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RESEARCH PAPER

Elevated CO_2 levels affect the activity of nitrate reductase and carbonic anhydrase in the calcifying rhodophyte *Corallina officinalis*

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Abstract

The concentration of CO_2 in global surface ocean waters is increasing due to rising atmospheric CO_2 emissions, resulting in lower pH and a lower saturation state of carbonate ions. Such changes in seawater chemistry are expected to impact calcification in calcifying marine organisms. However, other physiological processes related to calcification might also be affected, including enzyme activity. In a mesocosm experiment, macroalgal communities were exposed to three CO_2 concentrations (380, 665, and 1486 µatm) to determine how the activity of two enzymes related to inorganic carbon uptake and nutrient assimilation in *Corallina officinalis*, an abundant calcifying rhodophyte, will be affected by elevated CO_2 concentrations. The activity of external carbonic anhydrase, an important enzyme functioning in macroalgal carbon-concentrating mechanisms, was inversely related to CO_2 concentration after long-term exposure (12 weeks). Nitrate reductase, the enzyme responsible for reduction of nitrate to nitrite, was stimulated by CO_2 and was highest in algae grown at 665 µatm CO_2. Nitrate and phosphate uptake rates were inversely related to CO_2, while ammonium uptake was unaffected, and the percentage of inorganic carbon in the algal skeleton decreased with increasing CO_2. The results indicate that the processes of inorganic carbon and nutrient uptake and assimilation are affected by elevated CO_2 due to changes in enzyme activity, which change the energy balance and physiological status of *C. officinalis*, therefore affecting its competitive interactions with other macroalgae. The ecological implications of the physiological changes in *C. officinalis* in response to elevated CO_2 are discussed.

Key words: Calcification, carbon dioxide, carbonic anhydrase, coralline algae, nitrate reductase, ocean acidification.

Introduction

Increasing atmospheric CO_2 emissions are changing the chemistry in the surface layer of global oceans. As more CO_2 dissolves into the seawater, changes in the speciation of inorganic carbon occur, resulting in more bicarbonate ions (HCO_3^-), more protons (H^+), and fewer carbonate ions (CO_3^{2-}). The consequences of these changes are a lower pH and CO_3^{2-} saturation state of the seawater. By the end of this century, the pH of surface oceans is expected to drop by 0.3–0.5 units (Caldeira and Wickett, 2003; Feely *et al*., 2004; Orr, 2005) due to increasing concentrations of atmospheric CO_2 that could reach up to 970 µatm CO_2 (Houghton *et al*., 2001). Such changes in seawater chemistry could have severe impacts on calcifying organisms, which rely on inorganic carbon for producing their shells and skeletons, which consist of calcium carbonate ($CaCO_3$).

Several studies have shown negative responses of corals, macroalgae, and molluscs to elevated seawater CO_2 concentrations (Anthony *et al*., 2008; Jokiel *et al*., 2008;

Martin *et al*., 2008; Martin and Gattuso, 2009; Albright *et al*., 2010; Diaz-Pulido *et al*., 2011; Rodolfo-Metalpa *et al*., 2011; Hofmann *et al*., 2012*b*). However, due to the increase in ocean acidification research in the past few decades, it is now clear that calcifing marine organisms show a variety of responses, due to differences in the substrate (HCO_3^- or CO_3^{2-}) used for calcification, their ability to control the pH at the location of calcification, the crystalized form of $CaCO_3$ deposited, and the production of protective organic layers that prevent dissolution (Ries, 2009, 2011; Hurd *et al*., 2011; Jokiel, 2011*a*,*b*; Rodolfo-Metalpa *et al*., 2011; Roleda *et al*., 2012). Furthermore, studies have shown that physiological processes other than calcification, such as photosynthesis, nutrient assimilation, and growth, are also affected by elevated CO_2 concentrations (Magnusson *et al*., 1996; Mercado, 1999; Gordillo *et al*., 2001, 2003; Israel and Hophy, 2002; Zou, 2005; Connell and Russell, 2010; Zou *et al*., 2011; Hofmann *et al*., 2012*b*). The physiological and ecological responses of calcifying organisms to elevated CO_2 is therefore species specific, and also depends on local conditions, such as nutrient availability (Ries, 2009; Russell *et al*., 2009; Fabricius *et al*., 2011; Price *et al*., 2011; Hofmann *et al*., 2012*a*). It is nevertheless important to understand how all processes, not just calcification, will be affected by elevated CO_2, and what implications these changes will have for calcifiying organisms.

In calcifying primary producers, photosynthesis is also affected by increasing CO_2 levels. However, the responses of these organisms are again variable, because of different mechanisms and efficiencies of obtaining CO_2 for photosynthesis. Because the ambient seawater concentration of HCO_3^- is much higher than that of CO_2, marine algae have mechanisms called carbon-concentrating mechanisms (CCMs) which transport HCO_3^- across cell membranes using ion transporters, or catalyse the dehydration of HCO_3^- to CO_2 via the membrane-associated external carbonic anhydrase (Johnston, 1991; Badger and Price, 1994; Raven, 1997, 2003; Raven *et al*., 2002). In non-calcifying algae, CCMs have been shown to be down-regulated under elevated CO_2 conditions. This down-regulation relieves algae from the high energy demands of producing ion transporter proteins and enzymes (Raven, 2011; Raven *et al*., 2012). However, in calcifying algae, this enzyme may play an additional role in calcification, and has been shown to increase under elevated CO_2 (Isenberg *et al*., 1963; Hofmann *et al*., 2012*b*).

