Received Date : 20-Dec-2012
Accepted Date : 08-May-2013
Article type : Regular Article

Effects of elevated pCO_2 on the metabolism of a temperate rhodolith *Lithothamnion corallioides* grown under different temperatures[1]

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Editorial Responsibility: C. Hurd (Associate Editor)

ABSTRACT

Coralline algae are considered among the most sensitive species to near future ocean acidification. We tested the effects of elevated pCO_2 on the metabolism of the free living coralline alga *Lithothamnion corallioides* ("maerl") and the interactions with changes in temperature. Specimens were collected in North Brittany (France) and grown for 3 months at pCO_2 of 380 (ambient pCO_2), 550, 750 and 1000 µatm (elevated pCO_2) and at successive

temperatures of 10°C (ambient temperature in winter), 16°C (ambient temperature in summer) and 19°C (ambient temperature in summer + 3°C). At each temperature, gross primary production, respiration (oxygen flux) and calcification (alkalinity flux) rates were assessed in the light and dark. Pigments were determined by HPLC. Chl *a*, carotene and zeaxanthin were the three major pigments found in *L. corallioides* thalli. Elevated pCO_2 did not affect pigment content while temperature slightly decreased zeaxanthin and carotene content at 10°C. Gross production was not affected by temperature but was significantly affected by pCO_2 with an increase between 380 and 550 µatm. Light, dark and diel (24 h) calcification rates strongly decreased with increasing pCO_2 regardless of the temperature. Although elevated pCO_2 only slightly affected gross production in *L. corallioides*, diel net calcification was reduced by up to 80 % under the 1000 µatm treatment. Our findings suggested that near future levels of CO_2 will have profound consequences for carbon and carbonate budgets in rhodolith beds and for the sustainability of these habitats.

Keywords: ocean acidification, coralline algae, maerl, calcification, photosynthesis, pigment

Abbreviations: dry weight, DW; Intergovernmental Panel on Climate Change, IPCC; the Service d'Observation des Milieux LITorraux, SOMLIT

INTRODUCTION

Since the beginning of the industrial revolution, the atmospheric CO_2 partial pressure (pCO_2) has continuously and unprecedentedly increased (Sabine et al. 2004) from 280 ppm to today's level of about 390 ppm. This climb in pCO_2 has enhanced the greenhouse effect and has led to an annual rise in temperature of 0.2-0.3°C (Solomon et al. 2007). Over the next century, seawater surface temperatures have been predicted to increase by 3°C and pCO_2 to reach 1000 ppm (Solomon et al. 2007). Increased CO_2 concentrations will cause a pH

decrease termed "ocean acidification" of 0.1-0.4 units in the surface ocean by the end of the century (Caldeira & Wickett 2003). This would result in a decrease in carbonate ions (CO_3^{2-}) concentration (Orr et al. 2005), and in the calcium carbonate saturation state (Ω; Feely et al. 2004) which is a parameter controlling the calcification process (Millero et al. 2006, Millero2007). Precipiting $CaCO_3$ could therefore become less efficient, making calcifying organisms particularly sensitive to ocean acidification (Hoegh-Guldberg 2009, Doney et al. 2009).

Coralline algae (Corallinaceae, Rhodophyta) are the most common group of calcareous algae. They are widespread around the world from tropical to polar oceans and at all photic zone depths (Nelson 2009). They are considered ecosystem engineers (Nelson 2009) being major framework builders and carbonate producers especially in temperate and cold water benthic ecosystems (Basso 2012). Among coralline algae, rhodoliths are the free-living non-geniculate species, which form extensive beds by accumulating live and dead thalli. These so-called "maerl beds" constitute one of the four world's largest macrophyte-dominated benthic communities together with kelp-beds, seagrass meadows and crustose coralline algal reefs (Foster 2001). Thanks to the three-dimensional lattice formed by their branches crossed, rhodolith beds have several key ecological roles (Foster 2001) and provide ecosystem services. They represent microhabitats for cryptofauna (Grall et al. 2006), settlement places for invertebrate larvae (Kamenos et al. 2004a) and nursery for commercial invertebrate and fish juveniles (Kamenos et al. 2004a,b). Rhodolith beds support a highly diversified fauna and fleshy macroalgae (Cabioch 1969, Foster 2001, Barbera et al. 2003, Grall et al. 2006, Pena & Barbara 2010) making them a hot-spot of biodiversity. They also make a large contribution to global carbon production (Martin et al. 2005). In the Bay of Brest, France, annual primary production of maerl beds is 2-fold higher than annual

phytoplanktonic production, contributing about a third of the bay total productivity (Martin et al. 2007). Although temperate rhodoliths have very slow growth rates (around 1 mm $year^{-1}$) (Potin et al. 1990, Blake & Maggs 2003), they are major carbonate producers with a production rate around 490 g $CaCO_3 \cdot m^{-2} \cdot y^{-1}$ in Brittany waters (Martin et al. 2007) which is close to that reported for tropical reef environments (Bosence & Wilson 2003, Gherardi 2004, Amado-Filho et al. 2012).

Coralline algae precipitate high magnesium-calcite (Mg-calcite) to form their thalli, with the highest mol% $MgCO_3$ rate at low latitudes and warm temperature (Andersson et al. 2008). High Mg-calcite is the most soluble form of biogenic $CaCO_3$ when mol% $MgCO_3$ is higher than 12% (Andersson et al. 2008, Morse et al. 2006). The physiological mechanism of calcification is poorly understood in coralline algae. The rate of calcification can vary as a function of the CO_3^{2-} concentration (Borowitzka 1981, Gao et al. 1993, Raven 2011), although HCO_3^- ions may also be a substrate for calcification (Digby 1977, Koch et al. 2012). Due to the solubility of their skeleton, coralline algae appear to be the most sensitive calcifying organisms to near future ocean acidification (Kroeker et al. 2010, Basso 2012). Among the first studies conducted in the 80's on the effects of pH on coralline algae, Borowitzka (1981) demonstrated that a decrease in pH (from 9 to 7) increases photosynthesis and calcification in the light. Conversely, most of the recent studies have shown a general negative impact of elevated pCO_2 on coralline algae on recruitment (Kuffner et al. 2008), abundance (Martin et al. 2008, Porzio et al. 2011), growth (Jokiel et al. 2008), calcification (Semesi et al. 2009, Gao & Zheng 2010). Furthermore, bleaching can lead to mortality (Anthony et al. 2008, Diaz-Pulido et al. 2012) and bleaching has been found to increase in response to high pCO_2. In addition, Ries et al. (2009) found that in some coralline algae, calcification has a parabolic response to pCO_2 with the highest calcification rate under

intermediate pCO_2 levels (600 and 900 µatm). The majority of these measurements were conducted in mesocosms, but recent open field and in situ flume experiments agree that increasing pCO_2 causes reduced abundances (Hall-Spencer et al. 2008, Porzio et al. 2011) and increased dissolution (Kline et al. 2012) of coralline algae exposed to naturally decreased pH levels.

The interacting effect of temperature has to be considered together with the effect of increased pCO_2 because these two environmental variables fundamentally influence the physiology of algae. Algal photosynthesis and respiration are usually enhanced under warmer temperatures (Steller et al. 2007). In various studies on coralline algae, temperature has been shown to emphasize the negative effects of ocean acidification on algae (Anthony et al. 2008, Diaz-Pulido et al. 2012, Martin & Gattuso 2009), although the mechanism is not well understood. The interaction of elevated pCO_2 and increasing temperature, which will inevitably come in parallel to ocean acidification, should induce various species specific responses. The aim of this study was to investigate the interactive effects of pCO_2 and temperature on *Lithothamnion corallioides*, the most common rhodolith species of Brittany coasts, in France. Because photosynthetic calcifying marine organisms use DIC as substrate for both photosynthesis and calcification, the response of these metabolic activities were explored.

