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Physiological responses of three temperate coralline algae from contrasting habitats to near-future ocean acidification

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ABSTRACT

Coralline algae are major calcifiers of significant ecological importance in marine habitats but are among the most sensitive calcifying organisms to ocean acidification. The elevated pCO_2 effects were examined in three coralline algal species living in contrasting habitats from intertidal to subtidal zones on the north-western coast of Brittany, France: (i) *Corallina elongata*, a branched alga found in tidal rock pools, (ii) *Lithophyllum incrustans*, a crustose coralline alga from the low intertidal zone, and (iii) *Lithothamnion corallioides* (maerl), a free-living form inhabiting the subtidal zone. Metabolic rates were assessed on specimens grown for one month at varying pCO_2: 380 (current pCO_2), 550, 750 and 1000 μatm (elevated pCO_2). There was no pCO_2 effect on gross production in *C. elongata* and *L. incrustans* but *L. incrustans* respiration strongly increased with elevated pCO_2. *L. corallioides* gross production slightly increased at 1000 μatm, while respiration remained unaffected. Calcification rates decreased with pCO_2 in *L. incrustans* (both in the light and dark) and *L. corallioides* (only in the light), while *C. elongata* calcification was unaffected. This was consistent with the lower skeletal *m*Mg/Ca ratio of *C. elongata* (0.17) relative to the two other species (0.20). *L. incrustans* had a higher occurrence of bleaching that increased with increasing pCO_2. pCO_2 could indirectly impact this coralline species physiology making them more sensitive to other stresses such as diseases or pathogens. These results underlined that the physiological response of coralline algae to near-future ocean acidification is species-specific and that species experiencing naturally strong pH variations were not necessarily more resistant to elevated pCO_2 than species from more stable environment.

1. Introduction

Coralline algae (Corallinaceae, Rhodophyceae) are the most dominant group of calcareous algae. They are abundant and widespread around the world from tropical to polar oceans and throughout the photic zone (Nelson, 2009). In benthic coastal areas, they are major framework builders and carbonate producers (Cabioch et al., 1992; Nelson, 2009). Corallinaceae developed different morphologies: geniculate (articulated) algae have erected, branched thalli with uncalcified joints between calcified segments; non-geniculate (non-articulated) algae are crusts attached to the substratum or occur as free-living forms called rhodolithes (Cabioch et al., 1992). In shallow waters where they develop, they have important biological and ecological roles (Foster, 2001) and are considered ecosystem engineers (Nelson, 2009). They participate in reef accretion acting as cement (Adey, 1998; Jokiel et al., 2008) or can build large habitats as coralligenous or rhodolith beds. They favor larval recruitment and settlement of marine invertebrates (Adey, 1998), acting as nurseries for some commercial molluscs and fishes (Kamenos et al., 2004a,b). They increase benthic biodiversity, providing hard substratum to settle and microhabitats for shelter (Foster, 2001; Grall et al., 2006; Pena and Barbara, 2010).

In temperate waters, coralline algae can be found at various depths, from the intertidal to the subtidal zone. In the intertidal zone, they frequently inhabit rock pools, forming dense mats at the edges or covering the bottom of the pools (Cabioch et al., 1992). Because rock pools are disconnected from the open sea at low tide, large pH variations are common in this habitat (Morris &Taylor, 1983). Diurnal variation often exceeds one pH units, as a result of photosynthesis and respiration (Björk et al., 2004; Morris and Taylor, 1983; Truchot and Duhameljouve, 1980). In the low intertidal zone where channels are formed in shallow waters, understory coralline algae develop on rocks and pebbles under the dense macroalgal canopy. Under the canopy, pH fluctuates according to depth, photosynthetic production, wave exposure, flow or irradiance and is likely to present strong diurnal variations of more than one unit (Middelboe and Hansen, 2007). In the subtidal zone, rhodoliths form large beds. These rhodolith beds grow in stable environments with reduced flow (Foster, 2001) and relatively low pH

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variation (Teichert et al., 2012). In all these habitats, coralline algae are important contributors to the global carbon budget and carbonate production through their high community primary production and respiration and their high calcium carbonate production (Amado-Filho et al., 2012; Bensoussan and Gattuso, 2007; Martin et al., 2005, 2007).

Photosynthesis, respiration and calcification are linked metabolic processes that can influence each other (Borowitzka, 1981; De Beer and Larkum, 2001; Gao et al., 1993; Martin et al., 2013). Via CO_2 uptakes and outputs, photosynthesis and respiration processes cause increase and decrease of pH respectively, in the intracellular medium and in the diffusive boundary layer (Raven and Hurd, 2012). These variations will increase the rate of calcification in the light and decrease it in the dark. Very few studies have investigated these processes all together, especially in coralline algae. Coralline species precipitate calcium carbonate ($CaCO_3$) containing magnesium (i.e. high magnesian calcite, Mg-calcite) to form their thallus. This biogenic $CaCO_3$ is more soluble than aragonite at mole percentage (mol%) $MgCO_3$ higher than 12% (Andersson et al., 2008). In the Corallinales order, the mean mol% $MgCO_3$ in calcite is 13% but varies depending on the taxa considered from 14% in *Corallina* genus or 25% in the *Lithothamnion* genus (Smith et al., 2012). Due to the solubility of their skeleton, coralline algae might be among the most sensitive organisms to CO_2-driven ocean acidification (Basso, 2012; Kroeker et al., 2010).

The ocean acidification phenomenon is generated by the constant increase in atmospheric CO_2 partial pressure (pCO_2) since the 1800s (Sabine et al., 2004). Surface ocean pH is predicted to decrease by 0.3–0.4 units by 2100 and by 0.7 units by the year 2300 (Caldeira and Wickett, 2003). Simultaneously, the concentration of bicarbonate ions (HCO_3^-) is predicted to increase and carbonate ions (CO_3^{2-}) concentration to drop by 30% by the end of the century (Orr et al., 2005). The $CaCO_3$ saturation state (Ω), which is dependent on the CO_3^{2-} concentration and influences $CaCO_3$ precipitation, is consequently expected to decrease (Feely et al., 2004). Such changes in seawater chemistry may have direct impacts on metabolic processes, particularly ones using dissolved inorganic carbon (DIC) as a substrate, and thus affect both calcifying and photosynthetic marine organisms such as coralline algae.

