MACROALGAL PERFORMANCE AND COMPETITION UNDER ELEVATED CO_2

BY

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Summary

Since the industrial revolution, atmospheric carbon dioxide (CO_2) concentrations have been increasing, and the surface waters of the global oceans have absorbed 30% of the anthropogenic CO_2 released into the atmosphere. A higher CO_2 concentration in surface ocean waters shifts the carbon chemistry, resulting in higher concentrations of bicarbonate ions (HCO_3^-) and protons (H^+) and lower concentrations of carbonate ions (CO_3^{2-}). Such a shift in ocean carbon chemistry decreases the pH and the saturation state of the seawater with respect to CO_3^{2-} thereby making the precipitation of $CaCO_3$ less kinetically favorable. These changes in ocean chemistry (termed ocean acidification) are expected to have negative impacts on marine calcifying organisms, which deposit $CaCO_3$ in the form of aragonite, calcite and high-magnesium calcite into their shells and skeletons. Because calcifying marine primary producers are very important to the carbon cycle and for rocky shore habitat structure and stability, investigating how they will respond to future oceanic CO_2 levels is a relevant and important topic of research. Therefore, two calcifying marine macroalgae were chosen as the central organisms for investigation in this thesis. I investigated the physiological responses of the temperate calcifying coralline rhodophyte alga *Corallina officinalis* (L.) and the tropical calcifying chlorophyte alga *Halimeda opuntia* (L.) J. V. Lamouroux to elevated CO_2 concentrations which are expected to occur by the end of this century. Furthermore, the effect of elevated CO_2 on the competitive interactions between these two calcifiers and their noncalcifying counterparts was investigated in order to predict how macroalgal communities will respond to future surface ocean CO_2 levels in both temperate and tropical environments. Because CO_2 concentrations are increasing in surface ocean in parallel with other abiotic stressors, I also chose to investigate the response of *H. opuntia* to the combined effect of elevated CO_2 and inorganic nutrients, which replicates a likely scenario for the condition of some eutrophied tropical coral reefs at the end of this century.

The studies carried out during this thesis revealed that there are differences in the physiological responses of calcifying macroalgae to elevated CO_2, but similar patterns of competitive interactions between calcifiers and noncalcifiers occur under elevated CO_2 regardless of species and latitude. I found that the temperate coralline alga *C. officinalis* was highly sensitive to elevated CO_2, as shown by lower growth and

photosynthetic rates and less calcified cell walls than under normal conditions. On the other hand, the tropical calcifying chlorophyte alga *H. opuntia* was only moderately sensitive to elevated CO_2 concentrations, as this species had lower growth rates but maintained normal calcification rates and increased electron transport rates. Enzyme activity (external carbonic anhydrase and in situ nitrate reductase) in both species was affected by CO_2 indicating that external carbonic anhydrase plays an important role in calcification by regulating the speciation of inorganic carbon, and that nitrogen assimilation in these species is affected by elevated CO_2. The effect of CO_2 on energy balance in these two species is also discussed. The different calcification mechanisms utilized by these two species is likely to account for some of the observed differences in physiological responses, and is discussed in detail below. While these two species showed different susceptibilities to elevated CO_2 in isolation, they both showed similar sensitivity to overgrowth and outcompetition by noncalcifying algae when grown with their natural communities under elevated CO_2 conditions. This trend was amplified under conditions of inorganic nutrients. The results of this thesis indicate that calcifying macroalgae show differences in their susceptibility to ocean acidification, but regardless of their sensitivity in isolation, both temperate and tropical species are likely to be outcompeted by noncalcifying macroalgae under elevated CO_2 conditions. Tropical systems are especially susceptible to a shift in community composition (from calcifier- to noncalcifier-dominated) when eutrophication and ocean acidification occur simultaneously.

Zusammenfassung

Seit Beginn der Industrialisierung ist die Konzentration von Kohlendioxid (CO_2) in der Atmosphäre stetig angestiegen. Bis zu 30% dieser anthropogenen Kohlendioxidemissionen wurden vom Oberflächenwasser des Weltozeans aufgenommen. Erhöhte CO_2-Gehalte im Oberflächenwasser des Weltozeans verändern die Kohlenstoffchemie des Wasserkörpers. In der Folge führt dies zu einer Erhöhung der Bikarbonat (HCO_3^-)- und Protonen-Konzentration (H^+), sowie zu geringeren Konzentrationen der Karbonationen (CO_3^{2-}). Solch eine Änderung der Kohlenstoffchemie verringert den pH-Wert des Oberflächenozeans, sowie das Sättigungsgleichgewicht in Bezug auf CO_3^{2-}. Dies wiederum führt zu einer kinetisch weniger begünstigten Ablagerung von $CaCO_3$. Es wird vermutet, dass diese Änderungen in der Ozeanchemie, welche unter dem Begriff „Ozeanversauerung“ zusammengefasst werden, negative Folgen auf kalkbildende Meeresorganismen, die $CaCO_3$ in Form von Aragonit, Kalzit oder Hochmagnesiumkalzit in ihre Gehäuse und Skelette einbauen, haben werden. Da kalkbildende marine Primärproduzenten eine Schlüsselstellung im Kohlenstoffkreislauf einnehmen und auch für die Stabilität und Vergesellschaftungsstruktur felsiger Küsten eine zentrale Rolle spielen, ist es wichtig zu verstehen, wie eben diese Organismen auf erhöhte zukünftige CO_2-Konzentrationen in der Atmosphäre und somit auch im Oberflächenozean reagieren werden. Aus diesem Grund wurden für die vorliegende Arbeit zwei kalkbildende marine Makroalgen zur näheren Untersuchung ausgewählt, welche unterschiedlichen Klimazonen zugeordnet werden können. An der kalkbildenden, korallenartigen Rhodophyt-Alge *Corallina officinalis* (L.), welche den gemäßigten Breiten zugeordnet wird, und der tropischen kalkbildenden Chlorophyt-Alge *Halimeda opuntia* (L.) J. V. Lamouroux, habe ich mich auf die physiologischen Reaktionen konzentriert, die bei einer Exposition mit erhöhten CO_2-Gehalten auftreten, die bis zum Ende dieses Jahrhunderts erwartet werden. Weiterhin wurde der Effekt von erhöhten CO_2-Gehalten auf das konkurrierende Verhalten zwischen diesen beiden kalkbildenden und ihren nicht-kalkbildenden Entsprechungen untersucht, um dadurch vorhersagen zu können, wie Makroalgenvergesellschaftungen in tropischen und gemäßigten Klimaten auf erhöhte CO_2-Gehalte im Oberflächenwasser reagieren könnten. Da parallel zu erhöhten CO_2-

Konzentrationen auch andere abiotische Stressfaktoren im Oberflächenwasser wirken, habe ich mich weiterhin dazu entschieden, die Reaktionen von *H. opuntia* in Bezug auf kombinierte Wechselwirkungen von erhöhten CO_2-Gehalten und inorganischen Nährstoffen zu untersuchen. Diese Wechselwirkungen könnten ein wahrscheinliches Szenario für die Bedingungen in eutrophierten tropischen Korallenriffen darstellen, die sich bis zum Ende dieses Jahrhunderts einstellen könnten.

Die Untersuchungen, die während der Erstellung dieser Arbeit ausgeführt wurden, konnten zeigen, dass sich bei kalkbildenden Makroalgen, die erhöhten CO_2-Konzentrationen ausgesetzt waren, Unterschiede in ihren physiologischen Reaktionen eingestellt haben. Jedoch konnte auch gezeigt werden, dass sich, unabhängig von Art und geographischer Breite, unter erhöhten CO_2-Konzentrationen ähnliche Muster im konkurrierenden Verhalten kalkbildender und nicht-kalkbildender Algen abgezeichnet haben. Ich konnte außerdem herausstellen, dass die in gemäßigten Breiten vorkommende korallenartige Alge *C. officinalis* sehr empfindlich auf erhöhte CO_2-Konzentrationen reagiert hat, was sich durch geringeres Wachstum und photosynthetische Raten, sowie durch geringer kalzifizierte Zellwände geäußert hat. Die tropische kalkbildende Chlorophyt-Alge *H. opuntia* wiederum zeigte nur geringe Reaktionen auf erhöhte CO_2-Gehalte. Es stellten sich zwar geringere Wachstumsraten ein, jedoch konnte diese Art unter erhöhten CO_2-Gehalten eine normale Kalzifizierungs- und erhöhte Elektronentransportrate aufrecht erhalten. Die Enzymaktivität (externe Carboanhydrase und in-situ Nitratreduktase) wurde in beiden Arten von CO_2 beeinflusst, was die externe Carboanhydrase als wichtigen Baustein im Kalzifizierungsprozess herausstellt, da sie die Speziation von inorganischem Kohlenstoff reguliert. Weiterhin beeinflussen erhöhte CO_2-Gehalte auch die Stickstoffaufnahme dieser Arten. Im Folgenden wird auch der Effekt von CO_2 auf die Energiebilanzen in diesen beiden Arten diskutiert. Die unterschiedlichen Kalzifizierungsmechanismen, die von diesen beiden Arten angewendet werden, sind wahrscheinlich der Grund für die beobachteten Unterschiede physiologischer Reaktionen (nähere Erläuterungen dazu siehe unten). Während beide Arten in Isolation voneinander abweichende Empfindlichkeiten in Bezug auf erhöhte CO_2-Gehalte gezeigt haben, konnte eine ähnliche Anfälligkeit bezüglich übermäßigem Wachstum und dominantem Verhalten von Seiten der nicht-kalkbildenden Algen festgestellt werden, wenn sie in ihrer natürlichen Wachstumsumgebung, jedoch unter erhöhten CO_2-

Bedingungen, gewachsen sind. Dieser Trend wurde verstärkt, nachdem die Konzentration von inorganischen Nährstoffen angehoben wurde. Die Ergebnisse dieser Arbeit weisen darauf hin, dass kalkbildende Makroalgen unterschiedliche Empfindlichkeiten auf die Versauerung der Ozeane aufzeigen, jedoch, und das unabhängig von ihren jeweiligen Anfälligkeiten in Isolation, beide Algenarten (tropische und in gemäßigten Breiten vorkommend) unter erhöhten CO_2-Konzentrationen von ihren nicht-kalkbildenden Entsprechungen dominiert werden. Tropische Systeme scheinen hierbei besonders anfällig auf Änderungen in der Vergesellschaftungsstruktur (vormalige Dominanz kalkbildender Organismen zu folgender Dominanz nicht-kalkbildender) zu reagieren, falls Eutrophierung und Ozeanversauerung gleichzeitig auftreten.

Introduction

Ocean acidification and its effect on ocean chemistry

Since the industrial revolution, atmospheric carbon dioxide (CO_2) concentrations have been increasing, and the surface waters of the global oceans have absorbed one third of the anthropogenic CO_2 released into the atmosphere (Siegenthaler & Sarmiento 1993; Sabine et al. 2004; Sabine & Feely 2007). Before the industrial revolution, the partial pressure of CO_2 in the atmosphere was about 280 parts per million volume (ppmv), and the current partial pressure of CO_2 in the atmosphere is 380-390 ppmv (Solomon et al. 2007). The predicted CO_2 emmissions scenarios for the end of the 21st century, according to the most conservative and the most liberal models from the International Panel on Climate Change (IPCC), range from 540 to 970 ppmv CO_2 (Houghton et al. 2001). As global CO_2 emissions continue to rise due to fossil fuel burning, the surface ocean waters will continue to absorb more CO_2 in order to maintain equilibrium with the atmosphere. Increasing dissolved CO_2 into ocean surface waters alters the seawater chemistry, which is a cause for concern for all marine organisms living in the surface layers as well as those living in deep layers due to potential changes in carbon cycling. As more CO_2 dissolves into surface oceans, it reacts with seawater to produce carbonic acid, which dissociates into bicarbonate ions (HCO_3^-) and protons (H^+), resulting in a lower pH. In order to buffer the lower pH, CO_3^{2-} ions react with the extra protons to produce more HCO_3^-. Therefore the seawater becomes undersaturated with carbonate ions, which are the building blocks for calcium carbonate ($CaCO_3$) shells and skeletons of marine organisms (Figure 1).

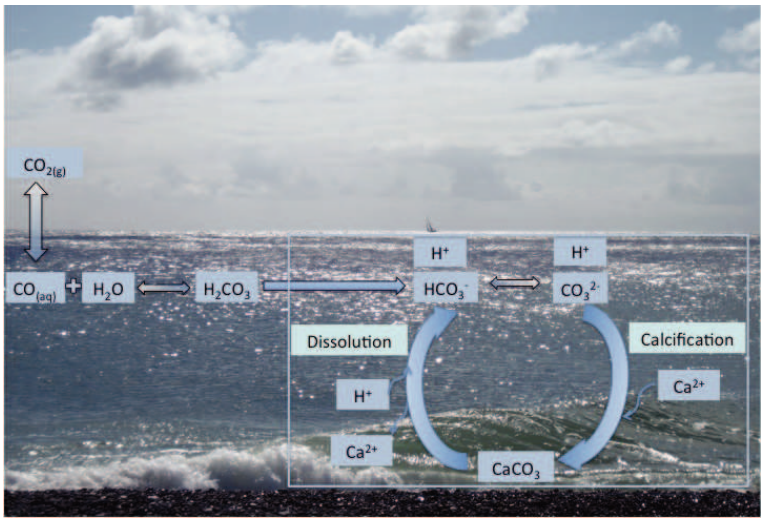

Figure 1. Diagram showing the chemical reactions that occur when CO_2 from the atmosphere dissolves into surface ocean waters, and their relationship to precipitation and dissolution of $CaCO_3$. Carbon dioxide reacts with water to form carbonic acid (H_2CO_3), which immediately dissociates into HCO_3^- and H^+. In order to buffer the additional H^+ produced, CO_3^{2-} ions combine with H^+ to form more HCO_3^-. The result is a lower pH (due to higher [H^+]) and fewer CO_3^{2-} ions, (decreased saturation state of CO_3^{2-}) which favors $CaCO_3$ dissolution rather than precipitation.

Calcification in the marine environment

Many marine organisms produce $CaCO_3$ skeletons and shells, including mollusks, echinoderms, corals and algae. Among the diverse calcifying taxa, $CaCO_3$ is precipitated in three crystal forms: aragonite, calcite and high-Mg calcite. Calcite crystals have a rhomboidal shape and this mineral is the least soluble form of $CaCO_3$ (Figure 2). However, when the Ca^{2+} ions are replaced by Mg^{2+} ions and the ratio of Mg/Ca becomes greater than 0.04, the $CaCO_3$ mineral is considered high-Mg calcite, which is the most soluble $CaCO_3$ crystal in seawater. Aragonite crystals have an orthorhombic shape, and are more soluble in seawater than calcite, but less soluble than high-Mg calcite. Some calcifying marine organisms deposit only aragonite or only calcite, while others deposit both crystal forms. Furthermore, some organisms are able to alter their Mg/Ca ratios depending on abiotic factors such as temperature, seawater carbonate saturation state and light (Chave 1954; Mackenzie et al. 1983; Andersson et al. 2008; Ries 2011).

a) b)

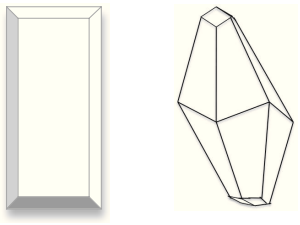

Figure 2. Crystal structures of a) aragonite (orthorhombic) and b) calcite (rhomboidal).

Calcifying organisms play important ecological roles in marine environments, particularly with respect to coral reef accretion, sediment production, carbon cycling, and habitat formation. The massive calcium carbonate structures that are formed by coral skeletons are the basis of highly productive and diverse coral reef systems, while calcifying coralline algae act as cement to keep reef structures intact, and bioherms produced from the dead skeletons of calcifying green algae contribute to carbonate sediment (Littler 1972; Hillis-Colinvaux 1980; Drew 1983; Littler & Littler 1984; Davies & Marshall 1985; Drew & Abel 1988; Littler et al. 1988; Diaz-Pulido et al. 2007; Rees *at al.*, 2007; Hughes 1994; Rees et al. 2007). Futhermore, calcifying microalgae are important food sources for marine herbivores, and they act as huge carbon sinks and oxygen sources (Harris 1994; Milliman 1993; Holligan & Robertson 1996).

Due to the ecological importance of marine calcifying organisms, it is important to understand how they will respond to increasing surface ocean CO_2 concentrations (termed ocean acidification). In the past few decades, there has been an explosion of research in the field of ocean acidification, particularly regarding coral reefs, but there is no consensus as to how marine calcifiers as a whole will be affected by the expected changes in ocean chemistry, as different organisms have been shown to respond differently. Many organisms are sensitive to elevated CO_2-induced low pH (Langdon et al. 2000; 2003, Leclercq et al. 2000; Guinotte et al. 2003; Albright et al. 2008; 2010; Jokiel et al. 2008; Andersson et al. 2009) while others have shown mixed responses (Andersson et al. 2009; Ries 2009; Fabricius et al. 2011; McCulloch et al. 2012; Rodolofo-Metalpa et al. 2011). Despite the recent increase in ocean acidification research, there are still gaps in our understanding of calcification mechanisms, physiological responses of single organisms, community responses, and the combined effects of ocean acidification and other global abiotic stressors (i.e. temperature,

ultraviolet radiation, sea level rise, eutrophication). Particularly lacking is our understanding of how calcifying macroalgae will respond to elevated CO_2 levels and how their competitive interactions with other macroalgae will change under future CO_2 conditions.

Calcifying macroalgae are particularly interesting organisms to investigate with respect to ocean acidification because of the closely linked processes of photosynthesis and calcification that occur in these organisms. While calcification is expected to suffer in these organisms under elevated CO_2 due to decreased bicarbonate saturation states, the substrate for carbon fixation in photosynthesis is CO_2, and therefore a higher CO_2 concentration might be expected to stimulate photosynthesis. However, noncalcifying macroalgae have shown a variety of responses when grown under elevated CO_2, indicating that they are not necessarily carbon limited at the ambient oceanic CO_2 concentration (Gao et al. 1991; 1993; García-Sánchez et al. 1994; Israel et al. 1999; Kübler et al. 1999; Zou 2005). Although the bicarbonate concentration in seawater is approximately 200 times that of the CO_2 concentration at the ambient oceanic pH of about 8.1, many macroalgae have the ability to convert bicarbonate to CO_2 via the enzyme carbonic anhydrase or can actively transport HCO_3^- across their cell membranes to be used in photosynthesis (Figure 3; Sültemeyer 1998; Raven 1997; 2003; Moroney & Somanchi 1999; Raven et al. 2012). Such mechanisms are called carbon concentrating mechanisms (CCMs), as they concentrate dissolved inorganic carbon inside the cell at the site of Ribulose-1,5-bisphosphate carboxylase oxygenase (RubisCO), the enzyme responsible for carbon fixation. These mechanisms reduce the competition of oxygen for the enzyme by increasing the intracellular CO_2:O_2 ratio, thereby making it more efficient by reducing photorespiration (see Raven 1997; 2003; Raven et al. 2012 for reviews). Carbon concentrating mechanisms have been reported in many macroalgal species, and are well documented and described among the green (Chlorophyte) algae, and many of the red (Rhodophyte) and brown (Phaeophyte) algae also contain CCMs (Axelsson et al. 1991; 1995; Drechsler & Beer 1991; Mercado et al. 1998; Moulin et al. 2011; Raven et al. 2012). Algae that do not contain efficient CCMs could be more stimulated by elevated CO_2 than those that contain highly efficient CCMs, although algae that use HCO_3^- could also benefit by downregulating their CCMs under elevated CO_2 because such CCMs have high energy demands (Cornwall et al. 2011).

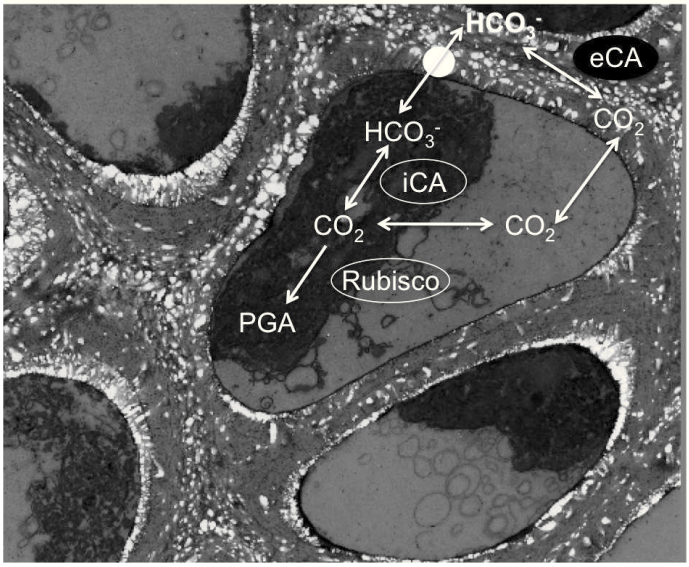

Figure 3. Example of how a carbon concentrating mechanism works in macroalgae. Bicarbonate ions can be transported across the cell membrane via an ion transporter in some algae, or it is converted to CO_2 via the enzyme external carbonic anhydrase (eCA). The CO_2 then diffuses into the cell due to the concentration gradient between the inside of the cell and the seawater. Internal carbonic anhydrase (iCA) converts the CO_2 inside the cell back into HCO_3^- in order to prevent it from diffusing out again. When the HCO_3^- is accumulated inside the cell, internal carbonic anhydrase converts it back to CO_2, which then diffuses into the chloroplast, where Rubisco can use it to fix carbon into phosphoglycerate (PGA), the first stable product of the Calvin cycle. Schematic design based on Moroney & Somanchi (1989).