MATERIAL & METHODS

Biological material

Lithothamnion corralioides P. L.Crouan & H. M.Crouan, 1867 thalli were collected by SCUBA diving on the 15th December 2010, in a maerl bed of the Bay of Brest (northwest Brittany, France), at the Roscanvel site (4°24'59"W/48°17'46"N), at 10 m depth below Chart

Datum. They were transferred directly in a cool box maintained at in situ temperature to the laboratory at the Station Biologique de Roscoff. Rhodolith thalli of around 3 cm in diameter were selected, gently cleaned to remove most epiphytes and biofilm-forming organisms. They were kept in natural unfiltered seawater until the beginning of the experiment. The thalli were softly brushed to take off epiphytes and biofilm prior to experiments. Dry weight (DW) of each thallus was measured at the end of the experiment after oven drying fresh samples at 60°C for 48 h.

Experimental conditions

Organisms were exposed for 3 months (20th December 2010 to 14th March 2011) to four pCO_2 treatments: an ambient pCO_2 of 380 µatm (pH_T = 8.07), and three elevated pCO_2 treatments of 550 µatm (pH_T = 7.94), 750 µatm (pH_T = 7.82) and 1000 µatm (pH_T = 7.77). Elevated pCO_2 treatments correspond to different scenarios predicted by the Intergovernmental Panel on Climate Change (IPCC) for the end of the century (Solomon et al. 2007) and were selected according to the recommendations of Riebesell et al. (2010).

Algae were grown at three successive temperature levels, according to *in situ* temperature data recorded in the Bay of Brest by the Service d'Observation des Milieux LITorraux (SOMLIT) in winter (9.49 ± 0.16°C) and in summer (16.36 ± 0.09°C). The initial experimental temperature was adjusted to the in situ temperature when algae were collected (10°C, winter temperature), and algae were grown at this temperature for 12 d at which time metabolic rate measurements (see below) were made. Then, the temperature was progressively increased over three weeks to reach 16°C (summer temperature), and algae were grown at this stable temperature for a further 12 d, and the metabolic rates were measured again. Finally, the temperature was increased over a one week period (0.5°C per

day) to reach 19°C (summer temperature elevated by 3°C, representative of the temperature predicted by the end of the century), and algae were grown for a further 12 d prior to making the last set of metabolic rate measurements.

Light was provided by 39 W fluorescent tubes (JBL Solar Ultra Marin Day, JBL Aquaria, Nelson, New Zealand) with a photoperiod of 12:12 h. Irradiance was adjusted to 15 µmol photons $\cdot m^{-2} \cdot s^{-1}$ using a quantum sensor (LiCor®, LI-192 SA). Irradiance reproduced a mean annual irradiance level in the Bay of Brest at 10 meters depth using an mean attenuation coefficient of -0.395 $\cdot m^{-1}$ determined according to those reported by Martin et al. (2006) for the Bay of Brest of -0.41 $\cdot m^{-1}$ in winter and -0.38 $\cdot m^{-1}$ in summer.

Experimental set up

Twenty four sets of 5-6 thalli were labeled with small plastic numbers attached with nylon wire and randomly distributed into twelve 10-L aquaria (2 sets of algae per aquarium; Fig. 1). In addition unlabelled thalli were kept in each aquarium for pigment analyses. The pCO_2 was adjusted by bubbling CO_2 free air (ambient pCO_2) or pure CO_2 (elevated pCO_2) in four 100 L header tanks supplied with unfiltered seawater pumped in front of the Station Biologique de Roscoff. Each of the four pCO_2 treatments had three 10 L replicate aquaria which continuously received CO_2-treated seawater at a rate of 9 $L \cdot h^{-1}$ (i.e., a renewable rate of 90% $\cdot h^{-1}$) from the mixing header tanks. Water velocity in the aquaria was around 0.5 cm $\cdot s^{-1}$. The 12 aquaria were placed in thermostated baths where temperature was controlled to within ± 0.2 °C using 100 and 150 W submersible heaters. pCO_2 and temperatures were monitored and controlled by an off line feedback system (IKS Aquastar, Karlsbad, Germany) that regulated the addition of gas in the header tanks and the on/off heater switch in thermostated bath. The pH values of the pH-stat system were adjusted from daily

measurements of pH on the total scale (pH_T) in the 12 aquaria using a pH meter (HQ40D, Hach Lange, Ltd portable LDO™, Loveland USA) calibrated using Tris/HCl and 2-aminopyridine/HCl buffers (Dickson et al. 2007). The different pCO_2 conditions were reached gradually (0.05 pH units per d) before the beginning of the experiment.

Seawater parameters

Seawater parameters were monitored throughout the experiment. pH_T and temperature were recorded daily in each of the twelve aquaria with a pH meter (HQ40D, Hach Lange, Ltd portable LDO™, Loveland USA). Total alkalinity was measured every three weeks. Salinity was also measured every three weeks, at each temperature, with a conductimeter (LF 330/ SET, WTW, Germany) and remained constant with a mean value of 35.1 ± 0.1. The carbonate chemistry of seawater, i.e., dissolved inorganic carbon (DIC), exact CO_2 partial pressure (pCO_2) and saturation state of aragonite (Ω_{Ar}; solubility of high Mg-calcite closer to aragonite than calcite) were calculated at each pCO_2 and temperature treatment using CO_2SYS software (Lewis & Wallace 1998) with constants of Mehrbach et al. (1973; refitted by Dickson & Millero 1987).

Metabolic rate measurements

Photosynthesis (net and gross production), respiration and net calcification rates were determined for *L. corralioides* in each pCO_2 treatment and at each of the successive temperature levels through short incubations in 185 mL acrylic respirometry chambers (Engineering & Design Plastics Ltd, UK), in both the light and dark. The labeled sets of 5-6 thalli were placed into the respirometry chamber that was filled with seawater from the 10-L aquarium. Thalli were put on a plastic grid above a stirring bar (speed 100 r.p.m.), which ensured water homogeneity. Respirometry chambers were kept in their respective aquarium

during the incubation to keep the temperature constant. Light incubations were carried out under the culture irradiance (15 µmol photon $\cdot m^{-2} \cdot s^{-1}$) and dark incubations were achieved by covering the aquaria with black plastic bags, and the fluorescent tubes switched off. Incubations varied between 4 h at 10°C and 3 h at 19°C in order to maintain oxygen saturation above 80% at the end of dark incubation. At the end of the incubation, pH in the chamber did not increase or decreased more than 0.1 unit from the value measured at the beginning of the incubation. Control incubations without algae were carried out to correct fluxes from any biological activity in seawater.

Net production (measured during light incubation) and respiration rates (dark incubation) were calculated by measuring oxygen molar concentration at the beginning and the end of the incubation period with a non-invasive optical fiber system (FIBOX 3, PreSens, Germany). The reactive oxygen spots were calibrated at the beginning of each incubation set (i.e., each temperature level) with 0% and 100% oxygen buffers. Net production (NP), respiration (R) and gross production (GP) rates (in µmol $O_2 \cdot g^{-1}$ DW $\cdot h^{-1}$) were corrected from controls and calculated as:

$$R = \frac{\Delta O_2 \times V}{\Delta t \times DW} \qquad NP = \frac{\Delta O_2 \times V}{\Delta t \times DW} \qquad GP = NP - R$$

where ΔO_2 is the difference between initial and final O_2 concentrations (µmol $O_2 \cdot L^{-1}$), V is the volume of the chamber (L), Δt is the incubation time (h), and DW is the dry weight of the algae (g).

Calcification rates were estimated using the alkalinity anomaly technique (Smith & Key 1975) based on a decrease of total alkalinity (A_T) by 2 equivalents for each mole of $CaCO_3$ precipitated (Wolf-Gladrow et al. 2007). This method is usually used to examine the changes in calcification rate that may occur over time (Chisholm & Gattuso 1991, Gattuso et

al. 1998). Seawater was sampled at the beginning of the incubation, directly in the aquaria just after the chambers were closed, and at the end, in the incubation chamber. Samples were filtered through 0.7 µm Whatman GF/F filters into 100 mL glass bottles and immediately poisoned with mercuric chloride (0.02 % vol/vol; Dickson et al. 2007). A_T value (in $\mu Eq \cdot L^{-1}$) were determined by HCl 0.01N potentiometric titration on an automatic titrator (Titroline alpha, Schott SI Analytics, Germany) and by using the Gran method (non-linear least-squares fit) applied to pH values from 3.5 to 3.0 (Dickson *et al.* 2007). Light and dark calcification rates (g, in $\mu mol\ CaCO_3 \cdot g^{-1}\ DW \cdot h^{-1}$) were corrected from controls and calculated as:

$$g = -\frac{(\Delta At) \times V)}{2 \times \Delta t \times DW}$$

where ΔA_T is the difference between initial and final total alkalinity concentrations (µmol Eq L^{-1}).