Responses to high pCO_2 of coralline algae belonging to different morphological or taxonomical groups are variable and species-specific (see Martin et al., 2013 for a review). Most of them are negatively affected with detrimental effects on recruitment (Kuffner et al., 2008), growth (Ragazzola et al., 2012), abundance (Martin et al., 2008), photosynthetic production (Anthony et al., 2008) and calcification (Gao and Zheng, 2010). Bleaching associated to mortality (Anthony et al., 2008; Diaz-Pulido et al., 2012) has also been found to increase in response to high pCO_2. Conversely, some authors reported a positive effect on photosynthetic (Borowitzka, 1981) and calcification processes (Martin et al., 2013) or parabolic responses of calcification to increased pCO_2 (Johnson and Carpenter, 2012; Ries et al., 2009). The variability of the algal responses also depends on the abiotic parameters applied during the experiments. For example, calcification of *Hydrolithon onkodes* measured under 336 μmol photons m^{-2} s^{-1} presented a parabolic response with the highest calcification rate under the intermediate levels of 530 μatm (Johnson and Carpenter, 2012) whereas a constant calcification decrease was measured under 1200 μmol photons m^{-2} s^{-1} on the same species (Diaz-Pulido et al., 2012). Hofmann et al. demonstrated with the same technique (PAM fluorometry) that photosynthesis in *Corallina officinalis* can decrease (Hofmann et al., 2012a) or remain stable (Hofmann et al., 2012b) with an increase in pCO_2. As responses of living organisms are so varying, applying the same abiotic parameters is required to compare species-specific responses.

In the present study, the metabolic processes of photosynthesis, respiration and calcification were investigated simultaneously in different algal species from contrasting habitats in response to elevated pCO_2. Assuming that organisms inhabiting highly variable environments are likely to be more robust to ocean acidification (Harley et al., 2012; Raven et al., 2012) and able to tolerate high pH/pCO_2 fluctuations, we hypothesized that coralline algae living in fluctuating habitats (intertidal rock pools and channels) will be less affected by elevated pCO_2 than algae from more stable subtidal environments. We investigated the physiological responses of three different algal species: *Corallina elongata*, an erected coralline alga from rock pools; *Lithophyllum incrustans*, a pink thick crustose coralline alga (CCA) which covers the pebbles in tidal channels and *Lithothmanion corallioides*, also called "maerl", a key species forming rhodolith beds in the subtidal zone.

2. Methods

2.1. Biological material

Three coralline algal species living in contrasting environments from the intertidal to the subtidal zone on the north-western coast of Brittany were selected for this experiment.

(1) *C. elongata* Ellis and Solander, 1786, is a geniculate alga erected from a basal crust, composed of numerous articulated calcareous branches. It is a perennial species from the intertidal zone, abundant on exposed shores, which forms a continuous mat at rock pool edges along Atlantic and Mediterranean coasts (Cabioch et al., 1992). Specimens of *C. elongata* were sampled on October 11th, 2010 in a shaded rock pool on the low intertidal shore of "Les Amiets", Cléder (48°41.45′N, 4°7.26′W). Algal fronds free of epiphytic organisms were selected for the experiment and carefully separated from their substrate to obtain their encrusting base. In October 2012, under sunny conditions, temperature fluctuated by about 1 °C, from 16.4 °C just after disconnection from the sea (pool emersion) to 17.5 °C just before immersion at rising tide. Changes in temperature between the night and day can reach 4 °C in such low intertidal shaded rock pools (see Egilsdottir et al., 2013). The pH on the total scale (pH_T) in such a rock pool can vary locally according to the depth of the pool and the presence of other macroalgae between 8.61 and 7.82, corresponding to 70 and 1000 μatm, respectively (see Egilsdottir et al., 2013). The photosynthetic active radiations (PAR) measured using a flat quantum sensor (LiCor®, LI-192 SA) at midday under sunny conditions at the surface of the pool was around 30 μmol photons m^{-2} s^{-1}. This low light was due to a large rock overhanging the pool and shading the pool area along the day.

(2) *L. incrustans* Philippi, 1837, is a non-geniculate coralline alga forming thick pink/grey crusts covering the substrate. The thallus surface is variable in terms of color, thickness and shape, the oldest ones forming thick, rippled and peeled off margins (Steneck, 1986). This species is usually immersed (Littler, 1972) and can be found in rock pools and in the sub-canopy in the low intertidal. Small pebbles entirely covered by *L. incrustans* were collected on October 13th, 2010 in the middle of the Green Island Channel, front of the *Station Biologique de Roscoff* (48°43.73′N, 3°59.22′W). Selected thalli were completely pink, (without white patches) characteristic of healthy crusts. In this channel, depth can vary between a few centimetres to meters between high and low tides and spring and neap tides. Abiotic parameters were measured in October 2012 at low water mean spring tide (similar environmental conditions as during the algal collection). Temperature varied from 17.5 °C at midday on a sunny day to 16.2 °C during the night. Under the dense *Sargassum muticum* canopy, pH_T at low tide fluctuated between 7.83 ($pCO_2 \approx 700$ μatm) during the night and 8.74 ($pCO_2 \approx 50$ μatm) during the day, under sunny

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conditions. Incident irradiance measured at the surface at midday during low tide using a flat quantum sensor (LiCor®, LI-192 SA) was around 800 μmol photons m^{-2} s^{-1}. Using an under-canopy extinction coefficient calculated in laboratory (coefficient ≈ 16.2; Noël, unpublished data), incident irradiance was estimated less than 50 μmol photons m^{-2} s^{-1} in the understory.

(3) *L. corralioides* crouan & crouan, 1867 is a non-geniculate free-living form of coralline algae. This species forms extensive beds, called rhodolith or "maerl" beds, by accumulating live and dead thalli (Foster, 2001). *L. corralioides* thalli were collected by SCUBA diving on October 13th, 2010, in a maerl bed of the Bay of Morlaix at the Guérhéon site (48° 42.66′N, 3°57.06′W), at 7 m depth below Chart Datum. Individuals between 1.5 and 3 cm in diameter were selected for the experiment. Abiotic parameters at the Guérhéon site were characterized in October 2012. Temperature was stable around 16.3 °C. pH_T varied between 8.12 and 8.18 units before and after high tide (290–340 μatm). Irradiance measured with a PAR spherical sensor (biospherical QSP200PD) at 9 m depth reached 27 μmol photons m^{-2} s^{-1} in the midday under cloudy (but bright) conditions which corresponds to 15 μmol photons m^{-2} s^{-1} with a flat sensor (factor conversion: 0.57 see Ouisse et al., 2011).

