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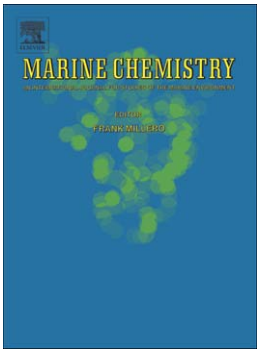

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Light and brominating activity in two species of marine diatom

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1. Abstract

Marine organisms mediate the formation of volatile inorganic (e.g. HOBr) and organic halogens (e.g. $CHBr_3$) and contribute to the sea-to-air emission of bromine and iodine. This air-sea halogen exchange has implications for atmospheric chemistry. It is important to establish the physiological function of halogen metabolism in key groups of marine organisms to permit predictive model development. In this study a series of laboratory experiments was performed to investigate the link between the availability of photosynthetically active radiation (PAR) and brominating activity, as measured by the bromination of phenol red, in two cold-water marine diatoms (*Thalassiosira antarctica*, CCAP 1085/25; *Porosira glacialis*, CCMP 668). Brominating activity in *T. antarctica* was found to change in response to short term changes in photon flux density and to have a strong positive linear relationship with gross photosynthetic rate up to 260 μmol O_2 (mg chla)$^{-1}$ hr^{-1}. Experiments performed across multiple diel cycles showed that light-phase brominating activities in *T. antarctica* were a factor of 2.8 (±1.0) higher than those measured in the dark. Whilst *P. glacialis* showed no response to short term changes in PFD, measurements across a number of diel cycles revealed that light-phase brominating activities in this diatom were significantly higher than those in the dark by a factor of 1.3 (±0.3). The addition of 0.1 μM H_2O_2 to the medium of *T. antarctica* cultures led to a significant increase in brominating activity by a factor of 2.4 (±0.3) relative to no-addition controls but no such response was seen in *P. glacialis*. These results suggest that there is a link between PAR light availability and brominating activity in marine diatoms but that the nature of this relationship differs between species. By establishing a potential link with common ecosystem model state variables (light and photosynthesis) this work provides the first step towards developing a predictive capability for brominating activity in the marine environment. More work is needed to assess the potential for developing generalised parameterisations between PAR light availability and brominating activity in diatom species representative of a wider range of ocean regions.

2. Introduction

A wide range of marine organisms are known to be involved in the production of halogenated organic (e.g. $CHBr_3$, CH_2I_2) and inorganic species (I_2, HOI) including bacteria (e.g. Fuse *et al*., 2003), cyanobacteria (e.g. Smythe-Wright *et al*., 2006; Hughes *et al*., 2011), microalgae (e.g. Moore *et al*., 1996; Hill and Manley, 2009; Hughes *et al*., 2013), seaweeds (e.g. Goodwin *et al*., 1997; Manley and Barbero, 2001) and invertebrates (Fielman *et al*., 1999). Despite this, many questions remain regarding the physiological and ecological functions of these halometabolites (Manley *et al*., 2002; Johnson *et al*., 2011). There is also still considerable uncertainty regarding the biogeochemical importance of biogenic volatile halogens; most specifically those that have a relatively low molecular weight (e.g. $CHBr_3$, I_2) and contribute to the transfer of halogens from the ocean reservoir to the atmosphere (McFiggans *et al*., 2004; Hughes *et al*., 2012; Ziska *et al*., 2013). This is of interest as once in the atmosphere the halogens are involved in ozone cycling (Platt and Honninger, 2003), new particle formation and can control the formation of cloud condensation nuclei (CCN) by precursors such as dimethyl sulphide (DMS, von Glasow *et al*., 2004). Whilst their biological and biogeochemical roles are often considered separately, predicting spatial and temporal variability in sea-air volatile halogen fluxes and hence the importance of this for atmospheric processes now and into the future requires an understanding of how environmental conditions control halogen metabolism in key groups of organisms.

Diatoms are an important microalgal group which contribute about one fifth of global photosynthesis (Ambrust, 2009) and are known to be involved in the production of volatile organic and inorganic halogenated species (e.g. Hill and Manley, 2009; Hughes *et al*., 2013). Given their widespread distribution and large contribution to marine primary productivity it is important from both an ecological and biogeochemical perspective to establish the physiological and ecological functions of halogen metabolism in diatoms. Some work has been done to understand how environmental factors control the rate of halocarbon formation by marine diatoms. Halocarbon production in diatom cultures has been found to be higher during the logarithmic growth phase (Moore *et al*., 1996; Hughes *et al*., 2013), at higher growth irradiance (Moore *et al*., 1996) and following antibiotic treatment to reduce bacterial activity (Hughes *et al*., 2013). Hughes *et al*. (2006) also show that halocarbon production is not induced when diatoms are exposed to levels of light which cause high levels of oxidative stress leading to strong and irreparable photo-inhibition (i.e. F_v/F_m <0.2). These studies provide important process information but they do not necessarily allow us to establish the reasons why diatoms put metabolic investment into driving reactive halogen formation. This is not least because halocarbon formation in seawater is believed to be controlled by both the production of reactive halogens and the availability of organic precursors (Lin and Manley, 2012) which may have external sources. Thus resolving the physiological and ecological functions of halogenating activity requires studies which probe the biochemical processes that drive this halogen metabolism.

It is well-established that some marine diatom species (and other marine organisms) produce a group of enzymes known as the haloperoxidases (chloro-, bromo-, iodoperoxidases) that are involved in the formation of hypohalous acids (HOI, HOBr) by the two electron oxidation of halide ions through the breakdown of hydrogen peroxide (Butler and Walker, 1993; Wever and van der Horst, 2013). Haloperoxidases are either vanadium or haem dependent and their nomenclature refers to the least electronegative halide they are able to oxidise. For example, bromoperoxidases oxidise I^- and Br^- and iodoperoxidases oxidise I^- only. The hypohalous acids produced by haloperoxidase activity are known to have a number of possible fates including reactions with organic compounds to form di- and tri-halomethanes (e.g. $CHBr_3$, CH_2I_2, Moore *et al*., 1996), the formation of BrCN (Vanelslander *et al*., 2012), self reactions to form other inorganic halogen species (e.g. Br_2, I_2) and possibly direct sea-to-air emission (Hill and Manley, 2009). It is worth noting here that methyl halides such as CH_3Br and CH_3I that also drive significant air-sea halogen exchange are not thought to be produced via haloperoxidase-mediated pathways (Wuosmaa and Hager, 1990). Whilst the mode of action of the haloperoxidases is well-established, their physiological and ecological functions are yet to be resolved. It has been suggested that the haloperoxidases are involved in oxidative stress defence in marine algae (Moore *et al*., 1996) and that their products act as deterrents against grazers, competing organisms, pathogens or epiphytes (e.g. BrCN, Vanelslander *et al*., 2012) but it is yet to be confirmed which, if any, of these functions explains the metabolic investment into the formation of these enzymes.