Calcification and photosynthesis in macroalgae

The relationship between photosynthesis and calcification in calcifying algae is complex and not completely understood. Photosynthesis has been shown to stimulate calcification rates in macroalgae (Borowitzka & Larkum 1976a; 1976b; Borowitzka 1981; Borowitzka 1984). The mechanism of stimulation is thought to be that the consumption of CO_2 by photosynthesis increases the pH of the surrounding seawater, thereby increasing the saturation state of CO_3^{2-} and favoring $CaCO_3$ precipitation (Digby 1977). Inversely, calcification may also stimulate photosynthesis by producing CO_2 (McConnaughey & Falk 1991; McConnaughey 1991). However, the so called “carbon dioxide utilization theory” proposed by Digby (1977) does not explain why some algae don’t calcify, as all algae take up CO_2 via photosynthesis and subsequently increase the extracellular CO_3^{2-} saturation state. In coralline algae, specific cell wall polysaccharides produced by these algae are thought to be nucleation sites for $CaCO_3$ deposition (Bilan & Usov 2001). It is hypothesized that noncalcifying algae produce $CaCO_3$ nucleation

inhibitors, for example herbivore deterrent phlorotannins, which would explain why they do not calcify (Borowitzka 1984; 1987). In other calcifying algae, like the calcifying chlorophytes, the location of the calcification process within the alga is an important factor that dictates $CaCO_3$ deposition. The calcifying chlorophyte algae in the genus *Halimeda* deposit aragonite crystals on the surface of intracellular spaces between specialized structures called utricles. The chemical environment between the utricles is semi-separated from the external seawater, and therefore the algae have biological control over this internal environment via photosynthetic and respiratory processes (Borowitzka & Larkum 1977; Figure 4). The combination of having an aragonite skeleton and a semi-isolated calcification locus suggests that *Halimeda* might be less susceptible to ocean acidification than the coralline algae, which deposit the highly soluble high-Mg calcite crystals directly on their cell walls. As the cell walls are in contact with the external seawater, the high-Mg calcite skeleton of coralline algae may be highly susceptible to dissolution under ocean acidification conditions.

a) b)

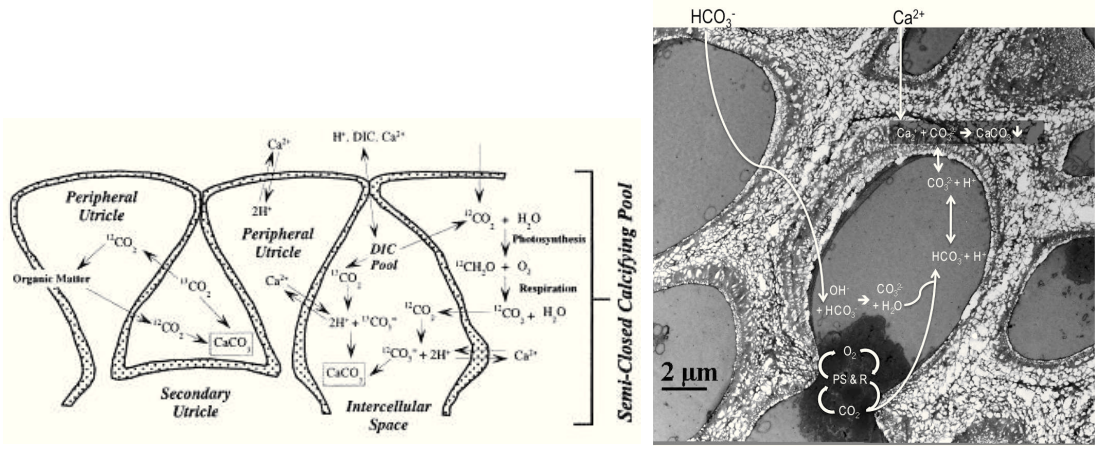

Figure 4. Calcification mechanism of a) *Halimeda* and b) coralline algae modified from Lee & Carpenter (2001) and Digby (1977). Calcification in *Halimeda* occurs between the utricles in the intracellular space, separated from the outer seawater. Calcification in coralline algae occurs in the intercellular region (seen as white areas in this transmission electron photograph of *C. officinalis* cells), which is in direct contact with the external seawater. Additional CO_3^{2-} ions produced by respiration (R) are used in calcification, and the uptake of CO_2 by photosynthesis (PS) results in increases in pH in the intracellular spaces.

The physiological and ecological purpose of calcification in macroalgae is not well described. It is suggested that calcification provides structural support against wave action, protection against high light and defense against herbivory (Littler 1976), but the

latter hypothesis is not strongly supported. Steneck (1983) suggests that calcifying macroalgae were prevelant before the most damaging feeding mechanisms (sea urchin teeth and parrotfish beaks) evolved, and studies have shown that some feeding generalists don't mind eating calcified tissue (Pennings & Svedberg 1993; Hay et al. 1994). An alternative hypothesis is that calcification serves as a proton source for nutrient and HCO_3^- uptake, which provides a competitive advantage over noncalcifiers in oligotrophic conditions (McConnaughey & Whelan 1997). A combination of these factors, in addition to the production of anit-herbivore secondary metabolites, most likely provide calcifiers with synergistic defenses against herbivory (Hay et al. 1994), structural stability and a physiological advantage over noncalcifiers under nutrient limiting conditions. Under elevated CO_2, these advantages for calcifying macroalgae may be at risk and could result in shifting the ecological relationships between calcifiers and noncalcifiers.

Macroalgal communities: competitive interactions

Macroalgal communities are diverse systems that provide habitat, food, nursery grounds, substrate, and protection from wave action for many marine organisms in temperate and tropical environments (e.g. Paine & Vada 1969; Lubchenco 1978; Lubchenco & Menge 1978; Littler & Littler 1984; Jenkins et al. 1999; Gibbons & Griffiths 1986; Eriksson et al. 2006). Within macroalgal communities, different species compete for space, light and nutrients, and compete with grazers for survival (Hawkins & Hartnoll 1983; Jenkins et al 1999). In temperate rocky intertidal environments, algal communities show strong zonation patterns dictated by wave action, tidal fluctuations and light intensity (Stephenson & Stephenson 1954; Southward 1958; Lewis 1964). Dessication and high light tolerant species generally occupy the highest intertidal zone, whereas species with efficient light harvesting complexes occupy the subtidal zone where light intensity is low (Stephenson & Stephenson 1949; Lewis 1964; Wahl 2009 and references therein). A more detailed summary of the macroalgal communities investigated in this thesis is given below.

Competitive interactions between calcifying and noncalcifying macroalgae are heavily dependent on abiotic factors, such as light, inorganic nutrients, temperature and dessication, as well as biotic factors, such as grazing (Doty 1946; Paine 1966; Connell

1972; Dayton 1971; Lewis 1977). Calcifying macroalgae have slower growth and lower productivity rates than noncalcifiers and therefore represent late successional species that are abundant in stable environments (Littler 1979; Littler and Littler 1980). In environments with high wave exposure, coralline algae are often dominant occupiers of space, and they are also abundant in tide pools (Stephenson & Stephenson 1949; Bartsch & Tittley 2004; Reichert et al. 2008). However, abiotic disturbances, such as changes in inorganic nutrients or temperature, can shift macroalgal community structure, leading to the dominance of more opportunistic species that have stimulated growth rates under higher temperatures and/or can efficiently assimilate nutrients under eutrophic conditions (Littler & Littler 1980).

The strongest factors affecting the ecological balance between calcifying and noncalcifying macroalgae are herbivory (top-down control) and nutrient enrichment (bottom-up control; see Masterson et al. 2008 and references therein). When herbivory is low due to over fishing and/or inorganic nutrient conditions are high due to anthropogenic nutrient enrichment of coastal environments, opportunistic noncalcifying algae are able to overgrow and shade the calcifying species because of their high growth rates (e.g. Lapointe et al. 1992; Delgado & Lapointe 1994; Belliveau & Paul 2002; Burkepile & Hay 2006; Masterson et al. 2008). Eutrophication (nutrient enrichment) is a local threat to the diversity, stability and productivity of coastal marine environments, and its combination with the current global threat of ocean acidification could be even more damaging to coastal communities. It is therefore important to investigate how calcifying organisms will respond as individuals to changing global and local conditions in isolation and in combination, as well as how their competitive interactions with noncalcifying organisms will be affected.

Macroalgal communities: collection sites for experimental work

The temperate macroalgae species that were investigated in this study were collected from the rocky coast of Helgoland in the German Bight. In this environment, the upper littoral zone is dominated by *Ulva* spp., while the upper eulittoral zone is dominated by *Fucus* spp. with seasonal prescence of *Ulva* spp. and an understory dominated by red algae (rhodophytes), including calcifying rhodophytes. The lower eulittoral is a transition zone between *Fucus* spp. and *Laminaria* spp., and the sublittoral is dominated by an

overstory of *Laminaria hyperborea*, *L. digitata* and *Saccharina latissima* and an understory of rhodophytes, including calcifying red algae (Markham & Munda 1980; Janke 1990; de Kluijver 1991; Jenkins et al. 1999; Reichert et al. 2008). An example of a diverse Helgoland macroalgal community is shown in Figure 5.

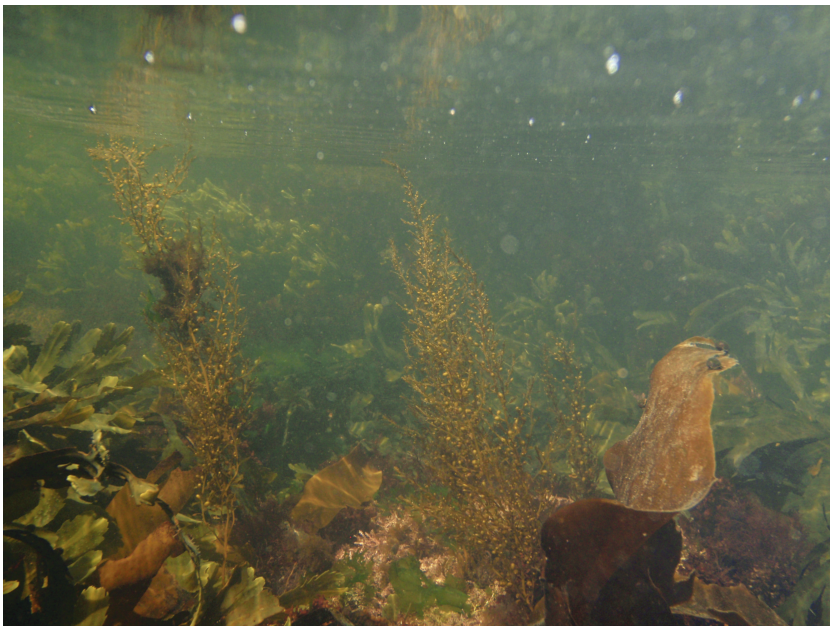

Figure 5. Diverse macroalgal community in the lower eulittoral zone on the rocky coast of Helgoland in the German Bight.

The tropical macroalgae species investigated in this study were collected from Curaçao, former Netherland Antilles close to the Caribbean Marine Biological Institute (CARMABI). The shallow reefs in this area have diverse macroalgal and coral communities that also show zonation patterns (van den Hoek et al. 1972; 1975). Fleshy and filamentous algae have the highest species richness in the lower eulittoral zone, accompanied by a diverse community of crustose coralline algae. *Halimeda* spp. are also common in the lower eulittoral zone, but can penetrate to deeper depths, as can many of the crustose corallines and a few fleshy algae (van den Hoek et al. 1972; 1978). *Halimeda opuntia* and other calcifying green algae are also abundant in lagoons and bays, mixed with sublittoral seagrass beds (van den Hoek et al. 1972; Kuenen & Debrot 1995). Eutrophication has become a serious problem in some of the fringing coral reefs in Curaçao due to sewage discharge, rainwater runoff, industrial waste, and groundwater seeping (Gast 1998). Therefore, the algae collected from this site were

exposed to both elevated CO_2 and inorganic nutrients, in order to determine how macroalgal communities might respond to future conditions in Curaçao.

Thesis objectives

The purpose of this thesis was to first identify the physiological responses of calcifying macroalgae to CO_2 concentrations expected by the end of the 21st century. Two calcifying macroalgae, the temperate calcifying coralline alga *Corallina officinalis* and the tropical calcifying chlorophyte alga *Halimeda opuntia* were focused on in this thesis. The potential synergistic effects of the global phenomenon of ocean acidification combined with more localized eutrophication events on these organisms was also investigated, because these anthropogenic changes are always occuring simultaneously, and it is difficult to predict how organisms will respond when only one factor is considered. In order to have both a physiological and ecological perspective in the thesis, I also investigated how the competition of calcifiers with neighboring noncalcifying organisms will change under future oceanic CO_2 conditions alone, and in combination with nutrient enrichment. Finally, a comparison of the physiological and competitive responses of a temperate and tropical calcifying macroalgae under elevated CO_2 is discussed.

Research Questions

General research questions

1. How are growth, photosynthesis and calcification in calcifying macroalgae affected by elevated CO_2?

2. How are growth and photosynthesis in noncalcifying macroalgae affected by elevated CO_2?

3. How are the comptetitive interactions between calcifying and noncalcifying macroalgae affected by elevated CO_2?

4. How will temperate and tropical macroalgae communities respond to elevated CO_2?

5. Is the combination of elevated CO_2 and eutrophication beneficial or stressful for macroalgae, and how do these factors affect their competition?

Publication-specific research questions

Chapter 1

1. How are growth, photosynthesis and calcification affected by CO_2 concentrations expected at the end of the 21st century and beyond in the calcifying rhodophyte alga *Corallina officinalis*?

2. How is the enzyme external carbonic anhydrase affected by elevated CO_2 in *C. officinalis*?

3. Does carbonic anhydrase play a role in calcification?

4. How is the skeletal structure of *C. officinalis* affected by elevated CO_2?

Chapter 2

1. How are carbonic anhydrase and nitrate reductase affected by elevated CO_2 over time?

2. How does seasonality affect enzyme activity in *C. officinalis* exposed to elevated CO_2?

3. How are inorganic carbon and nutrient uptake and assimilation affected by elevated CO_2 and seasonality?

Chapter 3

1. How will temperate rocky-intertidal macroalgal communities respond to elevated CO_2?

2. How will the competitive interactions between *C. officinalis* and its noncalcifying counterpart *Chondrus crispus* be affected by elevated CO_2?

Chapter 4

1. How will growth, photosynthesis and calcification in the calcifying chlorophyte alga *Halimeda opuntia* respond to future oceanic CO_2 concentrations?

2. How do the combined factors of elevated CO_2 and inorganic nutrients (nitrate and phosphate) affect the physiology of *H. opuntia*?

3. How are the enzymes involved in inorganic carbon and nitrate assimilation (external carbonic anhydrase and nitrate reductase) in *H. opuntia* affected by elevated CO_2 and inorganic nutrients?

Chapter 5

1. Will the competitive interactions between *H. opuntia* and its common noncalcifying epiphyte *Dictyota* (phaeophyta) be altered under elevated CO_2?

2. How does the combination of elevated CO_2 and inorganic nutrients effect the competitive interactions between *H. opuntia* and its epiphyte *Dictyota*?

3. How will tropical macroalgal communities respond to ocean acidification at the global level, and how will the localized problem of eutrophication interact with ocean acidification to affect these communities?

List of publications and declaration of own contribution

Publication 1. Physiological responses of the calcifying rhodophyte, *Corallina officinalis* (L.), to future CO_2 levels

Authors: Laurie C. Hofmann, Gamze Yildiz, Dieter Hanelt, Kai Bischof

Contributions: I designed the experiment, collected the algae with help from Gamze Yildiz and Kai Bischof, and maintained the experimental set-up. I measured all response variables with the help of Gamze Yildiz, and analyzed all the data. Dieter Hanelt provided transmission electron microscope images of the algae, and Kai Biscof supervised the thesis. I wrote the manuscript with the cooperation of the coauthors.

Publications 2 & 3: The same contributions apply to both manuscripts

2.The effect of elevated CO_2 and seasonality on the activity of two enzymes in the calcifying rhodophyte *Corallina officinalis*

3. Competitive interactions between calcifying and noncalcifying temperate marine macroalgae under elevated CO_2 levels: a mesocosm study

Authors: Laurie C. Hofmann, Sandra Straub, Kai Bischof

Contributions: I designed the experiment, collected the algae with help from Sandra Straub, and built the experimental set-up with the help of technicians from the Wadden Sea Station, who are mentioned in the acknowledgements. Sandra Straub measured enzyme activity, inorganic content, and growth in the algae and maintained the experimental set-up. I measured photochemistry and all community responses. I analyzed all data and wrote the manuscript with the cooperation of the coauthors. Kai Bischof supervised the thesis.

Publication 4. Physiological responses of the calcifying chlorophyte *Halimeda opuntia* to elevated inorganic nutrients and carbon dioxide

Authors: Laurie Hofmann, Jasmin Heiden, Mirta Teichberg, Kai Bischof

Contributions: I designed the experiment with help from Mirta Teichberg, built the experimental set-up with help from technicians from the Leibniz Institute for Marine Tropical Ecology, who are mentioned in the acknowledgements. I measured all response variables of the algae with the help of Jasmin Heiden and Mirta Teichberg, analyzed all the data and wrote the manuscript in cooperation with the coauthors. Both Jasmin Heiden and myself maintained the experimental set-up. Kai Bischof supervised the thesis.

Publication 5. The effect of elevated CO_2 and inorganic nutrients on competition between the calcifying chlorophyte *Halimeda opuntia* and its noncalcifying epiphyte *Dictyota* sp.

Authors: Laurie C. Hofmann, Mirta Teichberg, Cecilia Baggini, Andrew Johnson and Kai Bischof

Contributions: I designed the experiment with help from Mirta Teichberg, and we received technical help from Achim Meyer (acknowledgements) with the experimental set-up. Cecilia Baggini was a visiting PhD student who contributed to the experimental maintenance, data accumulation and data analysis. Andrew Johnson helped with experimental set-up and maintenance as well as preparing samples for analysis in the lab. I analyzed the data with help from Cecilia Baggini, who analyzed some of the photographs. I wrote the manuscript in collaboration with the coauthors, and Kai Bischof supervised the thesis.

Chapter 1

Physiological responses of the calcifying rhodophyte, *Corallina officinalis* (L.), to future CO_2 levels

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Physiological responses of the calcifying rhodophyte, *Corallina officinalis* (L.), to future CO_2 levels

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Abstract

Future atmospheric CO_2 levels will most likely have complex consequences for marine organisms, particulary photosynthetic calcifying organisms. *Corallina officinalis* L. is an erect calcifying macroalga found in the inter- and subtidal regions of temperate rocky coastlines and provides important substrate and refugia for marine meiofauna. The main goal of the current study was to determine the physiological responses of *Corallina officinalis* to increased CO_2 concentrations expected to occur within the next century and beyond. Our results show that growth and production of inorganic material decreased under high CO_2 levels, while carbonic anhydrase activity was stimulated and negatively correlated to algal inorganic content. Photosynthetic efficiency based on oxygen evolution was also negatively affected by increased CO_2. The results of this study indicate that *C. officinalis* may become less competitive under future CO_2 levels, which could result in structural changes in future temperate intertidal communities.

Key words: *Corallina officinalis*; ocean acidification; photosynthesis; carbon dioxide; calcification

Introduction

Ocean acidification, which is described as a pH decrease in ocean surface waters caused by the dissolution of anthropogenically produced atmospheric CO_2, is currently under heavy investigation due to its potential impacts on marine organisms. The future atmospheric CO_2 levels are predicted to reach 800 microatmospheres (µatm) by 2100,

based on the “business as usual” scenario or 1000 µatm based on the most liberal predictions released by the Intergovernmental Panel on Climate Change (IPCC). These atmospheric CO_2 concentrations correspond to a predicted 0.3-0.5 unit drop in pH of the surface ocean waters relative to current conditions (Caldeira and Wickett 2005; Orr et al. 2005). A corresponding shift in the speciation of dissolved inorganic carbon (DIC) occurs as the system attempts to buffer itself, and the result is a lower saturation state for calcium carbonate ($CaCO_3$) – the main skeletal building block for all marine calcifying organisms.