Diel calcification rates (in $\mu mol\ CaCO_3 \cdot g^{-1}\ DW \cdot h^{-1}$) were calculated from light (g light) and dark (g dark) calcification rates based on a 12:12 (light:dark, h) photoperiod as:

$$G = (g\ light) \times 12 + (g\ dark) \times 12$$

Pigment analyses

The pigment content of *L. corallioides* thalli were analysed by HPLC. Three individuals (distinct from those used in the measurements described above) were collected at each temperature level in each aquarium, gently brushed and immediately frozen at -20°C, pending analyses. Frozen samples were ground within a bead grinder (Retsch ®) and the resulting powder was precisely weighted, then frozen until pigment extraction.

The maerl powder (200-500 mg) was suspended in 100% methanol for 2 hours at -20°C and centrifuged at 20,000*g*. The supernatant was collected and centrifuged again to ensure total removal of particles and cell debris. The supernatant was then brought to 10%

Milli-Q water to avoid peak distortion (Zapata & Garrido 1991) and a volume of 100 µL of the pigment extract was immediately injected into an HPLC Hewlett-Packard HPLC 1100 Series system, equipped with a quaternary pump and diode array detector. Pigment separations were performed using a Waters Symmetry C_8 column (150 x 3 x 4.6 mm, 3.5 µm particle size) according to procedures published elsewhere (Zapata et al. 2000, Six et al. 2005) at a flow rate of 1 mL $\cdot$ min^{-1}. All sample preparations were made under subdued light at low temperature. Chlorophylls and carotenoids were detected by their absorbance at 440 nm and identified by diode array spectroscopy. Pigments were identified and quantified using standards derived from macroalgae and phytoplankton cultures by preparative HPLC (Repeta & Bjørnland 1997), using previously compiled extinction coefficients (Jeffrey et al. 1997).

Statistics

All statistical analyses were performed using the free software R 2.15.0 version (©The R Foundation for Statistical Computing). Three-way repeated analyses of variance (ANOVA) were performed with the GAD package to investigate the impact of pCO_2, temperature and aquarium on the different metabolic rates with repeated measures on the same individuals. pCO_2 and temperature were considered as fixed factors. To take in account spatial pseudoreplication, individuals were nested in their own aquarium and aquarium was considered as a random factor. Any changes in pigment content were assessed using a three-way non-repeated ANOVAs (pCO_2, temperature, and aquarium as factors). Normality of the data and homoscedasticity were checked by Kolmogorov-Smirnov's test and Levene's test respectively. Student-Newman-Keuls (SNK) post hoc tests were applied to establish differences among treatments with a confidence level of 95% when ANOVA showed significant results. All the results are presented as mean ± standard error.

RESULTS

Seawater parameters

Temperature was maintained stable and showed little variability (± 0.5°C; Table 1; Fig. 2). The different pCO_2 levels were kept close to the selected values of 380, 550, 750 and 1000 µatm (Figure 2). The ambient pCO_2 showed little increase from 365 µatm at 10°C to 440 µatm at 19°C. Elevated pCO_2 showed variations of about 100 µatm, ranging from 516 to 607 µatm, 705 to 830 µatm, and 961 to 1049 µatm in the 550, 750 and 1000 µatm treatments, respectively (Table 1). Total alkalinity ranged from 2322 to 2367 µEq $\cdot$ kg^{-1}. The carbonate saturation state with respect to aragonite (Ω_{Ar}) never decreased under 1, even at 1000 µatm.

Photosynthesis and respiration

The mean rates of gross primary production varied from 0.50 (19°C, 750 µatm) to 0.69 µmol $O_2 \cdot g^{-1}$ DW $\cdot$ h^{-1} (16°C, 550 µatm; Fig. 3a). The gross production was not significantly affected by the aquarium or temperature. Conversely, rates significantly differed among pCO_2 treatments (Table 2) with values being 17% higher at 550 µatm relative to 380 µatm (Fig. 3a). No significant difference in gross production were detected between pCO_2 treatments of 750 and 1000 µatm but the rates were higher than at 380 µatm.

The mean respiration rates ranged from 0.25 to 0.37 µmol $O_2 \cdot g^{-1}$ DW $\cdot$ h^{-1} (Fig. 3b). They were significantly affected by temperature, being lower at 10°C (0.31 µmol $O_2 \cdot g^{-1}$ DW $\cdot$ h^{-1}) than at 16°C and 19°C (0.36 µmol µmol $O_2 \cdot g^{-1}$ DW $\cdot$ h^{-1}; Table 2). No effect of the aquarium or pCO_2 were detected.

Pigment analyses

A large number of pigments were detected in the HPLC analyses of maerl thalli, with most of them present as traces, and only the major pigments were identified (Fig. 4). Even

though thalli were brushed before extraction, pigment analyses revealed the presence of epiphytes on *L. corallioides* thalli: chl *b*, neoxanthin and siphonaxanthin derivatives are characteristic pigments of green seeweeds (Ulvophyceae). In particular, siphonaxanthin related compounds are common in siphonous green algae and *Umbraulva* species that commonly occur in maerl bed ecosystems (Cabioch 1969). Furthermore, fucoxanthin and diadinoxanthin were often detected, showing that brown plastid bearing organisms were also present on the maerl thalli (Phaeophyceae, diatoms, etc; Roy et al. 2011). These epiphyte pigments were generally found in low amounts and their quantities were very variable from one sample to another, with no relation to the experimental setup.

Three pigments, zeaxanthin, chl *a* and carotene, were systematically present in relatively high and stable amounts and were therefore attributed to *L. corallioides* (Figure 5). Chl a was the dominant pigment with concentrations varying between 37.81 ± 5.75 and 72.80 ± 4.42 $\mu g \cdot g^{-1}$ FW. Chlorophyllid a, a degradation product of chl *a* was detected at all temperature and pCO_2 conditions, with concentrations ranging from 14.18 ± 2.34 to 27.48 ± 2.76 $\mu g \cdot g^{-1}$ FW. Carotene molecules, usually associated to chl *a* in photosystem reactive centers were also detected, with concentrations varying from 0.85 ± 0.18 at 16 °C to 1.53 ± 0.24 $\mu g \cdot g^{-1}$ FW at 19°C (Fig. 5b). The shouldered shape of the peak suggests the presence of both α- and β-carotene. The last characteristic pigment was the xanthophyll zeaxanthin whose contents ranged from 0.90 ± 0.07 to 1.35 ± 0.20 $\mu g \cdot g^{-1}$ FW at 16 and 10°C, respectively (Fig. 5c). Interestingly, this zeaxanthin-based pigmentation does not agree with the chemosystematic pigmentation described by Schubert et al. (2006) for the Corralinales order to which *L. corallioides* belongs, and whose representative species usually contain lutein or antheraxanthin as major xanthophyll.

To examine the pCO_2 effect, chl *a* and chlorophyllid *a* were grouped as chl *a* compounds (Fig. 5a). pCO_2 and aquarium had no detectable effect on pigment contents (Table 3), whereas temperature impacted all pigments, except chl *a* compounds (Table 3, p = 0.732). Carotene content was higher at 10°C than at 16 and 19°C. The zeaxanthin content was significantly different only between 10 and 16°C. The interaction between pCO_2 and temperature was never significant (Table 3).

Calcification

Net calcification rate measured in the light was higher than calcification in the dark (Fig. 6). The aquarium effect was non-significant for both light and dark calcification rates as well as diel calcification (Table 2).