Here we report results from a series of experiments designed to establish if brominating activity in marine diatoms is controlled by the availability of photosynthetically active radiation (PAR). Brominating activity was measured by the bromination of an organic substrate using a standard phenol red incubation assay designed to determine haloperoxidase activity (Hill and Manley, 2009). Individual experiments involved determining how brominating activity changes in two polar marine diatoms in response to short-term and diel transitions in photon flux density. The two diatom strains included in this study (*Porosira glacialis*, CCMP 668; *Thalassiosira antarctica*, CCAP 1085/25) have both been found to produce some organobromines (e.g. $CHBr_3$, CH_2Br_2, Moore *et al*., 1996; Hughes *et al*., 2013) in previous laboratory studies and one (*P. glacialis*) has tested positive for haloperoxidase activity (Moore *et al*., 1996).

3. Methods

a. Cultures

Cultures of *Porosira glacialis* (CCMP 668, axenic) and *Thalassiosira antarctica* (CCAP 1085/25, non-axenic) were grown in batch cultures using autoclaved ESAW artificial seawater (Berges *et al*., 2001) enriched with f/2-levels of nitrate, phosphate, silicate and vitamins (Guillard and Ryther, 1962), and L1 trace metals (Guillard and Hargreaves, 1993). The choice of trace metal supplement was made as the L1 formulation contains vanadium, which is important for some haloperoxidases (Butler and Walker, 1993), but the f/2 trace metal mix does not. Cultures were grown at low light intensities at around 10 µmol photons m^{-2} s^{-1} under a 12:12 light:dark cycle at 4°C in an incubation cabinet. The low growth irradiances used in this study are consistent with those found in sea-ice habitats in the polar spring/summer (Smith *et al*., 1989). Diatom biomass was measured as *in vivo* fluorescence using a Turner Trilogy fluorometer and extracted chlorophyll *a* using the acidification method of Holm-Hansen *et al*., (1965). Cultures were handled in a biosafety cabinet at all times to prevent contamination. Bacterial activity and cell density were not measured in this study but a previous study (Hill and Manley, 2009) has concluded that the bacterial contribution to brominating activity in cultures is very low compared to that from the diatoms.

b. Brominating activity assays

Following the methods of Hill and Manley (2009), we used an *in situ* phenol red incubation assay to determine rates of brominating (haloperoxidase) activity in the diatom cultures. The assay is based on the bromination of phenol red to bromophenol blue by hypobromous acid (HOBr) produced by the action of haloperoxidase enzymes. For the assay, diatom cells were harvested by pipetting from the cultures. Using this method it was possible to collect dense biomass (1-4 mg chla L^{-1}) as *T. antarctica* cells collected at the bottom of the culture flask and *P. glacialis* formed mucilaginous accumulations during all stages of growth. The harvested cells (alongside a small volume of medium) were added to 0.1 M phosphate buffer (KH_2PO_4, K_2HPO_4) containing 827 µM Br^- (as KBr) and 35 g L^{-1} NaCl (all Fisher, reagent grade). Tests involving the additions of 0.45 µm (Millex) filtered culture to the assay solution revealed very little activity confirming suggestions from previous studies (Hill and Manley, 2009) that brominating activity is cell-associated. Following addition of the culture to the buffer solution, the assay was initiated by the addition of 25 µM phenol red (Fisher) and 0.4 mM H_2O_2 (Fisher, analytical reagent grade). The assay mixture was then incubated in the dark at room temperature (22°C). Absorbances at 433nm (phenol red) and 592 nm (BPB) were monitored in 0.45µm (Millex) hand filtered aliquots of the assay mixture at regular intervals for up to 30 minutes using a Perkin Elmer UV/VIS Lambda 25 spectrophotometer. Regular checks on the pH of the assay solution were also performed at a range of cell densities and treatments. The pH of the assay solution was found to be consistent throughout all of our experiments with an average (±standard deviation) of 6.38±0.02 (n=33). Absorbances at 750 nm were also measured in all samples to allow us to correct for any scattering and absorbance by any particulates remaining after filtration. Brominating activity is reported here as the chlorophyll *a*-normalised increase in BPB over time (µmol BPB [mg chla]$^{-1}$ hr^{-1}) which is calculated from the initial linear rate of change in absorbance at 592nm and the molar absorption coefficient for BPB (67.4 [mM]$^{-1}$ cm^{-1}; Hill and Manley, 2009).

As colorimetric assays such as the phenol red technique used here can be subject to interference, the technique was scrutinised using a number of approaches. Firstly, a range of tests were performed to ensure that the observed change in absorbance over time was due to haloperoxidase activity and was not due to dissolved organic matter release or other processes which could cause spectral interference. These include the incorporation of controls with no H_2O_2 added, no phenol red added and no cells added. In all controls the change in absorbance at the relevant wavelengths (433 and 592 nm) was negligible compared to that in the full assay (see Figure 1). These findings confirm that there was no spectral interference from microalgal exudates and confirm that the observed phenol red

bromination was due to haloperoxidase activity. The sensitivity of the method was also checked using solutions with BPB to PR ratios that could be obtained during an assay (i.e. PR decreasing from 25µM and BPB proportionally increasing to 25µM). The PR solutions used in this test were made up as described above and BPB was produced from PR by the action of commercially available bromoperoxidase (*Corallina officinalis*, Sigma). Using this approach it was possible to detect BPB absorbance at <0.05 µM (against a background of 24.95 µM PR) which is well below the levels of BPB encountered in this study (data not shown).