A decreased saturation state of $CaCO_3$ could make calcification more difficult for marine calcifying organisms, and some organisms have already been reported to decrease calcification rates under ocean acidification conditions (Gao et al. 1993; Langdon et al. 2000; 2003; Riebesell et al. 2000; Jokiel et al 2008; Kuffner et al. 2008). However, despite the large degree of effort that has already been made to investigate the effects of ocean acidification, the biological and ecological consequences of this scenario are not well understood because of the highly variable responses of different organisms (Ries et al. 2009). These variable responses are likely due to physiological processes other than calcification that are also affected by higher CO_2 levels, lower pH and lower $CaCO_3$ saturation states that accompany ocean acidification.

Calcifying autotrophic organisms are especially interesting to investigate due to the complex physiological processes of photosynthesis and calcification that play a large role in the fitness of these organisms, and the important ecological niches that they occupy. In tropical environments, corals are heavily studied in ocean acidification research due to the symbiotic relationship between the photosynthesizing zooxanthellae and the heterotrophic coral (Reynaud et al 2003; Langdon and Atkinson 2005; Krief et al 2010). In temperate environments, calcifying micro- and macroalgae are crucial organisms to investigate because of their high primary production and contribution to global biogeochemical cycles (Thierstein and Young 2004) and importance in providing structural support, refugia and substrata for inter- and subtidal marine communitites (Coull and Wells 1983; Hicks 1986; Akioka et al. 1999). Calcifying algae are unique organisms that conduct both photosynthesis and calcification – two processes that are intricately linked to DIC (CO_2, HCO_3^-, CO_3^{2-}). Because the speciation of DIC and subsequently the seawater carbonate system will be altered under ocean acidification

conditions, photosynthesis and calcification in calcifying algae could be heavily impacted.

Carbon dioxide is the main substrate for photosynthesis. The availability of CO_2 for photosynthesis is lower in seawater than in air due to its slower rate of diffusion in seawater. Therefore marine photosynthetic organisms have acquired efficient methods for carbon uptake called carbon concentrating mechanisms (CCMs). These mechanisms enhance photosynthetic efficiency by concentrating CO_2 at the reaction site of the carbon fixing enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (RubisCO), thereby decreasing competition by oxygen molecules (photorespiration). These mechanisms also compensate for the fact that bicarbonate ion (HCO_3^-) concentrations are higher than dissolved CO_2 concentrations, and therefore involve the enzyme carbonic anhydrase that increases the interconversion of HCO_3^- and CO_2. Some macroalgae even have the ability to use HCO_3^- directly via anion exchange processes (Drechsler et al. 1993; Axelsson 1995; Larsson et al. 1997). As a result, photosynthesis is very efficient at ambient DIC conditions, but it is not always saturated, as several macroalgal species have shown increased photosynthetic and growth rates at increased CO_2 concentrations (Gao et al. 1991; 1993; Kübler et al. 1999; Zou 2005). On the other hand, some macroalgae have even shown decreased photosynthetic performance and/or growth (García-Sánchez et al. 1994; Israel et al. 1999) or no response at all (Israel and Hophy 2002). Clearly, the photosynthetic responses of macroalgae to high CO_2 concentrations are variable and complex, and for calcifying macroalgae, the responses may be even more complex due to the process of calcification.

Calcifying macroalgae must balance both photosynthesis and calcification. Some studies have indicated that these two processes are linked (Borowitzka and Larkum 1976b) and that both are strongly light dependent (Pearse 1972; LaVelle 1979; Borowitzka and Larkum 1976a; Borowitzka 1981). The mechanism of calcification in *Halimeda* spp. has been heavily studied (Borowitzka and Larkum 1976a; b; c; De Beer and Larkum 2001), and is reported to occur as the deposition of aragonite crystals in the intercellular spaces of the alga. Photosynthesis is thought to stimulate calcification by removing CO_2 from the intercellular spaces and thereby increasing the local pH and carbonate ion concentration (Borowitzka and Larkum 1976b). The coralline red algae (Corallinaceae) have no intercellular spaces like *Halimeda* spp., but their cell walls have

an organic matrix that is thought to provide a nucleation site for calcite crystals (Borowitzka 1981; Pueschal et al. 1992; Bilan & Usov 2001), and the mechanism for calcification is also thought to be related to the localized increase in pH at the cell-seawater interface created by photosynthetic uptake of CO_2 (Digby 1977). Coralline algae deposit $CaCO_3$ as high magnesium-calcite, which is the most soluble form of $CaCO_3$. As a result, coralline algae could be some of the first calcifying organisms to be negatively impacted by ocean acidification. Some authors have already reported the negative effects of high CO_2 on coralline algae (Gao et al. 1993; Gao and Zheng 2010; Kuffner et al. 2008; Martin and Gattuso 2009), but there is a definite need for a better understanding of how different species will respond to increased CO_2 concentrations. Therefore, the goal of this study was to increase our understanding of how the coralline alga *Corallina officinalis* will respond to the levels of CO_2 predicted to be in future ocean surfaces.

Corallina officinalis is an upright calcifying alga found in the inter- and subtidal 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 turfs that cover large areas of the intertidal and provides substratum, habitat and refugia for a number of important marine organisms (Coull and Wells 1983; Hicks 1986; Akioka et al. 1999; Kelaher 2002; 2003). On the rocky coast of the island of Helgoland in the North Sea, *C. officinalis* is abundant and is an important species in the macroalgal community. It can be found in isolated patches and in extensive turfs, and is often closely associated with the other red algae *Chondrus crispus* and *Mastocarpus stellatus* and under a dense cover of *Fucus serratus* and *Ascophyllum nodosum*. The main goals of the current study were to determine the physiological responses, including growth, photosynthesis, and calcification, of *Corallina officinalis* to increased CO_2 concentrations expected to occur within the next century and beyond. Changes in the physiology of this species could have significant impacts on the surrounding communities in rocky tidal environments.

Materials and Methods

Experimental set-up

Corallina officinalis specimens were collected from the intertidal zone of the northern coast of Helgoland, Germany in October, 2009 and stored in bottles containing natural seawater until they were transferred into culture at the University of Bremen, Germany. Cultures were maintained at 15 °C, 33 psu (Reef Salt, ab Aqua Medic GmBH Bissendorf, Germany), 50 µmol photons·m^{-2}·s^{-1} light intensity (Osram Luminux Plus Daylight L18W/11-860), and a 12:12 hour light:dark cycle in artificial seawater containing ¼ strength Provasoli enrichment medium (Provasoli, 1968). Calcium chloride ($CaCl_2 \cdot 2H_2O$) was added to the enrichment medium to a final concentration of 10 mM to ensure the presence of calcium for the calcifying algae.

Three CO_2 concentrations were used for experimental treatments: 384 µatm (ambient atmospheric CO_2 level), 1313 µatm and 1939 µatm. The three treatments were aimed to achieve ambient CO_2, 1x and 2x the A1F1 scenario for 2100 from the IPCC Special Report on Emissions Scenarios, but the medium CO_2 treatment was higher than expected due to technical restraints. Four replicate tanks containing 400 mg of algae were used for each CO_2 treatment level. One additional tank without algae was monitored at each CO_2 level to detect any potential effects of the algae on the water chemistry, but they were not used in the statistical analysis. The desired CO_2 concentrations were manipulated using the Aquamedic CO_2 Set Professional (Bissendorf, Germany), which consisted of computer-controlled gas valves that were set to open and close when the recorded pH rose above or below, respectively, the desired pH. The CO_2 was bubbled directly into each 20-liter treatment tank until the pH level corresponding to the desired CO_2 concentration was reached. Once the desired pH was reached, the gas valve was automatically closed and the CO_2 bubbling stopped. Manual monitoring of the pH in each tank was conducted using a WTW 3310 pH meter, electrode model SenTix Mic (Weilheim, Germany) calibrated with standard buffer solutions pH 4 and 7 AVS Titrinorm (BDH Prolabo from VWR International, Dublin, Ireland). The pH electrode was calibrated with standard NBS buffers. The pH units were corrected to the total scale using a calibration with Tris buffers at 1°C intervals over a temperature range of 5-22°C. The pH was measured twice a day and before and after every water change. Water samples (25 ml) were taken weekly for Total alkalinity (TA)

analysis. The seawater carbon chemistry was calculated using the CO2SYS program (version 14) originally designed by Lewis and Wallace (1998). The data input included pH (total scale) and TA, and the constants from Mehrbach et al. (1973) were used. The artificial seawater was filtered through a Whatman polycap capsule filter (0.45 µm pore size) and circulated in each tank using magnetic stir bars. The tanks were covered with clear plastic to limit gas evaporation, and nutrients were added after each water change (every other day) during a total exposure time of four weeks.

Growth, Inorganic Content, and Carbonic Anhydrase

Growth rates were determined by weighing the fresh weight of one marked algal thallus in each treatment tank over time after blotting the thalli dry with paper towels. Relative growth rates (RGR) expressed as percent of fresh weight per day (%FW•day^{-1}) were calculated as $\ln(W_t/W_0)/t \times 100$, where W_t and W_0 are the fresh weights at time t (days) and time 0. Inorganic content was determined by drying the algae at 70°C for 24 hours followed by burning for 24 hours at 400°C in a muffle furnace. The area of calcified material between cells was calculated from transmission electron microscopy images provided from the University of Hamburg, Germany. Transmission electron microscopy (TEM) was performed according to Quader (1985). Briefly, the algae were fixed with 2% glutaraldehyde in cacodylate buffer (75 mM, pH 7,0) for 1,5 h, postfixed with 1% osmium tetroxide at 4°C overnight. The samples were dehydrated through a series of graded acetone concentrations, 30% - 100%, and finally embedded in plastic according to Spurr (1964). Ultrathin sections were obtained with a ultramicrotome (Ultracut E, Leica-Reichert-Jung, Nußloch, Germany) and stained with uranyl acetate followed by lead citrate (Reynolds 1963). Sections were viewed with a LEO 906 E TEM (LEO, Oberkochen, Germany) equipped with the MUltiScan CCD Camera (Model 794) of Gatan (Munich, Germany) using the Digital Micrograph 3.3 software from Gatan to acquire, visualize, analyze, and process image data. Images were analyzed with ImageJ (Image Processing and Analysis in Java, National Institutes of Health, Bethesda, Maryland, USA). Carbonic anhydrase activity was measured using the method described by Haglund *et al*. (1992). Samples of frozen (-80°C) *C. officinalis* (50-100 mg) were ground in extraction buffer (50 mM Tris, pH 8.5, 25 mM dithiothreitol, 25 mM isoascorbic acid, 5 mM EDTA) with a chilled mortar and pestel. The reaction was started by adding 3 ml of algal extract or buffer with no algae (blank) to 2 ml of ice-cold

CO_2 saturated water (substrate) in a glass tube. The time it took for the pH to drop 0.4 units within the pH range of 8.1-7.1 was recorded. Enzyme activity was calculated as $(t_b/t_s -1)/FW$, where t_b and t_s are the time in seconds it took for the pH to drop 0.4 units for the blank and the sample, respectively, and FW is fresh weight of the algal sample. Several aliquots of each algal extract were measured sequentially in order to ensure reproducibility of the assay.

Photosynthesis measurements

Photosynthesis parameters were measured using Pulse Amplitude Modulated (PAM) fluorescence (PAM 2100, Heinz Walz GmbH, Effeltrich, Germany) at time 0, 3 days, 9 days, 2 weeks and 4 weeks following the methods by **(**Bischof et al. 1999). Specimens were dark adapated for 5 minutes in order to obtain maximum photosynthetic efficiency values (Fv/Fm) and rapid light curves consiting of 30 second light intervals (0-2100 µmol photons m^{-2} s^{-1}) were conducted to measure relative electron transport rates (rETR). Oxygen production was measured using a Hansatech Chlorolab 3 System consisting of a S1 clark type polarographic oxygen sensor, DW3 electrode chamber, Oxylab electrode control unit, LH36/2R red LED light (660 nm), Quantitherm PAR/Temperature sensor, and Oxylab32 Windows software (Hansatech Instruments Ltd, Norfolk, England). A range of 50-100 mg fresh weight (FW) of algal tissue was placed in the electrode chamber containing 15 ml of filtered, artificial seawater at the respective treatment levels. Photosynthesis-Irradiance curves were produced by measuring oxygen production for 10 minutes (following 15 minutes of dark adaptation) at a range of increasing light intensities (0-1000 µmol photons m^{-2} s^{-1}) produced by the LH36/2R LED light source during a series of automated steps. During the incubation, the culture medium was aerated with a small magnetic stir bar. Oxygen measurements were made before and after the four-week experiment. Total oxygen production was calculated with respect to chlorophyll *a* content, which was extracted in 2 ml dimethyl fluoride (DMF) for 24 hours at 4 °C in the dark and calculated according to Inskeep and Bloom (1985).

Statistics

For each of the response variables including growth, inorganic content, and carbonic anhydrase activity, a treatment effect of CO_2 was tested using a one-way analysis of variance (ANOVA) in the statistics program PASW Statistics 18.0 (IBM SPSS Statistics

Inc.). Normality and homogeneity of variances were tested using Kolmogorov-Smirnov and Levine tests, respectively. The photosynthesis-irradiance curves generated from PAM fluorescence and oxygen evolution were fit to the Eilers and Peeters (1988) model. The following photosynthetic parameters were calculated from the model: maximum photosynthetic rate ($rETR_{max}$ or P_{max}), photosynthetic efficiency (alpha, α) and saturation irradiance (I_k). The curve fit parameters were calculated for each individual replicate and averaged for statistical analysis. Linear regressions and Pearson correlations were conducted to investigate relationships between photosynthetic performance and CO_2/pH.

Results

The seawater chemistry parameters for each of the three CO_2 treatments are shown in Table 1. A one way ANOVA followed by a Tukey´s post-hoc test for multiple comparisons showed that there was a significant difference between the pH units of the three treatments during the 4 week experiment ($p<0.01$).

Table 1. Seawater chemistry for the three treatments, including mean (±SE) pH, pCO_2, HCO_3^-, CO_3^{2-} and $\Omega_{calcite}$. Only the starting conditions (without algae) for pCO_2, HCO_3^-, CO_3^{2-} and $\Omega_{calcite}$ are shown; they decreased slightly after algal addition, but only by 2-7%. Different letter superscripts represent significant treatment effects of pH ($p<0.001$).

pH	pCO_2 (µmol kg SW^{-1})	HCO_3^- (µmol kg SW^{-1})	CO_3^{2-} (µmol kg SW^{-1})	$\Omega_{calcite}$
8.300^a (0.0089)				11.24
n= 155	384 (0.82)	3271 (7.0)	465 (0.99)	(0.02)
7.843^b (0.0117)				4.62
n= 124	1313 (1.46)	3878 (4.3)	191 (0.21)	(0.01)
7.668^c (0.0125)				3.12
n= 124	1939 (0.21)	3870 (0.42)	129 (0.01)	(0.00)

Growth, Inorganic Content, and Carbonic Anhydrase

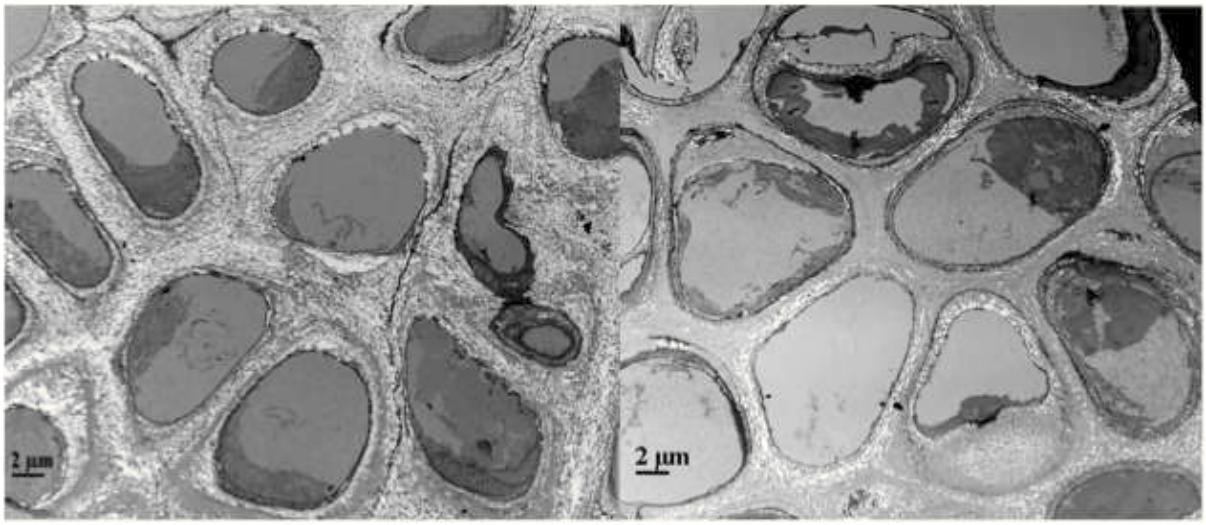
Relative growth rates (%FW•day^{-1}) showed no significant decline with respect to exposure time, so growth rates measured during the duration of the experiment were combined to obtain an overall mean at each pH condition. A multiple comparison test showed that growth rates in the low pH treatments (pH 7.67, pH 7.84) did not differ significantly from each other, but growth rates at both low pH treatments were significantly lower than those in the ambient pH treatment (Table 2, $p=0.005$, $p=0.056$, respectively).

A one-way ANOVA indicated that pH had a significant treatment effect on the percent inorganic material in *C. officinalis* (Table 2, p**=**0.017). The algae exposed to ambient pH conditions had significantly higher inorganic content (81.8 % ± 0.54) than the algae exposed to the lowest pH condition (79.3 % ± 0.38; Tukey´s post hoc test, p=0.048). Furthermore, the amount of calcium carbonate deposited as calcite on the cell walls was significantly lower at pH 7.67 (13.7 % ± 1.8) than at pH 8.30 (18.8 % ±1.7), which was clearly visualized in transmission electron microscopy images (Figure 1a**)**. Despite a trend, carbonic anhydrase activity was not significantly affected by pH (p = 0.08), but was significantly negatively correlated to inorganic content after 28 days of exposure (Figure 1b, pearson correlation = -0.057, p = 0.027, n = 12).

Table 2. Mean (±SE) relative growth rates, inorganic material, carbonic anhydrase acitivty and F_v/F_m ratios for the three CO_2 treatments after 28 days. Unshared superscript letters signifiy significant treatment effects.

pCO_2 (µmol kg SW^{-1})	Relative Growth Rate (% day^{-1})	Inorganic Material (% of Dry Weight)	CA Activity (units g FW^{-1})	F_v/F_m
384	1.97 (0.15)^{a}	81.8 (0.54)^{a}	43.42 (1.82)	0.61 (0.01)
1313	1.11 (0.15)^{b}	80.1 (0.72)^{ab}	76.37 (15.57)	0.62 (0.01)
1939	1.35 (0.24)^{b}	79.3 (0.38)^{b}	70.89 (5.43)	0.61 (0.02)

a)



b)

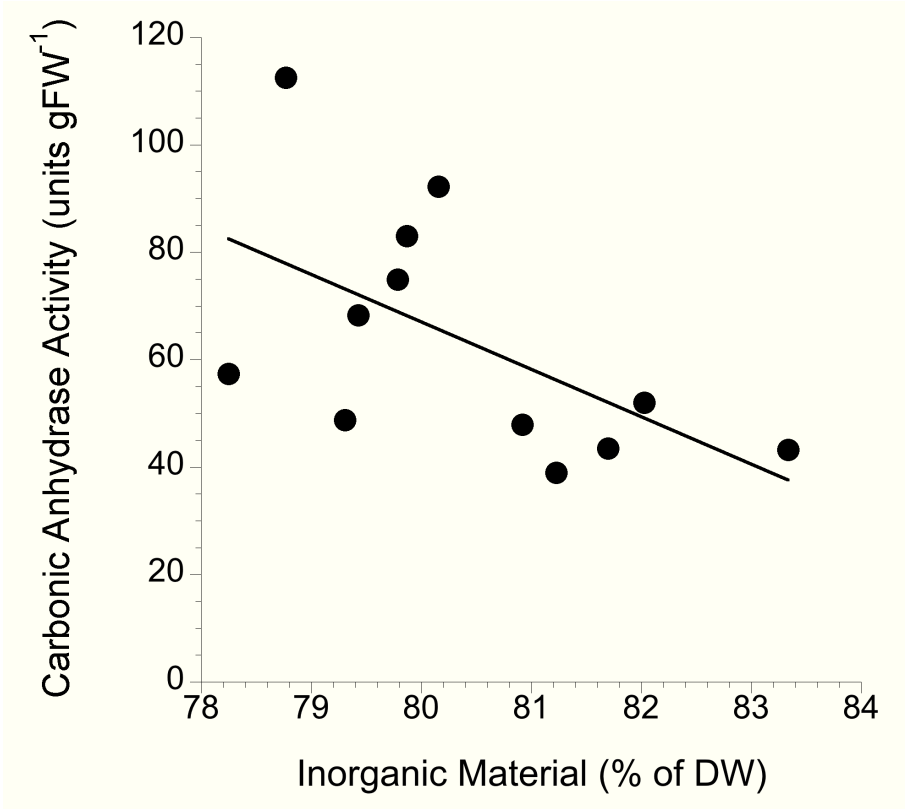


Figure 1. a) Transmission electron microscopy images of longitudinal sections through the youngest tip of each *C. officinalis* thalli. Calcite deposition (white material) between the cells is higher in algae grown under ambient (left) versus high (right) CO_2. b) Significant negative correlation between carbonic anhydrase activity and inorganic material after 28 days of exposure to CO_2 treatments (pearson correlation = -0.057, p = 0.027, n = 12).