2.2. Experimental conditions and set-up

After collection, all samples were transferred directly to a cool box maintaining *in situ* temperature and carried to the laboratory at the *Station Biologique de Roscoff*. Specimens were selected, gently cleaned to remove most epiphytes and biofilm forming organisms and were maintained in natural unfiltered seawater until the beginning of the experiment.

Sets of 4–6 *C. elongata* fronds, 5–6 *L. incrustans* pebbles and 4–5 *L. corralioides* thalli were labeled with small plastic numbers attached with nylon wire. Two sets of each algal species were randomly distributed to each of the twelve 10-L aquaria composing the experimental set up. In addition, unlabelled thalli were kept in each aquarium for chlorophyll analyses. The thalli were softly brushed to take off epiphytes and biofilm before proceeding to the different measurements. Dry weight (DW) of each alga was determined at the end of the experiment after oven drying fresh samples at 60 °C for 48 h. Then, thalli were burned for 4 h at 450 °C to obtain ash-free dry weight (AFDW).

At the beginning of the experiment, (October 19th to 26th, 2010), pH was progressively decreased by 0.05 pH units per day by gradually increasing the pCO_2 to avoid algae any drastic stress. Then, the organisms were acclimated for one month (October 26th to November 23th, 2010) to the different pCO_2/pH_T conditions reached, selected according to the recommendations of Riebesell et al. (2010): 380 μatm (pH_T = 8.07) was selected as the current pCO_2, and 550 μatm (pH_T = 7.94), 750 μatm (pH_T = 7.82) and 1000 μatm (pH_T = 7.77) as three elevated pCO_2 corresponding to different scenarios predicted by the Intergovernmental Panel on Climate Change (IPCC) for the end of the century (Solomon et al., 2007). The pCO_2 were adjusted by bubbling CO_2-free air to increase pH (current atmospheric pCO_2) or pure CO_2 to decrease pH (elevated pCO_2) in four 100 L header tanks. These tanks were continuously supplied with unfiltered seawater pumped in from the 1800 m^3 water reservoir of the *Station Biologique de Roscoff* that fills up at high tide. Seawater was delivered from each tank to a triplicate of aquaria at a rate of 100 ml min^{-1} (i.e. a renewal rate of 60% h^{-1}). The 12 aquaria were placed in temperature controlled baths regulated by 100 and 150 W submersible heaters at 16 °C ± 0.02 °C (October mean *in situ* temperature). pCO_2 and temperature were monitored and controlled by an off line feedback system (IKS Aquastar, Karlsbad, Germany) that regulated the addition of gas in the tanks and the on/off heater switch in the temperature controlled bath. The pH values of the pH–stat system were adjusted from daily measurements of pH on the total scale (pH_T) in the aquaria using a pH meter (HQ40D, Hach Lange, Ltd portable LDO™, Loveland, Colorado, USA) calibrated using Tris/HCl and 2-aminopyridine/HCl buffer (Dickson et al., 2007). Light was provided by 39 W fluorescent tubes (JBL Solar Ultra Marin Day, JBL Aquaria, Nelson, New Zealand). Irradiance was fixed at a mean value of 30 μmol photons m^{-2} s^{-1}, representative of mean daily *in situ* irradiance in the three habitats, by adjusting the distance of the fluorescent tubes above the aquaria and using a quantum sensor (LiCor®, LI-192 SA). The photoperiod was adjusted to 9:15 (light:dark, h) corresponding to the mean photoperiod in Autumn.

2.3. 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, Colorado, USA). Total alkalinity (A_T) was measured in the four pCO_2 treatments by HCl 0.01 N potentiometric titration on an automatic titrator (Titroline alpha, Schott SI Analytics, Mainz, Germany) at the beginning of the acclimation period (28th October 2010) and during the acquisition of metabolic rates (23th–26th November 2010). Salinity was checked in each aquarium at the beginning, twice during the experiment and at the end of the experiment with a conductimeter (LF 330/SET, WTW, Weilheim, Germany). The carbonate chemistry of the seawater, *i.e.* dissolved inorganic carbon (DIC), exact CO_2 partial pressure (pCO_2) and saturation state of aragonite (Ω_{Ar}, because solubility of high Mg-calcite is closer to aragonite than calcite) were calculated for each aquarium using CO_2SYS software (Lewis and Wallace, 1998) with constants of Mehrbach et al. (1973) (refitted by Dickson and Millero, 1987). Mean values of the parameters in each pCO_2 condition (3 aquaria per condition) are presented in Table 1.

2.4. mMg/Ca, chlorophyll a and bleaching analyses

The mol% Mg/Ca (*m*Mg/Ca) ratio was analyzed in three samples per algal species, only in the control condition (380 μatm) at the end of the experiment. The algae did not grow enough in one month to produce sufficient quantity of carbonate to perform comparisons between pCO_2 treatments. Samples were cleaned with distilled water, dried by paper towel tapping, bagged and sent to *the Institute of Earth Sciences,*

Table 1
Mean temperature and parameters of the carbonate chemistry in each pCO_2 treatment. pH_T (on the total scale) and temperature were measured daily. Total alkalinity (A_T) was measured at the beginning of the acclimation time and during metabolic measurements. Other parameters were calculated using CO2sys software. pCO_2: CO_2 partial pressure; Ω_{Ar}: saturation state of seawater with respect to aragonite.

pCO_2 treatments	Temperature (°C)	pH_T	A_T (μEq)	pCO_2 (μatm)	Ω_{Ar}
	n = 32	n = 32	n = 35	n = 32	n = 32
380 μatm	16.0 ± 0.1	8.01 ± 0.01	2401.92 ± 3.78	450 ± 7	2.41 ± 0.03
550 μatm	15.9 ± 0.1	7.88 ± 0.01	2402.07 ± 3.08	637 ± 23	1.88 ± 0.05
750 μatm	15.9 ± 0.1	7.80 ± 0.01	2414.49 ± 4.18	790 ± 22	1.58 ± 0.03
1000 μatm	15.9 ± 0.1	7.70 ± 0.01	2418.53 ± 5.16	1002 ± 24	1.29 ± 0.03

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University of Iceland. Small samples (<0.01 g) of the skeletal material of each alga were placed in 20 ml polyethylene vials which had been cleaned by soaking for 3 days in 5% HNO_3 and then washed with distilled water and dried. To dissolve calcium carbonate, 1 ml of 65% HNO_3 suprapure acid (Merck, Germany) was added to the samples and left to dissolve for 14 h, after which 4 ml of deionised purified water (Milli-Q purification system, Millipore, USA) was added to the sample. The mol% Mg/Ca (*m*Mg/Ca) ratios were analyzed at the *University of Iceland Institute of Earth Sciences*, with an Inductively Coupled Plasma-Atomic Emission Spectrometer (ICP-AES Spectro Ciros™, Germany). The ICP-analysis was calibrated with mixtures of NIST-traceable single element solutions (Spex Industries Inc. NJ, USA).