Nutrient assimilation and uptake are further metabolic processes that may be affected by higher CO_2 concentrations. Because the speciation of inorganic nitrogen and phosphate is affected by pH, the preference and uptake of inorganic nutrients may be affected, as well as the enzymatic activity involved in nutrient assimilation. Non-calcifying macroalgae have been shown to decrease nitrate uptake under elevated CO_2 (García-Sánchez *et al*., 1994; Magnusson *et al*., 1996; Andria *et al*., 1999). Such changes in metabolism are likely to have significant effects on macroalgal nutritional content, which could have implications for grazers and competitive interactions between species. To date, however, there have not been many studies investigating how inorganic carbon and nutrient-related enzymatic activity in calcifying macroalgae will respond to elevated CO_2.

Seasonal changes in temperature, nutrient availability, and light are also likely to interact with the effect of CO_2 on metabolic processes in algae (Tyrrell *et al*., 2008; Martin and Gattuso, 2009; Mercado and Gordillo, 2011). As calcification, photosynthesis, nutrient uptake, growth, and other metabolic processes are affected by temperature, light, and nutrient availability, changes in these factors are likely to have a strong influence on the enzymatic response of macroalgae to increasing CO_2. Therefore, mesocosm studies such as this one are useful for monitoring CO_2 effects over time during natural temperature, nutrient, and light fluctuations.

Both calcifying and non-calcifying algae provide important habitat and shelter for many marine organisms, and erect calcifying algae such as *Corallina officinalis* contribute to the strength of the intertidal community structure and provide refugia for organisms in environments with high wave action (Dommasnes, 1968; Stewart, 1982; Coull and Wells, 1983; Kelaher, 2002, 2003). *Corallina officinalis* is an upright calcifying alga found in the inter- and subtidal zones on rocky coastlines, often at exposed locations and in tidal drainage channels. It is a late successional species with a complex morphological structure (Littler and Littler, 1980). *Corallina* spp. often form extensive macroalgal beds that cover large areas of the intertidal zone and provide substratum, habitat, and refugia for a number of important marine organisms (Coull and Wells, 1983; Hicks, 1977; Akioka *et al*., 1999; Kelaher, 2002, 2003). The important ecological roles served by this alga could be interrupted under high CO_2 conditions, as its skeleton contains high-Mg calcite, the most soluble form of $CaCO_3$ found in calcifying marine macroalgae (Andersson *et al*., 2008). It is therefore important to understand how its metabolism may be affected in the future. Therefore, a mesocosm study was conducted with macroalgal communities containing the calcifying rhodophyte *C. officinalis* grown under three different CO_2 concentrations. The competitive interactions between *C. officinalis* and non-calcifying macroalgae as well as the overall macroalgal community response are discussed in a separate paper (Hofmann *et al*., 2012*a*). Here the focus is on inorganic nutrient uptake rates and the ezymatic activity of carbonic anydrase and nitrate reductase in *C. officinalis* grown under elevated CO_2 conditions.

Materials and methods

Experimental design and seawater chemistry

The experiment was conducted in 75 litre mesocosms on the German island of Sylt in the North Sea. Experimental conditions including mesocosm set-up, duration, and the inorganic carbon chemistry of the seawater are outlined in Hofmann *et al*. (2012*a*). Temperature, salinity, and pH were monitored daily. Seawater samples for inorganic nutrient analysis were taken weekly from the seawater source flowing into all tanks. Nutrient uptake rates were calculated based on a 3 h incubation of *C. officinalis* in 5 litre plexiglass chambers continuously bubbled with mixed gas (386, 665, or 1486 µatm CO_2). Rates were calculated after doubling the projected surface area of the algal thalli, which was measured using the imaging analysis software ImageJ (National Institute of Mental Health, Bethesda, MD, USA).

Tissue sampling and analysis

Corallina officinalis tissue samples were taken weekly for analysis of nitrate reductase and carbonic anhydrase activity, as well as total inorganic carbon content of the skeleton. Nitrate reductase activity of *C. officinalis* was determined based on the *in situ* method of Corzo and Niell (1991). Fresh algal tissue (200–400 mg) was placed into 5 ml amber vials containing 3 ml of anoxic assay buffer (0.1 M phosphate buffer, pH 8.0, 0.5 mM EDTA, 0.1% 1-propanol, 30 mM KNO_3, 10 µM glucose) that had been previously bubbled with N_2 gas for at least 5 min. Each vial was individually bubbled with N_2 gas for an additional 1 min before being placed into a 30 °C water bath in the dark for 30 min. After the incubation, 1 ml of the assay buffer was removed and the nitrite concentrations were determined colorimetrically (Snell and Snell, 1949) after the addition of 200 µl of 4% sulphanilimide and 300 µl of 0.1% *n*-(1-naphthyl) ethylenediamine dihydrochloride. Following the assay, the algal tissue was dried at 60 °C for 48 h to determine the dry weight (DW), and nitrate reductase activity was calculated as µmol NO_2^- g DW^{-1} h^{-1}. Prior to the experiment, nitrate reductase activity was measured hourly under ambient CO_2 conditions in the light (Fig. 1). Following this analysis, tissue was sampled for enzyme activity each week between 10:00 h and 14:00 h, when the activity was most stable, to ensure that the nitrate reductase activity measurements in *C. officinalis* were not confounded by daily fluctuations.