In the light (Fig. 6a), mean net calcification rates varied from 0.11 (10°C, 1000 µatm) to 0.35 µmol $CaCO_3 \cdot g^{-1}$ DW $\cdot h^{-1}$ (10°C, 380 µatm). Light calcification rates were affected by pCO_2 decreasing by 58% between 380 and 1000 µatm (Fig. 6a). In the light, calcification was similar at 380 and 550 µatm and higher at 380 µatm than at 750 and 1000 µatm (Table 2). Temperature had no significant effect on calcification in the light (p = 0.062).

In the dark (Fig. 6b), mean net calcification rates varied according to the temperature and pCO_2 conditions, from -0.10 to 0.19 µmol $CaCO_3 \cdot g^{-1}$ DW $\cdot h^{-1}$ (Fig. 6b). Net calcification was positive in all pCO_2 and temperature treatments except at 1000 µatm at temperatures of 10 and 16°C, where dissolution processes were more important than calcification ones. Dark calcification was affected by pCO_2, temperature and the interaction of the two factors (Table 2). Rates of dark calcification increased significantly when temperature as increased from 10°C to 16°C (p = 0.015), but this trend was not observed at 19°C. Dark calcification strongly decreased with increased pCO_2 (Table 2). It was negative at 1000 µatm, turning into

net dissolution at 10 and 16°C. The interaction between temperature and pCO_2 was marked at 1000 µatm with an increase in dark calcification with rising temperature (Fig. 6b).

Diel (24 h) net calcification rates calculated assuming a12:12 photoperiod were presented in Figure 7. They ranged from 0.14 (10°C, 1000 µatm) to 5.80 µmol $CaCO_3 \cdot g^{-1}$ $DW \cdot d^{-1}$ (10°C, 380 µatm). Diel calcification was significantly affected by pCO_2, decreasing by 50 and 80 %, respectively, at 750 and 1000 µatm relative to 380 µatm. No significant pCO_2 effect was detected between 380 and 550 µatm (Table 2). Temperature did not impact diel calcification. The interaction between pCO_2 and temperature was not significant but, at 1000 µatm, diel calcification increased from 0.14 µmol $CaCO_3 \cdot g^{-1}$ $DW \cdot d^{-1}$at 10°C to 2.20 µmol $CaCO_3 \cdot g^{-1}$ $DW \cdot d^{-1}$ at 19°C (Fig.7).

DISCUSSION

Studying ocean acidification in mesocosms represents a challenge because of the difficulties in reproducing field conditions in the laboratory with restricted material and time. Studies on the impact of elevated pCO_2 on coralline algae have shown strong species-specific responses (see Martin et al. 2013 for a review) which can vary between two studies on the same species. The different responses may be due to the timescale of the physiological measurement (short-term estimate vs integrated growth), the duration of the study (days vs months), the acclimation period or the time of the year (Hurd et al. 2009). In our study, we evaluated the impact of elevated pCO_2 at different temperatures on photosynthesis and calcification processes by measuring metabolic rates (gross primary production, respiration and calcification rates) in short-term measurements.

The gross primary production rate of *L. corallioides* measured under current pCO_2 (380 µatm) averaged 0.53 µmol $O_2 \cdot g^{-1}$ $DW \cdot h^{-1}$ at 10°C and 16°C. This production is estimated to be 0.45 µmol $C \cdot g^{-1}$ $DW \cdot h^{-1}$ by using a photosynthetic ratio of 1.17 (Martin et

al. 2006). It was close to the rates recorded *in situ* for the same species at 10 m depth in the Bay of Brest in winter (0.3 µmol C · g^{-1} DW · d^{-1}) and summer (2.1 µmol C · g^{-1} DW · d^{-1}; Martin et al. 2007). These values are slightly lower than the productivity calculated by Steller et al. (2007) at 10°C for *Lithothamnion margaritae* in the Gulf of California (3.45 µmol O_2 · g^{-1} DW · h^{-1}) and strongly lower than the rates reported for tropical coralline algae growing under higher irradiance and temperature (Payri 2000).

L. corallioides photosynthesis was not affected by temperature. This non responsiveness of photosynthesis to temperature was also observed in the rhodolith species *Phymatolithon calcareum* by Wilson et al. (2004). However, these findings contrast with previous results acquired *in situ* on *L. corallioides* (Martin et al. 2006) and *L. margaritae* (Steller et al. 2007) which showed strong variations in photosynthesis according to seasonal changes in temperature. In these previous studies, photosynthesis was higher in summer, when temperatures were the highest. In the present study, the seasonal cycle was not applied as temperature was increased in short steps, independently from light intensity, photoperiod and nutrient adjustments. It is likely that the changes in productivity observed by Martin et al. (2006) and Steller et al. (2007) were also related to the seasonal variations of other environmental parameters.

No pCO_2 effect was detected on *L. corallioides* respiration rate. However, gross production was significantly affected by pCO_2 with an increase at 550 µatm relative to 380 µatm. In addition, no interactive effect between increased temperature and pCO_2 was observed on photosynthesis and respiration. Macroalgal response to elevated pCO_2 has been investigated in numerous studies (see Wu et al. 2008 and Hurd et al. 2009 for a review). Responses varied among species, from an enhancement (Gao et al. 1991) to a decrease in

productivity (Gao & Zheng 2010) and many algae remained non impacted (Israel & Hophy 2002). As reported in *L. corallioides* under moderate pCO_2, other studies on coralline algae reported an increase in photosynthesis under elevated pCO_2 (Borowitzka 1981, Semesi et al. 2009, Cornwall et al. 2011, Hofmann et al. 2012). As a red coralline alga living in shaded environments, *L. corallioides* is likely to be a carbon concentrating mechanism (CCM)-lacking species (Giordano et al. 2005, Hepburn et al. 2011). Instead of using HCO_3^-, it may thus rely on CO_2 as photosynthetic substrate (Murru & Sandgren 2004, Hurd et al. 2009) and may be CO_2 limited in current pCO_2 (Kubler et al. 1999). The increased amount of CO_2 increases the affinity of the RuBisCO enzyme (Raven 2011) and may enhance gross primary production rate in *L. corallioides* under 550 µatm. This positive effect of increased pCO_2 decreased at 750 and 1000 µatm and may be attributed to the effect of lower pH level on periplasmic redox activity (Gao & Zheng 2010). Moreover, under elevated pCO_2, more energy can be required to cell maintenance, reallocated from down-regulating photosynthesis. The changes in photosynthesis may also be related to changes in algal photosynthetic pigments content according to pCO_2 and temperature treatments.

Among the main pigments determined in *L. corallioides* by HPLC, chl *a* compounds concentrations remained constant whatever the temperature and pCO_2 conditions. The absence of pCO_2 effect on algal chl *a* content was consistent with the results reported by Zou and Gao (2009) for *Gracilaria lemaneiformis*. Conversely, Gao and Zheng (2010) observed a down regulation of the chlorophyll content in *Corallina sessilis* under elevated pCO_2 that may be caused by a lower demand of energy for the HCO_3^- utilization mechanism. pCO_2 did not affect the carotenoids, but their concentrations decreased from 10°C to 16°C. This decrease between 10°C and 16°C may reflect an acclimation process from the *in situ* to the laboratory culture conditions. Such HPLC analyses do not allow the quantification of phycobiliproteins

which are hydro soluble proteins. Although Zou and Gao (2009) did not observe a CO_2 effect on phycobiliproteins in *G. lemaneiformis*, other authors have shown that they were negatively affected by elevated pCO_2 in the coralline algae *Corallina sessilis* (Gao & Zheng 2010). A down regulation of phycobiliprotein contents at 750 and 1000 µatm could explain the decrease in gross production at these elevated pCO_2 relative to the 550 µatm condition.