Chlorophyll *a* (Chl *a*) content was measured in the three algal species exposed to each pCO_2 treatment at the end of the experiment. Samples from the additive algal pool of each aquarium were removed and immediately frozen at −20 °C pending analyses. Branches of *C. elongata* fronds (≈50 mg) and pieces of *L. corallioides* thalli (≈1 g) were removed from the samples just taken out of the freezer. Pink surfaces of *L. incrustans*, around 1 cm^2 per thallus, were scratched with a scalpel to pick out the living cell layer. All the samples were weighed and then ground in 10 ml 90% acetone with a cold mortar pestle, on an ice bath, under dark conditions. The extract was poured into 15 ml centrifuge tubes and placed in the dark at 4 °C overnight. Samples were then centrifuged for 20 min at 4000 rpm. Total Chl *a* concentration in the supernatant was determined according to the method of Ritchie (2008), using a spectrophotometer (Helios Gamma, Thermo Electron Corporation, England). Two successive extractions were necessary for a complete Chl *a* extraction.

Bleaching was evaluated at the end of the experiment. White patches of thalli, characteristic of bleaching only occurred in *L. incrustans*. The surfaces of incubated alga sets were photographed at the end of the experiment. Images were analyzed with ImageJ software (Rasband, version 1.37) to calculate the percentage of the bleached thallus surfaces.

2.5. Metabolic rates measurements

Each set of labeled thalli was incubated between the 23th and the 26th November, once in the light and once in the dark, in 80 mL (*L. incrustans* and *L. corallioides*) or 190 mL (*C. elongata*) acrylic respirometry chambers (Engineering & Design Plastics Ltd, Cambridge, UK) filled with the aquarium seawater. Water homogeneity was insured by hand shacking and temperature was kept constant. Light incubations were carried out under culture irradiance (30 µmol photon m^{-2} s^{-1}) and dark incubations by covering the aquaria with black plastic bags with fluorescent tubes switched off. Incubations lasted around 3 h in order to avoid oxygen saturation greater than 120% during light incubation and maintain oxygen saturation above 80% at the end of the dark incubation. In parallel, control incubations without algae were carried out to correct fluxes from any biological activity in seawater.

Net production (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, Regensburg, Germany). The reactive oxygen spots in the chambers were calibrated just before the beginning of the measurements with 0% and 100% oxygen buffers. Net production (NP), respiration (R) and gross production (GP) rates (in µmol O_2 g^{-1} AFDW h^{-1}) were corrected from controls and calculated as:

$$NP = (\Delta O_2 \times V)/(\Delta t \times AFDW)$$
$$R = (\Delta O_2 \times V)/(\Delta t \times AFDW)$$
$$GP = NP - R$$

where ΔO_2 is the difference between initial and final O_2 concentrations in µmol O_2 L^{-1}; V is the volume of the chamber in liters; Δt is the incubation time in hour and AFDW is the ash-free dry weight of the algae in grams.

Calcification fluxes were estimated by 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). Seawater was sampled directly in the aquaria at the beginning of the incubation and in the incubation chamber at the end. 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 µEq L^{-1}) were determined by HCl 0.01 N potentiometric titration on an automatic titrator (Titroline alpha, Schott SI Analytics, Mainz, Germany) and by using the Gran method of 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 light and g dark, in µmol $CaCO_3$ g^{-1} DW h^{-1}) were corrected from controls and calculated as:

$$g = -(\Delta A_T \times V)/(2 \times \Delta t \times DW)$$

where ΔA_T is the difference between initial and final total alkalinity concentrations in µEq L^{-1}; V is the volume of the chamber in liters; Δt is the incubation time in hour and DW is the dry weight of the algae in grams.

2.6. Statistics

All statistical analyses were performed using the free software R 2.15.0 version (©The R Foundation for Statistical Computing). Before applying each test, normality of the data and homoscedasticity were checked by Shapiro's test and Levene's test respectively. Differences in *m*Mg/Ca ratio between the three algal species at 380 µatm and percentage of bleaching among the different pCO_2 treatments in *L. incrustans* were explored by one-way analysis of variance (ANOVA) following by post hoc Student–Newman–Keuls (SNK) test. Because of heterogeneous variances, chlorophyll contents among the algal species and among the pCO_2 conditions were compared by two different Kruskal–Wallis tests followed by post hoc Dunn's tests. The effect of pCO_2 on metabolic rates was investigated with the GAD package independently for each alga. All the metabolic rates were explored through nested two-ways ANOVA considering "pCO_2" as a fixed factor with 4 levels (390, 550, 750 and 1000 µatm) and "aquarium" as a 3 level random factor nested in the "pCO_2" one to deal with spatial pseudo-replication. In cases of significant differences between treatments, a post hoc Student–Newman–Keuls test was applied to explore them. All results are presented as mean ± standard error.

3. Results

3.1. Seawater parameters

Salinity remained stable at 35.2 ± 0.1 during the experiment. Mean values of the seawater parameters i.e. temperature, pH_T, alkalinity, pCO_2 and calcium carbonate saturation state relative to aragonite, in each pCO_2 condition (3 aquaria per condition) are presented in Table 1.

