

## Research article

# Temperature stress-induced bleaching of the coralline alga *Corallina officinalis*: a role for the enzyme bromoperoxidase

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Coralline algae are important components of coral reefs and are involved in reef building via calcification, cementation, the synthesis of anti-fouling compounds and the synthesis of allochemicals to aid recruitment, settling and metamorphosis of reefs species. Using *Corallina officinalis* we have shown that these algae undergo temperature-related bleaching at similar temperatures to those known to cause bleaching in corals. The bleaching appears to be associated with considerable increases in the vanadium-containing enzyme bromoperoxidase (VBPO). This enzyme is involved in hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) elimination and generates the powerful brominating/oxidizing agent hypobromous acid (HOBr, probably present as  $\text{Br}^+$ ). This is used to synthesize volatile halogenated organic compounds (VHOCs) from a pool of organic acceptor molecules. Earlier *in vitro* work has shown this enzyme to be effective in bleaching the phycobilin photosynthetic accessory pigments and to be partly located in chloroplasts. The data presented here supports the suggestion that increases in temperature lead to an increase in the cellular production of  $\text{H}_2\text{O}_2$  and other reactive oxygen species that result in an increase in VBPO, a subsequent increase in HOBr/ $\text{Br}^+$  followed by pigment bleaching when the capacity to produce VHOCs has been exceeded. Addition of the exogenous antioxidant mannitol decreases both pigment bleaching and VBPO induction. A scheme is presented to illustrate proposals for the involvement of VBPO in the bleaching of coralline algae such as *C. officinalis*. The importance of these species in reef building and rebuilding is discussed.

**Key words:** *Corallina officinalis*, coral reef, bromoperoxidase, phycobilins, bleaching, temperature stress.

## Introduction

Coral reefs are under considerable threat globally from anthropogenic and natural stress.<sup>1</sup> A notable symptom of stress is bleaching of the pigments present in the coral polyps and their symbiotic photosynthetic zooxanthellae.<sup>2</sup> The symptoms and causes of coral bleaching have been studied extensively and it is generally accepted that temperature increases of 1–3°C above the mean long-term annual maximum for the geographical area induce oxidative stress and the generation of reactive oxygen species (ROS) such as singlet oxygen, hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and the superoxide radical anion.<sup>3–5</sup> Interaction of ROS with photosynthetic pigments leads to bleaching and Lesser<sup>6</sup> has shown that the addition of exogenous antioxidants such as ascorbic acid and mannitol can lessen or prevent bleaching. In spite of

the considerable volume of work carried out, there has been no definitive identification of the ROS directly responsible for pigment bleaching, although singlet oxygen generated during the photosynthetic light reactions is a prime suspect.<sup>5</sup> The immediate effects of pigment bleaching appear to be reversible given a return to lower ambient sea temperatures, as pigment loss appears to be due to either expulsion of zooxanthellae or a loss of their photosynthetic pigments. Sustained temperature elevation, however, leads to polyp death, precluding any recolonization by zooxanthellae.

Coralline algae, with their calcium carbonate outer coat, constitute major structural components of coral reefs and are important in calcification, reef cementation and anti-fouling processes.<sup>7–9</sup> However, the effects of environmental stress on these reef species have been little studied.

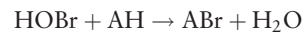
*Corallina officinalis*, of the order Corallinales,<sup>10</sup> is related to many important reef species. It has a crustose, discoid holdfast with erect, calcareous segmented and branched fronds, giving the alga a 'feather-like' appearance. Frond colour is variable, with purple, red, pink and yellow recorded, sometimes with white knuckles and extremities. Colours are often paler in more brightly lit sites<sup>11</sup> and colour alterations are considered to represent light-induced stress and subsequent pigment degradation. *Corallina* growth rate is slow, becoming stunted when cold, and ceasing altogether at elevated temperatures.<sup>12</sup>

*C. officinalis* is recorded widely throughout the northern Atlantic from northern Norway southwards to Morocco in the east and Greenland as far down as Argentina in the west, with scattered reports as far afield as Japan, China and Australasia. The alga is found across a wide range of habitats, ranging from exposed, open areas coastline to calm, sheltered embayments, and has been recorded on the shore from the mid-littoral down to a maximum depth of almost 30 m, across a wide range of natural and artificial hard substrates.<sup>10</sup> The morphology of *Corallina* varies, dependent upon its position on the shore, occurring as a cushion, compact turf or as scattered clumps.<sup>12, 13</sup> *Corallina* is often the dominant organism in the habitat and provides structure in the community, supporting diverse invertebrate communities<sup>10</sup> and acting as substrata for various epiphytes,<sup>13</sup> sometimes resulting in overgrowth with subsequent mortality, particularly during summer months.