In macroalgae, under light conditions, photosynthetic activity lead to a pH increase in the intercellular spaces and in the diffusion boundary layer. As *L. corallioides* precipitate $CaCO_3$ in their cell wall (Giraud & Cabioch 1979), the pH increase in intercellular spaces shifts the equilibrium toward an increase in CO_3^{2-} concentration (Borowitzka 1981, Koch et al. 2012) and promotes precipitation of $CaCO_3$. Conversely, in dark conditions, respiration leads to a decrease in the intercellular pH, and hinder precipitation of $CaCO_3$. This "vital effect" demonstrated by numerous authors (Borowitzka 1979, Gao et al. 1993, Hurd et al. 2009, Raven 2011) may explain the higher rates observed in the light than in the dark. The persistent diffusion boundary layer at the surface of coralline algae creates a pH microenvironment very different from the mainstream seawater (Hurd et al. 2009, Hurd et al. 2011) with strong diel pH variations (ΔpH_{NBS}: 7.64 – 8.52; Hurd et al. 2011).

Calcification measured in *L. corallioides* at 380 µatm ranged between 0.58 and 0.60 mg $CaCO_3 \cdot g^{-1}$ DW $\cdot d^{-1}$, it was close to the rate recorded in situ for *L. corallioides* at 10 meters depth of 0.1 mg $CaCO_3 \cdot g^{-1}$ DW $\cdot d^{-1}$, in winter and 0.3 mg $CaCO_3 \cdot g^{-1}$ DW $\cdot d^{-1}$, in summer (Martin et al. 2006). The consistence between laboratory and *in situ* calcification data confirmed the good health and development of *L. corallioides* under experimental conditions.

Only dark calcification was positively affected by increased temperature from 10 to 16°C at 380 and 550µatm, and from 10 to 19°C at 1000 µatm. The effect of temperature on calcification rate was already underlined by several authors in various coralline algal species (Steller et al. 2007, Budenbender et al. 2011) and in particular in *L. corallioides* (Martin et al. 2006), in which calcification rates were the highest in summer, under warmer temperature. In our experiment, the temperature factor could not be dissociated from the time effect because temperature was progressively increased. Thus, even if temperature acclimation was quite long at each step (10, 16, 19°C), temperature impact on algal physiology has to be considered with caution. Particularly at 1000 µatm, increased calcification at 19°C may result from an acclimation of the physiology of *L. corallioides* to stressful pCO_2 condition.

The pCO_2 effect was more pronounced than the temperature effect on calcification with a decrease of the different calcification rates with increasing pCO_2. Diel calcification was lowered by 50% at 750 µatm to 80% at 1000 µatm. This general trend was already observed in several tropical coralline algae (Semesi et al. 2009, Anthony et al. 2008, Diaz-Pulido et al. 2012). In polar species, net calcification may even turn into net dissolution under elevated pCO_2 (Budenbender et al. 2011). This sensitivity of coralline algae to elevated pCO_2 is attributed to the high Mg-calcite they precipitate, which is the most soluble form of $CaCO_3$. Light calcification in *L. corallioides* was less impacted by pCO_2 than dark calcification, as reported for *Corallina pilulifera* (Gao et al. 1993). This may be attributed to the changes in pH at the site of calcification due to photosynthesis and respiratory activities. Temperature and pCO_2 acted antagonistically at 1000 µatm, where diel net calcification increased with temperature from 10 to 19°C. Although most of the studies show that temperature exacerbated the negative impacts of ocean acidification in coralline algae (Anthony et al. 2008, Martin & Gattuso 2009, Diaz-Pulido et al. 2012), other authors reported that

calcification remain unaffected under elevated temperature and pCO_2 (Johnson & Carpenter 2012). The mechanisms of the interactive effects between temperature and pCO_2 are yet not well understood and in our case can result from an acclimation process over time.

Rhodolith beds have low resilience in the face of major disturbances and are predicted to rapidly decline across the globe, at faster rates than those expected for coral reefs (Amado-Filho et al. 2012). Under the most optimistic future pCO_2 scenario (550 µatm), we showed that the gross production of rhodolith beds may be enhanced by 20% and that their calcification may be maintained at rates similar to current rates. Accordingly, rhodolith beds may contribute in a larger way to the productivity of the ecosystem. In the Bay of Brest, this may lead to an increase in productivity from 241 to 289.2 g $C \cdot m^{-2} \cdot y^{-1}$ (Martin et al. 2007). Moreover, soft red algae are largely present on rhodolith beds particularly microscopic stages of macroalgae as shown by the HPLC data. These life cycle stages are highly productive forms of macroalgae and may contribute in a large part to the global gross production of maerl beds. In summer, increase epiphytic macroalgal biomass has been observed on the rhodolith beds of the Bay of Brest (Guillou et al. 2002). Under elevated temperature, soft macroalgae could become more important in rhodolith beds. Some authors have shown that, fleshy algae are favored against calcareous algae under elevated pCO_2, (Kuffner et al. 2008, Anthony et al. 2008). In a context of global change, soft macroalgae may be favored in detriment of rhodolith, leading to major changes in rhodolith bed functioning and productivity.

It is likely that the 550 µatm level will be exceeded and pCO_2 will reach 750 to 1000 µatm by the end of the century (Gattuso & Hansson 2011). Under the previous pCO_2 levels, *L. corallioides* primary production may remain constant but calcification is likely to decrease

by up to 80% at 1000 µatm, relative to the ambient conditions. This may lead to a reduction in $CaCO_3$ precipitation from 490 (current pCO_2, Martin et al. 2007) to 97 g $CaCO_3 \cdot m^{-2} \cdot y^{-1}$ in the most pessimistic scenario (1000 µatm) in the Bay of Brest. The calcification process ($Ca^{2+} + 2HCO_3^- \leftrightarrow CaCO_3 + CO_2 + H_2O$) releases one mole of CO_2 for each mole of $CaCO_3$ precipitated (Wolf-Gladrow et al. 2007). In that way, the CO_2 released by maerl net calcification, currently estimated to 39 g $C \cdot m^{-2} \cdot y^{-1}$ (Martin et al. 2007), may be reduced to 8 g $C \cdot m^{-2} \cdot y^{-1}$ at 1000 µatm. Such changes in carbonate production induced by elevated pCO_2 will thus have major implications for carbon and carbonate budgets in rhodolith beds. The decrease in calcification in rhodolith is also likely to cause major habitat losses for numerous species (Amado-Filho et al. 2012) and main changes in the ecosystem services they provide as habitat, food provision, predation sheltering for early life stages of numerous marine species and nurseries for commercial invertebrate and fishes (Kamenos et al. 2004a,b).

ACKNOWLEDGMENTS

The authors thank the Marine Operations and Services Department (SMO) from the Station Biologique de Roscoff for all the diving sampling and the help for system building. In addition we are grateful to the SOMLIT (Service d'Observation en Milieu LITtoral, INSU-CNRS) program for the temperature and nutrient datasets provided to calculate seawater carbon parameters. We also thank the co-editor and two anonymous reviewers for their helpful and constructive comments on the manuscript. This work was supported by the CALCAO project, which received funding from the Region Bretagne and contributes to the ''European Project on Ocean Acidification'' (EPOCA) which received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement n_ 211384.

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Table 1: Mean seawater temperature and parameters of the carbonate system in each pCO_2 treatment (3 aquaria per treatment) and at each temperature level. The pH_T (on the total scale) and total alkalinity (A_T) were measured while other parameters were calculated. pCO_2 : CO_2 partial pressure; DIC : dissolved inorganic carbon; Ω_{Ar} : saturation state of seawater with respect to aragonite