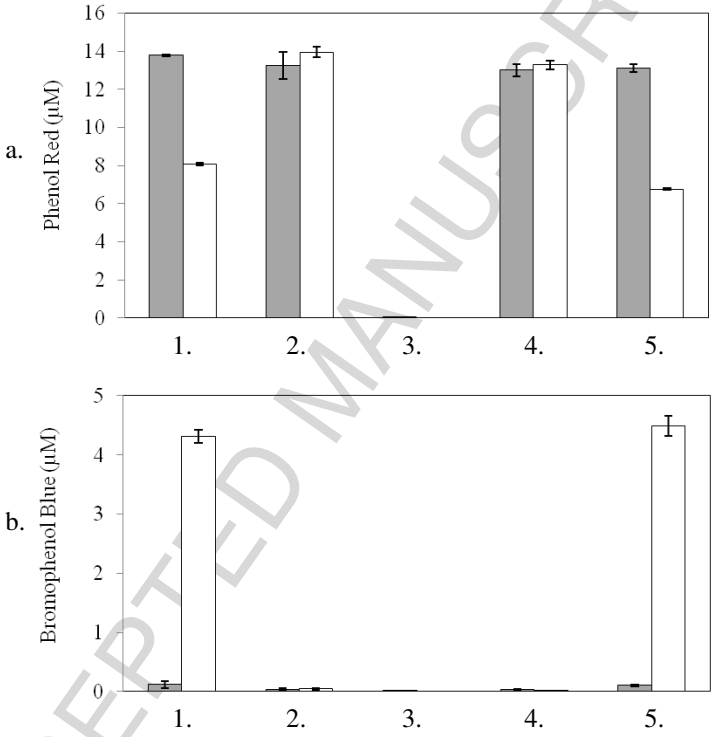

Figure 1. Phenol red in situ activity assay tests. Concentrations of a) phenol red and b) bromophenol blue at T=0 (grey bars) and T=60 minutes (white bars) in a series of assays containing 0.1M phosphate buffer with 35g L^{-1} NaCl, 827 µM Br^- and 1. *Thalassiosira antarctica* (CCAP 1085/25) cells, 0.4 mM H_2O_2 and 14 µM phenol red, 2. *T. antarctica* cells and 14 µM phenol red, 3. *T. antarctica* cells, 4. 14 µM phenol red and 5. purified bromoperoxidase (*Corallina officinalis*, Sigma), 0.4 mM H_2O_2 and 14 µM phenol red. Error bars show the standard deviations of three replicate treatments.

c. Experimental

All of the experiments performed within this study involved exposing the diatoms to a range of treatments, allowing appropriate periods of time for the cells to respond to the applied treatment and subsequently measuring brominating activity using the techniques described in *Section 3b*.

i. Photon flux density (PFD) and brominating activity

A set of experiments was performed to test the hypothesis that brominating activity increases with PFD in *T. antarctica* and *P. glacialis*. At least duplicate (logarithmic-phase) cultures of diatoms were exposed to a range of PFD levels ranging from 0-500 µmol photons m^{-2} s^{-1} using a Hansatech LED light source. The specific light source used (LED1/R) is one that is commonly employed for studying photosynthesis and has a peak wavelength centred on 627nm. The degree of replication in these experiments was limited by the time needed to cover the PFD range. Cultures were gently mixed during light exposure using a small magnetic stirrer to ensure that light levels were consistent throughout the cultures. Brominating activity was measured (as described in *Section 3b*) in all

cultures after 30 minutes exposure at each PFD. This timescale was appropriate as it is known that physiological changes at the cellular level occur in diatoms immediately after the transition to a different PFD (Post *et al*., 1985).

If a change in brominating activity with PFD was observed, the link between brominating activity and photosynthesis was investigated. Photosynthetic rates were determined at each PFD used in the brominating activity experiment described above by measuring oxygen evolution with a Hansatech Chlorolab II instrument fitted with a Clarke-type oxygen electrode. Gross photosynthetic rates (P_g) were calculated by correcting the oxygen evolution rates measured in the light for oxygen consumption by respiration and the oxygen electrode. The relationship between photosynthetic rate, and brominating activity was assessed using least squares regression analysis.

ii. Diel variability

Two different experiments were performed to test the hypothesis that brominating activity is higher in *T. antarctica* and *P. glacialis* in the light phase of a diel cycle. The first involved measuring brominating activity (as in *Section 3b*) at various times just before and after the start of the light period during one diel cycle in at least duplicate cultures of both diatoms. The degree of replication in these experiments was dictated by the biomass levels available. Independent T-tests were used to determine if there were significant differences in the brominating activities measured in each culture in the light and dark phases in this experiment. The second set of experiments involved measuring brominating activity 1 hour before and 3 hours after the start of the light period for 4 days in triplicate *T. antarctica* and *P. glacialis* cultures, respectively. Paired T-tests were used to determine if there was a significant difference between dark and light brominating activity rates in individual cultures.

iii. H_2O_2 additions

Experiments were performed to investigate if the physiological responses to increased extracellular H_2O_2 concentrations in *T. antarctica* and *P. glacialis* include a change in brominating activity. Depending on their composition, some cell membranes are permeable to H_2O_2 (Bienert *et al*., 2006) so additions to the external medium could also lead to increased levels of intracellular reactive oxygen species. H_2O_2 (0.35%, Fisher reagent grade) was added to triplicate cultures of each diatom in microliter volumes to achieve final concentrations of 0, 0.1, 0.2, 0.5 and 1.0 µM. These H_2O_2 concentrations are orders of magnitude lower than those used in the *in situ* incubation assay and so did not interfere with the brominating activity measurements. It is worth noting here that this experiment differs to that performed by Hill and Manley (2009) in which H_2O_2-dependent changes in brominating activity were investigated. Whereas Hill and Manley (2009) investigate the optimum H_2O_2 concentration for the assay, the present study investigates how H_2O_2-induced changes in cell physiology impacts brominating activity. In order to achieve this, the cells were incubated at the treatment H_2O_2 concentrations for a set period of time before any of the phenol red assay reagents (including H_2O_2) were added and the standard assay (detailed in *Section 3b*) was performed. The treatment H_2O_2 concentrations (0-1.0 µM) used in the present study were chosen as they are lower than the H_2O_2 level (2 µM) previously found to be lethal to diatoms but are known to induce a physiological response (Hunken *et al*., 2009). Following the H_2O_2 additions, all cultures were incubated at 4°C in the dark for 2 hours and the *in situ* brominating activity assay (as in *Section 3b*) performed at the end of this incubation period. This timescale is appropriate as, following exposure to 0.5 and 1.0 µM H_2O_2, Hunken *et al.* (2009) observed an immediate reduction in photosynthetic efficiency in the marine diatom *Amphiprora kufferathii*. Differences in brominating activity at the different H_2O_2 concentrations were assessed using a one-way ANOVA with a Tukey HSD post-hoc test.

4. Results

a. *Brominating activity*

Figure 2 shows that logarithmic phase cultures of both *P. glacialis* and *T. antarctica* were found to drive the disappearance of phenol red (PR) and mediate the formation of bromophenol blue (BPB) in the *in situ* incubation assays performed within this study. It is not appropriate to quantitatively compare the brominating activity rates observed here to previous studies (Hill and Manley, 2009) due to some differences in assay approaches and culture conditions. The most significant differences are that: i) Hill and Manley (2009) use a centrifugation step to concentrate the cells but, due to the aggregation in the diatoms cultures used in the present study, this was not required here; and, b) the use of L1 trace metals in the growth medium in the present study (*Section 3a*) compared to f/2 trace metals in Hill and Manley (2009). We can however compare results obtained within controlled experiments performed as part of this study to assess how changes in PAR light conditions alter brominating activity (see *Sections 4b-d*).