3.2. mMg/Ca ratios, chlorophyll a contents and bleaching

*m*Mg/Ca ratios were 0.169 ± 0.002, 0.202 ± 0.006 and 0.202 ± 0.009 mol for *C. elongata*, *L. incrustans* and *L. corallioides* respectively. The three algal species showed differences in their skeletal composition (Table 2). *L. incrustans* and *L. corallioides*, the two species which have the closest morphotypes, had similar percentages (Table 2) of about 20% $MgCO_3$. *C. elongata* precipitated calcite with less magnesium than the other species (Table 2) with 17% $MgCO_3$.

Chlorophyll *a* contents in the living part of the algae (Table 3) did not differ among pCO_2 treatments regardless of algal species (Table 2) while differences appeared between species (Table 2). Chlorophyll *a* content

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Table 2
Results of the one-way ANOVAs testing the differences of *m*Mg/Ca ratios between the three algal species at 380 µatm and the effects of pCO_2 on bleaching in *L. incrustans*. Chlorophyll contents (algal and pCO_2 effects) were explored by two Kruskall–Wallis tests.

		*m*Mg/Ca ratio		Bleaching		Chorophyll contents	
	df	F	p	F	p	H	p
Factor: pCO_2	3			7.337 *SNK test p < 0.05* 380 < 1000 380 < 750 550 < 1000	0.002	0.140	0.987
Factor: alga	2	9.241 SNK test p < 0.05 *C. elongata* < *L. incrustans* *C. elongata* < *L. corallioides* *L. incrustans* = *L. corallioides*	0.015			63.160 Dunn's test p < 0.05 *C. elongata* > *L. incrustans* > *L. corallioides*	<0.001

was the highest in *C. elongata*, intermediate in *L. incrustans* and the lowest in *L. corallioides*.

Bleaching occurred only in *L. incrustans* thalli and was observed in all pCO_2 treatments. The percentage of bleached surface at the end of the experiment was significantly affected by pCO_2 (Table 2) and increased with increasing pCO_2 from 1% of the whole surface of the thalli bleached at 380 µatm to more than 10% at 1000 µatm (Fig. 1).

3.3. Metabolic rates

In all the metabolic rates, no aquarium effect was detected ($p > 0.05$, Table 4).

In *C. elongata*, the mean rates of net production varied from 12.62 (550 µatm) to 17.02 µmol O_2 g^{-1} AFDW h^{-1} (380 µatm) while respiration and gross production rates reached maxima of −11.87 and 28.89 µmol O_2 g^{-1} AFDW h^{-1}, respectively (Fig. 2A). Gross production was slightly decreased at 550 µatm (−24% relative to 380 µatm) whereas net production and respiration were not affected by elevated pCO_2 (Table 4). Calcification rates in the light and dark were positive except at 1000 µatm in the dark (Fig. 2B). No pCO_2 effect was detected on calcification both in the light and dark. Mean net calcification ranged from 0.93 ± 0.71 µmol $CaCO_3$ g^{-1} DW h^{-1} at 550 µatm to 1.81 ± 0.70 µmol $CaCO_3$ g^{-1} DW h^{-1} at 1000 µatm in the light and from 0.78 ± 0.33 µmol $CaCO_3$ g^{-1} DW h^{-1} at 550 µatm to −0.28 ± 0.41 µmol $CaCO_3$ g^{-1} DW h^{-1} at 1000 µatm in the dark. Dissolution (negative net calcification) only occurred at 1000 µatm in the dark.

Net production of *L. incrustans* varied from −0.91 to 0.23 µmol O_2 g^{-1} AFDW h^{-1}. Gross production rates were not affected by elevated pCO_2 but conversely, respiration increased with increasing pCO_2 and net production was also affected (Table 4). Gross production ranged from 2.26 to 2.58 µmol O_2 g^{-1} AFDW h^{-1} and respiration rate increased from −2.04 (380 µatm) to −3.49 O_2 g^{-1} AFDW h^{-1} (1000 µatm). Calcification in the light and in the dark (Fig. 2B) decreased with increasing pCO_2, as we observed a drop of 185.7% between 380 and 1000 atm in the light (from 0.08 ± 0.03 to −0.07 ± 0.03 µmol $CaCO_3$ g^{-1} DW h^{-1}) and 250% in the dark (from −0.06 ± 0.01 to −0.21 ± 0.03 µmol $CaCO_3$ g^{-1} DW h^{-1}). Dissolution, as the net calcification rates were negatives, occurred in the light only under elevated pCO_2 (750 and 1000 µatm) and in all the conditions in the dark.

Table 3
Chlorophyll a content in the three coralline algal species in each pCO_2.

	Chlorophyll concentrations (mg chlorophyll g^{-1} AFDW)			
	380 µatm	550 µatm	750 µatm	1000 µatm
C. elongata	2.04 ± 0.10	1.74 ± 0.13	1.56 ± 0.24	1.73 ± 0.06
L. incrustans	1.46 ± 0.03	1.69 ± 0.18	1.89 ± 0.10	1.94 ± 0.24
L. corallioides	1.15 ± 0.04	1.06 ± 0.03	1.13 ± 0.07	1.10 ± 0.05

L. corallioides net production rates ranged between 6.39 and 9.23 µmol O_2 g^{-1} AFDW h^{-1}, at 750 and 380 µatm respectively. Gross production rates increased from 8.73 to 13.23 µmol O_2 g^{-1} AFDW h^{-1} and respiration rates ranged between −1.55 (750 µatm) and −4.00 O_2 g^{-1} AFDW h^{-1}(1000 µatm). Elevated pCO_2 affected respiration, net and gross production (Table 4) by enhancing primary production at 1000 µatm and decreasing respiration at 750 µatm (Fig. 2A). With a mean of 0.38 ± 0.07 µmol $CaCO_3$ g^{-1} DW h^{-1}, calcification measured in the light was much higher than calcification in the dark (Fig. 2). pCO_2 effects on light calcification were significant ($p = 0.043$, Table 4) even though post hoc comparison tests did not show any significant differences between pCO_2 conditions. Dark calcification was not significantly affected by pCO_2 (Table 2). However, a general trend showed a decrease in calcification rates from 0.14 ± 0.06 at 380 µatm to −0.03 ± 0.08 µmol $CaCO_3$ g^{-1} DW h^{-1} at 1000 µatm in the dark (Fig. 2B). Dissolution occurred in the two most elevated pCO_2 condition (750 and 1000 µatm) only in the dark.

4. Discussion

Calcifying marine algae show a large variety of responses to ocean acidification (Hurd et al., 2009) and species-specific responses may be developed by algae from the same family living in contrasting habitats to cope with abiotic changes (Harley et al., 2012).