		Temperature (°C)		pCO_2 (µatm)		pH_T		A_T (µEq · kg_{SW}^{-1})		DIC (µmol C · kg_{SW}^{-1})		Ω_{Ar}	
		n = 12		n = 12		n = 18		n = 12		n = 12		n = 12	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
10° C	380 µatm	10.3	0.1	365.19	11.07	8.09	0.01	2340.43	4.24	2138.96	8.74	2.28	0.04
	550µatm	10.3	0.1	516.42	9.32	7.95	0.01	2335.65	10.72	2189.47	2.68	1.73	0.02
	750 µatm	10.2	0.1	755.16	14.80	7.81	0.01	2350.56	4.63	2261.77	2.31	1.29	0.03
	1000 µatm	10.3	0.1	1023.54	23.32	7.68	0.01	2353.35	3.90	2300.56	2.67	1.00	0.02
16° C	380 µatm	16.1	0.0	378.83	5.62	8.07	0.01	2325.54	6.52	2085.34	3.81	2.67	0.02
	550µatm	16.2	0.1	606.95	12.84	7.89	0.01	2321.81	6.71	2162.50	3.55	1.90	0.03
	750 µatm	16.0	0.1	829.55	27.25	7.78	0.01	2339.50	3.63	2228.09	4.84	1.50	0.04
	1000 µatm	16.2	0.3	1048.81	19.60	7.68	0.01	2347.78	2.16	2267.13	2.39	1.25	0.02
19° C	380 µatm	18.5	0.1	440.33	23.66	8.02	0.02	2333.69	12.47	2117.93	18.00	2.71	0.08
	550µatm	18.9	0.1	547.73	8.64	7.94	0.01	2358.43	5.02	2158.41	2.95	2.33	0.03
	750 µatm	18.9	0.1	705.05	14.41	7.84	0.01	2355.13	6.25	2200.04	3.32	1.92	0.03
	1000 µatm	19.0	0.0	961.17	21.55	7.72	0.01	2366.73	2.55	2255.95	3.20	1.53	0.03

Table 2: Summary of three-way repeated measures ANOVA followed by SNK post hoc tests testing the effects of pCO_2, temperature and aquarium on the metabolic rates. Bold numbers indicate significant level < 0.05%

		Gross production	Respiration	Light calcification	Dark calcification	Diel calcification
		µmol $O_2 \cdot g^{-1}$AFDW $\cdot h^{-1}$	µmol $O_2 \cdot g^{-1}$AFDW $\cdot h^{-1}$	µmol $CaCO_3 \cdot g^{-1}$DW $\cdot h^{-1}$	µmol $CaCO_3 \cdot g^{-1}$DW $\cdot h^{-1}$	µmol $CaCO_3 \cdot g^{-1}$DW $\cdot h^{-1}$
	df	F p	F p	F p	F p	F p
pCO_2	3	4.771 **0.005***	1.151 0.337	15.700 **0.001*****	36.200 **0.001*****	31.201 **0.001*****
Temperature	2	1.381	3.590	2.919	3.660	1.990
	2	0.260	**0.034***	0.062	**0.032***	0.146
Aquarium		3.634	0.143	1.880	1.108	3.181
		0.158	0.873	0.296	0.436	0.181
pCO_2 x temperature	6	1.099	0.753	1.013	3.254	2.305
		0.375	0.610	0.427	**0.008****	0.050

**: p < 0.05, **: p < 0.01, ***: p < 0.001*

Post hoc SNK test for pCO_2 and temperature factors independently (p < 0.05)

pCO_2	550 > 380 µatm 550 > 750 > 380 µatm 550 > 1000 > 380 µatm		380 > 750 > 1000 µatm 550 > 750 > 1000 µatm	380 > 550 > 750 > 1000 µatm	380 > 750 > 1000 µatm 550 > 750 >1000 µatm
temperature		10°C < 16°C 10°C < 19°C		10°C > 16°C	

Table 3: Summary of three-way ANOVAs followed by SNK post hoc tests testing the effects of pCO_2, temperature and aquarium on pigment contents. Chlorophyll compounds are the sum of chl *a* and chlorophyllid *a*. Bold numbers indicate significant level < 0.05%.

		Chlorophyll *a* compounds		Carotene		Zanthophyll	
	df	F	p	F	p	F	p
pCO_2	3	0.467	0.499	1.673	0.204	0.001	0.979
Temperature	2	0.119	0.732	17.043	**<0.001*****	4.255	**0.009****

Aquarium	2	1.110	0.299	0.110	0.742		0.604	0.443
pCO_2 x temperature	6	0.000	0.999	0.359	0.553		0.007	0.936
*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$								
Post hoc SNK test ($p < 0.05$)								
Temperature				10°C > 16°C 10°C > 19°C			10°C > 16°C	

Figure 1: Schematic of laboratory experimental open set up with four pCO_2 conditions: 380 µatm as current pCO_2 condition and 550, 750 and 1000 µatm as elevated pCO_2 conditions. Arrows indicate ambient seawater flowing into the header tanks and subsequently treated water flowing through aquaria out of the system. All the aquaria were maintained at constant temperature in a thermostated bath. Each aquaria contained 18 *L. corralioides* thalli.

Figure 2: Evolution of pCO_2 and temperature during the experimental period in the four pCO_2 treatments. Grey bars represent the measurement periods were metabolic rates were assessed (10^{th} -12^{th} January; 16^{th} - 18^{th} February; 8^{th} - 10^{th} March 2011). Results are expressed as mean ± standard error, n = 3 (3 aquaria for each pCO_2 treatment).

Figure 3: Gross production (a) and respiration (b) rates in the four pCO_2 treatments at 10, 16 and 19°C. Results are expressed by mean ± standard error, n = 6.

Figure 4: Example of photosynthetic pigments analysis by high pressure liquid chromatography of a *Lithotamnion coralloides* thallus. Underlined pigments are associated to *L. coralloides*. Abbreviations are as suggested by Roy et al. (2001): Chlide a: chlorophillide a; Siph: siphonaxanthin; Fuco: fucoxanthin, c-Neo: 9'-cis-neoxanthin; Viola: violaxanthin;

Zea: zeaxanthin; Siph deriv: siphonaxanthin derivative; Chl: chlorophyll; Pheo: pheophytin; Car: carotene.

Figure 5: Chlorophyll compounds (a), carotene (b) and zeaxanthin (c) contents in the four pCO_2 treatments at 10°C, 16°C and 19°C. Results are expressed by mean ± standard error, n = 3 (or 6).

Figure 6: Calcification rates in the light (a) and dark (b) in the four pCO_2 treatments at 10°C, 16°C and 19°C. Results are expressed as mean ± standard error, n = 6.

Figure **7**: Diel calcification rates in the four pCO_2 treatments at 10, 16 and 19°C. Results are expressed as mean ± standard error, n = 6.