*C. officinalis* is one of a number of algal species that possesses phycobilisomes (PBS),<sup>14–18</sup> highly organized aggregations of photosynthetic accessory pigments that are found attached to the chloroplast thylakoid membranes in close proximity to photosystem II (PSII).<sup>16</sup> Within *Corallina* these serve to increase light capture efficiency and assist in unidirectional energy transfer.<sup>15, 16</sup> Structurally they consist of a series of peripheral rods surrounding a central core<sup>17</sup> and contain three types of pigment proteins, 'phycobiliproteins'; red fluorescent, phycoerythrins (PE,  $\lambda_{\max} = 540\text{--}570$  nm), blue fluorescent, phycocyanins (PC,  $\lambda_{\max} = 610\text{--}620$  nm), bright blue fluorescent allophycocyanins (APC,  $\lambda_{\max} = 650\text{--}660$  nm),<sup>14–16</sup> as well as associated linker polypeptides for structure and assembly.<sup>18, 19</sup> Captured light energy is transported from high energy PE, through intermediate energy PC, to low energy APC before being transferred to chlorophyll a at the reaction centre of PSII. The number, size and internal pigment ratio of the PBS are variable, and are known to be influenced by a number of environmental factors, allowing acclimation to various conditions.<sup>19, 20</sup>

One response to environmental stress in many algae, including *C. officinalis*, is the production of volatile halocarbons via vanadium bromoperoxidase (VBPO).<sup>21–24</sup> This enzyme catalyses the addition of bromine or iodine to organic substrates, producing a range of organic halogenated

products.<sup>22, 25</sup>  $\text{H}_2\text{O}_2$  produced in the cell is utilized to oxidize bromide anions ( $\text{Br}^-$ ) to a highly reactive intermediate, probably either hypobromous acid ( $\text{HOBr}$ )<sup>10, 26</sup> or bromonium cations ( $\text{Br}^+$ ).<sup>25</sup> The reactive species can then react in a number of ways, including to halogenate nucleophilic organic compounds,<sup>24, 27</sup> or to react with excess  $\text{H}_2\text{O}_2$  to yield singlet oxygen in the absence of suitable substrates.<sup>28</sup> The typical reaction that occurs is illustrated below:<sup>21</sup>



(A is representative of an organic, nucleophilic acceptor)

VBPO activity is considered to be rate limited by  $\text{H}_2\text{O}_2$  concentration, with increases shown to be inducible by high light stress,<sup>23, 29</sup> elevated pH<sup>29</sup> and oxidative stress,<sup>22</sup> suggesting that environmental stressors capable of elevating  $\text{H}_2\text{O}_2$  levels are capable of causing elevated VBPO activity.

The main physiological roles suggested for VBPO are halohydrocarbon production, primarily as compounds for defence and competition,<sup>7, 23, 27</sup> elimination of excess  $\text{H}_2\text{O}_2$ <sup>23</sup> and assistance in halide uptake.<sup>30</sup> In a recent review,<sup>4</sup> Dring has pointed to the two main locations of algal cells that generate increased ROS in response to disease and stress. These are the chloroplast (via photosynthesis) and the plasma membrane (via the FADH-dependent NADPH oxidase). Interestingly, VBPOs have been shown to be located within the chloroplast and on the outer cell wall.<sup>31, 32</sup> The pigments bleached during stress (phycobilins, carotenoids, chlorophylls) are also located in the chloroplast. Recent work in our laboratory suggests that HOBr is involved in pigment degradation and bleaching, with both hypo-osmotic shock and ultraviolet (UV) capable of altering accessory pigment concentrations, and elevating VBPO activity.<sup>33, 34</sup> In addition, *in vitro* studies have shown VBPO to be capable of producing rapid bleaching responses in *C. officinalis* accessory pigments.<sup>35</sup> This would suggest that elevated temperatures, known to be capable of causing oxidative stress and associated excess  $\text{H}_2\text{O}_2$  production,<sup>3, 4, 36, 37</sup> may be capable of inducing increased VBPO activity, increased production and accumulation of HOBr and subsequent degradation and bleaching of pigments.

Whilst in the UK we are located in a geographically remote location from major reef systems, coralline algae represent important local species. The aim of this study was to carry out preliminary investigations into the effects of elevated seawater temperatures and the presence of an exogenous anti-oxidant mannitol on *C. officinalis*, exploring the responses of accessory pigment concentrations and VBPO activity. The temperature range we chose was 5–35°C, the upper value being representative of temperatures causing bleaching in coral reefs in warmer oceanic regions.<sup>2</sup>

## Materials and methods

### Sample collection

*C. officinalis* was collected in autumn 2006, from rock pools in the mid to lower littoral zone, of Wembury Beach, Devon, UK (OS Grid Reference SX 517484). The *Corallina* was transported directly from Wembury Beach to the University of Plymouth where it was transferred to aerated seawater tanks and maintained at  $\sim 15^{\circ}\text{C}$  for three days prior to its use to allow any transportation stress to subside.

### Standard experimental set-up

Water baths were set up, at  $5^{\circ}\text{C}$ ,  $15^{\circ}\text{C}$ ,  $20^{\circ}\text{C}$ ,  $25^{\circ}\text{C}$ ,  $30^{\circ}\text{C}$  and  $35^{\circ}\text{C}$ . These were equilibrated for several days prior to the start of the experiment, until the chosen temperatures had become settled and constant. Two fluorescent tubes (one 18 W and one 20 W) were suspended on light frames 6 in. above the tops of the tanks containing the *Corallina*, and set on a 12 h light, 12 h dark light regime. The units were then covered with black plastic to eliminate incident light. For each temperature, 35 g of *Corallina*, representative of the overall condition of the material collected and, where possible, free from epiphytic growth, were selected and placed in 1 l, clear glass beakers, filled with natural seawater. Full water changes were performed every other day, ensuring that the temperatures were equal prior to exchange to prevent thermal shock.

