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Physiological response of temperate microphytobenthos to freezing temperatures

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Microphytobenthos (MPB) contributes up to half the primary production of estuaries. These microorganisms are sensitive to changes in sediment temperatures, particularly the extreme temperatures during exposure periods. This study investigates the physiological responses of MPB to freezing temperatures at two locations near Hobart, Tasmania during winter. Photosynthetic parameters were measured at 2 mm intervals to a depth of 10 mm. FV/FM values at three different distances from the shoreline at Kings Beach and Browns River in winter were between 0.584 and 0.617. rETRmax values were between 24.696 and 20.773. Maximum α values peaked in the subsurface rather than at the sediment surface. In vitro *laboratory experiments (down to −5°C) showed little difference in response between the control and treatment groups, indicating no apparent effects of short term freezing on the MPB. Little change in photosynthetic parameters in response to freezing was probably associated with the resistance of light-harvesting reactions to freezing temperatures, recovery of the plasmalemma integrity or cryoprotection. Sediment composition and species composition were similar at both sampling sites. Therefore, responses of MPB were not due to species and grain size composition.*

Keywords: microphytobenthos, photosynthesis, temperature, diatom, thermoinhibition, sediment surface, maximum relative electron transport rate, primary production, fluorescence

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INTRODUCTION

Microphytobenthos (MPB) is a vital component of most shallow marine ecosystems, contributing between 20% and 50% of annual primary production (Underwood & Kromkamp, 1999; Guarini *et al.*, 2000; Underwood & Provot, 2000; Blanchard *et al.*, 2002; Herlory *et al.*, 2004). MPB, which is usually dominated by diatoms, can attain a biomass of ~300 mg chlorophyll-*a* m^{-2} (Kromkamp *et al.*, 2006; Koh *et al.*, 2007). Much of this biomass is concentrated in shallow water environments where it can be exposed to extremes of temperature at low tide from freezing in winter at less than 0°C to around 35°C in summer. There have been many studies of the effects of temperature on MPB, identifying it as one of the main environmental factors regulating production (Blanchard & Guarini, 1996; Barranguet *et al.*, 1998; Du *et al.*, 2012), but very few have investigated the effects of extreme temperatures. Responses to sub-zero temperatures in the northern hemisphere have been measured in macroalgae (Davison *et al.*, 1989; Dudgeon *et al.*, 1989, 1990; Pearson & Davison, 1993) and microalgal studies have been undertaken in polar areas (Raymond, 2000; Raymond & Knight, 2003; Sabacka & Elster, 2006; Bayer-Giraldi *et al.*, 2010; Bayer-Giraldi *et al.*, 2011; Foreman *et al.*, 2011). This study is the first to investigate the effects of freezing temperatures on MPB from temperate regions of the southern hemisphere.

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In many cool temperate areas, frost is likely to form in intertidal areas during winter under freezing temperatures. In these circumstances MPB use two freezing-tolerance mechanisms in response to freezing temperatures; freezing resistance and cryoprotection. Freezing resistance is strongly related to the ability of the plasmalemma to recover from freezing events (Davison *et al.*, 1989; Dudgeon *et al.*, 1989; Pearson & Davison, 1993). Loss of cell contents occurs following freezing events due to the breakdown of the plasmalemma. Release of amino acids indicates a breakdown of plasmalemma integrity (Davison *et al.*, 1989). Antifreeze production, or cryoprotection, is a mechanism providing freezing tolerance in some microalgae. In diatoms, cryoprotection is related to the occurrence and function of anti-freeze proteins (AFPs). AFPs are able to bind to ice and influence the formation of ice (Raymond, 2000; Bayer-Giraldi *et al.*, 2010, 2011). The characteristic features of AFPs are thermal hysteresis, i.e. the reduction of the freezing point of a solution below melting point, and inhibition of recrystallization (Raymond, 2000; Bayer-Giraldi *et al.*, 2010, 2011). Raymond & Knight (2003) also found that in the sea ice diatoms, *Berkeleya* sp. and *Navicula* sp., there were substances functioning like AFPs, called the ice-active substances (IASs), with a function resembling that of glycoproteins in polar fish AFPs (Raymond *et al.*, 1989; Bayer-Giraldi *et al.*, 2010, 2011), but they were not able to lower the freezing point. Raymond & Knight (2003) also showed that the presence of IASs in the temperate marine diatom, *Navicula frustulum*, greatly enhanced their freeze–thaw survival. The absence of free water molecules within the cell is also critical for the survival of algae under freezing conditions, as ice crystals can readily form when free water is present (Davey, 1989; Harding *et al.*, 2004). Active transfer of free water out of the cell provides another

mechanism of cryoprotection in algae. Harding *et al.* (2004) stated that this type of cryoprotection has colligative consequences, as the solute composition within the cells becomes increasingly concentrated, preventing the formation of ice crystals.

Microphytobenthos can also respond behaviourally to extreme environmental conditions, by migrating vertically within the sediment, ensuring they do not experience photodamage (Guarini *et al.*, 2002; Jordan *et al.*, 2005). Motile or epipelic diatoms secrete a considerable percentage of photosynthetically fixed carbon as extracellular polymeric substances (EPS), which help in this migration within the sediment. EPS largely consists of carbohydrates, which allows diatoms to embed within the sediment by attaching themselves to sediment particles (Staats *et al.*, 1999; De Brouwer *et al.*, 2005). Several studies have shown that MPB migrated further into the sediment when the surface temperatures were unfavourable (Serodio & Catarino, 2000; Blanchard *et al.*, 2001, 2002, 2004; Jesus *et al.*, 2006).