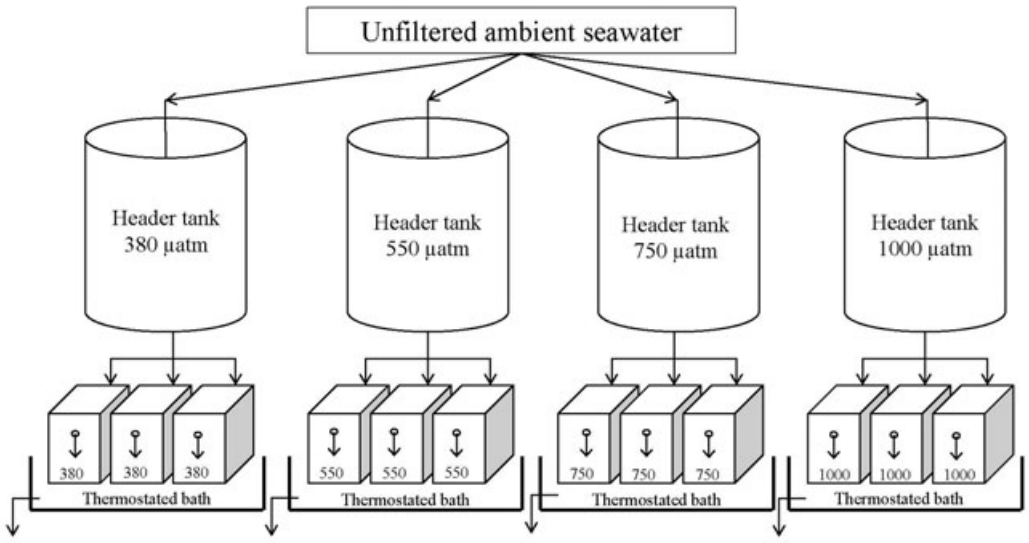

Figure 1

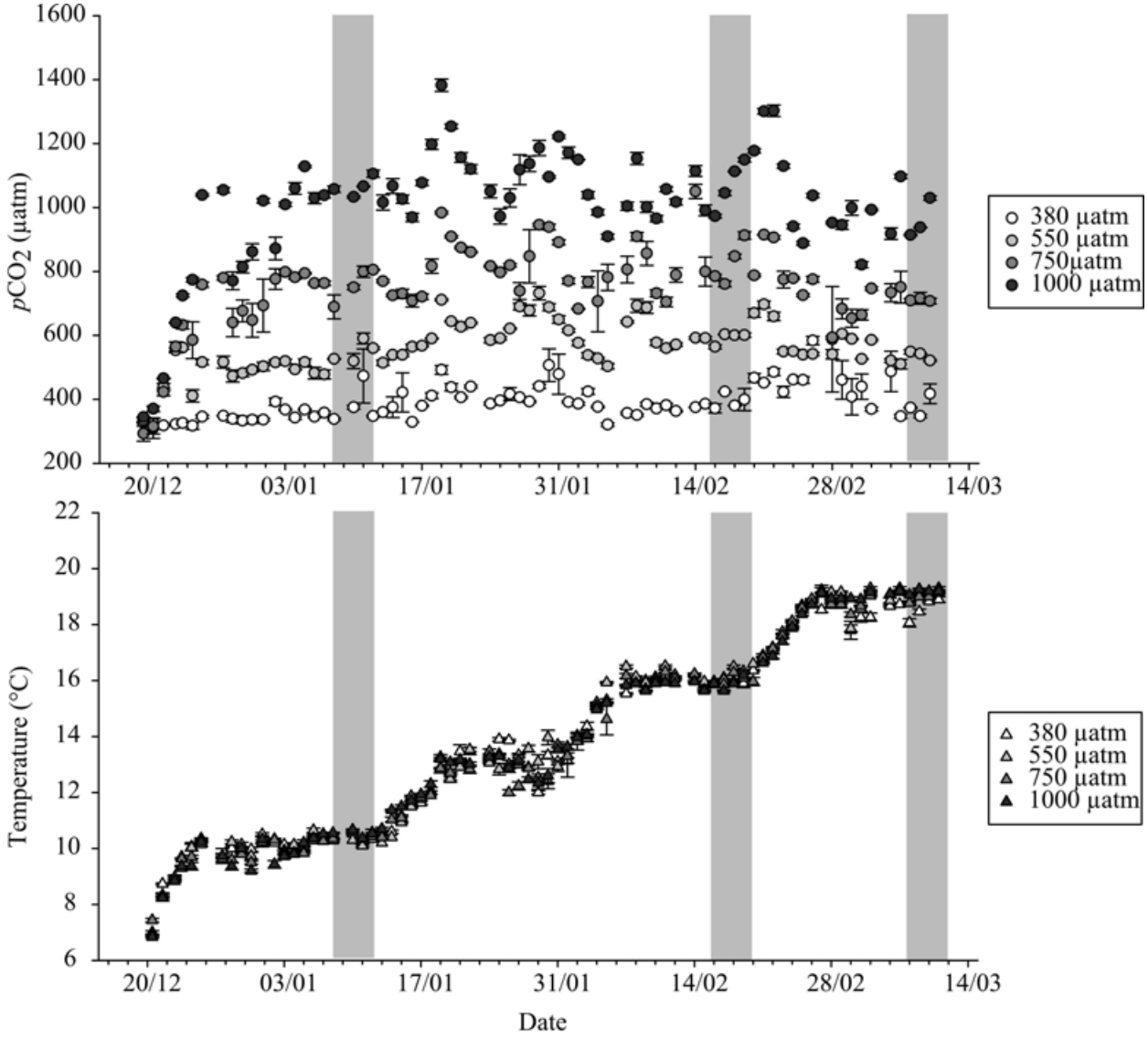

Figure 2

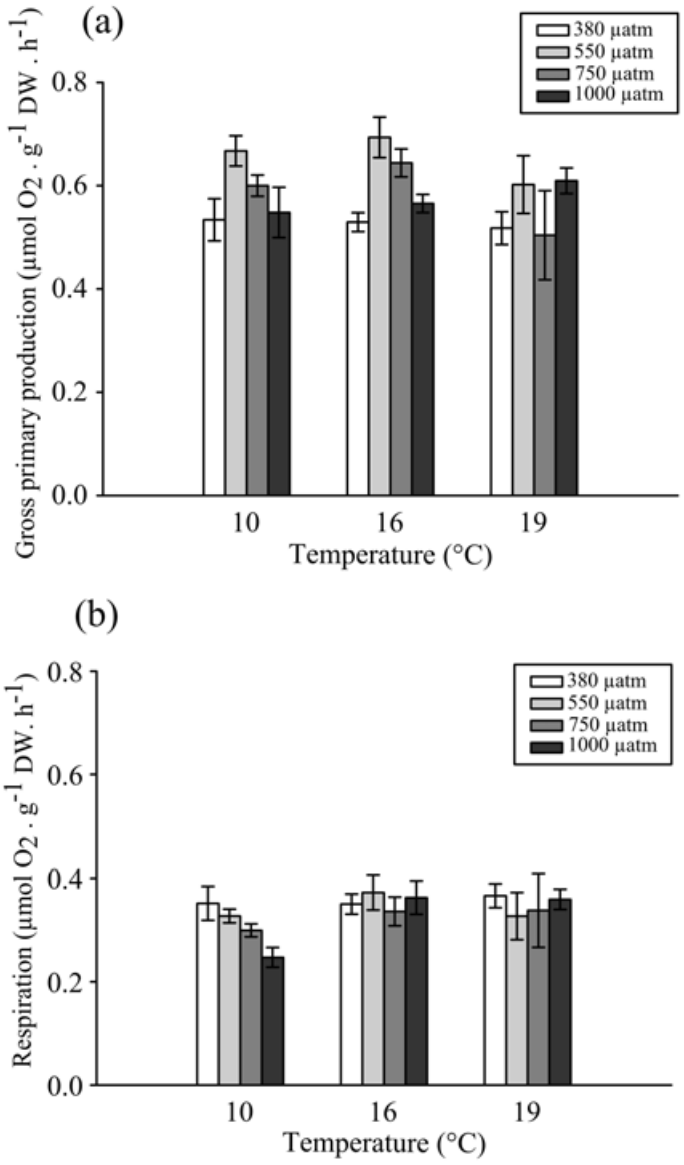

Figure 3

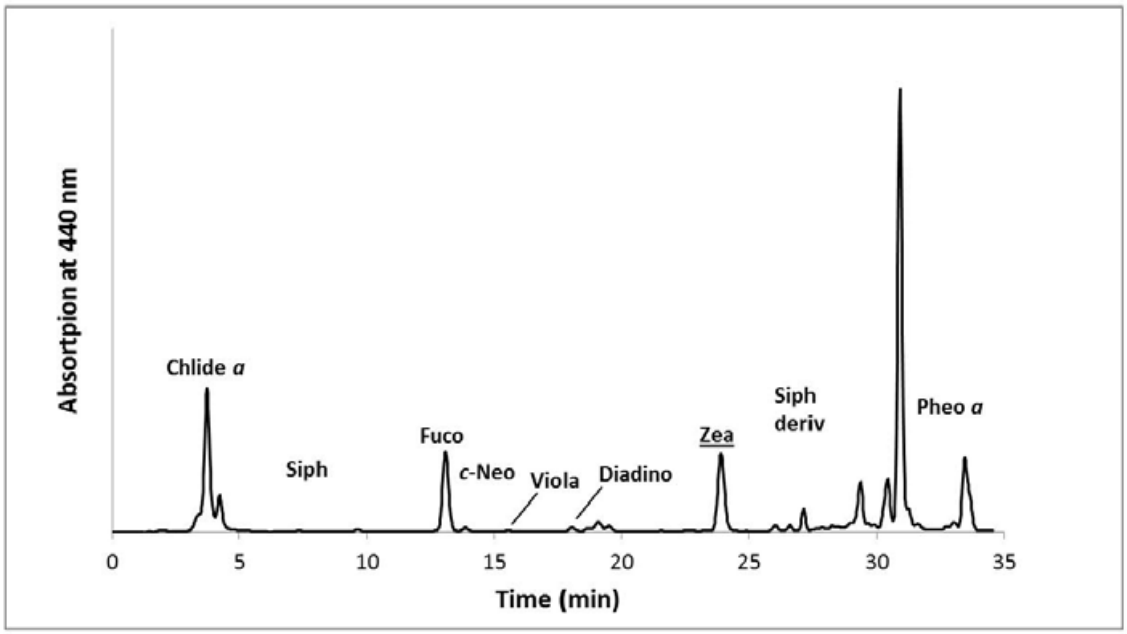