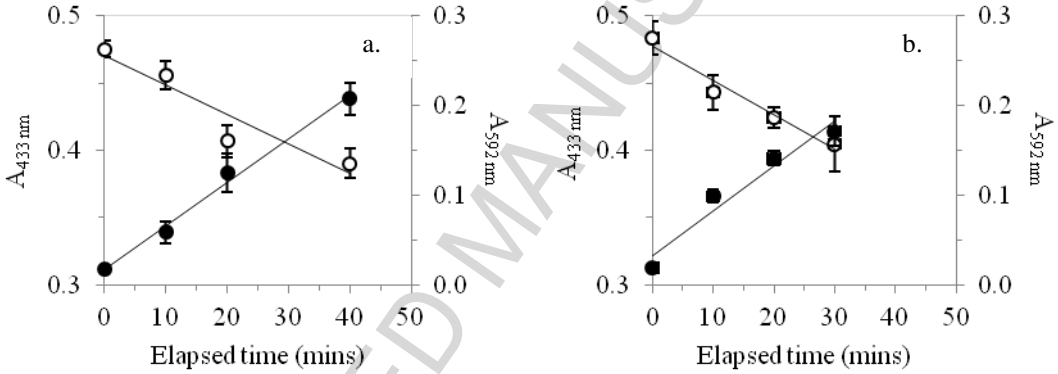

Figure 2. Change in absorbances at 433nm (A_{433nm}, open circles) and 592nm (A_{592nm}, closed circles) in phenol red *in situ* incubation assays containing a) *Thalassiosira antarctica* (CCAP 1085/25) and b) *Porosira glacialis* (CCMP 668) cells. A_{433nm} is the peak absorbance for phenol red and A_{592nm} is that for its brominated product bromophenol blue. Trend lines are least squares regression relationships with R^2 values consistently ≥0.89 and p-values <0.05. Errors show the standard deviation of three replicate cultures. As in Figure1 no significant change in A_{433nm} and A_{592nm} was observed in controls where no H_2O_2 and/or phenol red was added and those where no cells were added to the assay mixture.

b. Link between brominating activity and photon flux density (PFD)

Figure 3a shows how brominating activity changed in cultures of *Thalassiosira antarctica* in response to varying PFD in two experiments (TA1 and TA2). The results from the TA1 experiment reveal an increase in brominating activity with PFD from 3.8±0.1 µmol BPB (mg chla)$^{-1}$ hr^{-1} in the dark to 8.0±0.3 µmol BPB (mg chla)$^{-1}$ hr^{-1} at 50 µmol photons m^{-2} s^{-1} and 4.5±0.2 µmol BPB (mg chla)$^{-1}$ hr^{-1} at 200 µmol photons m^{-2} s^{-1} . Despite achieving higher chlorophyll-normalised brominating activities for a given PFD, this same pattern of an initial increase in brominating activity at lower PFD followed by a reduced response at higher PFD was repeated in a second experiment performed on *T. antarctica* cultures (TA2, Figure 3a). The discrepancy in the levels of brominating activity observed at the different PFD in TA1 and TA2 (Figure 3a) can be explained when the results are compared against the photosynthetic rates measured in the two experiments. Figure 4 presents the net oxygen evolution rates measured at a range of PFD in TA1 and TA2. The hyperbolic tangent curves (Falkowski and Raven, 2007) fitted to the data (R^2>0.74) reveal a clear difference in the maximum photosynthetic rate P_{max} for TA1 (129 µmol O_2 [mg chla]$^{-1}$ hr^{-1}) and TA2 cultures (210 µmol O_2 [mg chla]$^{-1}$ hr^{-1}; Figure 4). The curves shown in Figure 4 have photosynthetic efficiencies (α) of 0.03 to 0.06 mg C (mg chla)$^{-1}$ hr^{-1} (µmol photons m^{-2} s^{-1})$^{-1}$ (assuming a photosynthetic quotient of 1.3, Falkowski and Raven, 2007) in TA1 and TA2, respectively. These values are within the range

of those (0.023±0.009 to 0.039±0.041 mg C [mg chla]$^{-1}$ hr^{-1} [µmol photons m^{-2} s^{-1}]$^{-1}$) reported previously for shade-adapted Arctic and Antarctic ice algae (Cota, 1985).

It is clear that TA1 and TA2 cultures had different photosynthetic characteristics. Previous research has shown that this can occur in phytoplankton grown at different irradiances (e.g. Stuart *et al*., 2000), so this discrepancy is most likely due to variability in the growth irradiances in TA1 and TA2 cultures, possibly resulting from differing placement in the incubator. When the results from TA1 and TA2 are combined a significant linear relationship between brominating activity and gross (i.e. dark-corrected) photosynthetic rate (P_g) up to around 260 µmol O_2 (mg chla)$^{-1}$ hr^{-1} is apparent (Figure 3b; R^2=0.67, p<0.05, n =11; µmol BPB [mg chla]$^{-1}$ hr^{-1} = [0.056 x P_g] + 1.858, where P_g is in units of µmol O_2 [mg chla]$^{-1}$ hr^{-1}).