Fluorescence methods have become one of the main tools utilized to examine the environment responses of MPB (White & Critchley, 1999; Ralph & Gademann, 2005; Jordan *et al.*, 2010; Du *et al.*, 2012). Here we use a fluorescence approach to examine the response of MPB to freezing temperatures in shallow marine environments of Southern Tasmania during periods of low tide. Our aim was to determine the physiological response of MPB to freezing temperatures; testing the hypothesis that freezing inhibits photosynthesis. This was undertaken both in the field and under laboratory conditions.

MATERIALS AND METHODS

Study sites

The photosynthetic response of MPB under ambient conditions was examined at two neighbouring sites, at a sandier site at Kings Beach, Sandy Bay (42°93′S 147°30′E) and at a muddier site at Browns River in Kingston Beach (42°96′S 147°51′E). Sampling occurred on frosty winter mornings at 6.00 am on 25 July 2011 at Browns River and on 5 August 2011 at Kings Beach. Sampling was performed before sunrise and during dawn at both sites during low tide period when the surface sediment is exposed. Ambient temperatures were −3°C with a thick frost (2 mm) on the sediment surface and the ambient light was approximately 90 μmol photons m^{-2} s^{-1}.

Physical and chemical parameters of the study sites

The surface irradiance (PAR) at each sampling site was measured as μmol photons m^{-2} s^{-1} using a Biospherical QP radiometer with 2π sensor.

The vertical temperature profile within the sediment was measured by inserting a temperature probe (HI 766C) (K-type thermocouple thermometer, HI935005, Hanna Instruments, Woonsocket, Rhode Island, USA) vertically into the sediment until 120 mm deep. Triplicate measurements were determined at three distances (0 m, 1 m and 2 m) above the low tide mark. The same profiling approach was performed at both sampling sites.

Sediment grain size analysis was undertaken at each site. Only one sediment core was collected from each sampling site for the grain size analysis. Samples for the analysis were sieved at 2360 μm, 1400 μm, 710 μm, 500 μm, 335 μm, 250 μm, 180 μm, 90 μm and 62.5 μm using standard sieving methods (Folk, 1974).

Relative species abundance was determined at each study site. One 150 mm long sediment core (diameter 45 mm) was collected from each sampling site, and the top 10 mm was removed and preserved in Lugol's iodine solution (10 ml) to stain the diatom frustules. Samples were cleaned in hydrogen peroxide (H_2O_2) for three days and mounted in Naphrax mounting medium for diatoms. The slides were examined with a compound microscope (Zeiss Axioskop, Jena, Germany) using a 100 ×, oil immersion objective. Photographs were taken using a Zeiss AxioCam digital camera and Zeiss Axiovision software. Species identification was carried out following Saunders *et al.* (2010).

MPB sampling

Additional cores (45 mm diameter) were collected for fluorescence analysis of the sediment. Nine core samples were collected from each sampling site, with three replicate cores taken from three distances from the low tide mark (0 m, 1 m, 2 m above low water level). Clear polycarbonate tubes were manually pushed into the sediment and stoppered using a rubber bung and immediately returned to temporarily established working place. The top 10 mm of each core was sectioned on site into 2 mm intervals for pulse amplitude modulated (PAM) fluorometry analysis of the photosynthetic parameters of MPB. Each section was mixed with filtered seawater, the temperature of which was adjusted to be similar to the measured sediment temperatures at each depth, and transferred into a vial wrapped in aluminium foil to protect them from light and to dark-adapt the samples for 20 min. The samples were shaken vigorously and then allowed to settle for approximately 10 s before analysis. The chlorophyll fluorescence of the MPB was determined following Jordan & McMinn (2008) and Jordan *et al.* (2010) using a PAM fluorometer (Water PAM; Walz, Effeltrich, Germany).

Fluorescence parameters and rapid light curves

Chlorophyll fluorescence was measured using a PAM fluorometer (Water-PAM, Waltz). The initial fluorescence (F) was measured by applying a weak measuring light (<1 μmol photons m^{s-2} s^{-1}) and a saturating pulse (>3000 μmol photons m^{-2} s^{-1} for 0.8 s) was applied to determine the maximum fluorescence (F_m'). The ratio of the change in fluorescence ($\Delta F = F_m' - F$) and the maximal fluorescence, $\Delta F/F_m'$, is a measure of the effective quantum yield of PSII in the illuminated sample. The relative photosynthetic electron transport rate (rETR) was calculated as the product of the effective quantum yield and quantum flux density of photosynthetically active radiation (PAR) (Genty *et al.*, 1989).