Total carbonic anhydrase activity of *C. officinalis* was measured according to Haglund *et al.* (1992). Algal tissue [50–100 mg fresh weight (FW)] was ground with liquid nitrogen using a chilled mortar and pestle and immersed in 15 ml of chilled assay buffer (50 mM TRIS, pH 8.5, 25 mM dithiothreitol, 25 mM isoascorbic acid, 5 mM EDTA). Aliquots of 3 ml of the extract were added to clean tubes followed by 2 ml of ice-cold CO_2-saturated water. The time it took for the pH to drop 0.4 units during continuous mixing was recorded. Three aliquots from each extract were measured and the mean of these measurements was considered as one replicate. External carbonic anhydrase activity was measured using the same method, but with intact algal thalli (200–400 mg FW) immersed in assay buffer rather than algal extract. Total and external carbonic anhydrase activity were calculated as $(T_b/T_s–1)/FW$, where T_b=the time it took for a blank sample with just assay buffer to drop 0.4 pH units, T_s=the time it took for the algal extract (total) or buffer with an intact thallus (external) to drop 0.4 pH units, and FW=fresh weight of the algae in grams. External carbonic anhydrase activity was normalized to the dry weight of the thalli. The internal carbonic anhydrase activity was calculated by subtracting the external from the total carbonic anhydrase activity.

The percentage of *C. officinalis* tissue made of $CaCO_3$ was determined in fragments that were used in the enzyme activity analysis and was measured by determining the ash free DW of the dried tissue after removing the organic material by burning at 400 °C for 12 h. No effect of the enzyme assay buffers was apparent on the skeletal material, as the amount of $CaCO_3$ in algae grown under 385 µatm CO_2 did not differ from previous measurements on algae that had not been exposed to any enzyme buffer. Furthermore, the $CaCO_3$ content of algae exposed to the nitrate reductase assay buffer did not differ from that of algae exposed to the carbonic anhydrase assay buffer, and algae exposed to the assay buffers showed the same trend with respect to CO_2 concentration.

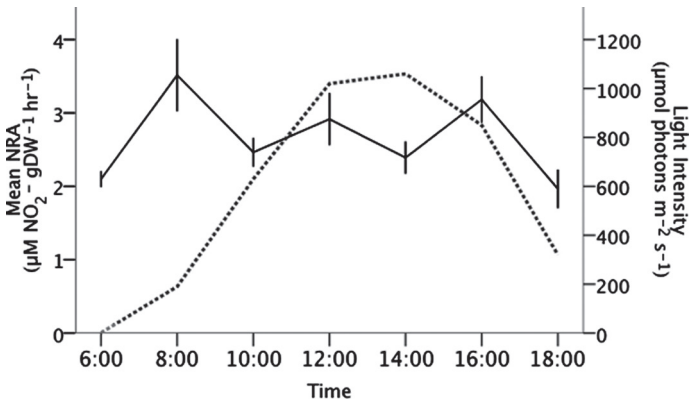

Fig. 1. Daily cycle of mean (±SE, *n*=3) nitrate reductase activity in *C. officinalis* in the light in April 2011. The hourly light intensity is shown by the dotted lines.

Results

Seasonal variability of temperature and inorganic nutrients

The mean seawater temperature in the mesocosm tanks during the experimental period is shown in Fig. 2. Temperature increased linearly with time from the end of March to the beginning of July 2011, and ranged from 6 °C to 19 °C. The seawater concentrations of nitrate, nitrite, ammonium, phosphate, and silicate are shown in Fig. 3. Nitrate concentrations in the seawater ranged from 6.7 µM to 38.9 µM and were highest in March, at the beginning of the experiment, and declined rapidly to a minumum 40 d after the experiment began. Nitrate levels then began to increase again, but only reached 37% of the initial concentration by the end of the experiment. Silicate concentrations followed a similar pattern, but reached higher than initial levels at the end of the experiment. On the other hand, phosphate concentrations increased during the experiment, and ammonium concentrations ranged from 1.03 µM to 3.06 µM.

Nutrient uptake rates and nitrate reductase activity

Nutrient uptake rates of nitrate, ammonium, and phosphate by *C. officinalis* were measured after 35 d of exposure to the CO_2 treatments and are shown in Fig. 4. There was a negative correlation between nitrate uptake and CO_2 concentration (Pearson's correlation coefficient= –0.81, P=0.002). There was no significant treatment effect of CO_2 on ammonium or phosphate uptake rates.