The extractions, pigment assays and VBPO assays were performed on Day 0, to establish control pigment concentrations and VBPO activities prior to exposure to thermal stress. The  $20^{\circ}\text{C}$ ,  $25^{\circ}\text{C}$ ,  $30^{\circ}\text{C}$  and  $35^{\circ}\text{C}$  extractions, pigment assays and VBPO assays were performed on Days 1–4 and Day 7, as it was not possible to access the laboratories over the weekend (Days 5 and 6) to extract samples. The  $5^{\circ}\text{C}$  and  $15^{\circ}\text{C}$  samples, representative of ‘normal’ temperature ranges experienced by the *Corallina* collected, were sampled only on Day 0 and Day 7. Following the extractions, pigment assays and VBPO assays, the extracts were mixed with glycerol, in a 50:50 extract: glycerol ratio and stored at  $-20^{\circ}\text{C}$  for later analysis of protein.

Photographs were taken on a Nikon Coolpix 5700 digital camera utilizing the flash, with all camera settings remaining unchanged throughout the duration of the experiment.

### Extraction

The method used for phycobilin extraction was based on that of Rosenberg and Ramus.<sup>38</sup> Two,  $\sim 1$  g, samples, each representative of the overall condition of the *Corallina* batch, were removed from the containers, blotted dry, photographed and the exact weight of the samples recorded. The samples were individually placed in a cooled mortar and freeze-thawed three times with liquid nitrogen while being ground to a fine powder. Each powdered sample was carefully

transferred to a mortar at room temperature, where 10 ml of 0.05 M (pH 6.7) potassium phosphate buffer was added and the sample was ground for a further minute. The liquid extract was pipetted into microcentrifuge tubes. The extract was then centrifuged in a microcentrifuge for 4 min at 13 000 rpm. The supernatant was removed, its exact volume recorded, and transferred into labelled storage tubes for use in assays.

### Accessory pigment assay

The pigment assay used to analyse PE, PC and APC concentrations was based on that used by Rosenberg and Ramus.<sup>38</sup> A Phillips PU8720 spectrophotometer was set up to scan and record absorbance levels between 400 and 700 nm. Potassium phosphate buffer, 0.05 M (pH 6.7), was used to perform a baseline scan. Two, 1 ml pigment samples were taken from each extraction, scanned and the absorbance values for 565, 615 and 650 nm were recorded. Pigment concentrations were calculated from the absorbencies recorded.<sup>38</sup> The *Corallina* extraction process is time-consuming therefore only basic one-way ANOVA statistical analyses were performed on the data.

### Bromoperoxidase (VBPO) assay

The VBPO assay used was based on that used by de Boer *et al.* (1987),<sup>21</sup> which follows the bromination of phenol red at 580 nm. Each assay contained 2 ml of 100  $\mu\text{M}$  phenol red, 0.2 ml of 1.0 M potassium bromide and 0.2 ml of 10 mM  $\text{H}_2\text{O}_2$ . Enzyme (0.1 ml) was added to start the reaction.

### Bradford protein assay

Protein was assayed using the Bradford dye binding assay<sup>39</sup> using bovine serum albumin as the standard reference protein.

### Effect of exogenous antioxidants

To investigate whether the addition of an antioxidant would prevent or delay pigment bleaching, two water baths were set up, as for the previous experiment, at the two highest temperatures,  $30^{\circ}\text{C}$  and  $35^{\circ}\text{C}$ . Each water bath contained two 1-l clear glass beakers, one containing just natural seawater and the other made up to a 10 mM mannitol seawater solution. Six grams of *Corallina*, representative of the condition of the collected material, were transferred to each beaker. The seawater and mannitol solutions were exchanged daily, to prevent bacterial build up in the mannitol solutions, with the solutions brought up to equal temperatures prior to exchange to prevent thermal shock. The experiment was run for a total of four days and the extractions, pigment assays and VBPO assays were all performed prior to the experiment start, on unexposed samples, to give control values. They were then performed daily for the remaining three days, utilizing the same methods as detailed previously.

### Statistical analyses

Results for both pigment analyses and VBPO activity were checked to ensure that the data confirmed to the assumptions required for one-way ANOVA analysis. Probability plots, drawn daily for the data sets, showed them all to be normally distributed ( $P \geq 0.05$ ). As inequalities within the variances may affect the outcome of the ANOVA, the significance level used was reduced to  $P = 0.01$ , to reflect this possible source of error. This reduction in significance level should reduce the likelihood of a type I error. Due to inequalities in the number of samples across the days, it was not possible to utilize a two-way ANOVA. It was considered to be most appropriate to perform five separate one-way ANOVAs, across the five experimental days (Days 1–4 and 7).

The null hypotheses under test are that there are no significant differences present between the means of the phycobilin concentrations ( $H_0$ )<sub>1</sub> or the mean VBPO-specific activities ( $H_0$ )<sub>2</sub> of the 20°C, 25°C, 30°C, 35°C, and control data sets.