To obtain the rapid light curves (RLC) samples were placed in the cuvette as quickly as possible. The RLC were obtained by illuminating the samples for 10 s before each $\Delta F/F_m'$ measurement at each of a series of eight irradiances; 0, 83, 122, 186, 277, 393, 545, 890 and 1299 μmol photons m^{-2} s^{-1} (White & Critchley, 1999; Ralph & Gademann, 2005). The rETR data generated by the rapid light curves

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were fitted to the following equation with a multiple non-linear regression (Platt *et al.*, 1989):

$$rETR = rETR^{*}_{max}[1 - \exp(-\alpha E_d / rETR_{max})] \exp(-\beta E_d / rETR_{max})$$

$rETR_{max}x$ represents the maximum potential rETR in the absence of photoinhibition. α is the initial slope of the light curve before the onset of saturation and represents the efficiency of light utilization. E_d is the irradiance (in the general formula 400–700 nm). β is the parameter characterizing photoinhibition. In the absence of photoinhibition in the light curves, where β = 0, the function becomes:

$$rETR = rETR_{max}[1 - \exp(-\alpha E_d / rETR_{max}]$$

where $rETR_{max}$ is the maximum rETR at light saturation and thus represents the photosynthetic capacity. Standard RLCs only generate nine points in their P vs E function, unlike traditional ^{14}C-based P vs E functions, which typically have 20 or more data points (Lewis & Smith, 1983). This low number of data points makes correctly estimating both alpha and beta unreliable. Therefore, because none of the communities in this study were inhibited at their maximum ambient irradiance, we removed any 'inhibited' data points (rarely more than one per RLC) from the multiple non-linear regressions. In this way we achieved a more robust estimate of rETRmax and α. The PM-gain was adjusted to be between 5 and 15 before each measurement to keep the measurements consistent between samples. Red light-emitting diodes (LED) provided the measuring light, actinic light and saturating pulses used in the RLC.

Laboratory experiments

A further six sediment cores, collected in the same way as those for the field measurements, were taken at each site for each experiment. Three cores served as the treatment group and three cores as the control group. The cores were collected on 22 August 2011 in Kings Beach and 17 August 2011 in Browns River at 7:00 am, when the sediment surface temperature was approximately 5°C and the ambient irradiance was ~100 μmol photons m^{-2} s^{-1}. The treatment cores were placed in a chest freezer for two hours until the surface of the sediment core reached −5°C, a temperature sufficient to cause freezing of the MPB. A spot light (Osram Vialox NAV(SON)-E, 400 W, Munich, Germany) was used to apply a low intensity irradiance, similar to the irradiance at the time of collection (~90 μmol photons m^{-2} s^{-1}) on the samples. The control cores were placed in an open polystyrene box inside the freezer where surface temperature was not allowed to drop below 0°C. The cores were then sectioned and the photosynthetic parameters measured. Photosynthetic parameters measuring protocols were the same as those used in the field component of the study.

Statistical analyses

The photosynthetic parameter data were compiled and organized in Microsoft Excel with statistical analysis performed with computing software, R (R Development Core Team, 2012). Three-way analysis of variances (ANOVA) was carried out to test the effect of the freezing treatment on each photosynthetic parameter. A significance level of $P <$ 0.01 was considered to be strongly significant, while $P <$ 0.05 was treated as significant. The analyses were undertaken on different spatial scales, with sampling sites, three distances from the low tide mark where the core samples were collected and sections of sediment at five different depths along core (2 mm per section down to 10 mm) as the fixed factors and core samples being collected as the random effects.

RESULTS

Environmental data

The surface irradiance before dawn at Kings Beach was 80 μmol photons m^{-2} s^{-1}, whilst the irradiance at Browns River was 90 μmol photons m^{-2} s^{-1}.

The sediment surface temperatures at Kings Beach were 2.400 ± 0.058°C at 0 m, 2.600 ± 0.058°C at 1 m and 2.467 ± 0.033°C at 2 m from the low tide mark, and increased with depth (Figure 1A).

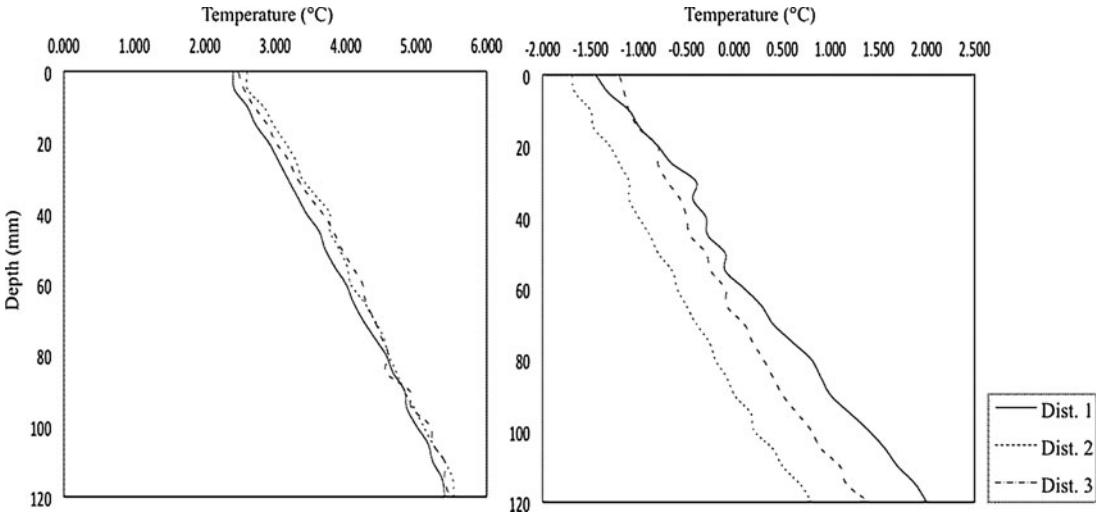

Fig. 1. Sediment temperature profiles as a function of depth at Kings Beach (A) and Browns River (B); distance 1 (Loc 1) was 0 m (R^2 = 0.997 and 0.992, respectively), distance 2 (Loc 2) at 1 m (R^2 = 0.997 and 0.994, respectively), and distance 3 (Loc 3) at 2 m from the low tide mark (R^2 = 0.991 and 0.992, respectively).