Throughout the experimental period, there was a significant effect of time on nitrate reductase activity, as it decreased in all CO_2 treatments after 12 weeks. There was also a significant effect of CO_2 on nitrate reductase activity (Table 1). Algae grown under ambient CO_2 levels had the lowest enzyme activity, while algae grown under 665 µatm CO_2 had the highest (Fig. 5A). The relationship between nitrate reductase activity and nitrate uptake rate differed between the CO_2 treatments (Fig. 6). The algae grown under elevated CO_2 had higher nitrate reductase activity, but lower nitrate uptake rates compared with algae grown in the ambient CO_2 treatment.

Carbonic anhydrase activity

All carbonic anhydrase activity (total, internal, and external) was significantly affected by time. External carbonic anhydrase activity was affected by CO_2, and internal and external carbonic anhydrase activity was affected by an interaction between time and CO_2 (Table 1). Internal carbonic anhydrase showed no observable pattern over time, except for two peaks

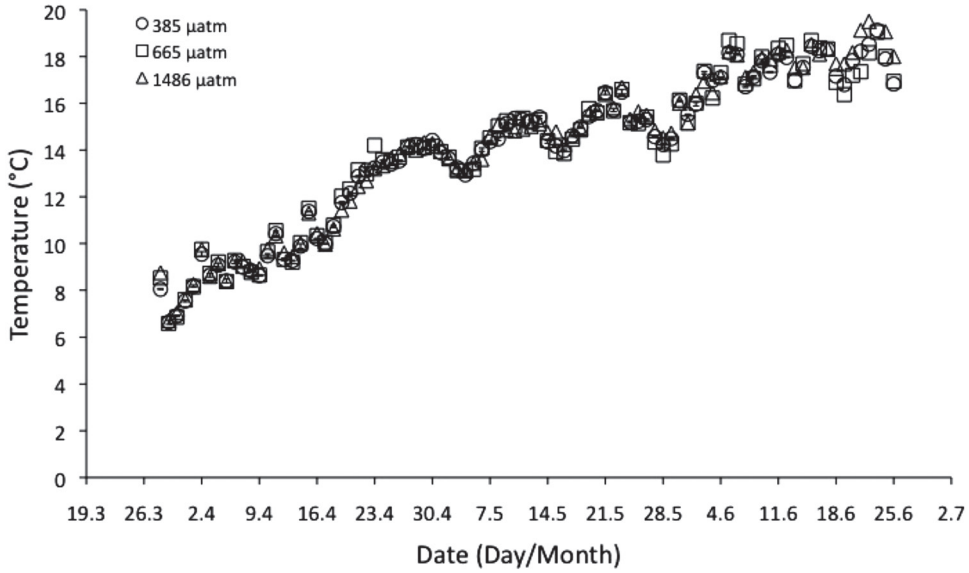

Fig. 2. Mean (±SE, *n*=4) seawater temperature in the mesocosm tanks during the experimental period. Circles, 385; squares, 665; and triangles, 1486 µatm CO_2.

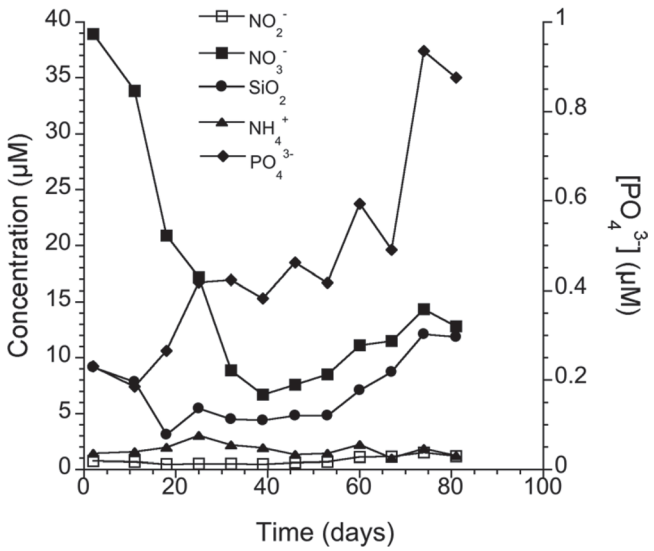

Fig. 3. Inorganic nutrient concentrations of the ambient seawater throughout the duration of the experiment, from 30 March to 17 June 2011.

in the 1486 µatm CO_2 treatment after 4 and 8 weeks, and a large drop after 12 weeks. On the other hand, external carbonic anhydrase increased equally in all treatments during the first half of the experiment until week 7 when all treatments levelled off, but the enzyme activity was highest in the 1486 µatm CO_2 treatment and subsequently decreased with decreasing CO_2 level (Fig. 5B, C).

$CaCO_3$ content

Inorganic carbon content of the *C. officinalis* skeleton peaked in all treatments after 3 weeks, and afterwards the CO_2 treatment effect became apparent, as the skeletal inorganic carbon content decreased with increasing CO_2 concentration (Fig. 5D). By the end of the experiment, there was a negative linear relationship between skeletal inorganic content (% DW of $CaCO_3$) and external carbonic anhydrase activity which was not apparent after shot-term exposure (36 d) to elevated CO_2 (Fig. 7A, B).