Figure 4

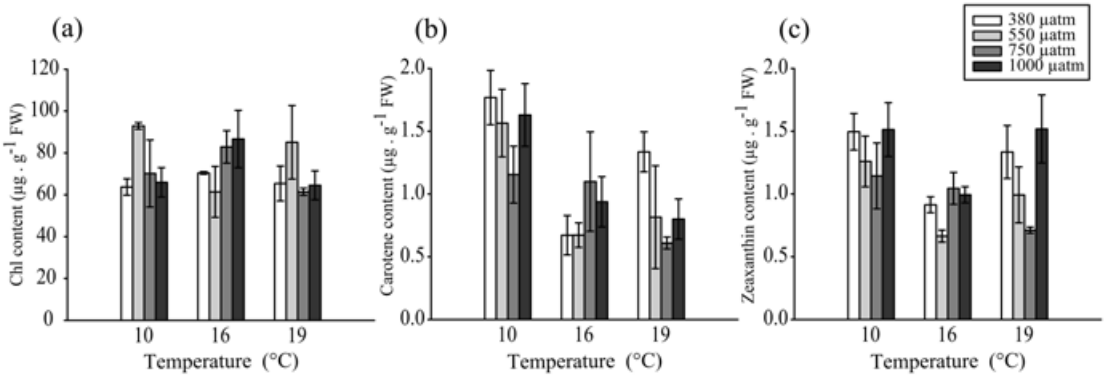

Figure 5

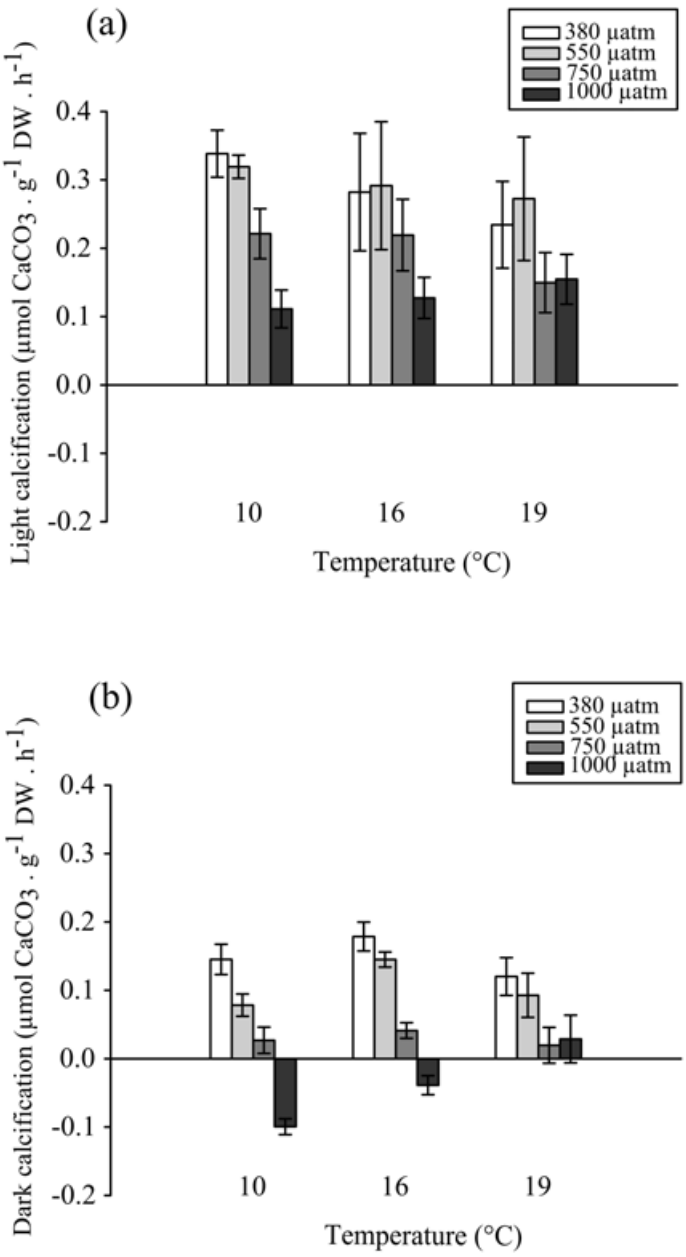

Figure 6

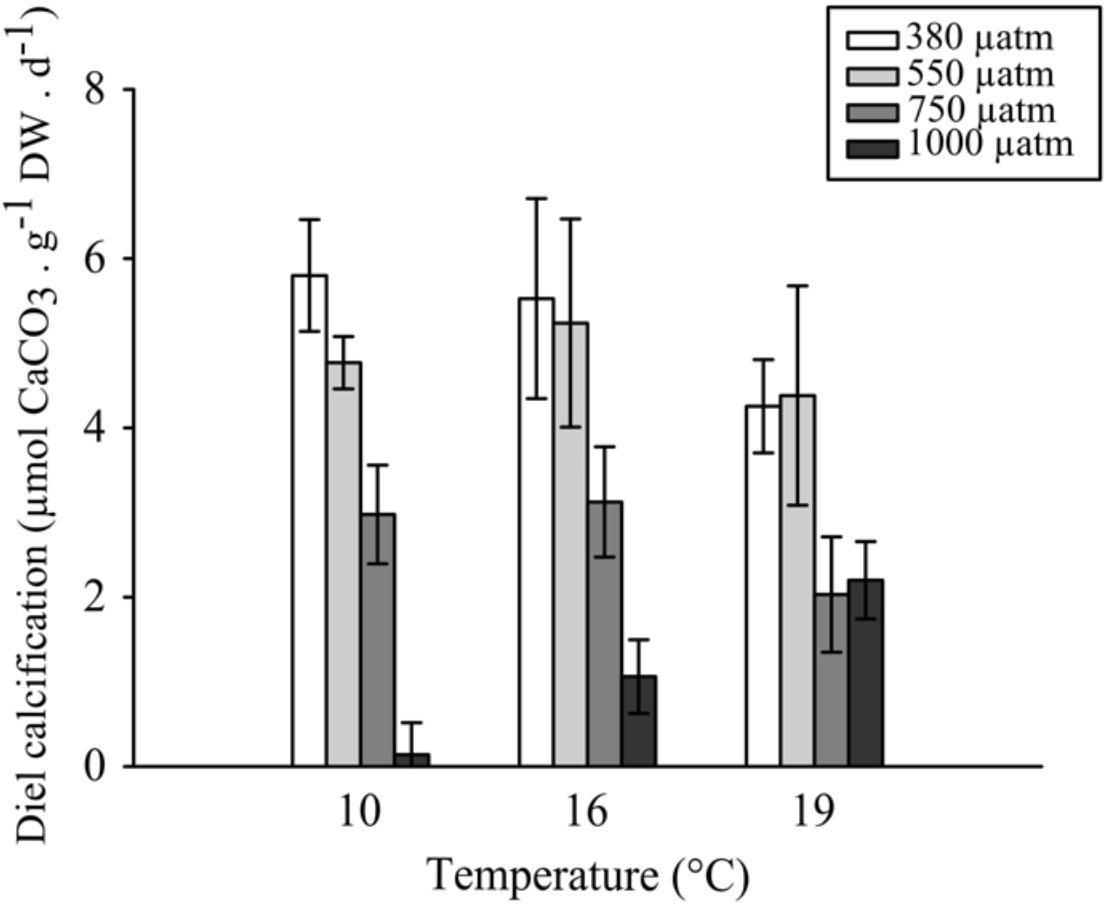

Figure 7