Photosynthesis

The mean Fv/Fm ratios of *C. officinalis* did not differ significantly with respect to pH condition (Table 2). The F_v/F_m values of *C. officinalis* after 28 days of exposure to high CO_2 levels were not significantly different from initial Fv/Fm values prior to exposure, indicating that there was no culture effect or pH effect on Fv/Fm. As a result, we concluded that the algae were not photosynthetically stressed under the higher CO_2 conditions.

Table 3. Mean (±SE) maximum photosynthetic rates (P_{max}), light compensation points and respiration rates from oxygen evolution measurements and the maximum relative electron transport rates ($rETR_{max}$), photosynthetic efficiencies (α) and light saturation points (I_k) for PAM fluroescence measurements of *C. officinalis* exposed to different CO_2 levels. Unshared subscript letters for respiration rates respresent significant differences at the 95% confidence level.

Treatment	O_2 Evolution			PAM Fluorescence		
pCO_2 (μmol kg SW^{-1})	P_{max} (μmol O_2 mg Chl *a*$^{-1}$ hr^{-1})	Light Compensation Point (μmol photons m^{-2} s^{-1})	Respiration Rate (μmol O_2 mg Chl *a*$^{-1}$ hr^{-1})	$rETR_{max}$ (μmol e^- m^{-2} s^{-1})	α (μmol e^- μmol photons)	I_k
384	2.66 (0.17)	17.4 (6.3)	-0.66 (0.07)^{ab}	68.6 (1.9)	0.315 (0.009)	217.9 (3.0)
1313	3.89 (0.70)	31.4 (8.1)	-1.24 (0.26)^{a}	86.2 (13.5)	0.321 (0.019)	265.7 (32.8)
1939	1.92 (0.73)	27.5 (5.2)	-0.34 (0.08)^{b}	92.2 (6.5)	0.332 (0.009)	279.2 (25.0)

Photosynthesis - Irradiance (P-I) curves generated from PAM measurements and photosynthetic O_2 evolution measurements show mean relative electron transport rates (rETR) and mean O_2 evolution, respectively, as functions of light intensity for *C. officinalis* 28 days after exposure to different pH conditions (Figure 2). At ambient pH, relative electron transport rate and oxygen evolution showed similar non-linear trends with respect to light intensity. However, the two P-I curves (rETR and O_2 evolution) deviate from each other with decreasing pH. The non-linear curve fit parameters for rETR were not significantly affected by pH and are shown in Table 3. In terms of oxygen evolution, maximum photosynthetic rate (P_{max}) and light compensation point were not significantly affected by pH, but there was a significant treatment effect of pH on respiration rates (Table 3, $p = 0.021$). Respiration rates were highest at pH 7.8 and lowest at pH 7.6, and overall they increased (became more negative) significantly with increasing maximum photosynthetic rates (Figure 3a, $R^2 = 0.622$, $p = 0.012$). Furthermore, a significant linear regression indicated that I_k decreased with increasing pH (Figure 3b, $R^2 = 0.547$, $p = 0.023$), and there was a significant linear correlation between α and pH (Figure 3c, pearson correlation = 0.586; $p = 0.049$).

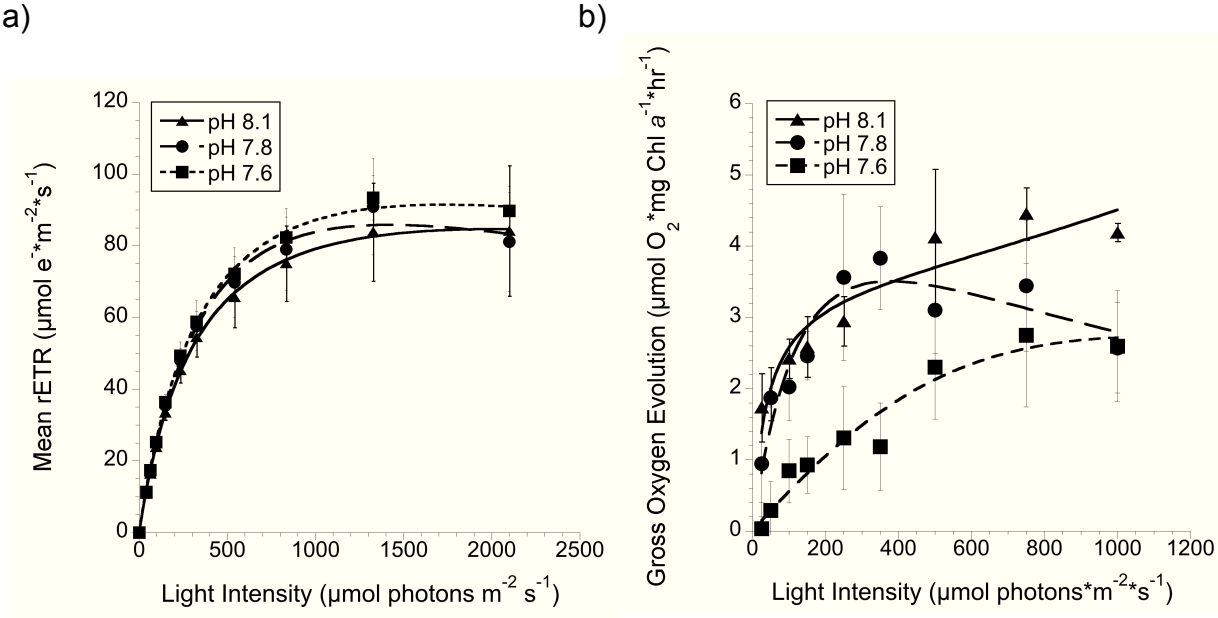


Figure 2. a) Mean (± SE, n=4) relative electron transport rates and b) mean (± SE, n=3) gross O_2 evolution as a function of light intensity for *C. officinalis* 28 days after exposure to the pH treatments.

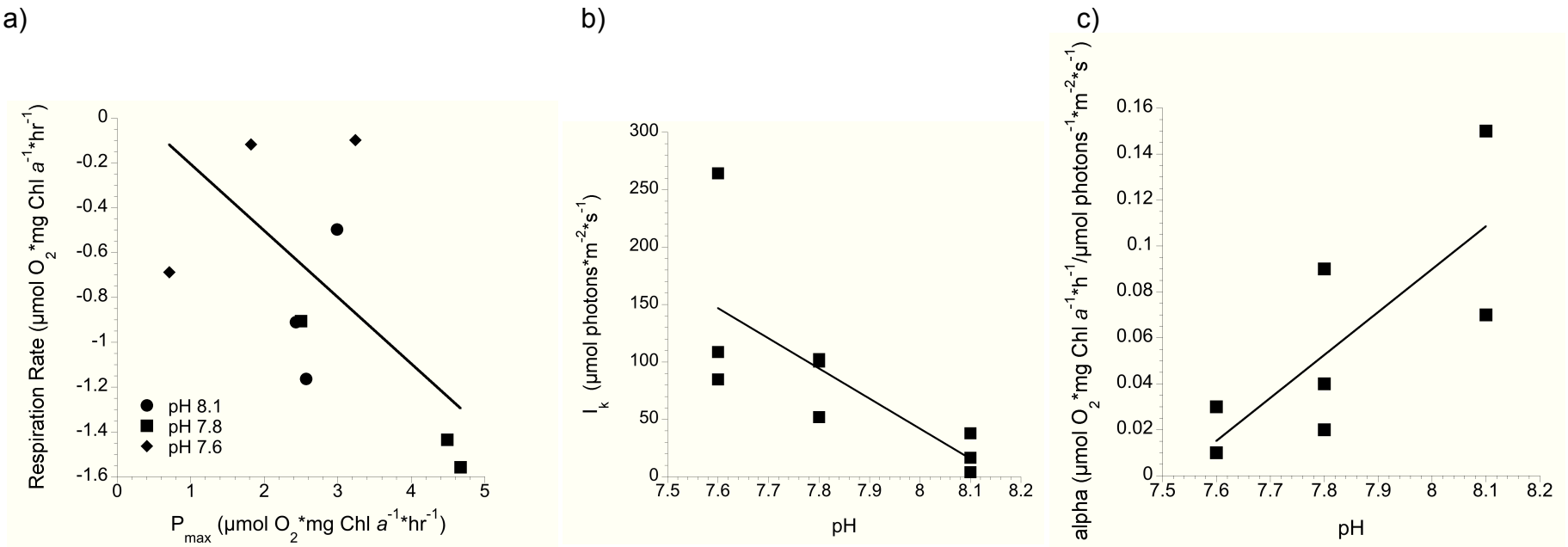


Figure 3. a) Respiration rates as a function of maximum photosynthetic rates; b) light saturation point and c) photosynthetic efficiency as a function of pH for *C. officinalis* after 28 days of exposure to the 3 pH levels. All data is from oxygen evolution measurements.

Discussion

The results of this study indicate that several aspects of *C. officinalis* physiology could be significantly affected by increasing surface seawater CO_2 concentrations. Growth of *C. officinalis* was affected by increased CO_2 concentrations, as both increased CO_2 treatments caused significantly slower growth rates than in the algae grown at ambient CO_2 concentrations. Our growth rates and the negative effect of CO_2 on growth complement those of Gao et al. (2010) who found that growth of *C. sessilis* was lower at 1000 ppmv CO_2 ($0.9\%\bullet day^{-1}$) than at 380 ppmv CO_2 ($2.1\%\bullet day^{-1}$). Because *Corallina officinalis* is a slow growing macroalga relative to non-calcifying species (Colthart and Johansen, 1973; Andrake and Johansen, 1980), significant reductions in growth could have large implications for reproduction, recruitment, and overall community structure in intertidal communities dominated by the calcifying alga.

In contrast to growth, which showed a clear negative response to increased CO_2 concentrations, photosynthetic parameters showed inconsistent responses to elevated CO_2 levels. The F_v/F_m ratios remained the same across all treatments, but the negative correlation between pH and $rETR_{max}$ indicated that at the light intensity used in this study, *C. officinalis* was not photosynthetically saturated with respect to inorganic carbon because maximum electron transport rates increased with increasing CO_2 concentration (decreasing pH). On the other hand, the O_2 evolution data suggested that *C. officinalis* photosynthesis was negatively affected in the low pH treatment, due to the low P_{max} and photosynthetic efficiency relative to the other treatments. The discrepancy between the photosynthetic responses detected in the PAM fluorometry measurements and the O_2 evolution measurements indicate that elevated CO_2 may increase non-assimilatory electron flow in Photosystem I, which is not detectable by PAM fluoresence. In general, the rETR and O_2 evolution data did not complement each other at high irradiances, which has been reported earlier to be a result of critically low yield values and photoinhibition at high irradiances (Hanelt and Nultsch 1995; Beer and Axelsson 2004). However, even at low irradiances, the rETR and O_2 evolution data for the two low pH treatments did not complement each other – they were only similar at the ambient pH treatment (8.3). Russel et al. (2009) found that elevated CO_2 had a negative effect on the effective quantum yield of coralline crusts, while Russel et al. (2011) found no CO_2 effect on their relative electron transport rate. The relative electron

transport rates may therefore not be a good indication of macroalgal health under elevated CO_2 conditions. In contrast, the O_2 evolution measurements directly measure a product of photosynthesis and are therefore more likely to represent the actual physiological response of the algae, compared to the relative electron transport rate. Futhermore, Gao et al. (2010) previously reported the inhibitory effect of elevated CO_2 (1000 ppmv) on photosynthesis and calcification in *C. sessilis*. Therefore, we conclude that the photosynthetic efficiency and maximum photosynthetic rate were negatively impacted at the lowest pH (7.6)/highest CO_2 concentration based on oxygen evolution. This decrease in photosynthetic efficiency may have been linked to the decreased physiological health of the algae as evidenced by decreased growth rates and lower calcite deposition under elevated CO_2 concentrations.

While growth and inorganic content were expected to decrease under high CO_2 concentrations, photosynthesis and carbonic anhydrase showed unexpected responses. We expected that photosynthetic efficiency would increase with increasing CO_2, as the algae would have more substrate for RubisCO. In parallel, we expected the carbonic anhydrase activity to be downregulated when more CO_2 was available, which has been found in previous algal studies under elevated CO_2 levels (Nelson et al. 1969; Garcia-Sánchez et al. 1994; Rost et al. 2003). While no significant treatment effect of pH was detected for carbonic anhydrase activity due to high variability within the assay measurements, the algae exposed to the two high CO_2 concentrations had roughly 40% higher CA activity than algae grown under ambient CO_2 conditions. We thus hypothesize that this stimulation of carbonic anhydrase activity may have been an attempt by the algae to compensate for decreased calcification/increased dissolution under high CO_2 concentrations. Carbonic anhydrase has been shown to play a role in the calcification process of many organisms, particularly corals, by regulating the internal and external cellular speciation of dissolved inorganic carbon (Kingsley and Watabe 1987; Niemer et al. 1994; Al-Horani et al. 2003; Rahman et al. 2007; Tambutté et al. 2007). Under high CO_2 concentrations, the following mechanism of external calcification in *C. officinalis* could be possible (originally proposed by Tambutté et al. 2007 for an azooxanthellate coral):

1. $CO_2 + H_2O \rightarrow H^+ + HCO_3^-$
2. $HCO_3^- \rightarrow CO_3^{2-} + H^+$
3. $Ca^{2+} + CO_3^{2-} \rightarrow CaCO_3$

In the first reaction, carbonic anhydrase catalyzes the conversion of CO_2 into HCO_3^- and during the process, two protons are produced, decreasing the pH outside of the cell. These protons could be removed from the site of calcification by a Ca^{2+}-ATPase, which catalyzes the exchange of $2H^+$ for Ca^{2+} in some algae, such as *Chara corallina* (McConnaughey and Falk 1991). It is also possible that carbonic anhydrase catalyzes the conversion of HCO_3^- to CO_3^{2-} in reaction 2, as suggested by Digby (1977). However, the stimulated CA activity could not completely compensate under the elevated CO_2 conditions, as inorganic content was low even when CA was stimulated. Furthermore, the question still remains why oxygen evolution and photosynthetic efficiency decreased under high CO_2 conditions compared to ambient, especially when CA activity was increased and CO_2 was abundant. Gao and Zeng (2010) found the same negative response of photosynthesis under elevated CO_2 concentrations in *C. sessilis*, and suggested increased nonphotochemical quenching and higher energy requirements were likely under CO_2 stress, resulting in lower growth and photosynthesis. In the current study, perhaps increased cyclic electron flow in Photosystem I created more ATP for the upregulation of CA activity, which therefore contributed to the decrease in photosynthetic efficiency and growth in *C. officinalis*.

In future studies with *C. officinalis* it will be necessary to separately measure both external and internal carbonic anhydrase activity in order to determine if both enzymes increase their activity in response to increased CO_2, or if only external CA is affected. Giordano and Maberly (1989) reported that *C. officinalis* only contained internal carbonic anhydrase. However, Mercado et al. (1997) reported the presence of CA in the related alga *Corallina elongata* after developing a more sensitive method for detecting the enzyme. Furthermore, Ragazzola (2009) found evidence for the presence of external CA in *C. officinalis* when photosynthesis was inhibited by the impermeable sulphonamide inhibitor acetazolamide (AZ), and the activity of external carbonic anhydrase in *C. officinalis* has been detected in our lab since this study was conducted. Therefore, future work will clarify how this enzyme is responding to increased CO_2 levels both inside and outside of the cells.

The clear response of decreased inorganic material production and lower growth rates in *C. officinalis* under elevated CO_2 conditions indicate that there are physiological impacts of ocean acidification on this alga that may impact the ecology of rocky

intertidal macroalgal communities. Slower growth rates and weaker (i.e. less calcium carbonate) skeletons may lower the ability of *C. officinalis* to compete with other macroalgae, particularly non-calcifying species, in its natural environment. Gao and Zeng (2010) suggested that the inorganic skeleton of *C. sessilis* provides protection from UV exposure, due to the exacerbated inhibition of photosynthesis they observed under the combined stress of elevated CO_2 concentrations and UV exposure in this species. Moreover as UV radiation as a single stress factor was shown to strongly affect competition between non-calcifying rhodophytes (Bischof et al. 2000; 2006), the combination of these two stressors might have strong influences on community structure. Furthermore, several studies have shown that biotic and abiotic stressors such as increased grazing pressure and high CO_2 levels lower recruitment of crustose calcifying algae (Belliveau and Paul 2002, Jokiel et al. 2008, Kuffner et al. 2008, Martin et al. 2008), while turf macroalgal cover increases (Russell et al. 2009; Connell and Russell 2010; Russell et al. 2011). If increased CO_2 levels weaken the skeletal structure of *C. officinalis*, the potential combination of increased grazing pressure, slower growth, higher UV stress and less recruitment will likely cause a phase shift in *C. officinalis* communities towards more fleshy, non-calcifying macroalgae, and could even amplify changes in competition between non-calcifying algae. While both calcifying and non-calcifying algae provide important habitat and shelter for many marine organisms, erect calcifying algae such as *C. officinalis* contribute to the strength of the intertidal community structure and provide refugia for organisms in environments with high wave action (Stewart 1982; Coull and Wells 1983; Kelaher 2002; 2003) – all important ecological roles that could be interrupted under high CO_2 conditions.

In conclusion, during our relatively short-term experiment conducted at CO_2 concentrations 1 and 2 times those that could be reached by 2100 (AIFI scenario, IPCC Special Report on Emissions Scenarios), *C. officinalis* growth was negatively affected by these conditions. Furthermore, inorganic carbon production and photosynthetic efficiency (based on O_2 evolution) were significantly negatively affected at the highest CO_2 concentration investigated in this study. The results indicate that *C. officinalis* will be physiologically disadvantaged if CO_2 concentrations in surface oceans reach above 1000 µatm, and could be negatively affected at even lower concentrations that are expected by the end of the century. The ecological implications of these physiological disadvantages are that this calcifying species could be less competitive under future

CO_2 scenarios compared to fleshy macroalgae unless it is able to adapt. However, the exact ecological impacts on *C. officinalis* and the surrounding macroalgal community are still unknown, especially when considering adaptation strategies. Therefore future work should focus on identifying how the physiological impacts observed in this study may induce changes in ecological relationships among macroalgae in temperate macroalgal environments.

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Chapter 2

The effect of elevated CO_2 on the activity of two enzymes in the calcifying rhodophyte *Corallina officinalis*: a mesocosm study

The effect of elevated CO_2 on the activity of two enzymes in the calcifying rhodophyte *Corallina officinalis*: a mesocosm study

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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 lower saturation state of carbonate ions. Such changes in seawater chemistry are expected to negatively effect calcification in calcifying marine organisms. However, other physiological process related to calcification will also be affected, including enzyme activity. In a mesocosm experiment, we exposed macroalgal communities to three CO_2 concentrations (380, 665, 1486 µatm) to determine how the activity of two enzymes related to inorganic carbon and nutrient uptake in a calcifying macroalga will be affected by elevated CO_2 concentrations. 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 percent of inorganic carbon in the algal skeleton decreased with increasing CO_2. Our results indicate that the processes of inorganic carbon and nutrient uptake are affected by elevated CO_2 due to changes in enzyme activity, which changes 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.

Introduction

Increasing atmospheric CO_2 emmission 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 0.3 to 0.5 units by 2100 (Feely et al. 2004; Caldeira & Wickett 2003; Orr 2005) due to increasing concentrations of atmospheric CO_2 that could reach up to 970 ppmv 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 ($CaCO_3$).