## Results and discussion

### Visual bleaching

Figure 1 shows the visual evidence of bleaching at 35°C over the 7-day period of the experiment with the *Corallina* fronds changing from a dusky pink-purple at Day 0 to pale orange at Day 1 through to complete bleaching by Day 3. All other samples (20°C, 25°C, 30°C) remained a healthy pink-purple in colour throughout Day 2. By Day 3, the 30°C samples were beginning to bleach to a pale orange, mainly around the frond tips. The 20°C and 25°C, however, still remained the same healthy pink-purple as the Day 0 samples. By Day 4, the 30°C samples remain similar in colour to the Day 3 samples, with partially bleached fronds. There was no visible difference between the 20°C and 25°C samples of Day 4, and those on Day 0. By Day 7, the 35°C *Corallina* samples were completely bleached white, with no pale orange colouration visible, while the partial bleaching of the 30°C samples had reached a larger proportion of the fronds.

### Phycobilin concentrations

Both PE and PC concentrations showed clear trends of decreasing concentration with increasing temperature (Figure 2) confirming the visual observations.  $P$ -values of  $<0.01$  throughout the experiment were obtained, giving evidence for a significant difference between the data sets, allowing rejection of ( $H_0$ )<sub>1</sub>. At lower temperatures, 20°C and 25°C, only small initial decreases in PE and PC pigments are visible and, following the initial drop, they remain relatively steady throughout the remainder of the experimental period. It is probable that, at these temperatures, a small decrease in photosynthetic energy input, caused by partial degradation of the accessory pigments, may reduce the

production of ROS enough to enable the antioxidant defences of the *Corallina* to successfully regulate the oxidant/antioxidant balance within the cell.<sup>33–35</sup> Further investigation using intracellular measures of ROS and a variety of irradiances, would enable confirmation of the cause of this trend in the 20°C and 25°C PE and PC concentrations. It would allow verification of whether ROS levels were remaining stable, and establish that the pigment degradation was due to elevated temperature, not simply as a result of the light levels under which the *Corallina* was kept, which may also produce gradual elevations of H<sub>2</sub>O<sub>2</sub> concentrations<sup>4</sup> and subsequent pigment degradation.

The higher temperatures, 30°C and 35°C, show far more drastic losses of PE across the 7 day period, although the 30°C mean values do show signs of leveling out over Days 3 to 7. Again, this may be due to the ability of the *Corallina* defence mechanisms to cope with regulating ROS levels, once sufficient light energy input pathways have been disabled by accessory pigment degradation. The 35°C PE mean values show no signs of levelling off suggesting that once the temperature reaches this level, excessive production and accumulation of ROS has overwhelmed the antioxidant defences of the *Corallina*. These unregulated levels of ROS are likely to lead to complete pigment loss, damage to numerous cellular components, and cell death.<sup>40, 41</sup>

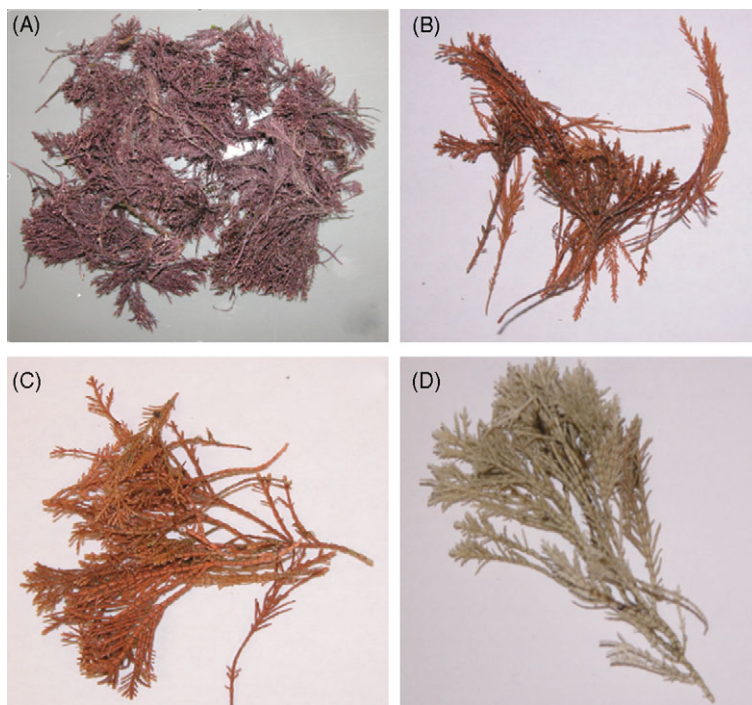
The APC mean concentrations do not produce the same clear trend as PE and PC pigment concentrations and, while the 35°C concentrations are consistently lowest out of the four temperatures, the 20°C, 25°C and 30°C APC concentrations actually increase in all but one mean (30°C; Day 7). However, ANOVA analysis showed a clearly significant fall in APC at 35°C over all other temperatures.