Discussion

The present results suggest that elevated CO_2 will significantly affect enzyme activity and subsequently many metabolic processes in *C. officinalis*, including photosynthesis, calcification, and inorganic nutrient uptake and assimilation. The enzyme external carbonic anhydrase is important in the CCM of many macroalgae. Although its activity has been shown to decrease in non-calcifying macroalgae in response to elevated CO_2 (Björk *et al*., 1993; García-Sánchez *et al*., 1994; Haglund and Pedersén, 2009), an increase in external carbonic anhydrase activity with increasing CO_2 concentration was observed. In non-calcifying macroalgae, there is evidence that less enzyme is produced because more CO_2 is available for photosynthesis, so less HCO_3^- must be converted to CO_2 (Giordano *et al*., 2005*a*, and references therein; Matsuda *et al*., 2011). However, in the case of calcifying macroalgae, it is likely that external carbonic anhydrase plays a role in metabolic processes other than photosynthesis, particularly calcification. In corals, carbonic anhydrase has been reported to be an important enzyme in the calcification process (Kingsley and Watabe, 1987; Nimer *et al*., 1994; Al-Horani *et al*., 2003; Rahman *et al*., 2008; Tambutté, 2007). Hofmann *et al*. (2012*a*) showed that calcification rates in *C. officinalis* had a parabolic relationship to CO_2 concentration, and Hofmann *et al*. (2012*b*) showed that external carbonic anhydrase showed an increasing trend with elevated CO_2 in the same species. As photosynthesis was not stimulated by CO_2 in this species, despite an increase in

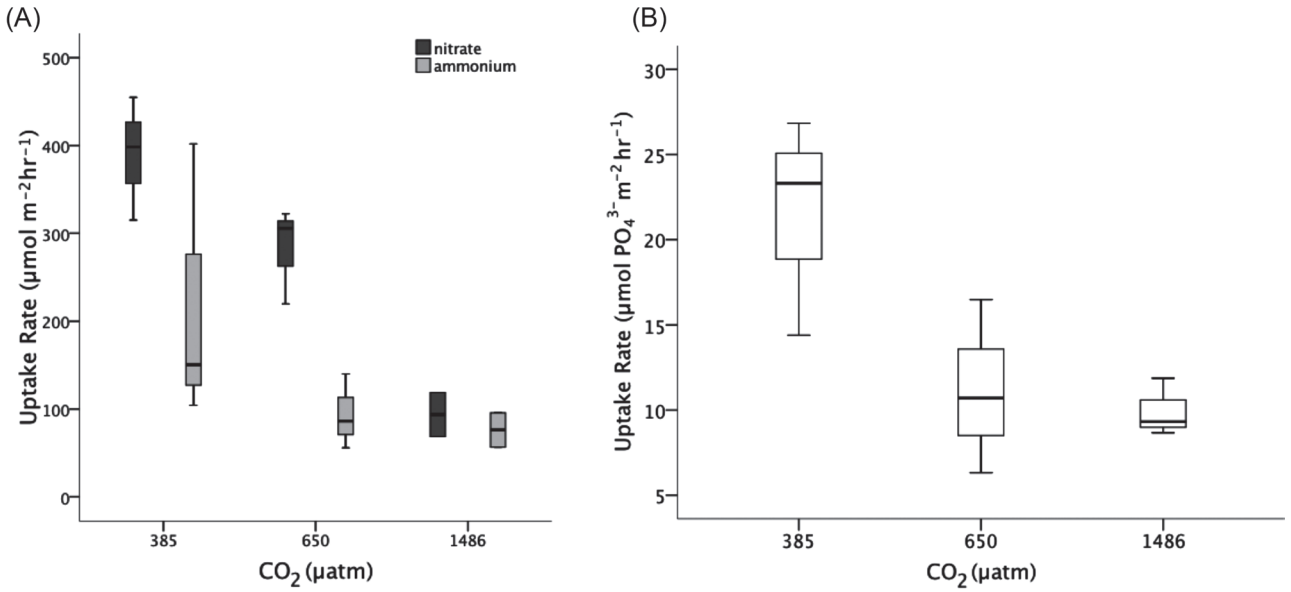

Fig. 4. Boxplots showing the median, minimum, maximum, and first and third quartiles of *C. officinalis* uptake rates for (A) nitrate and ammonium and (B) phosphate as a function of CO_2 concentration.

Table 1. Results from a MANOVA test on enzyme activity and $CaCO_3$ content (C_{inorg}) of *C. officinalis* with time as a within-subject factor and CO_2 as a between-subject factor. *F*-ratios are given with degrees of freedom in parentheses, followed by the *P*-values significant at the 95% confidence level.

