. The water samples (26.3 ml) were analyzed immediately after incubation. During injection

they were filtered through glass fiber filters (GF/F, Whatman™). The water was purged for 4 min in a purge chamber with ultra pure nitrogen, and the compounds were trapped on a solid support (Porapak N, Alltech Inc.). The trap was cooled with water to 5 °C during trapping, and electrically heated to 140 °C in the desorption mode. The chromatographic conditions were: start temperature of 35 °C at a hold time of 3 min, which was then raised to 60 °C at a rate of 5 °C min^{-1}. Thereafter the temperature was increased to 180 °C at a rate of 15 °C min^{-1}. The temperature was held at 180 °C for 3 min. The chromatographic separation was made on a 60 m narrow bore column, 502.2 (Restek™), with an inner diameter of 0.32 mm, and a film thickness of 1.8 μm. Ultra pure nitrogen was used both as carrier gas and make-up gas at flow rates of 6 and 10 ml min^{-1}, respectively. The detection limits of this method are in the fmol l^{-1} to pmol l^{-1} range, with a relative standard deviation of 1–3%. For further details concerning the analytical method, see Ekdahl and Abrahamsson (1997).

3.7. Data analysis

Principal Components Analysis (PCA; Jongman et al., 1987), implemented with the programme CANOCO 4 (ter Braak and Šmilauer, 1998), was carried out to detect overall patterns in the distributions of volatile halocarbon species in the samples, using pmol halocarbon (g ADW alga)$^{-1}$ h^{-1} as input data. Prior to analysis, a log-transformation was applied to the data. Additionally, multiple regression analysis was carried out on the PCA results to test if the variables H_2O_2 concentration, water temperature in the field during sampling, water temperature during incubation and the total sums of brominated, chlorinated and iodinated volatile halocarbons had a significant fit with the PCA results. Two PCA analyses were carried out. The first analysis included a comparison of 16 halocarbon species in the six algal species from 42 algal samples after 6 h incubations. The second PCA analysis was performed on the results of the cross-incubation experiment and included a comparison of 16 halocarbon species in 22 algal samples after 10 h incubations. Two out of the 24 samples had to be excluded from the analysis because they included extreme high concentrations for some halocarbons (possibly contaminations), which disrupted the pattern completely. Pearson's product moment correlation coefficient (r_P) was calculated with the MINITAB™ statistical package. Throughout this paper, significance was accepted at $P < 0.05$.

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