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Table 1. Diatom species composition at Kings Beach and Browns River based on a count of 500 cells.

Species	Abundance (%)	
	Kings Beach	Browns River
Achnanthes brevipes	23.2	20.6
Amphora subturgida	18.2	21.6
Cocconeis peltoides	12.6	12
Navicula punctulata	8.4	12.7
Navicula subinfladoides	7	10.9
Navicula salinarum	7.2	7.6
Opephora martyi	8	7.2
Paralia sulcata	4	5
Diploneis papula	1.4	2.4

At Browns River, sediment temperatures at the surface were below freezing, $-1.444 \pm 0.010^{\circ}$C at 0 m, $-1.700 \pm 0.100^{\circ}$C at 1 m, and $-1.200 \pm 0.100^{\circ}$C 2 m from the low tide mark (Figure 1B).

The grain size analyses of the sediment from Kings Beach and Browns River showed that they are mainly sandy clay, with 66.04% of sediments by weight <180 μm at Kings Beach and 71.47% at Browns River.

The diatom communities at Kings Beach and Browns River were similar and dominated by *Achnanthes brevipes* and *Amphora subturgida* (Table 1).

Photosynthetic parameters

The F_V/F_M values of the MPB at the surface at Kings Beach differed significantly from those at depth, with the highest values at the sediment surface (Tables 2 & 3). There was no significant difference between surface F_V/F_M values with distance from the low tide mark, although a slight increase was observed moving further from the low tide mark (Tables 2 & 3).

Table 3. Analysis of variance of microphytobenthos photosynthetic parameters under ambient conditions at Kings Beach on 22 August 2011 and Browns River on 25 July 2011 and variance between treatments in the laboratory experiment for both sampling sites; colon (:) indicating interaction. Significant differences are in bold with **indicates the significance of $P < 0.01$ and *indicating the significance of $P < 0.05$.

Field experiments				
Sites	**Analyses**	**Photosynthetic parameters**		
		F_V/F_M	$rETR_{max}$	α
Kings Beach	Depth	**<0.0001****	**<0.0001****	**0.0009****
	Distance	0.856	0.397	0.742
	Depth: distance	0.2631	0.462	0.326
Browns River	Depth	**<0.0001****	**0.020***	0.194
	Distance	0.321	0.331	0.493
	Depth: distance	0.766	0.337	0.011
Laboratory experiments				
Kings Beach	Depth	**<0.0001****	**<0.0001****	0.655
	Treatment	0.250	0.457	0.481
	Treatment: depth	0.717	0.971	0.764
Browns River	Depth	**<0.0001****	0.064	**0.010***
	Treatment	0.559	0.791	0.576
	Treatment: depth	0.026	0.769	0.603

At Browns River there were significant differences in F_V/F_M between depths within the core at all three distances from the low tide mark (Tables 2 & 3). Maximum F_V/F_M values were observed at the sediment surface, and minimum F_V/F_M at a depth of 8 mm at 0 m from the low tide mark, and at 10 mm at 1 and 2 m from the low tide mark (Table 2).

$rETR_{max}$ values at Kings Beach were highest at the sediment surface and decreased with depth. Minimum $rETR_{max}$ values were observed at a depth of 10 mm and at all three distances from the shore (Table 2; Figure 2).

Browns River $rETR_{max}$ values were highest at the surface, decreased with depth and were lowest at last section at 1 m and 2 m from the low water mark (Table 2). $rETR_{max}$ at

Table 2. Photosynthetic parameters of microphytobenthos under ambient conditions from Kings Beach on 22 August 2011 and Browns River on 25 July 2011; mean ± standard error ($N = 3$). Distance is the horizontal distance from the high tide line. Depth is the depth within the sediment cores.

Distance (m)	Depth (mm)	Photosynthetic parameters					
		Kings Beach			Browns River		
		F_V/F_M	$rETR_{max}$	α	F_V/F_M	$rETR_{max}$	α
0	0.0–0.2	0.584 ± 0.005	24.696 ± 3.443	1.015 ± 0.343	0.542 ± 0.024	20.132 ± 8.022	1.059 ± 0.486
	0.2–0.4	0.568 ± 0.013	18.325 ± 0.498	2.989 ± 1.009	0.498 ± 0.029	16.941 ± 5.047	1.147 ± 0.525
	0.4–0.6	0.506 ± 0.028	13.773 ± 1.419	1.521 ± 0.556	0.468 ± 0.047	13.900 ± 3.713	0.879 ± 0.524
	0.6–0.8	0.396 ± 0.059	9.658 ± 1.875	1.554 ± 0.626	0.450 ± 0.056	8.165 ± 1.337	2.182 ± 0.202
	0.8–1.0	0.286 ± 0.083	4.572 ± 2.054	0.332 ± 0.160	0.468 ± 0.078	10.517 ± 0.742	1.327 ± 0.892
1	0.0–0.2	0.610 ± 0.007	20.773 ± 2.385	1.666 ± 0.811	0.540 ± 0.007	25.945 ± 17.996	1.608 ± 0.683
	0.2–0.4	0.545 ± 0.018	13.323 ± 2.357	3.139 ± 0.462	0.507 ± 0.009	22.748 ± 9.679	1.932 ± 0.870
	0.4–0.6	0.447 ± 0.034	12.438 ± 2.821	1.803 ± 0.782	0.466 ± 0.011	23.570 ± 4.028	0.205 ± 0.010
	0.6–0.8	0.396 ± 0.046	7.260 ± 2.091	1.153 ± 0.297	0.423 ± 0.025	19.527 ± 10.210	0.663 ± 0.390
	0.8–1.0	0.305 ± 0.083	3.939 ± 2.241	1.053 ± 0.572	0.384 ± 0.041	7.967 ± 1.232	2.067 ± 0.209
2	0.0–0.2	0.617 ± 0.010	24.936 ± 0.385	0.767 ± 0.168	0.528 ± 0.010	23.272 ± 3.300	0.264 ± 0.035
	0.2–0.4	0.528 ± 0.051	13.315 ± 3.218	2.644 ± 0.431	0.446 ± 0.029	10.602 ± 1.398	1.755 ± 0.675
	0.4–0.6	0.460 ± 0.084	7.333 ± 3.906	1.739 ± 0.766	0.393 ± 0.029	7.912 ± 0.640	2.071 ± 0.209
	0.6–0.8	0.431 ± 0.087	6.512 ± 1.902	1.652 ± 0.494	0.346 ± 0.030	7.097 ± 2.045	1.940 ± 0.526
	0.8–1.0	0.440 ± 0.090	5.616 ± 1.972	1.409 ± 0.473	0.327 ± 0.015	6.790 ± 1.829	1.880 ± 0.499