### Bromoperoxidase-specific activity

The plot of the mean VBPO-specific activities at the different exposure temperature exposures reflects the trend shown in the ANOVA results (Figure 3a), showing the correlation between elevated temperatures and increasing specific activity. There is a slight increase in specific activity mean values in the 30°C samples, particularly towards the end of the experiment. The 99% confidence intervals suggested this difference not to be substantial.  $P$ -values of  $<0.01$  throughout the experiment were obtained, giving evidence for a significant difference between the data sets, allowing rejection of ( $H_0$ )<sub>2</sub>.

There was a considerable decrease in the amount of soluble protein extracted from the 35°C samples, giving rise to high VBPO-specific activities based on International Units (IU) per mg of protein. When specific activities were calculated based on IU per gram of *Corallina* extracted, the increases at 30°C became more significant (Figure 3b). All specific activities were declining by Day 7.

VBPO-specific activity has been shown to be primarily rate-limited by the H<sub>2</sub>O<sub>2</sub> concentration,<sup>29</sup> which has been



**Figure 1.** Photographs of bleaching *Corallina* treated at 35°C. Samples of *Corallina officinalis* were treated at 35°C as described in the methods. Photographs were taken at (a) prior to the start of treatment; (b) treatment at 35°C for 24 h; (c) treatment at 35°C for 48 h; (d) treatment at 35°C for 7 days (complete bleaching). The orange colour in (b) and (c) is due to remaining carotenoids.

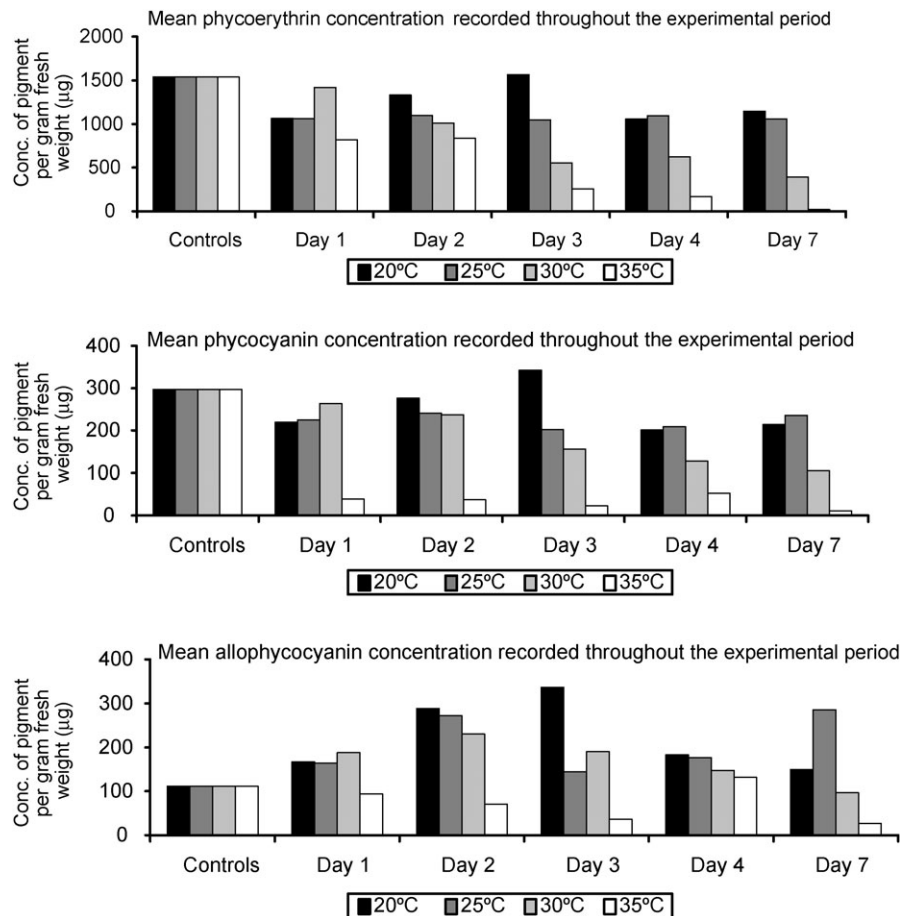
demonstrated to be elevated by numerous environmental stressors including, high visible irradiance, UV radiation, salinity variation, nutrient and mineral deficiencies, exposure to xenobiotics, heavy metal pollutants and extremes of temperatures,<sup>3, 6, 33, 35–37</sup> This would suggest that the rise in VBPO-specific activity recorded in the higher temperature samples is likely to be due to an increase in H<sub>2</sub>O<sub>2</sub> within the *Corallina*. Increases in VBPO activity, in response the environmental stressors of altered salinity and increased UV radiation, have been previously reported to occur in *Corallina* in work in this laboratory by Dent (2006)<sup>33</sup> and Vesty (2006).<sup>34</sup>