Lower saturation states of CO_3^{2-} in seawater are expected to have negative impacts of marine calcifying organisms. Indeed, several studies have already shown negative responses of corals, macroalgae, and mollusks to elevated seawater CO_2 concentrations (Anthony et al. 2008; Jokiel et al. 2008; Martin et al 2008; Martin & Gattuso 2009; Albright et al. 2010; Diaz-Pulido et al. 2011; Rodolfo-Metalpa et al 2011; Hofmann et al. 2012). 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; Rodolfo-Metalpa et al. 2011; Hurd et al. 2011; Jokiel 2011a; 2011b; 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. 1995; Mercado 1999; Gordillo et al. 2001; 2003; Israel & Hophy 2002; Zou 2005; Connell & Russell 2010; Zou et al. 2011; Hofmann et al. 2012). 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. in prep). It is nevertheless important to understand how all process, 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 is 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 CO_2, marine algae have mechanisms called carbon concentrating mechanisms (CCM), which transport HCO_3^- across cell membranes using ion transporters, or catalyze the dehydration of HCO_3^- to CO_2 via the membrane-associated external carbonic anhydrase (Johnston 1991; Badger & Price 1994; Raven 1997; 2002; 2003). In noncalcifying algae, carbon concentrating mechanisms have been shown to be downregulated under elevated CO_2 conditions because of the high energy demands of producing ion transporter proteins and enzymes (Raven 2011; 2012). However, in calcifying algae, this enzyme may play a role in calcification, and has been shown to increase under elevated CO_2 (Isenberg et al. 1963; Hofmann et al. 2012).

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. Noncalcifying 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 (Tyrell et al. 2008; Martin & Gattuso 2009; Mercado & 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 natrual 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 (Stewart 1982; Coull and Wells 1983; Kelaher 2002; 2003). *Corallina officinalis* is an upright calcifying alga found in the inter- and subtidal 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 and provides substratum, habitat and refugia for a number of important marine organisms (Coull and Wells 1983; Hicks 1977; Akioka et al. 1999; Kelaher 2002; 2003). It also serves as a buffer for meiofauna in areas with strong currents and wave action (Dommasnes 1968). The important ecological roles served by this alga could be interrupted under high CO_2 conditions, and it is therefore important to understand how its metabolism may be affected in the future. We therefore conducted a mesocosm study with macroalgal communities containing the calcifying rhodophyte *C. officinalis* grown under three different CO_2 concentrations. The competitive interactions between *C. officinalis* and noncalcifying macroalgae as well as the overall macroalgal community response are discussed in a separate paper (Hofmann et al. in press). Here we focus on inorganic nutrient uptake rates and the ezymatic activity of carbonic anydrase and nitrate reductase in *C. officinalis* grown under three different CO_2 conditions.

Materials and Methods

Experimental design and seawater chemistry

The experiment was conducted in 75 liter mesocosms on the German island of Sylt in the North Sea. Experimental conditions including mesocosm set-up, duration, and the carbonate chemistry of the seawater are outlined in Hofmann et al. (in press). Temperature, salinity and pH were monitored daily. Seawater samples for inorganic nutrient analysis were taken weekly from the mesocosm tanks. Nutrient uptake rates and calcification rates were calculated based on a 3 hour incubation of *C. officinalis* in 5 liter plexiglass chambers continuously bubbled with mixed gas (386, 665, or 1486 µatm CO_2). Calcification rates were calculated based on the alkalinity anomaly technique (Chisholm & Gattuso 1991).

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 by 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 minutes. Each vial was individually bubbled with N_2 gas for an addition minute before being placed into a 30°C water bath in the dark for 30 minutes. 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 4% sulfanilimide and 300 µl 0.1% n-(1-napthyl) ethylenediamine dihydrochloride. Following the assay, the algal tissue was tried at 60°C for 48 hours to determine dry weight (DW) and nitrate reductase activity was calculated as µmol NO_2^- g DW^{-1} hr^{-1}. A daily cycle of hourly nitrate reductase activity was measured under ambient CO_2 conditions in the light and in the dark to determine how the enzyme activity fluctuated. Following this analysis, we sampled tissue for enzyme activity each week based on the morning activity peak that was observed in *C. officinalis* (Figure 1).

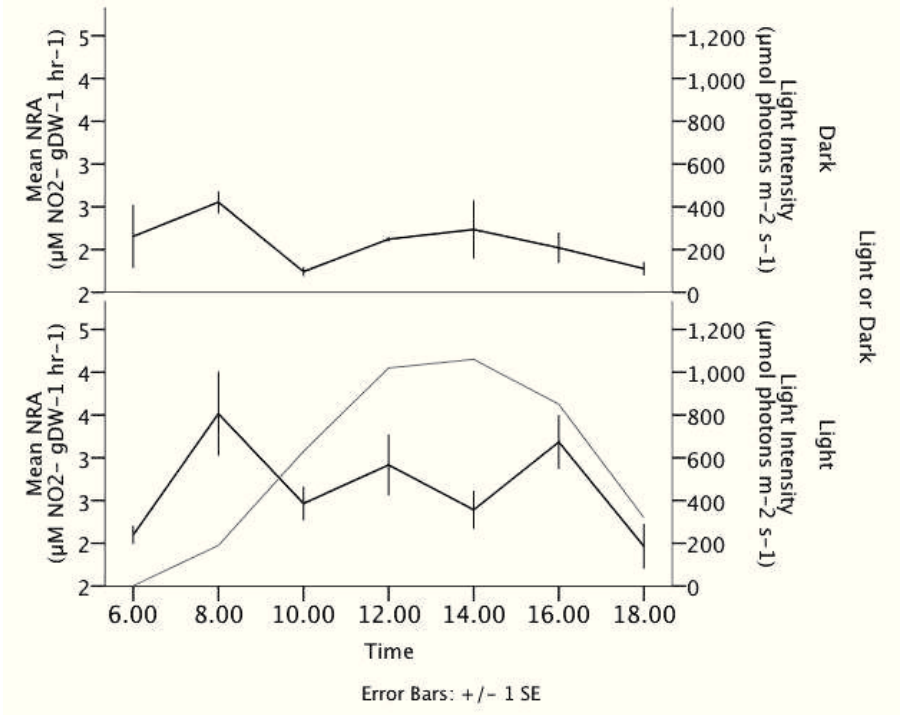

Figure 1. Daily cycle of nitrate reductase activity in *C. officinalis* in the dark(top panel) and light (bottom panel). The hourly light intensity is shown in the bottom panel in grey.

Total carbonic anhydrase activity of *C. officinalis* was measured according to Haglund et al. (1992). Algal tissue (50-100 mg fresh weight) was ground with liquid nitrogen in 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 were considered 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 $(T_b/T_s\text{-}1)/FW$, 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 dry weight (AFDW) of the dried tissue after removing the organic material by burning at 400°C for 12 hours.

Results

Seasonal variability of temperature and inorganic nutrients

The mean seawater temperature in the mesocosm tanks during the experimental period is shown in Figure 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 Figure 3. Nitrate concentrations in the seawater ranged from 6.7 to 38.9 µM and were highest in March, at the beginning of the experiment, and declined rapidly to a minumum 40 days 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 linearly during the experiment, and ammonium concentrations ranged from 1.03 to 3.06 µM.

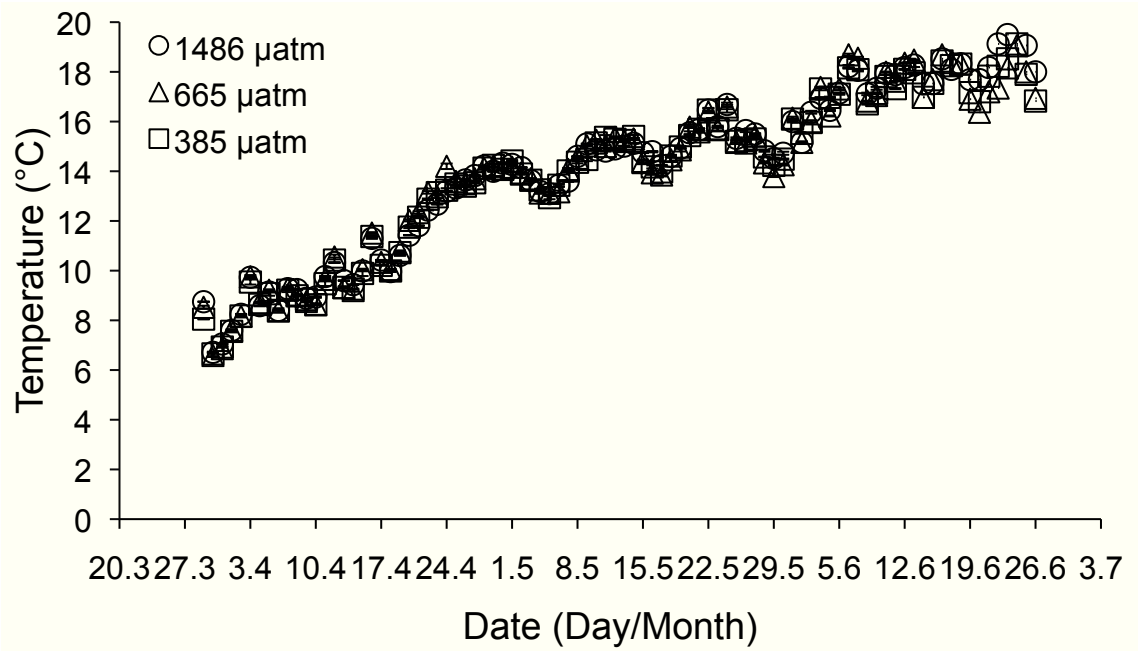

Figure 2. Mean (±SE, n = 4) seawater temperature in the mesocosm tanks during the experimental period.

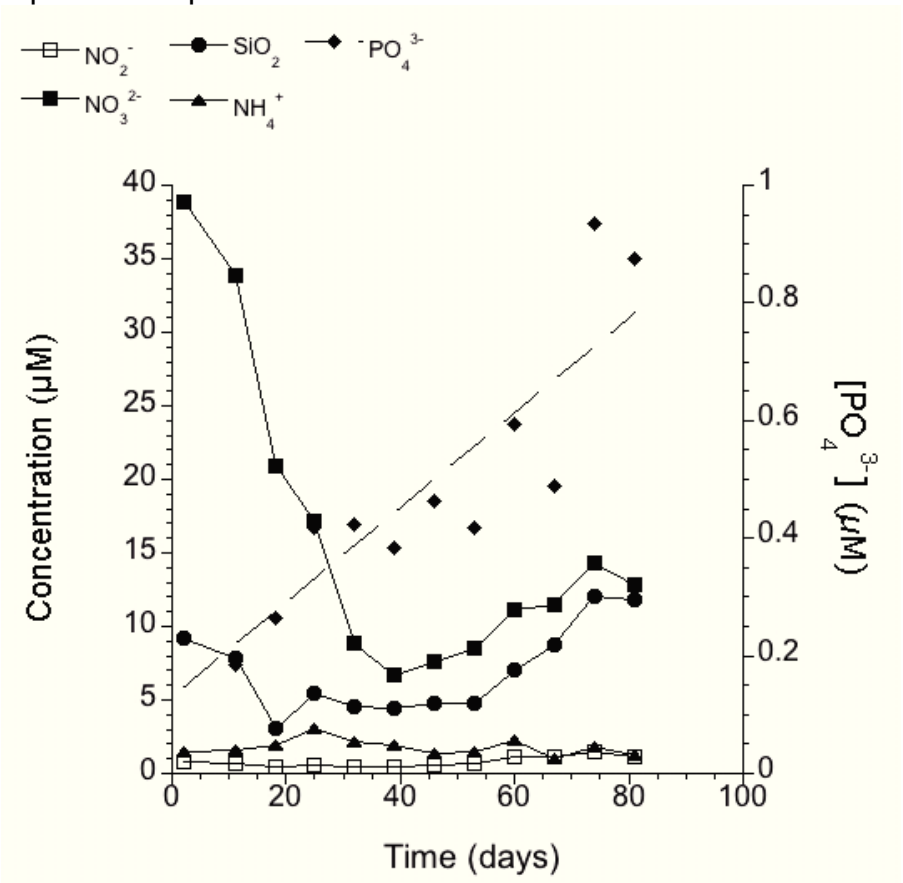

Figure 3. Nutrient concentrations of the ambient seawater throughout the duration of the experiment, from March 30th to June 17th, 2011. The dotted line indicates the linear fit of seawater phosphate concentrations as a function of time (R^2 = 0.79).

Nutrient uptake rates and nitrate reductase activity

Nutrient uptake rates of nitrate, ammonium, and phosphate by *C. officinalis* were measured after 35 days of exposure to the CO_2 treatments. There was a negative correlation between nitrate and phosphate uptake and CO_2 concentration (Pearson's correlation coefficient = -0.84, -0.62; p = 0.002, 0.037, respectively; Figure 4). There was no significant treatment effect of CO_2 on ammonium uptake rates (487 ± 183; 188 ± 49; 66 ± 90 μmol NH_4^+ m^{-2} hr^{-1}, respectively, for 385, 665 and 1486 μatm CO_2 treatments).

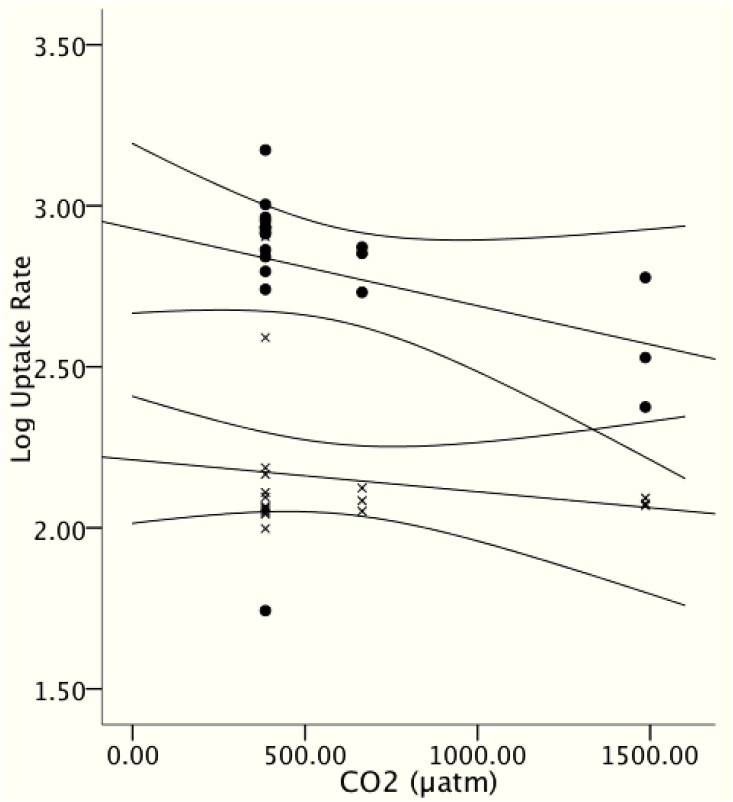

Figure 4. Phosphate (crosses) and nitrate (circles) uptake rates (log scale) as a function of CO_2 concentration. Linear correlations with 95% confidence intervals are shown. Data includes initial measurements. Mean (± SE) ammonium uptake rates are also shown for each CO_2 treatment.

A daily cylce of nitrate reductase showed that the enzyme activity showed little variation in the dark, except for a small peak in the morning that corresponded to the large peak in activity measured in the morning under light conditions. The enzyme activity fluctuated during the remainder of the light period until dropping off in the evening (Figure 1).

Throughout the experimental period, there was a significant effect of time on ntrate 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 (Figure 5a). Nitrate reductase was significantly negatively correlated to phosphate concentration at all CO_2 levels (Pearson's correlation coefficients for 385, 665 and 1486 µatm CO_2: -0.69, -0.61, -0.75; p = 0.007, 0.018, 0.003 respectively; Figure 6a), but the relationship between nitrate reductase activity and nitrate uptake rate differed between the CO_2 treatments (Figure 6b). The algae grown under elevated CO_2 had higher nitrate reductase activity, but lower nitrate uptake rates compared to algae grown in the ambient CO_2 treatment.

Table 1. Results from a MANOVA test on enzyme activity and $CaCO_3$ content (Cinorg) of *C. officinalis* with time as a within-subject factor and CO2 as a between subject factor. F-ratios are given with degreeds 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 x CO_2
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

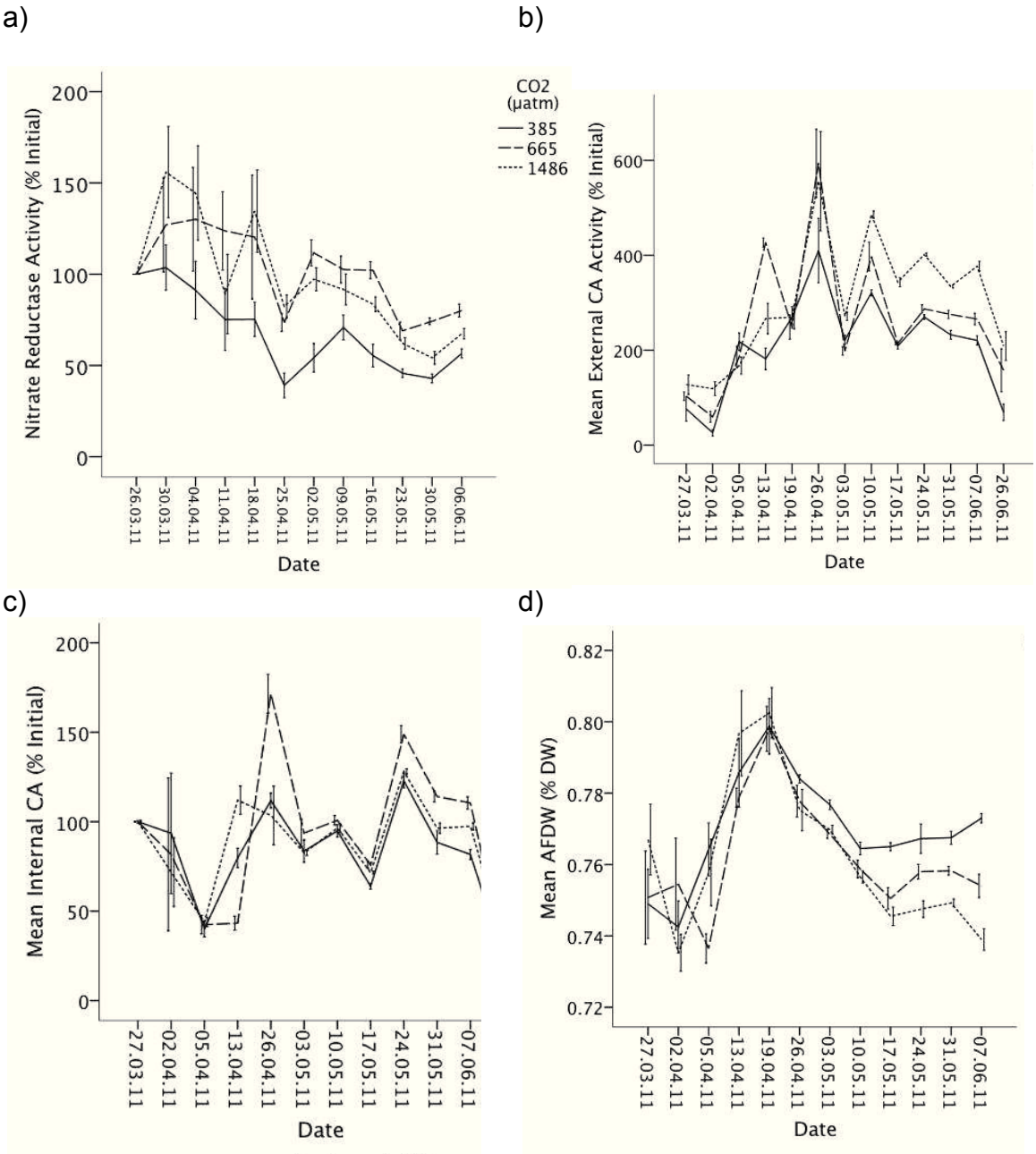

Figure 5. a) Time series of mean (± SE) a) nitrate reductase activity b) external carbonic anhydrase activity c) internal carbonic anhydrase activity and d) percent ash free dry weight of *C. officinalis* exposed to three carbon dioxide concentrations.