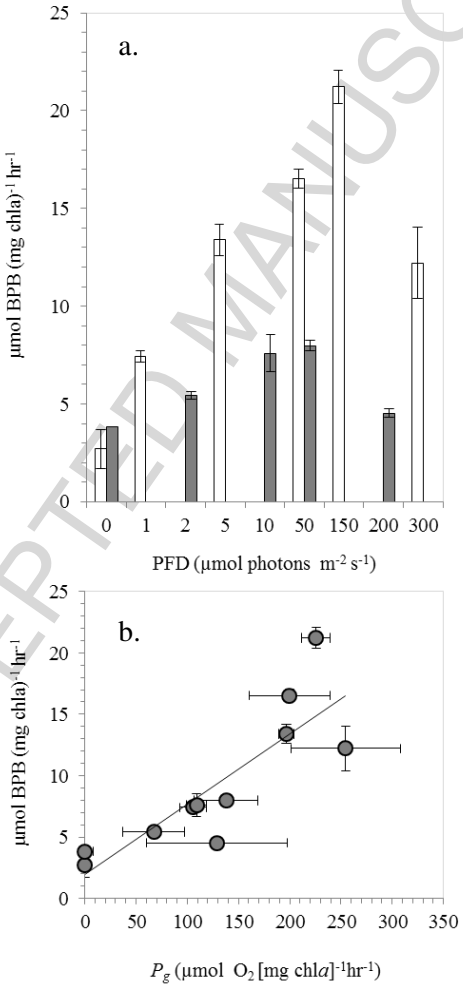

Figure 3. Change in brominating activity with a) photon flux density (PFD) in two independent experiments (TA1, grey bars and TA2, white bars) and b) gross photosynthetic rate in cultures of *Thalassiosira antarctica* (CCAP 1085/25). The data shown in b) include results from TA1 and TA2. The solid line in b) is a least squares regression line (R^2 = 0.67, p <0.05, n = 11; µmol BPB[mg chla]$^{-1}$ hr^{-1} = [0.056 x P_g] + 1.858, where P_g is in units of µmol O_2 [mg chla]$^{-1}$ hr^{-1}). Vertical error bars show the range of values from replicate cultures and the horizontal errors show the standard deviation of the O_2 evolution rates measured by the Hansatech Chlorolab II instrument during the measurement period (n=500).

Figure 5 shows there was no or very little change in brominating activity in cultures of *P. glacialis* in response to transitions in PFD ranging from 0 to 500 µmol photons m^{-2} s^{-1} in 3 individual experiments. A key observation here is that there is little or no difference in brominating activity in cultures incubated in the dark compared to those that are light-exposed. Whilst, as per our experimental plan (*Section 3c*) photosynthetic rates were not measured in this experiment, the

absence of a difference between light and dark activities confirms that the link between photosynthesis and bromination in *P. glacialis* is not as clear as it is for *T. antarctica*. Also, our previous work has revealed that photosynthetic rates in *P. glacialis* vary significantly within the light range studied (data not shown). It is, however, worth noting that higher brominating activities were observed at 25 and 50 µmol photons m^{-2} s^{-1} compared to 0 and 5 µmol photons m^{-2} s^{-1} in one experiment (PG2). In this case the ratio of light (25-50 µmol photons m^{-2} s^{-1}) to dark brominating activity was 1.4-1.5.

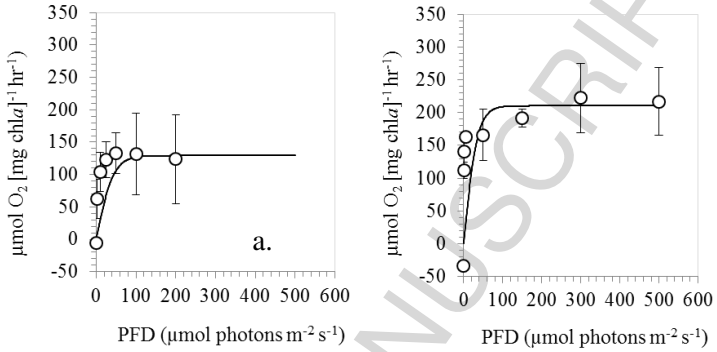

Figure 4. Net oxygen evolution rates observed at a range of photon flux densities (PFD) in two experiments (a. TA1 and b. TA2) performed on *Thalassiosira antarctica* (CCAP 185/25). The solid lines are hyperbolic tangent curves (Falkowski and Raven, 2007; R^2>0.74). Error bars show the standard deviation of the O_2 evolution rates measured by the Hansatech Chlorolab II instrument during the measurement period (n=500).

c. Diel variability in brominating activity

A set of experiments was performed to investigate if brominating activity in *T. antarctica* and *P. glacialis* changes on a diel cycle. A highly significant difference in brominating activity was observed between light and dark phases in *T. antarctica* (T-test, P=0.001, df = 19, t = 3.9; Figure 6a). Mean (± standard deviation) light and dark bromination rates in this culture were 8.0±2.8 and 3.4±0.7 µmol BPB (mg chla)$^{-1}$ hr^{-1}, respectively. In contrast, the difference in brominating activity in the dark and light phases in *P. glacialis* is only just significant (T-test, P=0.046, df=12, t=2.2; Figure 6b). *P. glacialis* bromination rates are 4.3±0.5 in the dark phase and 5.4±1.5 µmol BPB (mg chla)$^{-1}$ hr^{-1} in the light. There is clearly overlap in the bromination rates measured at the end of the dark phase and start of the light phase in *P. glacialis* (Figure 6b). However, rates measured later in the light phase (T= 12:00, 14:00, 16:00) are significantly higher (6.5 ± 0.7 µmol BPB [mg chla]$^{-1}$ hr^{-1}) than the maximum dark phase rate (4.7 ± 0.2 µmol BPB [mg chla]$^{-1}$ hr^{-1}). These results suggest that there is a difference in brominating activity in *P. glacialis* between light and dark phases but this is less pronounced than in *T. antarctica.* On average brominating activities approximately 3 hours after the beginning of the light phase were found to be a factor of 3.6 and 1.3 higher than those measured around one hour before the lights came on in the incubator in *T. antarctica* and *P. glacialis*, respectively.

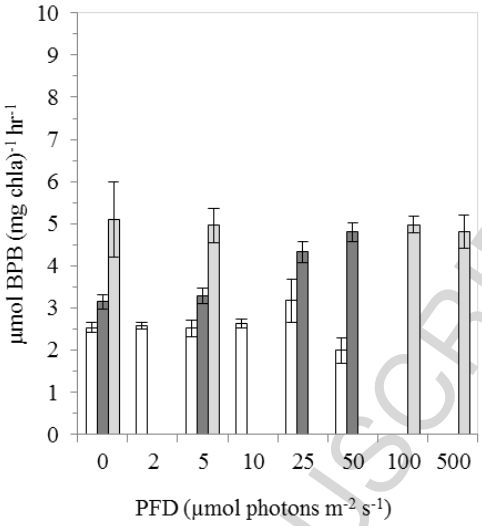

Figure 5. Change in brominating activity in cultures of *Porosira glacialis* (CCMP 668) with photon flux density (PFD) in three independent experiments (PG1, white bars; PG2, dark grey bars; PG3, light grey bars). Error bars show the range of values obtained from duplicate cultures in PG1 and PG3, and triplicate cultures in PG2.