The exact mechanisms by which elevated VBPO activity may cause bleaching within *Corallina*, and possibly other coralline algae, remains unconfirmed. However, a recent study in this laboratory by Thomas (2006)<sup>35</sup> showed that H<sub>2</sub>O<sub>2</sub>, Br<sup>-</sup> and VBPO can induce rapid bleaching *in vitro* in pigment extracts from *Corallina*, whereas with H<sub>2</sub>O<sub>2</sub> and VBPO in the absence of Br<sup>-</sup>, bleaching does not occur. It is known that the reaction of excess H<sub>2</sub>O<sub>2</sub>, produced by the cell under stress, plus bromine (Br<sup>-</sup>), taken up from seawater, is catalysed by VBPO, and leads to the production of a highly reactive intermediate, probably bromonium ions (Br<sup>+</sup>) but usually represented as HOBr. Under normal conditions, HOBr reacts with various organic compounds, either intra- or extracellularly, to produce a number of halogenated

organic products.<sup>21, 22, 25, 27, 31, 32</sup> It is possible that under stress conditions, increased VBPO activity could lead to excessive depletion of the pool of organic acceptor compounds drawn upon in the second stage of the reaction. Should this happen, and were the VBPO activity to remain elevated with continued supply of excess H<sub>2</sub>O<sub>2</sub> and ready availability of stored Br<sup>-</sup>, a build up of the highly oxidizing intermediate, HOBr, would occur.

Two routes are suggested, that may be possible mechanisms by which temperature-induced bleaching of the *Corallina* may occur (Figure 4). The HOBr may react directly with the pigments to cause bleaching, as suggested by Thomas (2006),<sup>35</sup> or the accumulated HOBr may react with excess H<sub>2</sub>O<sub>2</sub>, leading to the production of singlet oxygen,<sup>23, 26, 28</sup> which is also known to produce pigment bleaching.<sup>5</sup>

The dramatic difference between the reaction of the VBPO-specific activity at 30°C and at 35°C, with the higher temperature producing a much greater increase in activity suggests the presence of a ‘tipping point’—a temperature at which the defence mechanisms of the *Corallina* fail to regulate the production of ROS. Whether or not this tipping point varies as a result of acclimation to local conditions is unknown, although the distribution of *Corallina* across equatorial, as well as temperate waters suggests that this may be possible.<sup>10</sup> Further investigation of possible



**Figure 2.** Loss of phycobilins with increasing ambient temperature. Phycoerythrin and phycocyanin show a similar trend of decreasing pigment concentrations, with the 20°C and 25°C mean values showing small initial decreases that stabilize after Day 2, while the 30°C, and particularly the 35°C, mean values drop considerably. In contrast, allophycocyanin levels increase initially before starting to decline.

acclimation to thermal stress in *Corallina* and the possible effects that global warming may have on its distribution would no doubt yield interesting results.

#### Low temperature (5°C and 15°C) samples

A comparison, on Day 7, of the samples kept at 5°C and 15°C, with samples from the 20°C and 25°C *Corallina* showed little variation in response between the lower temperatures. The mean PE concentrations altered little over the 7 days, with relatively slight decreases noticeable across all four lower temperatures (results not shown). There was also little difference in mean VBPO activity over the lower temperature range (5°C to 25°C).

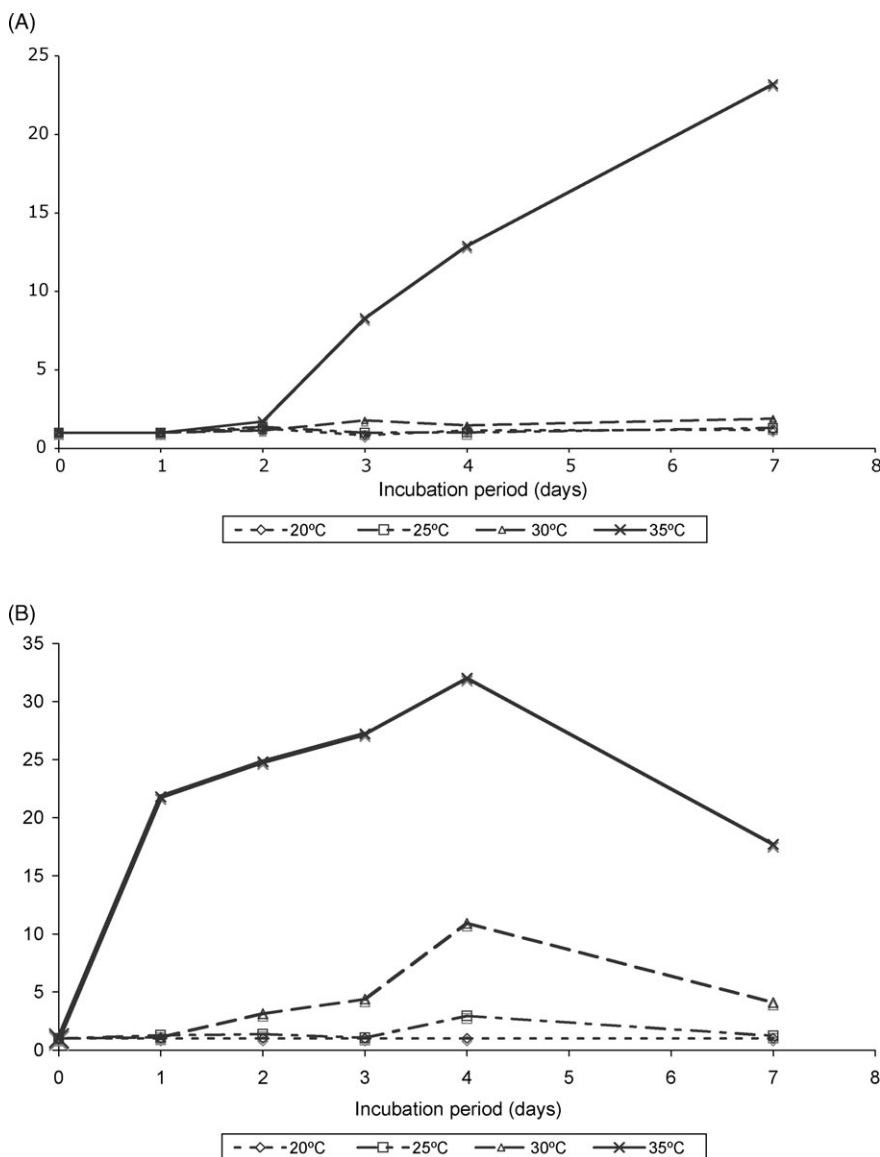
#### Effect of exogenous antioxidant (mannitol)