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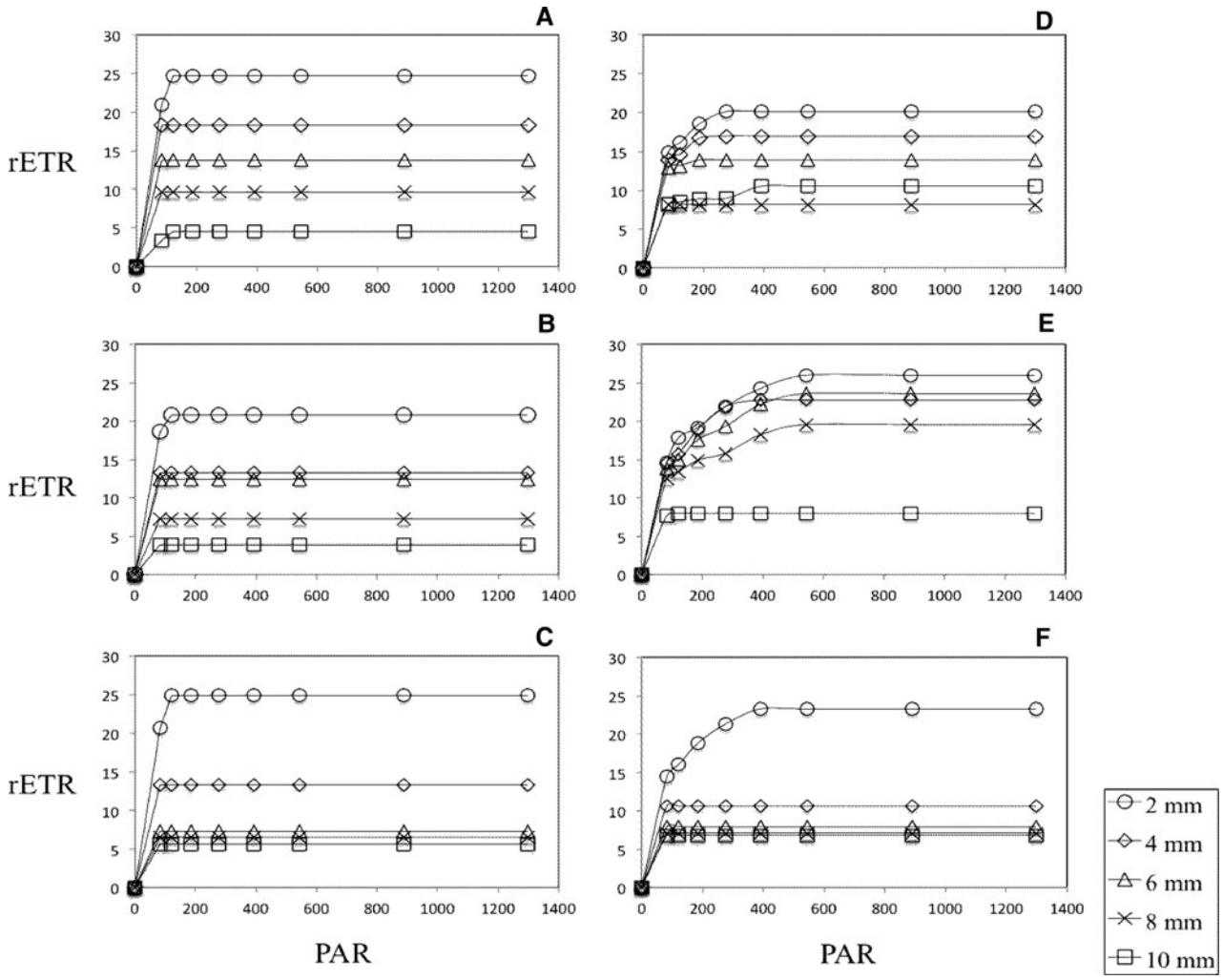

Fig. 2. Plots of rETR against PAR at Kings Beach at 0 m (A), 1 m (B), and 2 m (C) from the low tide mark and Browns River at 0 m (D), 1 m (E), and 2 m (F).