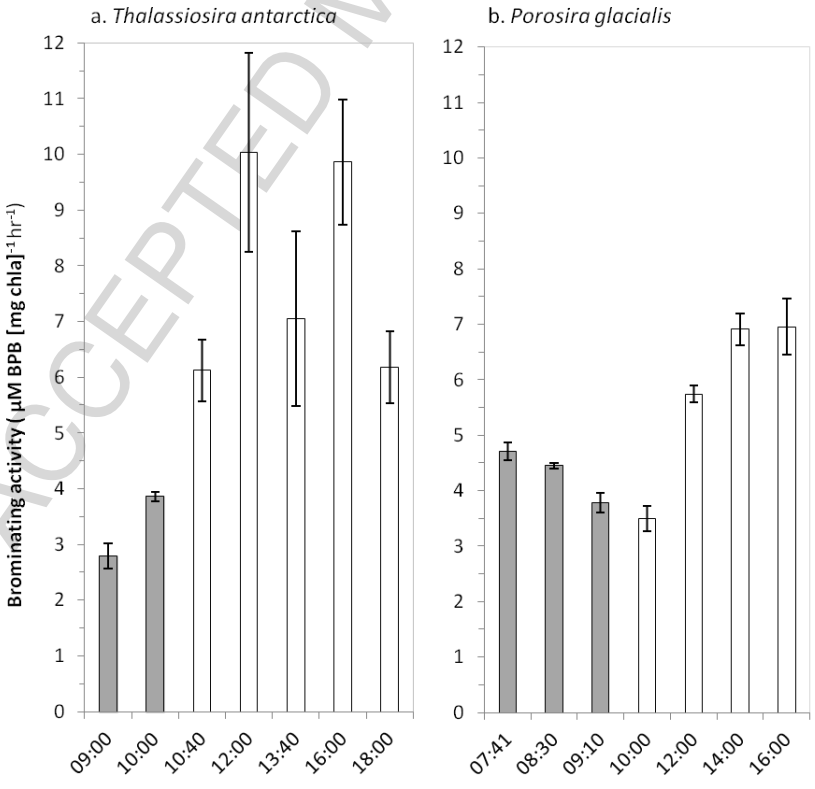

Figure 6. Brominating activity in cultures of a) *Thalassiosira antarctica* (CCAP 1035) and b) *Porosira glacialis* (CCMP 668) before (grey bars) and after (white bars) the transition between dark and light during one light:dark cycle. Error bars show the range of values obtained in triplicate and duplicate cultures of *T. antarctica* and *P. glacialis*, respectively.

Figure 7a confirms that differences in dark and light phase bromination rates occur consistently over multiple light:dark cycles in cultures of *T. antarctica*. Results from a paired T-test confirm that the brominating activities observed in light and dark phases were significantly different in individual cultures of *T. antarctica* (P=0.0001, df=11, t=5.7). Slightly higher light-phase brominating activities

were observed in 3 of the 4 light:dark cycles in *P. glacialis.* A paired T-test confirms that this difference was significant (Figure 7b; P=0.014, df=11, t=2.9) but Figure 7 shows the difference was clearly greater and more consistent in *T. antarctica* than in *P. glacialis*. On average, light phase bromination rates were found to be a factor of 2.8 (±1.0) higher than those measured in the dark phase in *T. antarctica* compared to 1.3 (±0.3) for *P. glacialis*. These ratios are consistent with those observed in the more detailed diel cycle experiments shown in Figure 6.

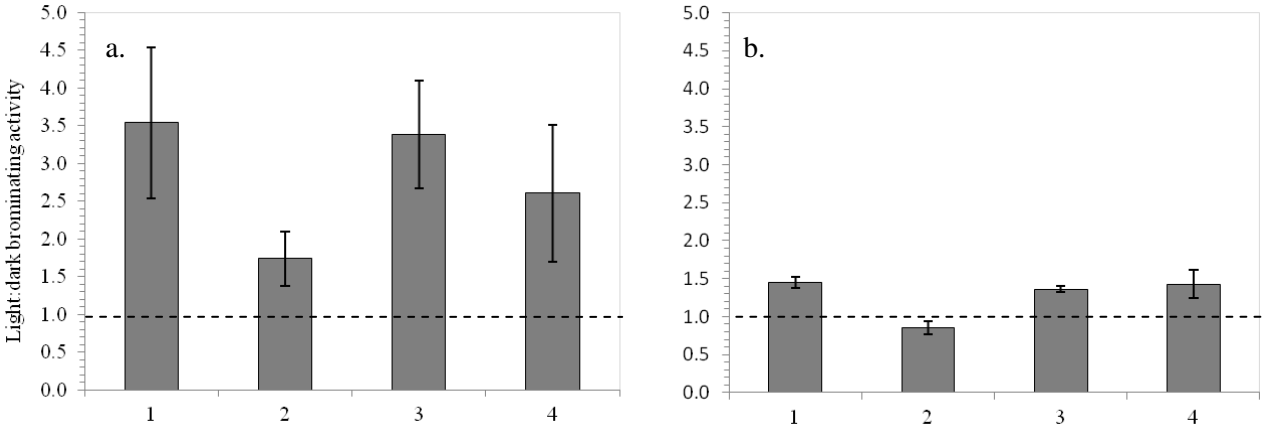

Figure 7. The ratio of light: dark brominating activity in cultures of a) *Thalassiosira antarctica* (CCAP 1085/25) and b) *Porosira glacialis* (CCMP 668) across 4 diel cycles. In (i) Error bars show standard deviations for triplicate cultures. The dashed lines highlight the 1:1 light/dark brominating activity ratio.

d. Brominating activity and extracellular H_2O_2

To more directly explore the theory that brominating activity is linked to oxidative stress mitigation a series of experiments were performed in which extracellular H_2O_2 concentrations were adjusted in cultures of *T. antarctica* and *P. glacialis.* It is apparent from Figure 8 that brominating activities in some of the H_2O_2 treatments are clearly different to the no-addition controls in *T. antarctica.* The differences are less clear for *P. glacialis*. The differing response in the two cultures is consistent with results from the light experiments described in *Section 4b* (Figures 3 and 5).

In *T. antarctica,* lower addition treatments (< 0.5 µM H_2O_2) induced an increase in brominating activities relative to the controls but higher H_2O_2 concentrations (≥ 0.5 µM) did not have any significant impact. Analysis of data collected in the *T. antarctica* experiment (Figure 8a) reveals that brominating activities at 0.1 µM H_2O_2 are significantly higher than those measured in the no-addition control and treatments at H_2O_2 concentrations ≥ 0.5 µM (One-way ANOVA, P<0.05, df = 11; Tukey HSD P<0.05). This analysis does not include the 0.2 µM H_2O_2 treatment as it is associated with large variability. Data from this treatment are still shown in Figure 8 as the large variability in brominating activities suggests that 0.2 µM H_2O_2 may be close to a physiological 'tipping point' for *T. antarctica*.