The effect of adding mannitol show clear trends in the 35°C data. Over Days 1–3, the mean VBPO activity recorded for the 35°C without mannitol samples is consistently higher than those treated with mannitol (Table 1). The 30°C data however, shows little difference between the standard

samples, the control Day 0 samples, and those with added mannitol, although the without mannitol mean values are, in all cases, very slightly higher than those from the mannitol treated samples.

The mean PE concentrations showed dramatic falls at 35°C with over 90% loss for the untreated samples against approximately 70% loss in the treated samples after 48 h showing the protective effect of mannitol. Losses were much less at 30°C for both treated and untreated samples.

The mannitol added to the seawater acts as an antioxidant, effectively removing ROS, preventing their accumulation, and associated oxidative stress.<sup>6</sup> The removal of ROS both lowers the concentration of H<sub>2</sub>O<sub>2</sub> and removes species that can be converted to H<sub>2</sub>O<sub>2</sub>, the main rate-limiting factor for VBPO.<sup>28</sup> Lowering the available concentration is likely to maintain the VBPO activity at low levels, restricting the formation of HOBr, and preventing, or constraining the extent of, pigment bleaching. Limiting VBPO activity would also help prevent excessive depletion of organic acceptor resources, ensuring sufficient substrate is available to react

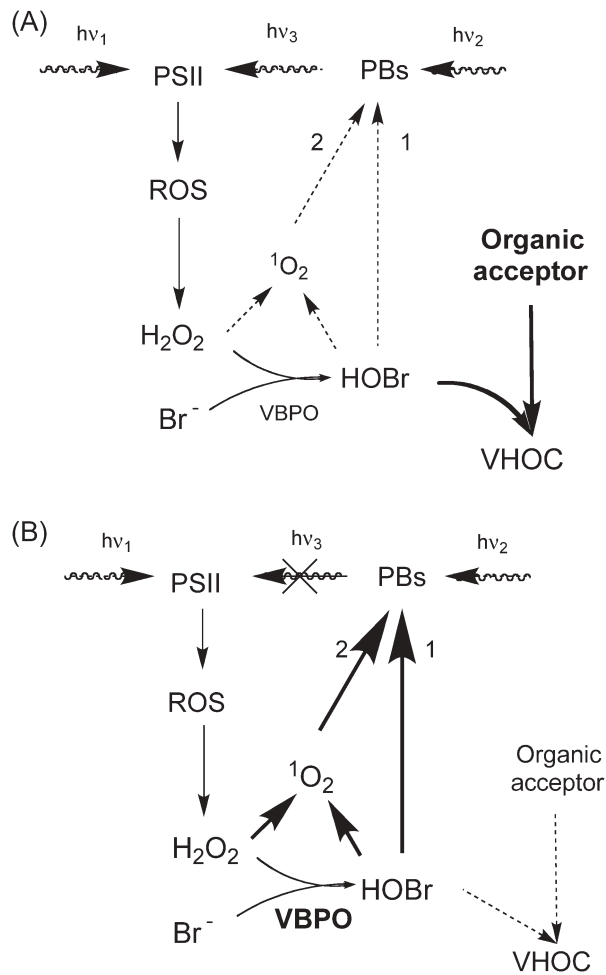


**Figure 3.** Temperature-induced increase in bromoperoxidase-specific activity. (a) Mean specific activities, based on IU per mg protein, at 35°C are far higher than all other samples, with an almost exponential rise in activity across the 7 days of the exposure. The 30°C mean specific activities also increase notably by Day 3 but the 99% confidence intervals suggest this difference is not statistically significant. However, at 35°C, there is a considerable reduction in the amount of soluble protein extracted, giving rise to very high specific activities based on protein. (b) Mean specific activities, based on IU per gram fresh weight of *Corallina*, at 35°C are higher than all other samples. The 30°C mean specific activities also increase notably by day 3 over the values at lower temperatures, showing a several fold increase in BPO catalytic activity. By day 7, all specific activities are declining.

with the HOBr, therefore allowing successful conversion to halohydrocarbons.<sup>22, 23</sup>