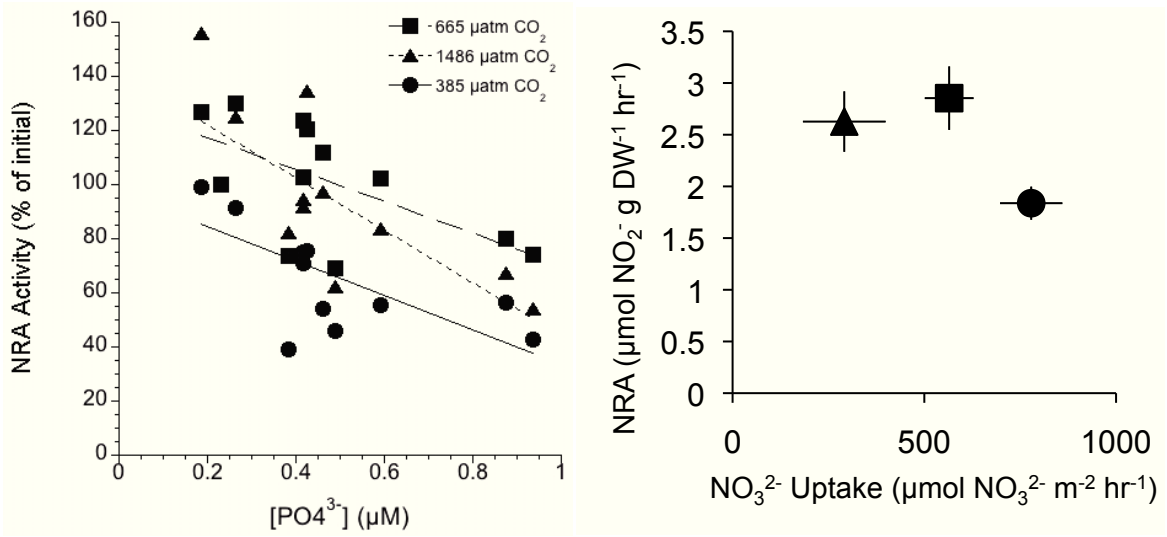

Figure 6. a) Nitrate reductase activity as a function of seawater phosphate concentration and b) mean nitrate reductase activity (± SE) as a function of mean nitrate uptake rates in *C. officinalis* exposed to the three CO_2 levels.

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 were affected by an interaction between time and CO_2 (Table 1). Internal carbonic anhydrase showed no observable pattern over time, except for two peaks in the 1486 pCO_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 seven when all treatments levelled off, but the enzyme activity was highest in the 1486 pCO_2 treatment and subsequently decreased with decreasing CO_2 level (Figures 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 (Figure 5d). By the end of the experiment, there was a negative correlation between skeletal inorganic content (% DW of $CaCO_3$) and external carbonic anhydrase activity (Figure 7).

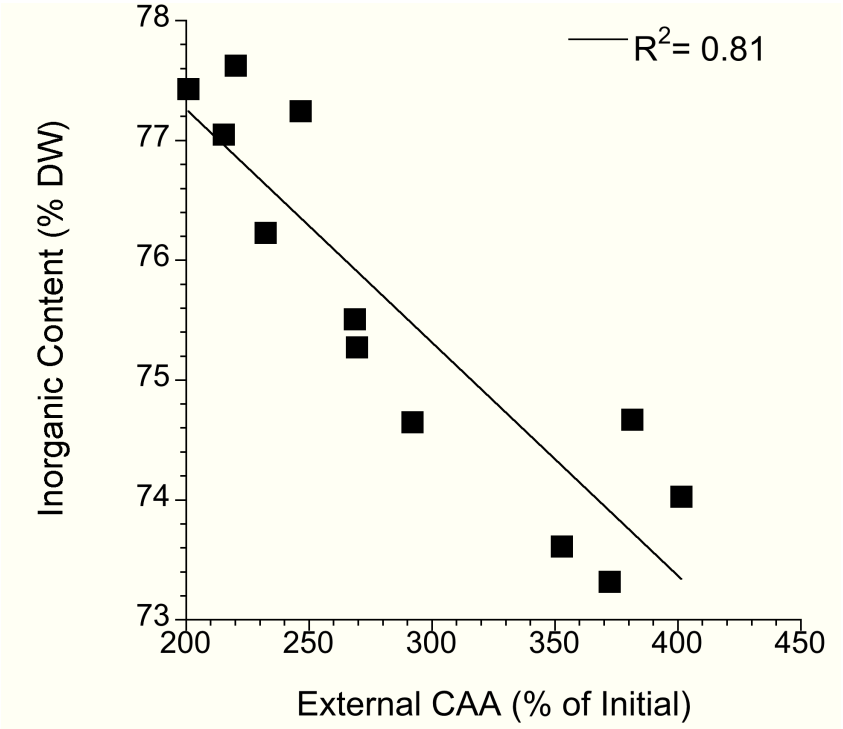

Figure 7. Inorganic content of *C. officinalis* as a function of external carbonic anhydrase activity after 71 days of exposure to 3 CO_2 concentrations

Discussion

Our results suggest that elevated carbon dioxide will significantly affect enzyme activity and subsequently many metabolic processes in *C. officinalis*, including photosynthesis, calcification, and inorganic nutrient uptake and assimilation. The enyzme external carbonic anhydrase is important in the carbon concentrating mechanism of many macroalgae. Although its activity has been shown to decrease in noncalcifying macroalgae in response to elevated CO_2 (Haglund & Pedersén 2009; Björk et al. 1993; García-Sánchez et al. 1994), we observed an increase in eCA activity with increasing CO_2 concentration. In noncalcifying macroalgae, it is hypothesized that less enzyme is produced because more CO_2 is available for photosynthesis, so less HCO_3^- must be converted to CO_2. However, in the case of calcifying macroalgae, it is likely that eCA plays a role in metabolic processes other than photosynthesis, particularly calcification. In corals, CA 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. 2007; Tambutté 2007). Hofmann et al. (in press) showed that calcification rates in *C. officinalis* had a parabolic relationship to CO_2 concentration, and Hofmann et al. (2012) showed that eCA 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

eCA activity, we hypothesize that eCA activity is related to calcification, and that its activity is upregulated under elevated CO_2 in order to control the calcification mechanism despite changes in seawater inorganic carbon chemistry that are unfavorable for $CaCO_3$ deposition. This response could be an attempt by the algae to compensate for higher dissolution rates under elevated CO_2 conditions.

The overall decrease in nitrate reductase activity in *C. officinalis* grown at all CO_2 concentrations during the first 6 weeks was most likely due to a decline in the seawater nitrate concentration, as the enzyme has been shown to be dependent on external nitrate availability (Solomonson & Barber 1990; Gordillo et al. 2006). This could also explain the inverse relationship between nitrate reductase activity and seawater phosphate concentration, as phosphate increased during the experimental period while nitrate decreased strongly during the first six weeks. The decrease in nitrate uptake rates by *C. officinalis* under elevated CO_2 conditions is consistent with other results found for noncalcifying macroalgae (García-Sánchez et al. 1994; Magnusson et al. 1996; Andria et al. 1999) as well as the observed increase in nitrate reductase activity (Mercado et al. 1999; Gordillo et al. 2001). Mercado et al. (1999) reported that the reduction and assimilation of nitrate in *Porphyra leucosticta* was uncoupled, which also seems to be the case in *C. officinalis*. The increase in nitrate reductase activity could be due to a change in the ratio of intracellular ATP:NADP+/NADPH. In *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 *C. officinalis* grown under elevated CO_2 have a lower ATP:NADPH requirement, the the excess NADPH could have stimulated nitrate reductase activity, as NADPH is needed as a reducing agent to convert nitrate to nitrite. The combination of stimulated nitrate reductase activity and decreased nitrate uptake rates in *C. officinalis* grown under elevated CO_2 indicate that the enzyme efficiency decreased because despite higher activity, the algae had a lower cellular concentration of proteins (and therefore a higher carbohydrate:protein ratio) under elevated CO_2 (Hofmann et al. in press). Although coralline algae are not the most nutritional food source for grazers due to the high inorganic content compared to noncalcifiers, some grazers still feed on these algae (Littler & Littler 1980; Maneveldt & Keats 2008). Therefore, such a change in cellular content could have nutritional implications for grazers, as algae with lower protein

content are of less nutritional quality for grazers (Horn & Neighbors 1984; Mattson 1980; Cruz-Rivera & Hay 2003).

The absoluate values and seasonal pattern of seawater temperature in the mesocosm tanks and ambient seawater nutrient concentrations was consistent with previously recorded seasonal trends in the Wadden Sea (van Beusekom et al. 2001; Müller et al. 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 six weeks of the experiment. The decrease in nitrate reductase activity is most likely due to the decrease in seawater nitrate concentration. Nitrate reductase has been shown to be dependent on external nitrate concentration in other algae (Solomonson & Barber 1990; Gordillo et al. 2006). Furthermore, algae generally prefer ammonium over nitrate, as it is less energy costly to assimilate (Losada & Guerro 1979; Syrett 1981), and the ammonium concentrations were sufficient to supply the algae with an alternative source of nitrogen when the nitrate concentrations decreased. Hofmann et al. (in press) showed that the carbohydrate:protein ratios in *C. officinalis* were affected by both CO_2 and time of exposure, indicating that the overall cellular status of the cells changed during the season, but the CO_2 effect remained constant. The increase in external carbonic anhydrase activity in all treatments during the first six weeks was most likely a response to increasing seawater temperature, as enzymes have optimum temperatures for maximum activity, and *C. officinalis* growth is optimal at temperatures between 12-18°C (Colthart & 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 & Miyachi 1985). This temperature effect could have masked the CO_2 effect during the first six weeks of the experiment, as there was no difference in eCA activity between the CO_2 treatments until after six weeks. Therefore, the enzymatic activity, relying metabolic mechanisms and cellular products of the calcifying red alga *C. officinalis* will be affected by CO_2, but will also depend on seasonal affects such as nutrient availability and temperature.

Our 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. (in press) 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, like *C. officinalis*, will be able to adapt to increasing CO_2 concentrations that will allow it to maintain its current competitive status within macroalgal communities. Their ability to adapt will most likely depend on other abiotic factors and seasonal patterns. Therefore, it will be important to conduct future experiment on different life 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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Chapter 3

Competitive interactions between calcifying and noncalcifying temperate marine macroalgae under elevated CO_2 levels: a mesocosm study

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Competitive interactions between calcifying and noncalcifying temperate marine macroalgae under elevated CO_2 levels: a mesocosm study

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Running head: Macroalgal competition under elevated CO_2

Abstract

Anthropogenic CO_2 production is changing the chemistry of surface ocean waters. Since pre-industrial times, uptake of CO_2 by surface oceans has caused a documented change of 0.1 pH units. Calcifying organisms have shown sensitivity to elevated CO_2 concentrations due to their calcium carbonate skeletons. In temperate rocky intertidal environments, calcifying and noncalcifying macroalgae make up diverse benthic photoautotrophic communities. These communities may change as calcifiers and noncalcifiers respond differently to rising CO_2. In order to test this hypothesis, we conducted an 86-day mesocosm experiment to investigate the physiological and competitive responses of calcifying and noncalcifying temperate marine macroalgae to 385, 665 and 1486 µatm CO_2. We focused on comparing two abundant red algae in the Northeast Atlantic: *Corallina officinalis* (calcifying), and *Chondrus crispus* (noncalcifying). We found an interactive effect of CO_2 concentration and exposure time on growth rates of *Corallina* and total protein and carbohydrate concentrations in both species. Photosynthetic rates did not show a strong response. Calcification in *Corallina* showed a parabolic response, while skeletal inorganic carbon decreased with increasing CO_2. Community structure changed, as *Chondrus* cover increased in all treatments, while *Corallina* cover decreased in both elevated CO_2 treatments. Photochemical parameters of other species are also presented. Our results suggest that CO_2 will alter the competive strengths of calcifying and noncalcifying temperate benthic macroalgae, resulting in different community structures, unless these species

are able to adapt at a rate similar to or faster than the current rate of increasing sea surface CO_2 concentrations.

Key words: macroalgae, competition, *Corallina officinalis*, *Chondrus crispus*, calcification, ocean acidification, CO_2

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Introduction

Anthropogenic CO_2 production is changing the chemistry of surface ocean waters and since pre-industrial times, uptake of CO_2 by the earths' oceans has caused a documented change of 0.1 pH units (Caldeira & Wickett 2003). The atmospheric CO_2 concentration is expected to climb to 800-1000 parts per million (ppm) by 2100 (IPCC 2007), and model simulations indicate they could even reach 1,900 ppm by 2300 (Caldeira & Wickett 2003, Orr et al. 2005). The atmospheric CO_2 levels expected in 2100 would result in a corresponding decrease of surface seawater pH of 0.3-0.5 units by 2100 (Orr et al. 2005, Caldeira & Wickett 2005). An abundance of recent research investigating the consequences of these changes in ocean chemistry on marine organisms has shown varied responses across multiple taxonomic levels (Fabry 2008, Ries 2009, Kroeker et al. 2010, Fabricius et al. 2011, Rodolfo-Metalpa et al. 2011). In general, calcifying organisms seem to be the most sensitive (Kroeker et al. 2010), but even among calcifiers, the response to elevated CO_2 has not been consistant (Fabry 2008, Ries 2009, Fabricius et al. 2011).

Calcifying benthic photoautotrophs may be particularly susceptible to the influence of elevated surface seawater CO_2 concentrations as they are sessile (as adults) and rely on CO_2 as the substrate for photosynthesis. As benthic marine macroalgae are ecologically important organisms that provide food, refugia, and substrata for diverse marine communities (Paine & Vada 1969, Lubchenco 1978, Lubchenco & Menge 1978, Littler & Littler 1984, Jenkins et al. 1999, Gibbons & Griffiths 1986, Eriksson et al. 2006), they are essential organisms to study within the context of future climate change. Several studies have already investigated the responses of noncalcifying macroalgae to elevated CO_2 concentrations (Gao et al. 1991, Gao et al. 1993, Kübler et al. 1999,

Mercado et al. 1999, Gordillo et al. 2001, Israel & Hophy 2002, Zou 2005, Porzio et al. 2011, Cornwall et al. 2012) and as with other taxonomic groups, have found varied responses. On the other hand, calcifying macroalgae, particularly high- magnesium calcite depositing coralline algae, show pronounced sensitivity to elevated CO_2 concentrations with respect to calcification rates, necrosis, mortality, and recruitment (Jokiel et al. 2008, Kuffner et al. 2008, Martin et al. 2008, Büdenbender et al. 2011, Porzio et al. 2011, Hofmann et al. 2012). Due to the variable responses of noncalcifying macroalgae and sensitivity of calcifying macroalgae to elevated CO_2, it is likely that macroalgal communities will show considerable changes in structure and diversity in future oceans. Indeed tropical community studies have already shown that crustose calcifying algae decrease growth rate, cover and recruitment, while noncalcifying algae show a subsequent increase in cover under elevated CO_2 conditions (Jokiel et al. 2008, Kuffner et al. 2008). Furthermore, Porzio et al. (2011) recently reported that macroalgal species diversity, abundance, and reproduction changes along a natural CO_2 gradient in the Mediterranean. In general, they found that calcitic species decreased in cover and species richness with decreasing pH. Such a change in macroalgal community structure could also have profound impacts on the marine fauna, to which coralline algae provide structural support, substrata, and refugia (Bak 1976, Stewart 1982, Coull & Wells 1983, Akioka et al. 1999).

While the above-mentioned works have shown significant impacts of CO_2 on warm water communities, little attention has been given to temperate macroalgal communities outside of the Mediterranean, despite the fact that both crustose and articulated coralline algae co-exist with noncalcifying species in diverse macroalgal communities (Hall-Spencer et al. 2008, Martin et al. 2008, Russell et al. 2011, Hepburn et al. 2011, Porzio et al. 2011). Therefore, such communities provide an ideal platform for investigating the physiological ecology of macroalgal responses to elevated CO_2 concentrations, particularly with respect to their competitive interactions. Being able to show changes in community structure in response to an external stress is often the main goal of large-scale mesocosm and field-manipulation experiments. However, it is also important to understand the physiology behind the species-specific responses of co-existing organisms to external stress, and how those responses affect their competitive interactions that are reflected in community structure and function. To date, no studies have directly linked elevated CO_2-related physiological responses of

temperate macroalgal communities to competition between calcifiers and noncalcifiers and the ecological consequences of such competition. Therefore, we have conducted an 86-day long mesocosm experiment to determine how the physiology of calcifying and noncalcifying benthic temperate macroalgae is affected by elevated CO_2 levels, how these physiological responses affect their competition, and finally if changes in competition strengths are reflected at the community level.

Materials and Methods

Experimental design and seawater chemistry

Macroalgal communities were collected from the coast of Helgoland, Germany on March 16th, 2011. The algae grow attached to red sandstone rocks in the intertidal, and during collection we chipped away the rocks using hammers and chisels. As a result, the experimental communities remained intact and attached to their natural substratum. All communities contained the calcifier *Corallina officinalis* and its associated counterparts, which at the time consisted of mostly the noncalcifying red algae *Chondrus crispus*, *Dumontia incrassata*, and *Polysiphonia fucoides* and red calcifying crustose algae. The communities were kept in running seawater overnight, and transported to the Wadden Sea Station of the Alfred Wegener Institute on the North Sea island of Sylt, where the study was conducted.

The algae were acclimated to the ambient Wadden Sea seawater in a large outdoor tank with filtered running seawater for one week before initial measurements were taken. The seawater was double filtered, first with a protein skimmer (Model III P with 2000 l/h flow rate, Sander Elektroapparatebau GmbH, Germany) and then with a UV filter (Model 4000/75 Watt, Wiegandt GmbH, Germany). During the acclimation week, the mesocosm treatment tanks were prepared and the seawater chemistry was monitored. The mesocosms were cylindrical plexiglass tanks 60 cm tall and 40 cm in diameter. They were surrounded by 2000 liters of continuously running seawater for temperature control. The mesocosms also received continuously running seawater and were rigorously bubbled with one of three CO_2 concentrations: 385 µatm (ambient), 665 µatm, and 1486 µatm CO_2. The CO_2 concentrations were achieved using an HTK five-

channel gas mixing system (Hamburg, Germany). Each CO_2 treatment contained five tanks, but one mesocosm per treatment was not filled with algae (n = 4).

Once the water chemistry was stable and the algae were acclimated to ambient light and seawater conditions, initial photochemical and photosynthesis measurements were taken, tissue samples were frozen in liquid nitrogen and stored at -80°C for later analysis, and photographs of each community were taken before the communities were sorted randomly into the 12 treatment tanks. Each tank contained one rock with exclusively *Corallina* for growth measurements, one rock with only *Corallina* and *Chondrus*, and one rock containing *Corallina* in a more diverse community (> three species).

The pH, temperature and salinity in each tank were monitored twice daily, and water samples for total alkalinity and nutrient analysis were taken weekly. Total alkalinitiy was measured using a TitroLine alpha 05 plus titrator with an automated sample changer and IoLine IL-Micro pH electrode (SI Analytics, Mainz, Germany). Salinity and temperature were measured using a Portamess 910 Cond conductivity meter and pH was measured using a WTW SenTix 41 pH electrode connected to a WTW pH 3310 portable pH-meter (Weilheim, Germany). The physiological response variables and analysis of community structure were measured monthly. The experimental period lasted for 86 days, from March 28th, 2011 to June 24th, 2011.

Growth and calcification of the calcifying rhodophyte *Corallina officinalis*

Growth of *Corallina* was measured by staining the algae thalli. Each treatment tank contained a rock on which only *Corallina* was growing. The algae growing on these rocks were stained for 12-24 hours in alizarin red stain for growth measurements based on length increase. Multiple tips of multiple thalli were measured and averaged for each tank, and this value was counted as one replicate.

Calcification rates were determined by measuring the total alkalinity (A_T) of seawater before and after two-hour incubations in small incubation chambers (10 cm diameter x 30 cm high). One rock containing only *Corallina* was placed into each chamber during the incubations, which were continuously bubbled with the respective premixed CO_2

treatment levels. Net calcification rates were calculated according to the equation G_{net} = $-0.5\rho_w$ (V/SA)*($\Delta A_T/\Delta t$) where G_{net} is net calcification rate in μmol $CaCO_3 \bullet m^{-2} \bullet hr^{-1}$, ρ_w is the seawater density ($kg \bullet liter^{-1}$), V is the seawater volume (liters), SA is the surface area of the algal assemblage (m^2), ΔA_T is the change in alkalinity during the incubation and Δt is the incubation period (hours). The percentage of the dry weight of *Corallina* thalli consisting of inorganic carbon was measured by determining the ash free dry weight (AFDW) of the tissue. Thalli fragments were dried for 48 hours at 50°C, weighed, placed into pre-burned and pre-weighed crucibles, and burned at 400°C for 24 hours. The relative percentage of the dry weight consisting of inorganic carbon was calculated as (AFDW/DW) x 100%.

Physiological responses of a calcifier (*Corallina*) versus a noncalcifier (*Chondrus*)

Photosynthesis

Respiration and oxygen evolution in *Corallina* and *Chondrus* were measured as outlined in Hofmann et al. (2012), with the modification of three-minute light intervals during photosynthesis-irradiance curve measurements. Respiration was measured in the dark for 15 minutes prior to the light steps, which consisted of light intensities ranging from 0-1,000 μmol photons m^{-2} s^{-1}. Oxygen consumption/production was measured using a Hansatech Chlorolab 3 System (Hansatech Instruments Ltd., Norforlk, England). Maximum photosynthesis rate (P_{max}), photosynthetic efficiency (alpha) and light saturation point (E_k) were calculated from nonlinear regression analyses based on the model from Eilers and Peeters (1988).