0 m from the low tide mark was highest at the surface but the lowest $rETR_{max}$ was at a depth of 8 mm (section 4); at the last section, it was 30% higher than at 8 mm (Table 2; Figure 2).

Alpha (α) values at Kings Beach did not change with increasing depth, although significant differences were observed between depth intervals (Table 2). The highest α values were consistently at a depth of 0.2–0.4 mm at all three distances from the low tide mark. The lowest α values were at a depth of 10 mm at 0 m and 1 m from the low tide mark, but this occurred in the first section of the core at 2 m from the low tide mark (Table 2).

Browns River α values did not change with increasing depth, although differences between depths were observed (Table 2). Additionally, the maximum and minimum α values fluctuated with depth at each of the three distances from the low tide mark (Table 2).

Laboratory studies

The surfaces of the sediment core collected from both sampling sites (Kings Beach, Browns River) were exposed to freezing temperatures of between −3°C and −5°C (Figure 3). The sediment temperature increased linearly with decreasing depth in both the control and freezing treatments (r^2= 0.929 and r^2 = 0.987, respectively).

PHOTOSYNTHETIC PARAMETERS

There were no significant differences in F_V/F_M between the controls and the sub-zero temperature treatments in the cores collected from Kings Beach (Table 3). Maximum F_V/F_M values occurred at the sediment surface, while minimum values occurred at 10 mm depth in both the control and sub-zero temperature treatments (Table 4). However, F_V/F_M did vary significantly with depth in both treatments (Table 4).

There were no significant differences in the Browns River cores in F_V/F_M between treatments (Tables 3 & 4). However, F_V/F_M decreased significantly with depth in both the treatment and control cores (Table 3). The maximum F_V/F_M occurred at the surface in both control and sub-zero temperature treatments (Table 4). The minimum F_V/F_M in the control treatments occurred at a depth of 8 mm, while in the sub-zero treatment it occurred at a depth of 10 mm (Table 4).

$rETR_{max}$ values of the Kings Beach cores were slightly higher in the sub-zero temperature treatment than in the control treatments, but the differences were not significant (Table 3). The highest values of $rETR_{max}$ were measured in the surface layers and the lowest values at 10 mm in both

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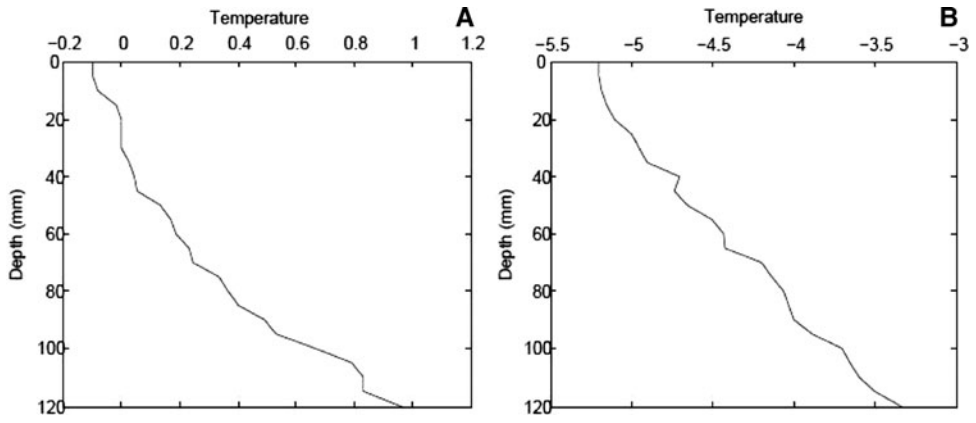

Fig. 3. Plots of the sediment temperature profiles against depths in laboratory experiments of Kings Beach and Browns River under control (A) and freezing (B) treatments.

control and sub-zero temperature treatments. The $rETR_{max}$ values showed significant differences between depths (Tables 3 & 4). Furthermore, the $rETR_{max}$ in the top two core sections were ~40% higher than those of the bottom sections (Figure 4).

At Browns River, there was no significant difference in $rETR_{max}$ between treatments (Tables 3 & 4). Maximum $rETR_{max}$ occurred at the sediment surface in both control and sub-zero temperature treatments, whilst the minimum $rETR_{max}$ of control treatment was observed at the depth of 10 mm and that of sub-zero temperature treatment was observed at the depth of 6 mm (Table 4).

There were no significant differences in α between treatment cores or between depths in the Kings Beach samples (Table 3). Maximum α values were observed at the depth of 6 mm in the control and the sub-zero temperature treatments. Minimum value was observed at the second section of the core in the control treatment, while that of sub-zero temperature treatment was observed at the depth of 10 mm (Table 4).

Significant difference in α between depth sections was observed at Browns River in both control and sub-zero treatments (Table 3). Maximum α were observed at the sediment surface in both control and sub-zero temperature treatments (Table 4). The minimum α occurred at the depth of 10 mm in the control treatment, while that of sub-zero temperature treatment occurred at the depth of 8 mm (Table 4).