The addition of mannitol to the *Corallina* samples produced results that appeared strongly dependent upon the sample temperature. In the 30°C *Corallina* samples the presence of mannitol in the seawater produced no real visible difference in VBPO activity across the 4 days, while in the 35°C samples the added mannitol showed a strong protective effect, with untreated sample mean values reaching much higher levels than the treated sample rates. The similarity in VBPO activities in the absence and presence of mannitol

at 30°C suggests that, at this temperature, the antioxidant defence mechanisms of the *Corallina* are efficiently scavenging and removing ROS produced within the cell, maintaining low H<sub>2</sub>O<sub>2</sub> concentrations and preventing induction of elevated VBPO activity. The large differences in VBPO activities at 35°C would suggest that, in the absence of mannitol, the antioxidant defence systems of the *Corallina* are failing, ROS are accumulating, and excess H<sub>2</sub>O<sub>2</sub> combined with an increased rate of VBPO<sup>38</sup> results in pigment bleaching.<sup>39</sup> At 35°C, a considerable rise in activity is still visible in the mannitol treated samples, this would suggest that the



**Figure 4.** Proposed mechanisms of phycobilin bleaching in *Corallina officinalis*. (a) Shows the elimination of hydrogen peroxide via the formation of HOBr (or Br<sup>+</sup>) by bromoperoxidase (VBPO). The HOBr/Br<sup>+</sup> reacts with an organic acceptor to produce a volatile halogenated organic compound (VHOC). Bleaching of phycobilin pigments is a minor side reaction. (b) Shows the impact of temperature-induced VBPO synthesis. The increased formation of HOBr/Br<sup>+</sup> overwhelms the capacity of cells to form VHOC and the bleaching of pigments becomes a major reaction, either by HOBr/Br<sup>+</sup> directly (1) or by singlet oxygen formed by the reaction of HOBr/Br<sup>+</sup> with hydrogen peroxide (2). Bleaching prevents energy transfer to PSII. Solid lines represent major reactions; dotted lines represent minor reactions; wavy lines represent incident light.

concentration of mannitol used, 10 mM, while sufficient to scavenge considerable levels of ROS, was not enough to completely quench the levels produced.

The PE, PC and APC pigment concentrations show similar trends in the 35°C mean pigment concentrations, with the mannitol treated samples maintaining consistently higher concentrations in comparison with the untreated samples. This loss of pigments is likely to be due to the inability of cells to control ROS concentrations, raising the VBPO activity and causing degradation and pigment loss.

## Conclusions

### Wider ecological effects of bleaching in coralline algae

Crustose coralline algae (CCA), the Corallinacea, contribute the most widespread and abundant benthic marine organisms to be found in the photic zone.<sup>9, 42</sup> Many species of CCAs are known to fulfill important roles in coral reef ecosystems, both in cementing the reef structure and laying down substantial amounts of calcareous material,<sup>7-9</sup> and also in inducing settlement of numerous benthic reef organisms,<sup>8</sup> including corals.<sup>43</sup> Though CCAs contribute significantly to the biotic cover and structure of a reef ecosystem, their ecological influences and the effects of environmental and anthropogenic impacts are relatively understudied,<sup>7</sup> with most of the investigative focus resting on the corals themselves.<sup>44</sup> The role of CCAs in recruitment of new corals also plays a vital role in recovery and resilience of the reef, to both natural and anthropogenic disturbances.<sup>43</sup> Therefore, if temperature can produce such significant bleaching effects within *Corallina*, further study into thermal bleaching of other coralline algae may prove immensely important in the future conservation of coral reefs, as adverse effects on the CCAs within a reef will subsequently affect the entire reef ecosystem.<sup>43</sup> Although the species studied here is not involved in reef building, other coralline algae such as *Lithophyllum yessoense* are. This species has been shown to contain VBPO and to produce volatile hydrocarbons such as bromoform<sup>23</sup> that are known to be involved in inducing settlement of larval stages of benthic organisms, and also in inducing metamorphosis of sea anemones.<sup>45</sup>

**Table 1.** Effect of mannitol on induction of bromoperoxidase in response to temperature

Temperature	30°C + Mannitol	30°C – Mannitol	35°C + Mannitol	35°C – Mannitol
Day	Fold increase in specific activity (IU/g fresh weight <i>Corallina</i> ) over Day 0			
0	1	1	1	1
1	1	1.28	1.11	21.8
2	1	1.38	3.14	24.8
3	1	1.06	4.38	27.2



Recognition of the importance of CCA as major reef building organisms warrants much more emphasis on the impact of environmental change on these organisms. They are clearly as important in the re-building of damaged reefs as they are in original reef construction. Their role needs to be more widely recognized. They deserve at least as much attention as the more charismatic corals and their symbiotic zooxanthellae.

## Acknowledgements

Many thanks to Les Jervis for continued support and guidance throughout the entire project. Also, thanks to Nick Crocker and Andy Atfield for advice on experimental design and techniques and assistance with experimental set-ups.

## Funding

Funding for this project was provided by the School of Biological Sciences, The University of Plymouth.

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Submitted on 30 September 2007; accepted on 28 January 2008; advance access publication 22 April 2008