Response variable	Time (within-subject factor)	CO_2 (between-subject factor)	Time×CO_2 (interaction)
tCAA	$F(9, 81)=15.5$, $P=2.1E-14$	–	–
eCAA	$F(11, 99)=45.6$, $P=7.8E-34$	$F(2, 9)=36.2$, $P=5.0E-5$	$F(22, 99)=3.4$, $P=1.6E-5$
iCAA	$F(10, 90)=15.5$, $P=1.0E-15$	–	$F(20, 90)=2.4$, $P=0.003$
NRA	$F(11, 99)=9.4$, $P=2.5E-11$	$F(2, 9)=5.0$, $P=0.034$	–
C_{inorg}	$F(11, 99)=21.4$, $P=1.5E-21$	$F(22, 99)=2.1$, $P=0.006$	$F(2, 9)=12.1$, $P=0.003$

tCAA, total carbonic anhydrase activity; eCAA, external carbonic anhydrase activity; iCAA, internal carbonic anhydrase activity; NRA, nitrate reductase activity.

external carbonic anhydrase activity, it is hypothesized that external carbonic anhydrase activity is related to calcification, and that its activity is up-regulated under elevated CO_2. In this way, the algae may regulate the calcification mechanism despite changes in seawater inorganic carbon chemistry that are unfavourable for $CaCO_3$ deposition. This hypothesis is supported by the relationship observed between external carbonic anhydrase activity and the skeletal inorganic carbon content of *C. officinalis*, which was only revealed after long-term exposure to elevated CO_2. Such a response of the algae could be to compensate for higher dissolution rates under elevated CO_2 conditions (Ries, 2011; Rodolfo-Metalpa *et al.*, 2011). If external carbonic anhydrase does indeed play a role in calcification, higher dissolution rates would explain why an increase in skeletal inorganic carbon was not seen despite an increase in external carbonic anhydrase activity under elevated CO_2.

The overall decrease in nitrate reductase activity in *C. officinalis* grown at all CO_2 concentrations during the first 6 weeks was most probably due to a decline in the seawater nitrate concentration, as the enzyme has been shown to be dependent on external nitrate availability (Solomonson and Barber, 1990; Gordillo *et al.*, 2006). Algae generally prefer ammonium over nitrate as their nitrogen source, as it is less energy costly to assimilate (Losada and Guerro, 1979; Syrett, 1981). The ammonium concentrations were sufficient to supply the algae with an alternative source of nitrogen when the seawater nitrate concentrations, and subsequently nitrate reductase activity, decreased.

The decrease in nitrate uptake rates by *C. officinalis* under elevated CO_2 conditions is consistent with other results found for non-calcifying macroalgae and seagrass (García-Sánchez *et al.*, 1994; Magnusson *et al.*, 1996; Andria *et al.*, 1999; Alexandre *et al.*, 2012) as well as the observed increase in nitrate reductase activity (Mercado *et al.*, 1999; Gordillo *et al.*, 2001; Alexandre *et al.*, 2012). Mercado *et al.* (1999) reported that the reduction and assimilation of nitrate in *Porphyra leucosticta* grown under elevated CO_2 was uncoupled, which also seems to be the case in *C. officinalis*. Changes in the intracellular ATP:NADP+/NADPH ratio could affect