DISCUSSION

Freezing had little impact on most of the photosynthetic parameters of the MPB measured in the field. An exception was F_V/F_M, which was significantly lower at Browns River, which experienced lower temperatures than Kings Beach. Other photosynthetic parameters did not show significant differences. While it is difficult to determine whether the lower F_V/F_M values of MPB at Browns River were due to the direct effect of freezing or merely the result of lower temperatures, the laboratory experiments, in which there were no significant differences between treatment and control in any photosynthetic parameters, suggest that the MPB have high freezing tolerance. This implies that the MPB were capable of withstanding short term sub-zero temperatures with minimal impact on photosynthetic capacity. Studies on sea ice algae by Ralph *et al.* (2005), which were also mostly pennate diatoms, reported somewhat similar results, with the microalgal cells able to photosynthesize normally at temperatures down to −5°C. They only displayed lower photosynthetic responses when the temperature went down to −10°C

Table 4. Photosynthetic parameters of freezing experiment on microphytobenthos for Kings Beach and Browns River; mean ± standard error (N = 3). Depth is the depth within the sediment cores.

Treatment	Depth (mm)	Photosynthetic parameters			Photosynthetic parameters		
		Kings Beach			Browns River		
		F_V/F_M	$rETR_{max}$	α	F_V/F_M	$rETR_{max}$	α
Control	0.0–0.2	0.565 ± 0.021	26.693 ± 3.308	1.941 ± 1.368	0.476 ± 0.032	4.623 ± 1.861	1.217 ± 0.527
	0.2–0.4	0.494 ± 0.004	18.063 ± 4.262	1.069 ± 0.660	0.386 ± 0.057	3.605 ± 2.663	0.912 ± 0.661
	0.4–0.6	0.427 ± 0.018	9.203 ± 2.068	2.345 ± 0.466	0.185 ± 0.096	2.085 ± 1.297	0.389 ± 0.353
	0.6–0.8	0.412 ± 0.019	7.731 ± 1.400	1.820 ± 0.283	0.132 ± 0.040	1.904 ± 0.163	0.351 ± 0.168
	0.8–1.0	0.402 ± 0.014	8.442 ± 1.734	1.315 ± 0.387	0.200 ± 0.051	1.844 ± 1.844	0.035 ± 0.035
Treatment	0.0–0.2	0.568 ± 0.006	30.571 ± 2.442	1.017 ± 0.399	0.458 ± 0.078	7.404 ± 4.517	1.657 ± 0.905
	0.2–0.4	0.514 ± 0.006	18.893 ± 2.224	1.659 ± 0.939	0.397 ± 0.089	3.513 ± 1.498	0.933 ± 0.407
	0.4–0.6	0.460 ± 0.008	13.027 ± 3.780	1.759 ± 0.789	0.349 ± 0.097	2.431 ± 1.226	0.627 ± 0.315
	0.6–0.8	0.414 ± 0.017	10.038 ± 2.963	1.358 ± 0.564	0.291 ± 0.109	2.549 ± 1.362	0.620 ± 0.326
	0.8–1.0	0.403 ± 0.011	9.298 ± 2.317	0.724 ± 0.225	0.229 ± 0.118	3.226 ± 0.861	0.838 ± 0.229

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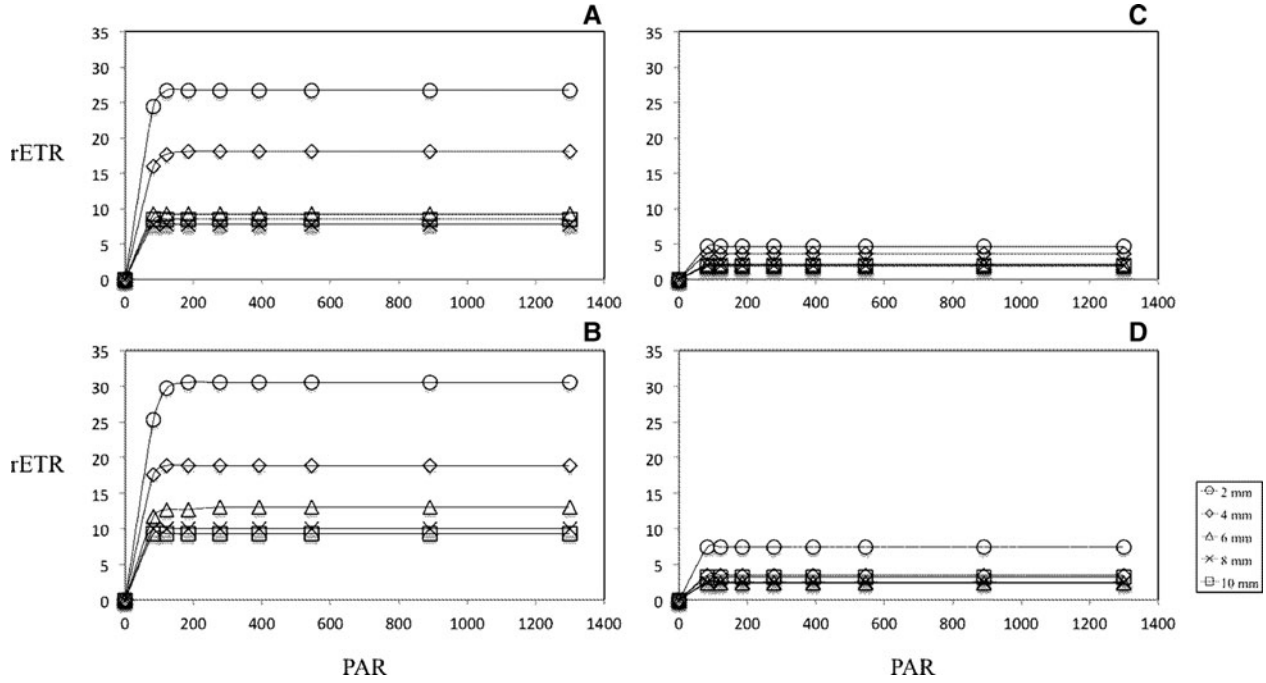

Fig. 4. Plots of rETR against PAR of laboratory experiments of Kings Beach under control (A) and freezing (B) treatments, and Browns River under control (C) and freezing (D) treatments.