5. Discussion

This study involved a series of experiments designed to investigate the relationship between the availability of photosynthetically active radiation (PAR) and brominating activity in two cold-water marine diatoms, *Thalassiosira antarctica* (CCAP 1085/25) and *Porosira glacialis* (CCMP 668). Cells of both diatoms were found to mediate the conversion of phenol red to bromphenol blue (BPB) in the presence of H_2O_2 suggesting for the first time that *T. antarctica* produces haloperoxidases and confirming previous findings for *P. glacialis* (Moore *et al*., 1996; Hill and Manley, 2009). Brominating activity in *T. antarctica* was found to change in response to both variability in PFD and the transition between light and dark phases. Brominating activity in *P. glacialis* was only found to

be significantly different between light and dark phases of the diel light cycles studied. These findings have implications for both the physiological role of brominating activity in marine diatoms and the potential for parameterising the production of the biogenic organobromines known to be produced via haloperoxidase activity (i.e. $CHBr_3$, CH_2Br_2; and their sea-air emission rates) against ecosystem model state variables. This study does not deal with the marine CH_3Br source as this compound is thought to be produced by methyl transferase and not haloperoxidase activity (Wuosmaa and Hager, 1990). Given their differing responses to the applied treatments the results for *T. antarctica* and *P. glacialis* are discussed separately at first and these findings are then brought together in *Section 6* (*Conclusions*).

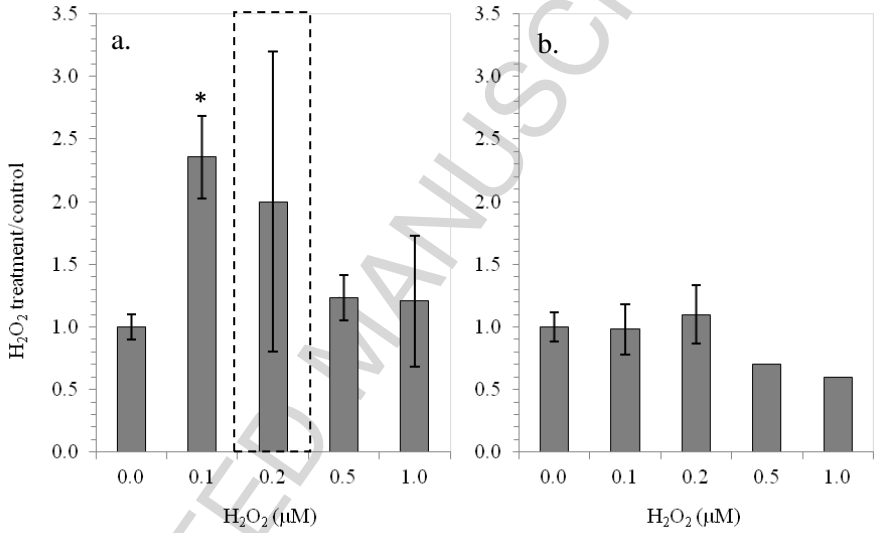

Figure 8. The ratio of brominating activity in H_2O_2 treatments and no-addition controls in cultures of a) *Thalassiosira antarctica* (CCAP 1085/25) and b) *Porosira glacialis* (CCMP 668). Error bars show the standard deviation from three replicate cultures. Where errors are not shown only single cultures were used in these treatments. The asterix indicates treatments that had significantly different brominating activities to the no addition controls and other treatments (ANOVA, Tukey HSD post hoc $p<0.05$).The dashed box in a) highlights a treatment with large variability which was not included in the statistical analysis.

5.1 Thalassiosira antarctica

A clear link between PAR light availability and brominating activity was found for *T. antarctica* in all experiments performed within this study. The responses observed to short term variability in PFD, significant positive relationship with photosynthetic rate and significant difference between light and dark phases suggest that brominating/haloperoxidase activity in *T. antarctica* is associated with a cellular protection mechanism aimed at moderating light-induced stress. The photosynthetic electron transport chain is a major source of the reactive oxygen species superoxide ($O_2^{\bullet-}$) and singlet oxygen ($^1O_2$) which are subsequently reduced to H_2O_2 and $OH^{\bullet}$. There is a clear link between photon flux density and photosynthetic rate, and algal H_2O_2 production (e.g. Milne *et al*., 2009). Hence, given their mode of action (Butler and Walker, 1993), our results can be explained by changes in the production of reactive HOBr as a by-product of the haloperoxidase-mediated breakdown of photosynthetic H_2O_2. As very little brominating activity was observed in the medium surrounding the cells (*Section 3b*) the haloperoxidases are likely to be cell-bound and most likely occur in the periplast (Hill and Manley, 2009). The significant increase in brominating activity observed in this study following the addition of 0.1 µM H_2O_2 to the medium of *T. antarctica* cultures supports the link with light stress, as do results from previous studies. For example, diel changes in the expression of peroxidase genes associated with the mitigation of light-induced oxidative stress have been shown in the marine diatom *Thalassiosira pseudonana* (Ashworth *et al*., 2013). Additionally, a

link between incident light levels and brominating activity has been previously proposed for diatoms (Hill and Manley, 2009) and demonstrated in macroalgae (Manley and Barbero, 2001). In particular, exposure to photosynthesis inhibitors (e.g. DCMU) has been found to reduce the rate of $CHBr_3$ formation in the green seaweed *Ulva lactuca* (Manley and Barbero, 2001) and the kelp *Macrocystis pyrifera* (Goodwin *et al*., 1997). Overall, there appears to be a clear link between the rates of brominating activity and primary metabolic processes in marine algae. Here we provide further evidence for the link between brominating activity and photosynthesis in diatoms, but other studies (e.g. Manley and Barbero, 2001) have shown that there is also a relationship between rates of respiration and organobromine formation in marine macroalgae. This, alongside persistence of the haloperoxidases in the medium, could explain the dark-phase brominating activity observed in our experiments.

High levels of stress appear to cause a reduction in brominating activity in marine algae. In this study, brominating activities in *T. antarctica* were reduced at higher light intensities (x 10 growth irradiance) compared to those at lower light levels (Figure 3a). These findings are consistent with those of Hughes *et al*. (2006) in which exposure to levels of PAR which led to strong and irreparable photoinhibition did not induce halocarbon formation in a range of microalgal species. Given that higher levels of stress are known to be associated with increased H_2O_2 (and other ROS) production (Milne *et al*., 2009), it might have been expected that brominating activity would be enhanced under high levels of light stress. Increasing levels of H_2O_2 have, however, been found to both activate and inhibit brominating activity depending on the concentration. In this study, brominating activity was not found to be significantly different compared to no-addition controls in *T. antarctica* cultures supplied with ≥ 0.5 µM H_2O_2 but was significantly higher in those supplied with 0.1 µM H_2O_2 (Figure 8a). Manley and Barbero (2001) observed a similar pattern in $CHBr_3$ production in cultures of *Ulva lactuca* as H_2O_2 levels were increased. The large variability in brominating activities in *T. antarctica* observed at 0.2 µM H_2O_2 (Figure 8a) suggest that this may be close to a 'physiological tipping point' for this diatom. Understanding the levels of an applied stressor at which reactive halogen production does not respond or declines will be essential for parameterisation development.