Concentration of phycobilins, soluble proteins, and carbohydrates

The method for measuring phycobilin, protein, and carbohydrate concentrations in a single algal extract was done according to Andria et al. (1999). Algal thalli (100-200 mg) previously frozen in liquid nitrogen and stored at -80°C were ground to a fine powder in pre-chilled shaking flasks using a Mikro-Dismembrator (B. Braun Biotech International, Goettingen, Germany). The algal powder was suspended in 5-10 ml of cold 0.1M phosphate buffer (pH 6.8) and kept in the dark at 4°C overnight. The resulting algal

extract was split into three supernatant fractions that were used for subsequent pigment, protein and carbohydrate analysis. The pellet was used to determine insoluble carbohydrate content. Methods for measuring phycoerythrin and phycocyanin were taken from Beer & Eshel (1985), protein precipitation was conducted according to Barbarino & Lourenço (2005) and protein concentration was determined by the Bradford method (Bradford 1976). The phenol sulfuric acid method was used to determine soluble and insoluble carbohyddrate concentrations (Kochert 1978).

Community analysis

Photochemistry of macroalgal communities

Photochemical parameters of the entire macroalgal communities were measured using a Maxi-Imaging-PAM (Pulse Amplitude Modulated) chlorophyll fluorometer (Walz GmbH, Effeltrich, Germany) equipped with a blue LED-Array illumination unit and a CCD Camera with 1392 x 1040 pixels (Pike, Allied Vision Techn.). The fluorescence signals measured by the PAM were digitized by the camera and transferred to a PC, which allows the user to see an image of the chlorophyll fluroescence for a large area, including mixed communities. The communities were immersed in a beaker containing their treatment water and dark adapted for five minutes prior to photochemical analysis. Following dark adaptation, the chlorophyll fluorometer measured the dark fluoresence yield (F_o) and maximum fluorescence (F_m) for the entire community. From these parameters the maximal photosystem II (PS II) quantum yield, F_v/F_m, was calculated according to the equation $F_v/F_m = (F_m-F_o)/F_m$. Then the communities were exposed to a series of pre-defined increasing photosynthesis-saturating light pulses at 20-second intervals and the effective PS II quantum yield (Y) was measured after each pulse. The relative electron transport rates (rETR) were then calculated using the equation rETR = 0.5 x Y x PAR, where PAR = the light intensity of each saturation pulse (µmol photons•m^{-2}•s^{-1}) and the factor 0.5 was used to account for the fact that two quanta must be absorbed for every electron transported due to the presence of two photosystems. The resulting values were used to produce rETR versus irradiance curves, from which we calculated the maximum rETR, the light saturation point (E_k), and the electron transport rate efficiency (alpha) using the non-linear model from Eilers and Peeters (1988).

Percent cover, diversity, and dominance

Digital photographs of each macroalgal community were taken monthly and analyzed for percent cover of individual species and community diversity and dominance indexes using the coral point count with excel extensions (CPCe) software (Kohler & Gill 2006). A 10 x 10 point grid was overlayed on each photograph, and the species that occurred at each point in the community was recorded for the analysis. The % percent change in cover was calculated by relating the percent cover of each species over time to the initial percent cover of that species according to the equation ((% $cover_t$ - % $cover_i$)/% $cover_i$ * 100), where t = day 36 or 86 and i = initial.

Statistical analysis

Statistical analyses were applied to test for significance at the 95% ($p < 0.05$) confidence level. When a single response variable for one species was analyzed, a one-way analysis of variance (ANOVA) was conducted followed by pairwise comparisons using a Tukey HSD test. When a single response variable was measured over time, a repeated measures ANOVA was conducted using time as a within subject factor and CO_2 as the between subject factor. For analysis of multiple response variables from multiple species over time, a mixed factorial multivariate analysis of variance (MANOVA) was conducted including time as a within-subjects factor and CO_2 and species as between-subject factors. A Tukey HSD test was used for pairwise comparisons. For the analysis of proteins, carbohydrates and phycobiliproteins, the MANOVA was followed by a discriminant analysis. If the data were not normally distributed, they were transformed to fit the assumptions of an ANOVA. When the data did not meet Mauchly's test of sphericity, the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity. Separate MANOVAs were conducted for P-E curve parameters, chlorophyll *a* fluorescence parameters, and tissue content because the sampling technique and time of these analyses were slightly different. Correlation analysis was conducted by calculating the Pearsons correlation coefficient of two variables using a one-tailed test for significance.

Results

Seawater Chemistry

Seawater chemistry parameters are outlined in Table 1. The mean CO_2 concentrations in the tanks containing algal communities were slightly lower than the mean values measured in control tanks without algae, indicating that the algal communities took up around 1% of the dissolved CO_2. The seawater in the highest CO_2 treatment tanks was undersaturated with respsect to aragonite ($\Omega_{aragoniate} < 1$), but not calcite ($\Omega_{calcite} > 1$). Weekly measurements of inorganic nitrogen and phosporous concentrations (nitrate and phosphate) showed seasonal fluctuations. Nitrate concentrations ranged from 7-40 μM, and were highest at the beginning of the experiment and lowest after 40 days. Phosphate concentrations started out at 0.19 μM and steadily increased during the experiment, with a maximum concentration of 0.94 μM. The increase in phosphate concentration over time was most likely due to the decomposition of spring phytoplankton blooms.

Table 1. Seawater chemistry parameters for control tanks without algae (italics) and treatment tanks with algae (bold) calculated from daily measurements throughout the duration of the experiment.

CO_2 Treatment	Mean pH	pCO_2 (µatm)	$[CO_2]$ (µmol kg SW^{-1})	$[HCO_3^-]$ (µmol kg SW^{-1})	$[CO_3^{2-}]$ (µmol kg SW^{-1})	$\Omega_{calcite}$	$\Omega_{aragonite}$
	8.22 (± 0.02)	*385 (± 20)*	*16.1(± 0.94)*	*1980 (± 24)*	*150 (± 8)*	*3.71 (± 0.2)*	*2.33 (± 0.1)*
Ambient	***8.25 (± 0.01)***	**371 (± 11)**	**16.5 (± 0.58)**	**2010 (± 28)**	**161 (± 4)**	**4.00 (± 0.1)**	**2.52 (± 0.1)**
	8.01 (± 0.02)	*665 (± 36)*	*28.1 (± 1.7)*	*2103 (± 19)*	*98 (± 6)*	*2.44 (± 0.2)*	*1.53 (± 0.1)*
Medium	**8.05(± 0.01)**	**602 (± 15)**	**25.4 (± 0.72)**	**2088 (± 10)**	**106 (± 3)**	**2.62 (± 0.1)**	**1.65 (± 0.1)**
	7.69 (± 0.02)	*1486 (± 73)*	*62.4 (± 3.4)*	*2230 (± 13)*	*49 (± 3)*	*1.22 (± 0.1)*	*0.77 (± 0.1)*
High	**7.73(± 0.01)**	**1380 (± 43)**	**58.3 (± 2.1)**	**2242 (± 30)**	**55 (± 2)**	**1.36 (± 0.1)**	**0.85 (± 0.03)**

Growth and calcification of the calcifying rhodophyte *Corallina officinalis*

The mean growth rate (mm day^{-1}) of *Corallina* after 74 days was influenced by a main effect of CO_2 concentration (one-way ANOVA, df = 2, F = 9.439, p = 0.008). The growth rate was highest at 385 µatm CO_2 and lowest at 1485 µatm CO_2 (Figure 1). Calcification rates showed a parabolic relationship to seawater aragonite saturation states ($\Omega_{aragonite}$), with the highest rate measured at $\Omega_{aragonite}$ = 1.65, which corresponded to the 665 µatm CO_2 treatment (Figure 2a). There was no significant difference between calcification rates at the highest and lowest CO_2 level. However, after 30 days, the skeletal inorganic carbon of *Corallina* was significantly positively correlated to aragonite saturation state (linear regression analysis r^2 = 0.535, p = 0.007), and this relationship remained consistent throughout the remainder of the experimental period (Figure 2b; time series data not shown). Furthermore, there was an inverse relationship between calcification rate and skeletal inorganic carbon when inorganic carbon was above 77.1% of the dry weight; below that point, there was no clear relationship between skeletal inorganic carbon and calcification rate (Figure 2c). In contrast, there was a positive correlation between inorganic carbon and maximum photosynthetic rate (Figure 2d).

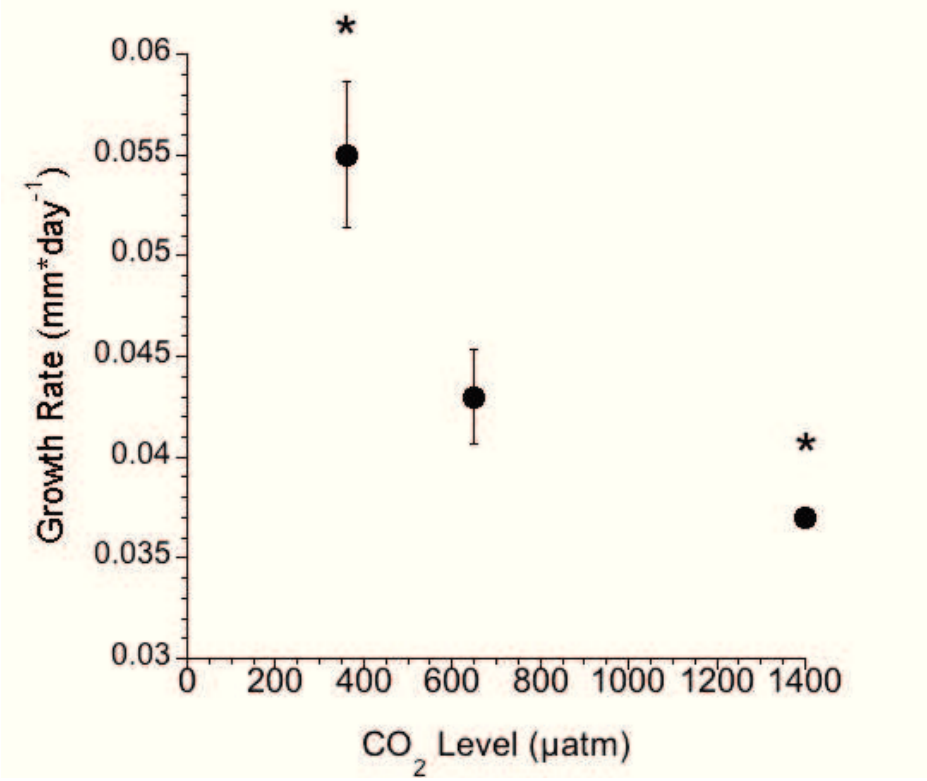

Figure 1. *Corallina officinalis* mean (± SE) growth rates after 74 days of exposure to the CO_2 treatments. Growth rates are based on length measurements of new material that appeared after staining with alizarin red. There was a significant main effect of CO_2 on growth rate ($F = 9.439$, $p = 0.008$). Significant differences in growth rates between CO_2 concentrations are indicated by asterics.

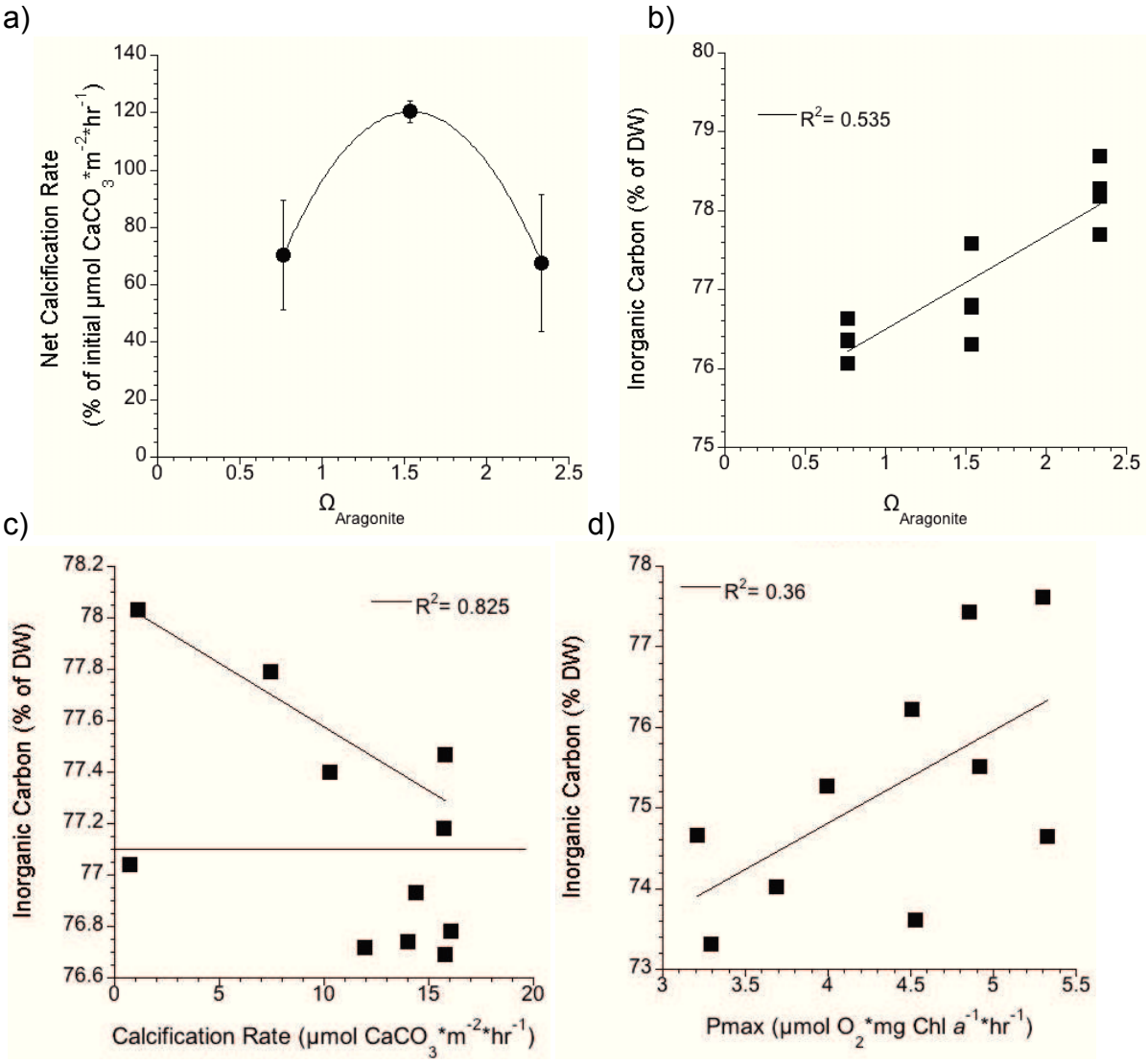

Figure 2 a) *Corallina* calcification rates after 38 days of exposure as a function of calcite saturation state. b) Percent inorganic carbon of *Corallina* thalli as a function of aragonite saturation state. c) Relationship between inorganic carbon and calcification rate showing a linear relationship until a tipping point at 77.1 %, and d) inorganic carbon versus maximum photosynthesis rate.

Physiological responses of a calcifier (*Corallina*) versus a noncalcifier (*Chondrus*)

Photosynthesis

Dark respiration rates E_k, P_{max}, and α of *Corallina* and *Chondrus* were not significantly affected by CO_2 concentration, but there was a significant main effect of time on α and respiration rate, a significant interaction between time and species with respect to E_k

and α, and a significant effect of species on α (Table 3, Figure 3). The interaction between time and species was due to the fact that light saturation points and photosynthetic efficiency of *Chondrus* were much higher and lower, respectively, after 88 days of exposure compared to the rates after 38 days, which was likely a seasonal effect in response to higher temperatures. On the other hand, photosynthetic efficiency of *Corallina* at the end of the experiment was higher than at the beginning, and was also higher than the photosynthetic efficiency of *Chondrus* after 88 days. The E_k values of *Corallina* after 88 days tended to decrease with increasing CO_2 concentration, but the trend was not significant (Pearsons coefficient = -0.567, p = 0.056). In contrast, the E_k values of *Chondrus* were higher at the end of the experiment than at the beginning, regardless of CO_2 concentration.

Table 2. Photosynthetic parameters of *Corallina officinalis* and *Chondrus crispus* measured after 37/38 and 87/88 days of exposure to the experimental CO_2 treatments. Parameters are based on oxygen evolution measurements at multiple light intensities ranging from 0-1000 µmol photons m^{-2} s^{-1}. Calculated parameters are maximum photosynthetic rate (P_{max}) light saturation point (E_k) and photosynthetic efficiency (α)

	CO_2 Level (µatm)	Days of Exposure	P_{max} (mmol O_2 mg Chl a^{-1} s^{-1})	E_k (µmol photons m^{-2} s^{-1})	α (mmol O_2 mg Chl a^{-1} µmol photons m^{-2})
Corallina	385	38	16.4 (± 4.2)	408(± 81)	0.046 (±0.015)
	665	38	17.1 (± 3.7)		0.050 (± 0.024)
	1486	38	10.8 (± 2.1)	396 (± 23)	0.023 (± 0.009)
	385	88	13.1 (± 1.5)	385 (± 145)	0.107 (± 0.071)
	665	88	13.8 (± 1.7)	165 (± 46)	0.200 (± 0.109)
	1486	88	11.7 (± 0.3)	93 (± 70)	0.487 (± 0.251)
Chondrus	385	38	11.3 (± 2.0)	205 (± 46)	0.066 (±0.018)
	665	38	11.9 (± 3.3)	280 (± 112)	0.050 (±0.009)
	1486	38	14.0 (± 2.3)	203 (± 62)	0.115 (±0.054)
	385	88	6.9 (± 2.0)	279 (± 63)	0.033 (±0.012)
	665	88	4.0 (± 0.8)	321 (± 97)	0.016 (±0.004)
	1486	88	4.6 (± 1.7)	208 (± 31)	0.021 (±0.005)

Table 3. Results from mixed factorial MANOVA tests conducted in SPSS using time as a within-subject variable and CO_2 and species as a between-subject variable. The F and p values are reported with degrees of freedom in parentheses. The lines separating groups of response variables indicate separate tests conducted due to differences in sampling dates or methods. A separate mixed factorial ANOVA was conducted for the percent change in cover because only data for days 36 and 86 could be analyzed.