(Ralph *et al.*, 2005). The ability of algae to withstand freezing conditions has been seen in a number of other studies. The red algae, *Chondrus crispus,* had the ability to acclimate to freezing conditions lasting up to three hours per day at −5°C, for 30 days; this occurred through the closure of reactions centres and an increase in the photosynthetic rate following the freezing events (Dudgeon *et al.*, 1990). This process enabled the acclimated fronds to maintain higher photosynthetic rates than the non-acclimated fronds. Similar work undertaken with another red alga, *Mastocarpus stellatus*, showed there was no significant effect on photosynthesis, indicating that *M. stellatus* has a greater freezing tolerance than *Chondrus crispus* (Dudgeon *et al.*, 1990).

Observation on the effects of freezing on the brown seaweeds, *Fucus spiralis* and *Fucus edentates.* showed that the photosynthetic rate of *F. spiralis* was unaffected, but that of *F. edentatus* was reduced by 97% after three hours at −20°C (Davison *et al.*, 1989; Pearson & Davison 1993). These variations in freezing tolerance resulted in a vertical zonation, where the most freezing tolerant species (*M. stellatus* and *F. spiralis*) were located in the upper intertidal zones, whilst those with less freezing tolerance (*C. crispus, F. edentatus* and *F. evanescens*) were located in the lower intertidal zones (Davison *et al.*, 1989; Dudgeon *et al.*, 1990; Pearson & Davison, 1993). Greater freezing tolerance in these seaweeds was mainly due to their ability to resume photosynthesis immediately following the freezing events (Davison *et al.*, 1989; Dudgeon *et al.*, 1990; Pearson & Davison, 1993). Similar studies have not been undertaken on MPB but comparable results would be likely as these macroalgae were located in intertidal zones and are likely to have been exposed to freezing temperatures during exposure at low tide.

The results of the present study for temperate MPB show that freezing does not have a large impact on photosynthesis, indicating that MPB are tolerant to freezing and they are able to resume photosynthesis immediately following periods of exposure to freezing. As with intertidal macroalgae, MPB is mostly confined to the intertidal zone, where it can be exposed to freezing temperatures during periods of exposure in winter. In this field study freezing temperatures occurred for a much shorter period, i.e. just for a few hours around sunrise, and this occurred relatively infrequently. Under these conditions it is likely that the cells would have been light-limited and photosynthetic activity would have been low. Therefore, the length of time the MPB was exposed to freezing would not have had a significant impact on the photosynthetic responses of the MPB.

Several factors need to be considered in determining the reason for the freezing tolerance demonstrated by the MPB in this study. Firstly, photoinhibition and/or cell bleaching might only occur after repeated freezing events over a relatively long period of time (i.e. several weeks) (Dudgeon *et al.*, 1990). Dudgeon *et al.* (1990) suggested that cumulative damage to membrane integrity from frequent freezing leading to permanent damage might require longer recovery periods. In the present study, the MPB did not experience prolonged periods of freezing due to the low number of frosty nights (~10 per annum) with minimum temperatures always greater than −5°C and mean daytime temperatures above 10°C, so even though damage to the plasmalemma may have occurred, freezing temperatures were not sustained and so did not prevent full recovery. Furthermore, it is not likely that high irradiance impacted MPB photosynthesis in winter because freezing temperatures only occurred around dawn when irradiance was low. Consequently, MPB are unlikely to experience photochemical stress caused by freezing temperatures. However, the relatively infrequent occurrence of freezing temperatures during winter might have had some negative impact because the MPB are probably not well adapted to an environment where freezing of sediment occurs.

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Although the sediment temperature increased with depth, temperature alone cannot be considered as the reason for the changes in photosynthetic parameters, as other factors, such as dissolved oxygen and light penetration, are also strongly correlated with sediment depth (Boudreau & Jorgensen, 2001). There was a significant negative relationship between F_V/F_M and $rETR_{max}$ and depth, with higher values at the surface in both the ambient conditions and *in vitro* studies. This was unexpected, as it was hypothesized that the freezing at the surface would most likely inhibit MPB and produce a downward migration of cells. This result was possibly an artefact of the sampling method as depth resolution was 2 mm and all migration may have occurred within this top interval (Jordan *et al.*, 2008). The photosynthetic parameter α is usually considered to be temperature independent (Falkowski & Raven, 1997). Lower values of this parameter at the surface therefore may provide evidence that MPB migrated away from the freezing sediment surface. However, similar trends in F_V/F_M and $rETR_{max}$ were not observed and so it was not possible to positively determine if the MPB had migrated to avoid freezing. Furthermore, Morris & Kromkamp (2003) showed that the α of the benthic diatom, *Cylindrotheca closterium*, decreased at extreme low temperature, but this was not seen here.

Results of the current study show that only F_V/F_M values were affected by freezing, and impacts on the other photosynthetic parameters were not apparent. Although it was expected that MPB would undergo acclimation under low temperature conditions, results from this study show that they were freezing tolerant rather than acclimating to the freezing conditions. This freezing tolerance may have been associated with the resistance of light-harvesting reaction centres to freezing, recovery of the plasmalemma integrity and cryoprotection.

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