Photosynthesis in the three investigated algal species was not strongly impacted by increasing pCO_2. Indeed, in *C. elongata*, gross primary production was not affected by increasing pCO_2 except for an inconsiderable decrease at 550 µatm. *L. incrustans* gross production remained constant

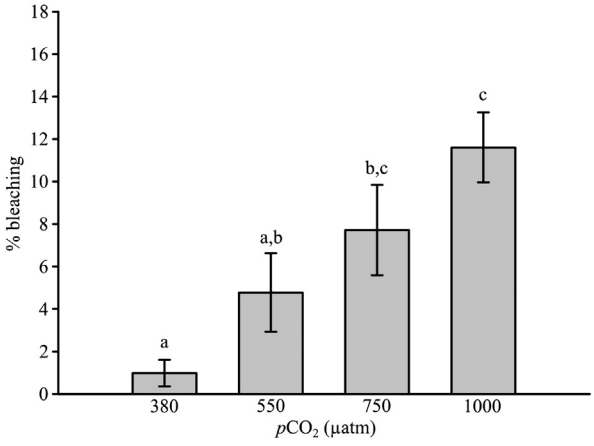

Fig. 1. Percentage of bleaching in *Lithophyllum incrustans* thalli in each pCO_2 treatment. Unshared letters above bars indicate significant differences between treatments ($p < 0.05$, SNK post hoc test), $n = 6$.

Table 4
Results of the one-way nested ANOVA testing the effects of pCO_2 on the metabolic rates in the three coralline algal species.

		Net production		Respiration		Gross production		Light calcification		Dark calcification	
		μmol O_2 g^{-1} AFDW h^{-1}		μmol O_2 g^{-1} AFDW h^{-1}		μmol O_2 g^{-1} AFDW h^{-1}		μmol $CaCO_3$ g^{-1} DW h^{-1}		μmol $CaCO_3$ g^{-1} DW h^{-1}	
pCO_2 effect	df	F	p	F	p	F	p	F	p	F	p
C. elongata	3	3.143	0.087	2.250	0.160	11.136	0.003**	0.659	0.600	1.431	0.304
L. incrustans	3	18.608	<0.001***	5.328	0.026*	0.133	0.938	18.262	<0.001***	11.908	0.003**
L. corallioides	3	6.612	0.015*	7.271	0.011*	9.573	0.005**	4.340	0.043*	1.211	0.366

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

at all the pCO_2 treatments while *L. corallioides* gross production increased at 1000 μatm. This general weak pCO_2 effect on coralline algal photosynthesis has already been demonstrated by several authors. For example, photosynthetic rates did not vary in response to increasing pCO_2 in the articulated coralline *C. officinalis* (Hofmann et al., 2012b) and in the crustose coralline alga *Hydrolithon* sp. (Semesi et al., 2009). Such non-responsiveness has been attributed to carbon-concentrating mechanisms (CCMs) present in many coralline algae (Giordano et al., 2005).

The CCMs transport bicarbonate ions (HCO_3^-) through the cell walls by using ion channels or catalyze the transformation of HCO_3^- in CO_2 via a carbonic anhydrase enzyme (Raven et al., 2012). Photosynthetic rates of macroalgae that have CCMs are not carbon-limited under current environmental conditions (Giordano et al., 2005) and a lack of response of photosynthesis is thus expected under near-future pCO_2. The presence of CCMs in *C. elongata* and *L. incrustans* has never been reported but these two algae are likely to have CCMs due to their gross production rate not enhanced by elevated pCO_2 (Raven and Hurd, 2012) and the presence of CCM in the taxonomically close species, *C. officinalis* (Hofmann et al., 2013). Non-CCM macroalgae are generally carbon-limited under current seawater concentration and may respond positively to elevated pCO_2 (Kubler et al., 1999). Red macroalgae without CCMs are most common in low light environments and subtidal habitats (Hepburn et al., 2011; Hurd et al., 2009; Middelboe and Hansen, 2007). This is most likely the case for *L. corallioides* which may have benefitted from the higher concentration of photosynthetic substrate (CO_2) at 1000 μatm, as demonstrated by its slightly elevated primary production.

In contrast to photosynthesis, respiration was differentially affected by pCO_2 among the three algal species. *C. elongata* and *L. corallioides* respiration rates remained unchanged regardless of pCO_2. This lack of response is consistent with the lack of pCO_2 effect shown in two other