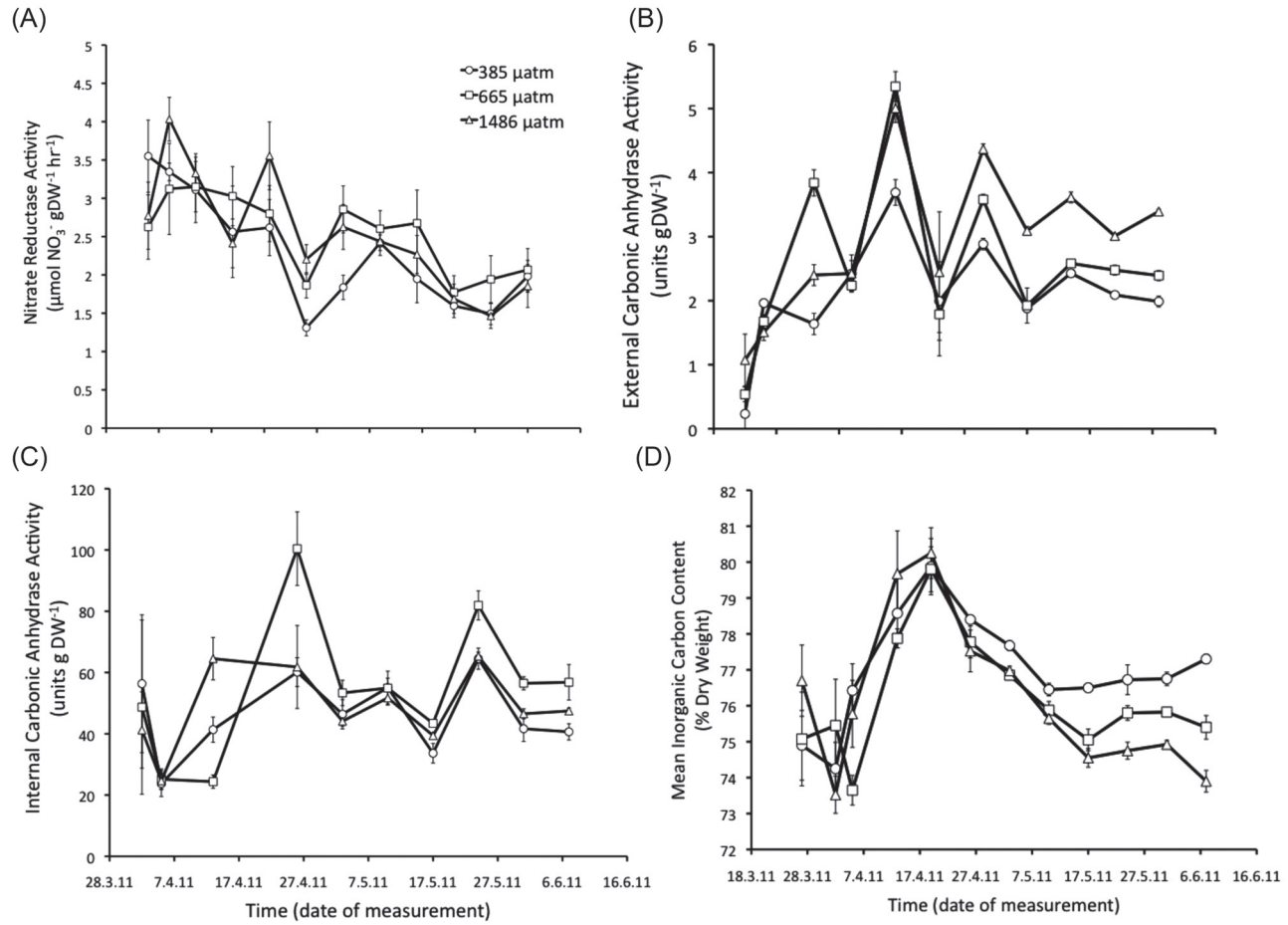

Fig. 5. Time series of mean (±SE, *n*=4) (A) nitrate reductase activity, (B) external carbonic anhydrase activity, (C) internal carbonic anhydrase activity, and (D) percentage inorganic carbon of *C. officinalis* exposed to three carbon dioxide concentrations. Circles, 385; squares, 665; and triangles, 1486 µatm CO_2.

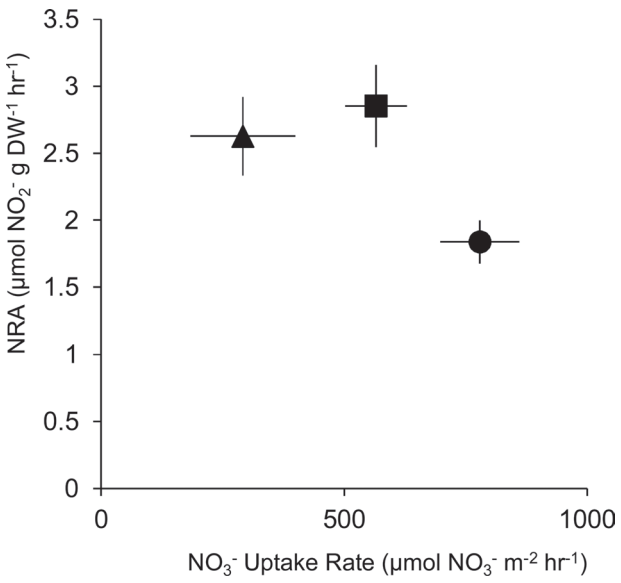

Fig. 6. Mean nitrate reductase activity (±SE, *n*=4) as a function of mean nitrate uptake rates in *C. officinalis* exposed to the three CO_2 levels. Circles, 385; squares, 665; and triangles, 1486 µatm CO_2.

nitrate reductase activity, due to the requirement for NADPH as a reducing agent to convert nitrate to nitrite (Corzo and Niell, 1991). *Chlamydomonas* sp. cells grown under normal CO_2 conditions require higher ATP:NADPH ratios for CO_2 assimilation than high CO_2-grown cells (Spalding *et al*., 1984). Therefore, if algae grown under elevated CO_2 have a lower ATP:NADPH requirement, the excess NADPH could stimulate nitrate reductase activity. However, this may only be the case when CCMs are down-regulated. In high CO_2-grown *C. officinalis*, the protein content decreases, indicating a likely decrease in Rubisco concentration. This reduction in Rubisco content could be interpreted as a partial down-regulation of the CCM in *C. officinalis*, despite the increase in external carbonic anhydrase activity. However, another possible reason for stimulation of nitrate reductase under elevated CO_2 is a change in the plastoquinone pool. Giordano *et al*. (2005*b*) reported that nitrate reductase activity is controlled by the redox state of the plastoquinone pool in *Chlamydomonas reinhardtii*, in that nitrate reductase activity is stimulated by a reduced plastoquinone pool. A reduced plastoquinone pool generally occurs under high light conditions, when the electron transport chain is saturated (Behrenfeld *et al*., 1998). The lower protein content in *C. officinalis* grown under elevated