There are several reasons why brominating activity may decline at higher levels of oxidative stress. It is well-established that high levels of H_2O_2 can lead to an apparent decline in haloperoxidase activity due to competing reactions between excess H_2O_2 and HOBr (Wever, 2001). However, the levels of H_2O_2 at which brominating activity showed no significant response compared to no-addition controls (≥ 0.5 µM) in our experiments are orders of magnitude below those at which BPB production has been found to decline under assay conditions (0.3 mM; Hill and Manley, 2009). Given that micromolar levels of H_2O_2 are known to induce physiological responses in the diatoms, it is more likely that our observations are due to a biological response rather than being attributed to these other artefacts. As H_2O_2 is damaging to cellular macromolecules, reduced brominating activity at ≥ 0.5 µM can be explained by membrane peroxidation leading to the loss of membrane-bound bromoperoxidases (Manley and Barbero, 2001) or damage to the cellular macromolecules involved in mediating the production of reactive bromine species (e.g. HOBr). Diatoms are also known to have a range of anti-oxidative enzymes such as catalase, ascorbate peroxidase and glutathione peroxidase and nonenzymatic oxidants such as α-tocopherol and beta-carotene which can also breakdown H_2O_2 (Hernando *et al*., 2011). Hence it is possible that these other antioxidant defence strategies become more important at higher H_2O_2 concentrations. We propose that the physiological responses that occur above a certain H_2O_2 concentrations restrict haloperoxidase antioxidant defence to lower levels of stress.

5.2 Porosira glacialis

Brominating activity in *P. glacialis* was found to be significantly higher during the light phase of the diel cycles studied here but, in contrast to *T. antarctica,* very little or no change was observed following short-term changes in photon flux density (PFD) and increases in extracellular H_2O_2. Moore *et al.* (1996) previously observed an increase in organobromine production (e.g. $CHBr_3$, CH_2Br_2) in *P. glacialis* cells grown for several weeks at a higher irradiance (40 µmol photons m^{-2} s^{-1}) compared to low light (12 µmol photons m^{-2} s^{-1}) treatments. Taken together these results suggest that changes in brominating activity in *P. glacialis* occurs relatively slowly following changes in the environment and/ or may be linked to long-term adaptations to light levels. The absence of a response to short-term variability in PFD and increasing extracellular H_2O_2 in *P. glacialis* may suggests that this species employs a different antioxidant network (Hernando *et al*., 2011) or have other adaptations to mitigate the impact of transient changes in the environment. Previous studies have shown that species-specific responses to increasing extracellular H_2O_2 in marine macroalgae can be explained by differences in the antioxidant networks of organisms adapted to a varying range of environmental conditions (e.g. light intensity, Dummermuth *et al*., 2003) and morphological variability (e.g. thallus thickness, Manley and Barbero, 2001). Any of the other antioxidant strategies that are known to occur in marine diatoms (Hernando *et al*., 2011) could be upregulated in response to the treatments we applied, thereby reducing the need for increased haloperoxidase-mediated H_2O_2 breakdown. It will be important to establish the position that brominating activity holds in the antioxidant networks of *T. antarctica* and *P. glacialis* in future studies, and explore other potential explanations for the observed disparity in response in these two diatom strains.

One observation that may be relevant is that under the culture conditions employed here *P. glacialis* (CCMP 668) secretes large amounts of extracellular mucilage which surrounds the cells and allows colonies to form. Mucilage sheaths are known to restrict the exchange of some chemical species between the microalgal phycosphere and bulk medium (Freire-Nordi *et al*., 2006). Hence, this mucilage could have interfered with the transport of H_2O_2 from the bulk medium to the cell membranes and the movement of reactive bromine (e.g. HOBr) from the cells to the external medium. A reduced rate of transport of H_2O_2 and HOBr between the *P. glacialis* phycosphere and the surrounding medium could provide a logical explanation for some of our observations including: the apparent lack of response to short term changes in light and H_2O_2; the lower light to dark brominating activity ratio in *P. glacialis* (1.3±0.3) compared to *T. antarctica* (2.8±1.0); the delayed increase in brominating activity in *P. glacialis* cultures in the first light-phase sample taken during the detailed diel cycle experiment (Figure 6, *Section 4c*). Uncertainties like this could, in part, be addressed in future studies by the incorporation of proteomic and genomic studies (e.g. Johnson *et al*., 2011) which would allow us to directly establish the upregulation of haloperoxidase activity in marine diatoms rather than relying on the reactions mediated by enzyme products.

6. Summary and conclusions

This study has found evidence for a link between the availability of photosynthetically active radiation (PAR) and brominating activity, as measured by phenol red bromination, in two cold water marine diatoms (*Thalassiosira antarctica* and *Porosira glacialis*). Given the mode of action of the haloperoxidase enzymes we propose that brominating activity is linked to the breakdown of H_2O_2 produced during photosynthesis, and has a diel cycle consistent with the mitigation of light-induced stress. Establishing a potential link between brominating activity in marine diatoms and common ecosystem model state variables such as light availability and photosynthetic rate is a first step towards developing the capability to accurately predict spatial and temporal variability in the sea-air emission of organobromines produced by haloperoxidase activity (e.g. $CHBr_3$). The differing responses reported for the two diatom strains indicates that it will be essential to explore how the link between light and brominating activity differs in a wider range of marine diatoms, and possibly other phytoplankton groups. These studies would benefit from the incorporation of proteomic and genomic approaches. In addition to having biogeochemical importance our studies also suggest that it may be

important to consider the position that brominating activity holds in the antioxidant defence network of marine diatoms in future research. Effort should also be made to understand the physiological function of the biogenic production of a wider range of marine organohalogens such as CH_3Br.

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Research Highlights

1. Brominating activity in marine diatoms was found to change in response to variability in the availability of photosynthetically active radiation.
2. A significant positive relationship was found between brominating activity and photosynthetic rate.
3. Brominating activity may be part of an antioxidant defence strategy in marine diatoms linked to the moderation of photosynthetic H_2O_2.