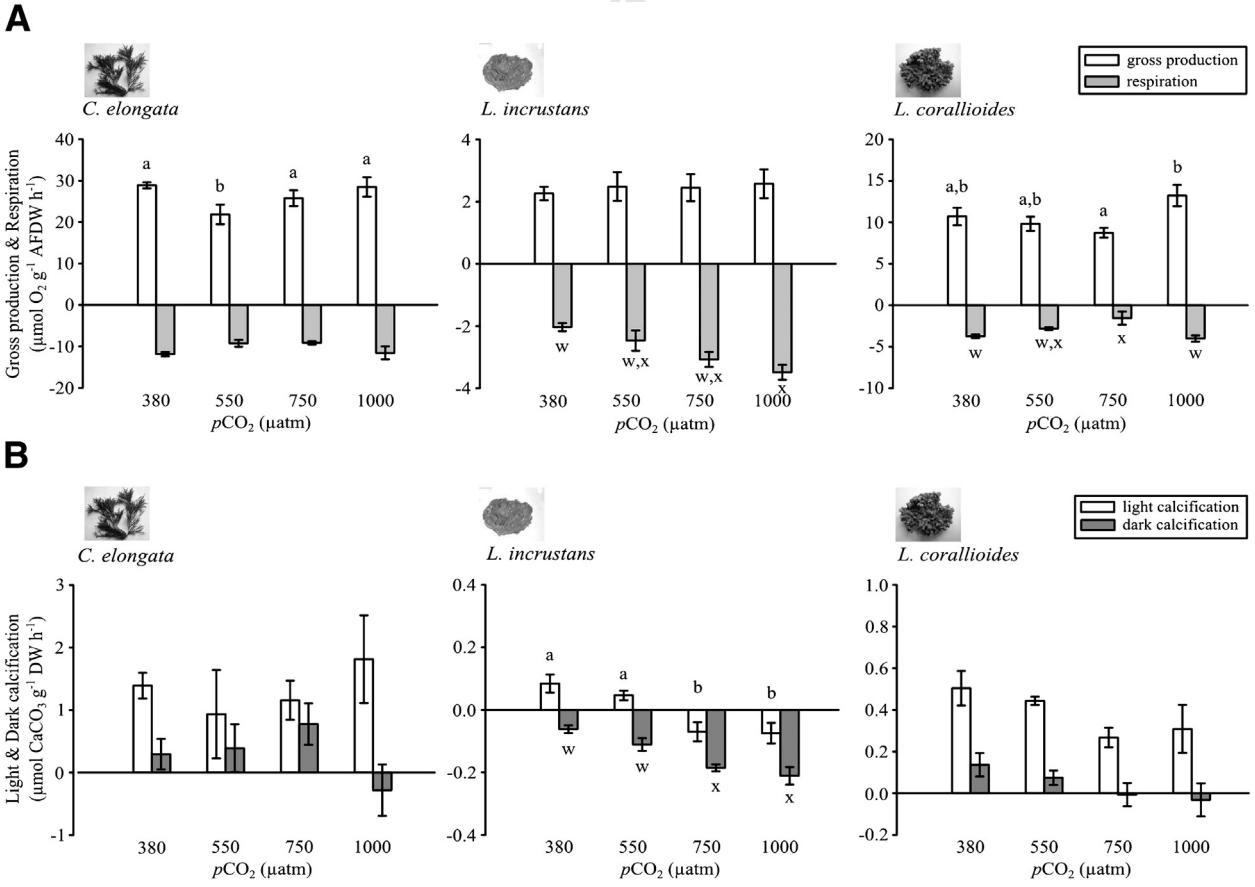

Fig. 2. Gross production and respiration rates (A) and net calcification rates in the light and dark (B) in each pCO_2 treatment. Unshared letters above bars indicate significant differences between treatments ($p < 0.05$, SNK post hoc test), $n = 6$.

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temperate coralline algal species, *C. officinalis* (Hofmann et al., 2012b) and *Lithophyllum cabiochae* (Martin et al., 2013) under similar pCO_2 ranges. The non-responsiveness of macroalgal respiration under elevated pCO_2 was attributed to the absence of changes in photosynthesis and chlorophyll content by Zou et al. (2011). This hypothesis is not supported by our findings since as *L. corallioides* gross production varied significantly without affecting respiration. In contrast, *L. incrustans* respiration strongly increased under elevated pCO_2. This may be related to the severe bleaching occurrence. With bleaching, the proportion of undamaged tissue decreased and necrosis (i.e. dead areas) increased. Non-photosynthetic organisms such as bacteria, fungi, boring organisms may have developed on top and within the dead surfaces (Figueiredo et al., 1997; Tribollet and Payri, 2001), contributing to the increase in respiration rate.

Although bleaching occurred in all the pCO_2 treatments, percentage of bleached surfaces increased with increasing pCO_2, covering more than 10% of the total thallus area under 1000 µatm. Elevated pCO_2 is known to increase bleaching in crustose coralline algae (Anthony et al., 2008; Diaz-Pulido et al., 2012; Martin and Gattuso, 2009). However, bleached surfaces were observed in *L. incrustans* thalli even in the control condition (380 µatm) suggesting poor health of this species under experimental conditions. Temperature (Martin and Gattuso, 2009) and desiccation (Martone et al., 2010) are known to be factors inducing crustose coralline algae bleaching. In our experiment, temperature was kept constant at 16 °C and crustose coralline algae were constantly immersed but other factors such as diseases or pathogens (widely known for tropical crustose coralline algae, Littler and Littler, 1998; Ballantine et al., 2005) may have caused *L. incrustans* bleaching. This understory species generally inhabits shaded environments, protected by the upper dense canopy from high light intensities (Irving et al., 2004). Although incident irradiance under the canopy may reach 50 µmol photon m^{-2} s^{-1} at low tide, the constant light of 30 µmol photon m^{-2} s^{-1} applied during the whole experiment may have been too high. In comparison, values reported by Figueiredo et al. (1997) did not exceed 8–24 µmol photons m^{-2} s^{-1} under a *Fucus* canopy (Isle of Man, UK) at high and low tide respectively. This constant illumination 9 h per day may have caused damages to cell tissues and led to partial bleaching of the thalli. In elevated pCO_2, the potentially negative effects of light may have been amplified at high CO_2 concentrations making the algae potentially more perceptive to diseases, increasing the bleaching.

Interestingly, no bleaching was observed in *L. corallioides* although it developed under dim irradiance (≈15 µmol photons m^{-2} s^{-1}). Comparisons between laboratory and *in situ* recorded metabolic rates suggest that *C. elongata* and *L. corallioides* were in good health and not negatively affected by experimental conditions. At 380 µatm, *C. elongata* and *L. corallioides* net production rates were higher than those recorded *in situ* in similar temperature and light conditions (7.7 µmol O_2 g^{-1} h^{-1}; Egilsdottir, pers. com. and 2.4 µmol O_2 g^{-1} d^{-1}; Martin et al., 2007, respectively). Daily and hourly net calcification rates in *L. corallioides* (3.1 µmol $CaCO_3$ g^{-1} d^{-1}; Martin et al., 2006) and *C. elongata* (3.5 µmol $CaCO_3$ g^{-1} h^{-1}, Egilsdottir, pers. com.) respectively, measured *in situ* were similar to those measured in our study. The consistency between field and laboratory data confirmed that *C. elongata* and *L. corallioides* were not stressed under the experimental conditions.