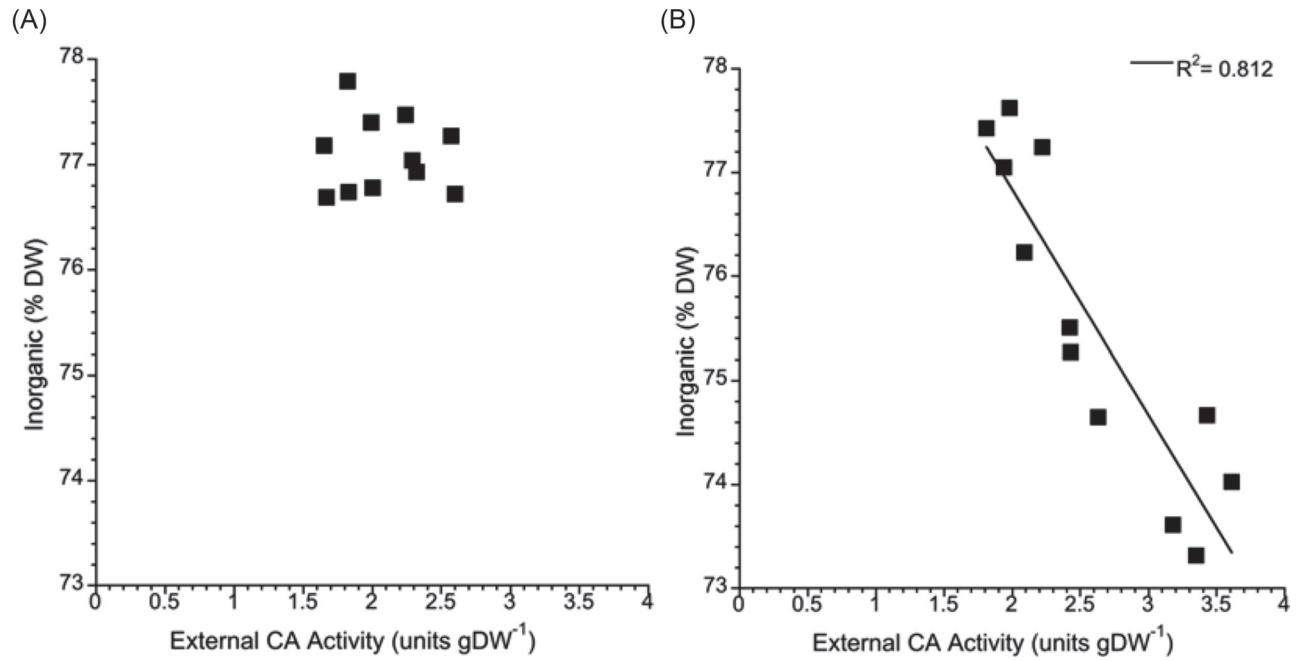

Fig. 7. Inorganic content of *C. officinalis* as a function of external carbonic anhydrase activity after (A) 36 d and (B) 71 d of exposure to three CO_2 concentrations

CO_2 (Hofmann *et al*., 2012*a*) suggests that Rubisco content may be lower in algae grown under high CO_2 conditions. When CO_2 concentrations are saturating for photosynthesis, activation of Rubisco can be rate limiting to photosynthesis rather than electron flow (Dietz and Herber, 1984). The combination of high light conditions during summer and lower Rubisco content under elevated CO_2 could have resulted in a more reduced plastoquinone pool in *C. officinalis* grown under elevated CO_2, causing the stimulation of nitrate reductase activity.

The absolute values and seasonal pattern of seawater temperature in the mesocosm tanks and ambient seawater nutrient concentrations were consistent with previously recorded seasonal trends in the Wadden Sea (van Beusekom *et al*., 2001, 2010). Therefore, the changes in both enzyme activities during the experimental period indicate that there was an effect of seasonally changing temperature and nutrient conditions on *C. officinalis* metabolism. However, the enzymes responded differently to seasonal fluctuations, as nitrate reductase increased and external carbonic anhydrase decreased during the first 6 weeks of the experiment. The increase in external carbonic anhydrase activity in all treatments during the first 6 weeks was most probably a response to increasing seawater temperature, as enzymes have optimum temperatures for maximum activity, and *C. officinalis* growth is optimal at temperatures between 12 °C and 18 °C (Colthart and Johansen, 1973), which were reached after the first half of the experiment. The stimulation of carbonic anhydrase by elevated temperature has been previously reported for *Chlorella vulgaris* (Shiraiwa and Miyachi, 1985). This temperature effect could have masked the CO_2 effect during the first 6 weeks of the experiment, as there was no difference in external carbonic anhydrase activity between the CO_2 treatments until after 6 weeks. Therefore, the enzymatic activity, reliant metabolic mechanisms, and cellular products of the calcifying red alga *C. officinalis* will be affected by CO_2, but will also depend on seasonal effects such as nutrient availability and temperature.

The present results indicate that the response of *C. officinalis* to elevated CO_2 is complex, and involves many metabolic processes other than just calcification and photosynthesis. The observed changes in enzyme activity, combined with changes in photosynthesis, calcification, and cell nutritional content reported by Hofmann *et al*. (2012*a*), will alter the competitive status of *C. officinalis* under future oceanic CO_2 conditions, which could have implications for macroalgal communities and their grazers. However, it is still unclear if calcifying coralline algae, such as *C. officinalis*, will be able to adapt to increasing CO_2 concentrations that will allow them to maintain their current competitive status within macroalgal communities. Their ability to adapt will most probably depend on other abiotic factors and seasonal patterns. Therefore, it will be important to conduct future experiments on different life history stages of this alga, as well as to follow the responses of multiple generations to elevated CO_2 under conditions which simulate seasonal changes.

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