Calcification in *L. incrustans* decreased along the pCO_2 gradient, both under light and dark conditions. Daily net calcification rate at 380 µatm was low (0.03 mg $CaCO_3$ g^{-1} DW d^{-1}) because of the net dissolution measured under dark conditions, most probably related to the bad health of this alga. Net dissolution was also observed at 750 and 1000 µatm in the light and in all the pCO_2 conditions in the dark. In contrast with *L. incrustans*, increasing pCO_2 did not affect calcification in *C. elongata*. In *L. corallioides*, calcification rate was significantly affected by elevated pCO_2 only in light conditions. In the dark, a general negative trend has been underlined but masked by a high inter-individual-response variability. This calcification decrease has already been shown in other rhodolith species (Büdenbender et al., 2011; Ragazzola et al., 2012) and crustose coralline algae (Anthony et al., 2008; Jokiel et al., 2008; Semesi et al., 2009). In some species of coralline algae, such as *L. cabiochae*, dissolution was related to necroses and bleaching (Martin and Gattuso, 2009). In *L. incrustans*, skeletal dissolution of dead surfaces may have been promoted under elevated pCO_2 and net calcification subsequently lowered. In the light, this phenomenon was partly buffered by the photosynthesis, which increased the pH in undamaged tissues and in the boundary layer (Borowitzka, 1981; Cornwall et al., 2013; Hurd et al., 2009). By increasing the pH, Ω was increased and may have favored the calcification process. Conversely, respiration in the dark released CO_2 leading to decrease in pH and Ω. Precipitation of $CaCO_3$ in undamaged tissues may thus be hindered and dissolution exacerbated. pH variations induced by photosynthesis and respiration in the surrounding medium of the algae were not likely to affect calcification in *C. elongata* as this alga may be able to cope with elevated pCO_2 by saving energy from down-regulating CCMs (Cornwall et al., 2012; Hurd et al., 2009) or by modifying enzymes contents such as carbonic anhydrase (Hofmann et al., 2012b; 2013) to maintain calcification rates.

Differences between the three algal species could also be partly explained by the high Mg-calcite they precipitate to form their thallus. The carbonate mineralogy is linked to the dissolution phenomenon and can influence calcification rates (Ries, 2011). Mg-calcite is the most soluble form of $CaCO_3$ and the mol% $MgCO_3$ in the algal skeleton may increase its solubility (Morse et al., 2006). *C. elongata* had a lower *m*Mg/Ca ratio (0.17) than the other two species (0.20), which can potentially reduce dissolution (Büdenbender et al., 2011). Although information on *m*Mg/Ca ratio is not sufficient to define the robustness of a calcareous structure (Ragazzola et al., 2012), a lower magnesium content in Mg-calcite can confer a greater resistance to elevated pCO_2. Besides, *C. elongata* has thin, branched thalli that are less calcified than thick crust thalli of *L. incrustans* and *L. corallioides*. Thallus morphology is known to influence the speed of corrosion (Ragazzola et al., 2012) and the thinnest thallus may be more resistant because of the higher surface to volume ratio that may allow more exchanges leading to a better chemistry regulation around the calcification site (Price et al., 2011).

This study has demonstrated that CO_2-driven effects varied between algal species from the same family but collected in habitats with varying abiotic conditions. Our original hypothesis that organisms naturally exposed to stressful conditions in their environment will be less sensitive to future pH/pCO_2 variations was partially supported. Indeed, *C. elongata* was the most resistant to elevated pCO_2 and may have developed adaptations to strong daily variations in pH, commonly occurring in tidal pools (Truchot and Duhameljouve, 1980). Surprisingly, *L. corallioides* from a more stable pH environment showed a better resistance than we expected with just a slight decrease in calcification observed under elevated pCO_2. This species may benefit from constant optimal temperature and light provided in the mesocosm and might maintain high metabolism even under elevated pCO_2. In contrast, *L. incrustans*, living in shallow-water dominated with macroalgae where pH fluctuations are high (Middelboe and Hansen, 2007), was the most sensitive to pCO_2 increase. However, physiological responses in *L. incrustans* are likely to be due to bleaching occurrence. Indeed, the bleaching was correlated to an increasing dissolution which implied a tissue deterioration affecting all the metabolic functions (Diaz-Pulido et al., 2012). The increasing bleaching could also be linked to productivity losses as shown on tropical crustose coralline algae (Anthony et al., 2008). The lack of canopy in our experimental set-up may have modify light intensity and quality that *L. incrustans* is used to, leading to bleaching that not occurs *in situ* in the understory. If individuals remained healthy and unbleached during the experiment, *L. incrustans* could prove to be more resistant and a lack of response to elevated pCO_2 may be expected but further investigation is needed.

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High pCO_2 sensitivity of coralline algae underlined by numerous authors, (Basso, 2012; Büdenbender et al., 2011; Gao and Zheng, 2010; Hofmann et al., 2012a) is counterbalanced by others studies that showed that calcification could be unaffected (Egilsdottir et al., 2013; Martin and Gattuso, 2009) or even increased (Martin et al., 2013; Ries et al., 2009) by moderate pCO_2. These contrasting results and the recent discovery of dolomite, a magnesium-rich stable carbonate less soluble than Mg-calcite, present in some crustose coralline algae led to a reappraisal of the sensitivity of coralline algae to near-future ocean acidification (Nash et al., 2013). However, many studies investigating coralline algae under elevated pCO_2 were carried out in mesocosms or laboratory experiments. In the field, pCO_2 is not the only stressor, and surely not the main one, impacting algal physiology. Under combined stresses (e.g. light, temperature, pCO_2), indirect pCO_2 effects could enhance the sensitivity of algae and facilitate disease development and bleaching occurrence even if algae are used to large and rapid pCO_2 variations in their habitat. As one stressor may limit organism ability to deal with another stressor, bleaching can induce a bias in the physiological responses to increasing pCO_2. As for *L. incrustans* in our study, bleaching induced dissolution that impacted the calcification balance even if calcification process in undamaged part of the thallus may not be affected by elevated pCO_2.

Resilience to elevated pCO_2 is probable but maybe at a cost (Martin et al., 2013). *C. elongata* was able to maintain a heavily calcified skeleton under elevated pCO_2 during our experiment (one month). In the long term, this ability could impact the general resistance of the organism by decreasing its fitness and could reduce their ability to compete with fleshy algae. *In situ* experiments along a natural pH gradient showed that even if coralline algae were able to withstand the effects of ocean acidification, they may suffer reductions in abundance (Hall-Spencer et al., 2008; Kroeker et al., 2013; Martin et al., 2008; Porzio et al., 2011). With the decrease of coralline algae in macroalgal-dominated communities, space could be released for fleshy algae (Kuffner et al., 2008) or turf which are generally favored by elevated pCO_2 (Connell and Russell, 2010). As turf and fleshy algae have different ecological roles than coralline algae, these algal community shifts could have considerable ecological and functional consequences for macroalgal communities from the intertidal and subtidal zones.

5. Uncited reference

Wood et al., 2008

Acknowledgments

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