				Factor or Interaction			
Response variable	Time (within-subject)	CO_2 (between-subject)	Species (between-subject)	Time x CO_2	CO_2 x Species	Time x Species	Time x CO_2 x Species
P_{max}	-	-	-	-	-	-	-
E_k	-	-	-	-	-	F(1, 11) = 6.4, p = 0.028	-
alpha	F(1, 11) = 233.8, p = 9.3E-9	-	F(1, 11) = 5.5, p = 0.038	-	-	F(1, 11) = 6.5, p = 0.027	-
respiration rate	F(1, 11) = 110.9, 4.4E-7	-	-	-	-	-	-
soluble carbohydrates	F(1, 15) = 99.4, p = 5.2E-8	-	F(1, 15) = 6.1, p = 0.026	-	-	-	-
insoluble carbohydrates	F(1, 15) = 119.5, p = 1.7E-8	F(2, 15) = 4.9, p = 0.023	F(1, 15) = 9.0, p = 0.009	F(2, 15) = 5.6, p = 0.016	F(2, 15) = 9.9, p = 0.002	F(1, 15) = 6.7, p = 0.021	F(2, 15) = 12.2, p = 0.001
total proteins	F(1, 15) = 4.6, p = 0.05	-	-	-	-	F(1, 15) = 22.1, p = 2.8E-4	-
phycoerythrin	F(1, 15) = 57.3, p = 1.7E-6	F(2, 15) = 4.4, p = 0.030	F(1, 15) = 17.3, p = 0.001	F(2, 15) = 7.3, p = 0.006	-	F(1, 15) = 25.0, p = 1.6E-4	-
phycocyanin	-	-	F(1, 15) = 13.1, p = 0.003	F(2, 15) = 5.1, p = 0.021	-	-	F(2, 15) = 3.7, p = 0.050
$rETR_{max}$	F(2, 16) = 50.1, p = 1.3E-7	F(2, 8) = 6.4, p = 0.022	-	F(4, 16) = 11.3, p = 1.6E-4	-	-	-
E_k	F(2, 16) = 21.4, p = 3.0E-5	-	-	-	-	-	-
alpha	F(2, 16) = 86.5, p = 2.6E-9	F(2, 8) = 6.8, p = 0.019	-	F(2.01, 8.04) = 6.6, p = 0.020	-	-	-
F_v/F_m	F(2, 16) = 25.7, p = 1.0E-5	-	-	-	-	-	-
percent change in cover	F(1, 61) = 183.6, p = 4.7E-20	F(2, 61) = 24.6, p = 1.5E-8	-	-	F(4, 61) = 2.7, p = 0.037	-	F(4, 61) = 2.7, p = 0.040
Shannon-Wiener Diversity Index	F(2, 18) = 7.6, p = 0.004	-	-	-	-	-	-
Simpsons Dominance Index	F(2, 18) = 5.7, p = 0.012	-	-	-	-	-	-

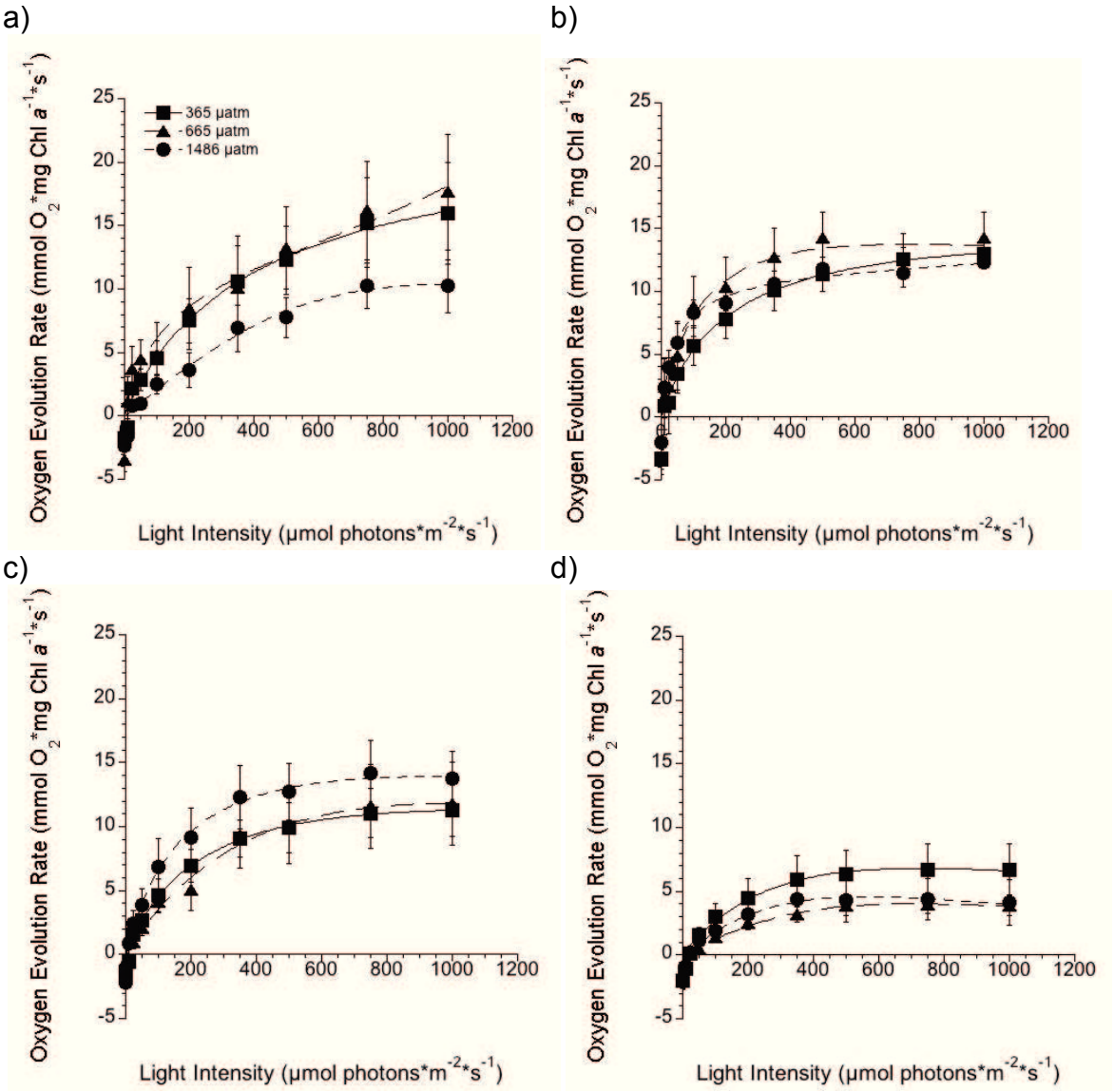

Figure 3. Oxygen evolution as a function of light intensity in *Corallina officinalis* after a) 37 and b) 87 days of exposure to the CO_2 treatments levels. c) Oxygen evolution in *Chondrus crispus* after 38 and d) 88 days.

Concentration of phycobilins, soluble proteins, and carbohydrates

A mixed factorial MANOVA test using species and CO_2 as between-subject factors, time as a within-subject factor, and soluble carbohydrates, insoluble carbohydrates, soluble proteins, and phycobiliproteins (phycoerythrin and phycocyanin) as dependent variables showed a significant main effect of time on all variables except phycocyanin. There was a significant interactive effect between all factors on insoluble carbohydrates and pycocyanin, while total proteins were only significantly affected by an interaction between time and species. Both phycobilisomes were affected by an interaction

between CO_2 and time, but only phycoerythrin was affected by an interaction between time and species (Table 3).

The phycobiliprotein content in *Corallina* and *Chondrus* was particularly affected by both time of exposure and CO_2 treatment (Figure 4a, b). While 35 days of exposure did not have a strong effect on phycobiliprotein content, the concentrations of both phycobiliproteins in *Chondrus* decreased with increasing CO_2 level after 85 days of exposure to the treatments. For *Corallina*, the response was not as strong, but phycoerythrin increased in the 665 µatm treatment from day 35 to 85, while the phycocyanin concentration was generally low in this treatment for both time measurements.

a) b)

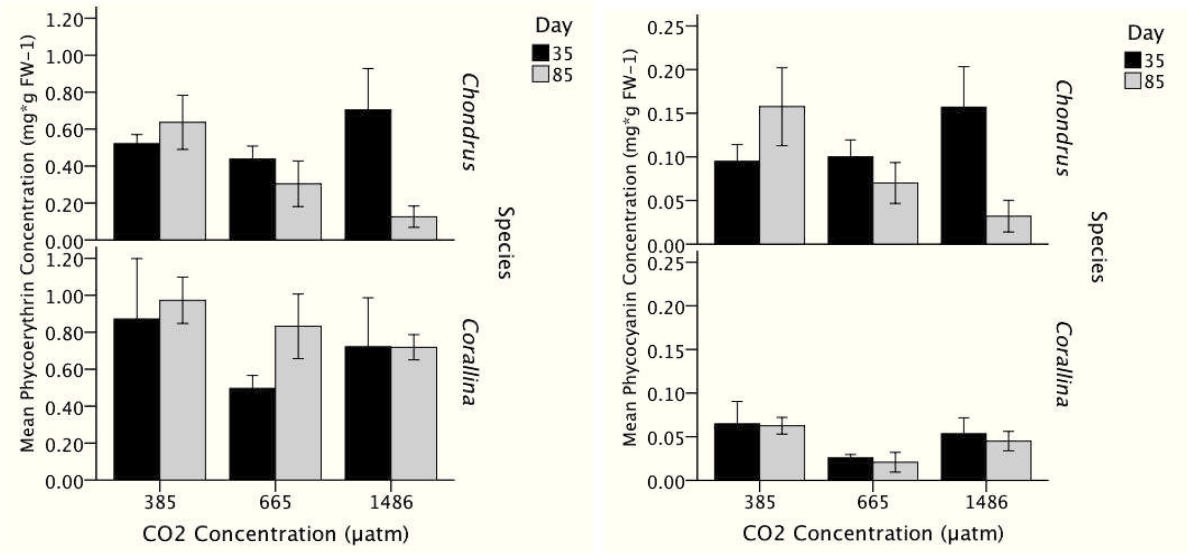

Figure 4. Phycobiliprotein content of *Chondrus* and *Corallina* after 35 and 85 days of exposure to the experimental CO_2 treatments. a) Concentration of phycoerythrin b) Concentration of phycocyanin.

During the 3-month experiment, the concentrations of soluble proteins, carbohydrates, and insoluble carbohydrates in *Corallina* and *Chondrus* changed over time and depended on species and/or CO_2 level (Figure 5). In general, *Chondrus* had higher levels of proteins and carbohydrates compared to *Corallina* after 35 days, and the total amount of insoluble carbohydrates was elevated in the two CO_2 treatments compared to the ambient treatment. However, after longer exposure, there was a sharp decline in protein and carbohydrates (both soluble and insoluble) content in the two high CO_2 treatments. This response may have been related to the combined stress of elevated

temperature and CO_2 during the warmest part of the summer (July). On the other hand, *Corallina* responded early to the CO_2 treatments after 35 days of exposure by decreasing protein levels. After 85 days, the protein concentrations in *Corallina* tissue increased in all treatments, but they did not differ significantly among CO_2 treatments.

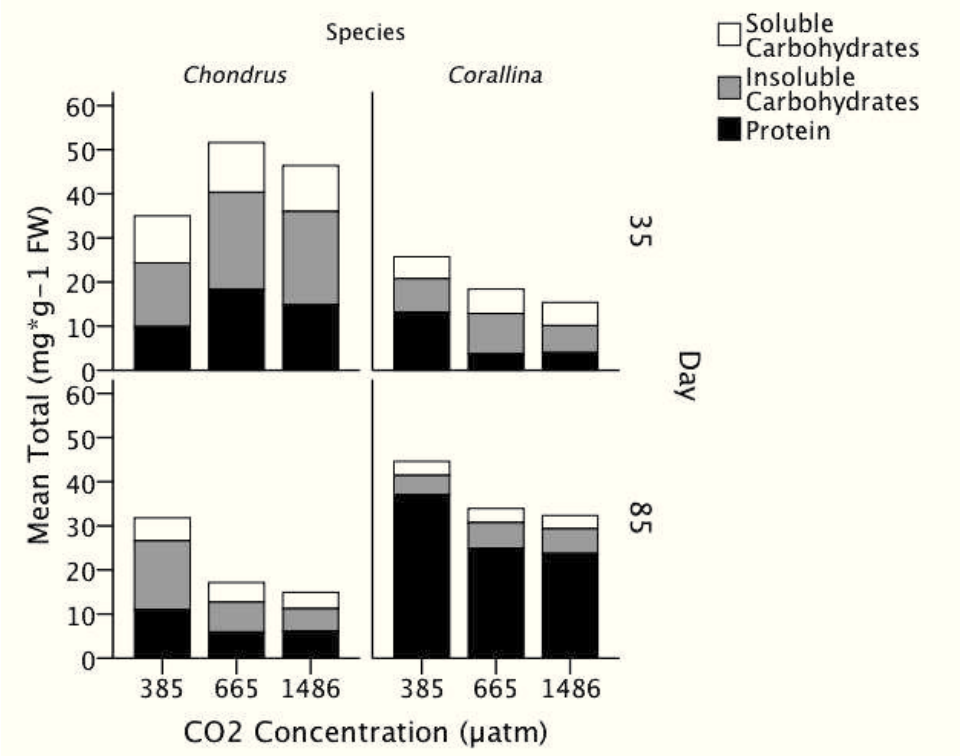

Figure 5. Total carbohydrate (soluble and insoluble) and protein concentrations in *Chondrus* and *Corallina* thalli after 35 and 85 days of exposure to the three experimental CO_2 treatments.

A discriminant analysis of the dependent variables listed above was conducted for better interpretation of the mixed factorial multivariate MANOVA. The analysis after 35 days revealed only one factor that significantly discriminated the treatment groups ((Λ = 0.067, χ^2 (25) = 47.2, p = 0.005) and explained 93.6% of the variance. However, the analysis after 85 days revealed two disciminate functions that explained 65.3 and 30.6 % of the variance, respectively (canonical R^2 = 0.90 and 0.81). These discriminant functions in combination significantly discriminated the treatment groups (Λ = 0.013, χ^2 (25) = 63.5, p < 0.001), and the second function further discriminated the treatment groups alone ((Λ = 0.123, χ^2 (16) = 30.4, p = 0.016). The correlations between the discriminating functions and the variables showed that after 35 days, all variables except phycoerythrin (*r* = -0.05) loaded equally low (*r* = 0.29-0.32) on function one. After

85 days, insoluble carbohydrates had the highest loading on function one (r = 0.51) while phycoerythrin and proteins had the highest correlations with function two (0.67, 0.53, respectively) The combined groups plots for both time periods are shown in Figure 6. The plot shows that after 35 days the first function discriminated between the control treatment and the elevated CO_2 treatmens for both species, while after 85 days, both functions strongly discriminated between the ambient and elevated CO_2 groups for the two species. Overall, this figure demonstrates that time, CO_2 and species had an effect on the protein, carbohydrate and phycobiliprotein concentrations of the algae investigated.

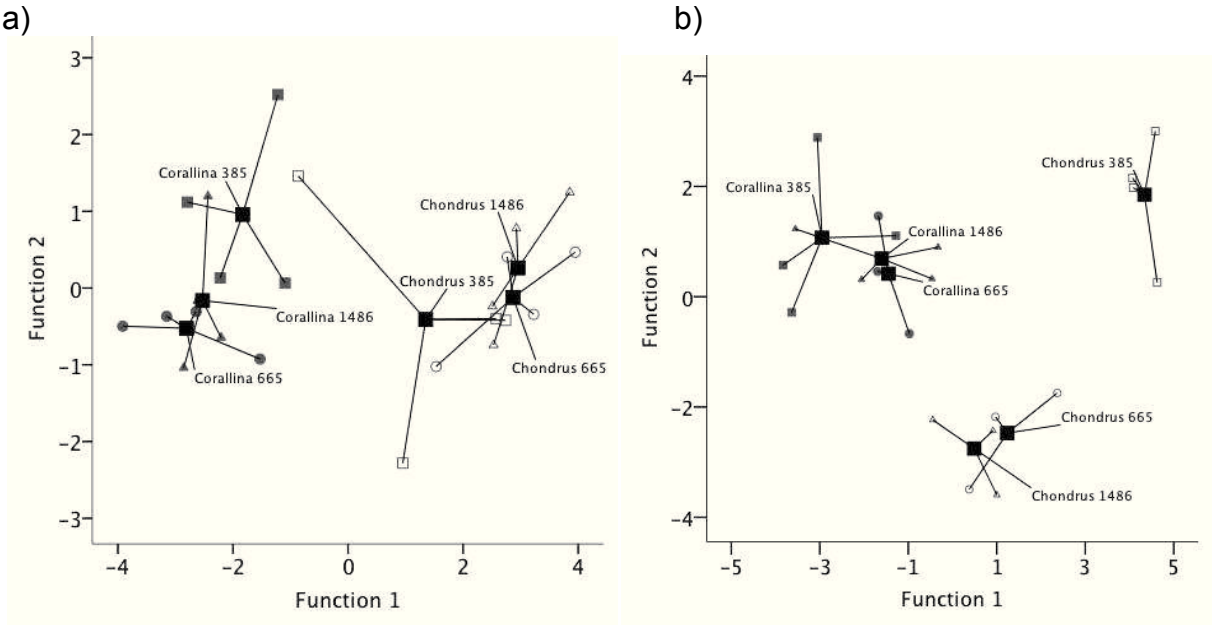

Figure 6. Combined groups plots generated by discriminant analysis of carbohydrate (soluble and insoluble), protein, and phycobiliprotein concentrations in *Corallina* and *Chondrus* after a) 35 and b) 85 days of experimental treatment. The response variables were grouped based on species and CO_2 treatment and the functions are canonical discriminant functions.

Community responses

Photochemistry

A mixed factorial MANOVA with species (*Chondrus* and *Corallina*) and CO_2 level as between-subject factors, time of exposure as a within-subject factor, and, maximum relative electron transport rate ($rETR_{max}$), electron transport rate efficiency (alpha), light saturation point (E_k) and F_v/F_m as dependent response variables indicated that there

was a significant effect of time on all four response variables, and a significant interactive effect between time and CO_2 on $rETR_{max}$ and alpha (Table 3). After 36 days of exposure to the experimental treatments, all algae investigated (with the exception of *Ulva* spp., which only appeared at the end of the experiment) had higher $rETR_{max}$ values in the ambient treatment than the two elevated CO_2 treatments (Figure 7). The lower $rETR_{max}$ rates exhibited by algae grown in the elevated CO_2 treatments resulted from higher nonphotochemical quencing and therefore lower chlorophyll fluorescence yields (Figure 8). However, by the end of the experiment, the CO_2 effect on chlorophyll fluorescence was no longer visible. The E_k values did not show a strong response to CO_2 after 36 days of exposure, but after 86 days, the values were highest in the 665 µatm treatment for the crustose coralline algae, *Chondrus* and *Corallina*.

The response of the maximum quantum yield (F_v/F_m) was not significantly affected by CO_2 (Table 3, Figure 7). The mean F_v/F_m of *Chondrus*, *Corallina* and *D. incrassata* showed a negative trend with increasing CO_2 after 36 days, but the F_v/F_m values recovered after 86 days in the 1486 µatm treatment for *Chondrus* and *Corallina*. The crustose corallina algae did now show a strong response after 36 days. *Ulva* sp. which appeared later during the course of the experiment, had the lowest mean maximum quantum yield in the 665 µatm treatment.

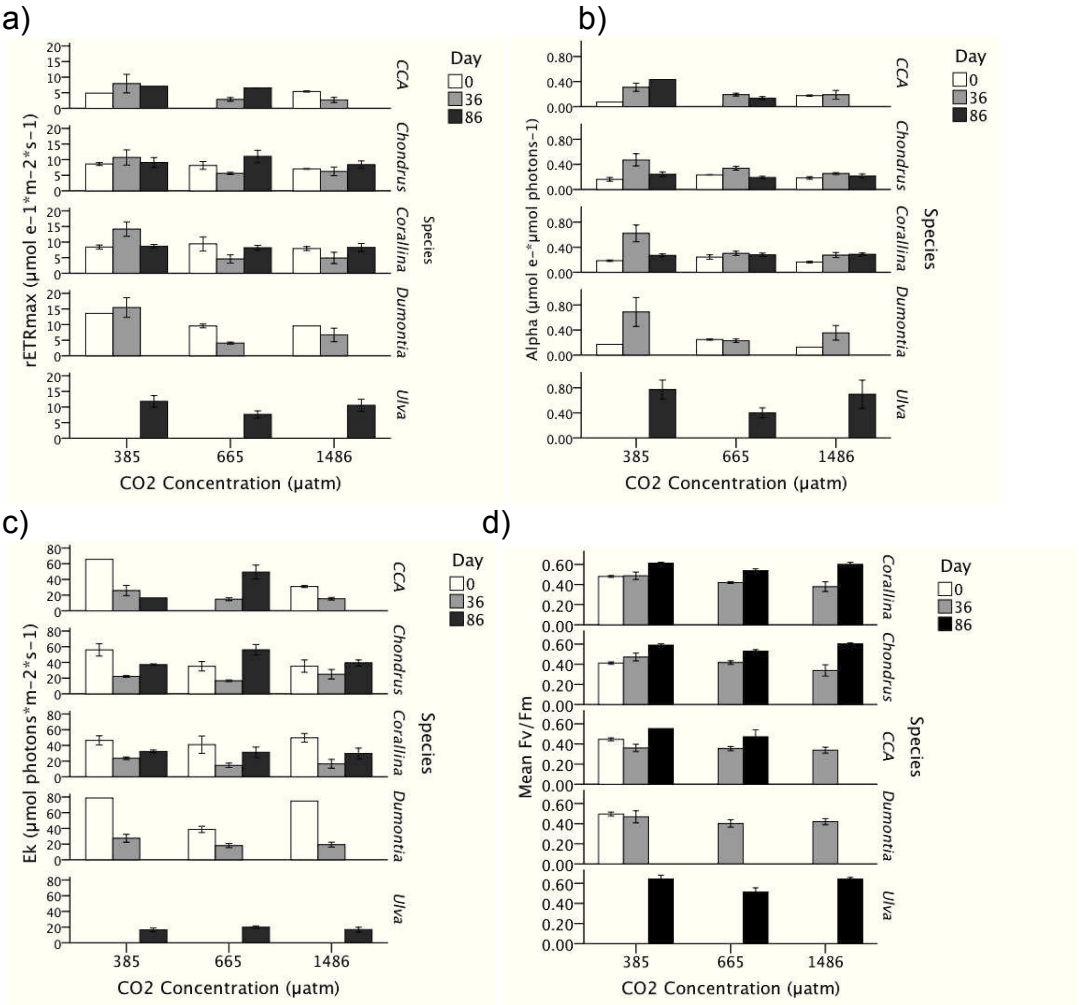

Figure 7. Photochemical parameters for five macroalgae present in the experimental communities based on chlorophyll fluorescence measurements at multiple light intensities ranging from 0-600 µmol photons m^{-2} s^{-1}. Mean values of a) relative maximum electron transport rates ($rETR_{max}$), b) electron transport efficiency (alpha), c) light saturation points (E_k) and d) maximum quantum yield of photosystem II (F_v/F_m) are shown for each species or algal group at time 0, 36, and 86 days of the experimental period.

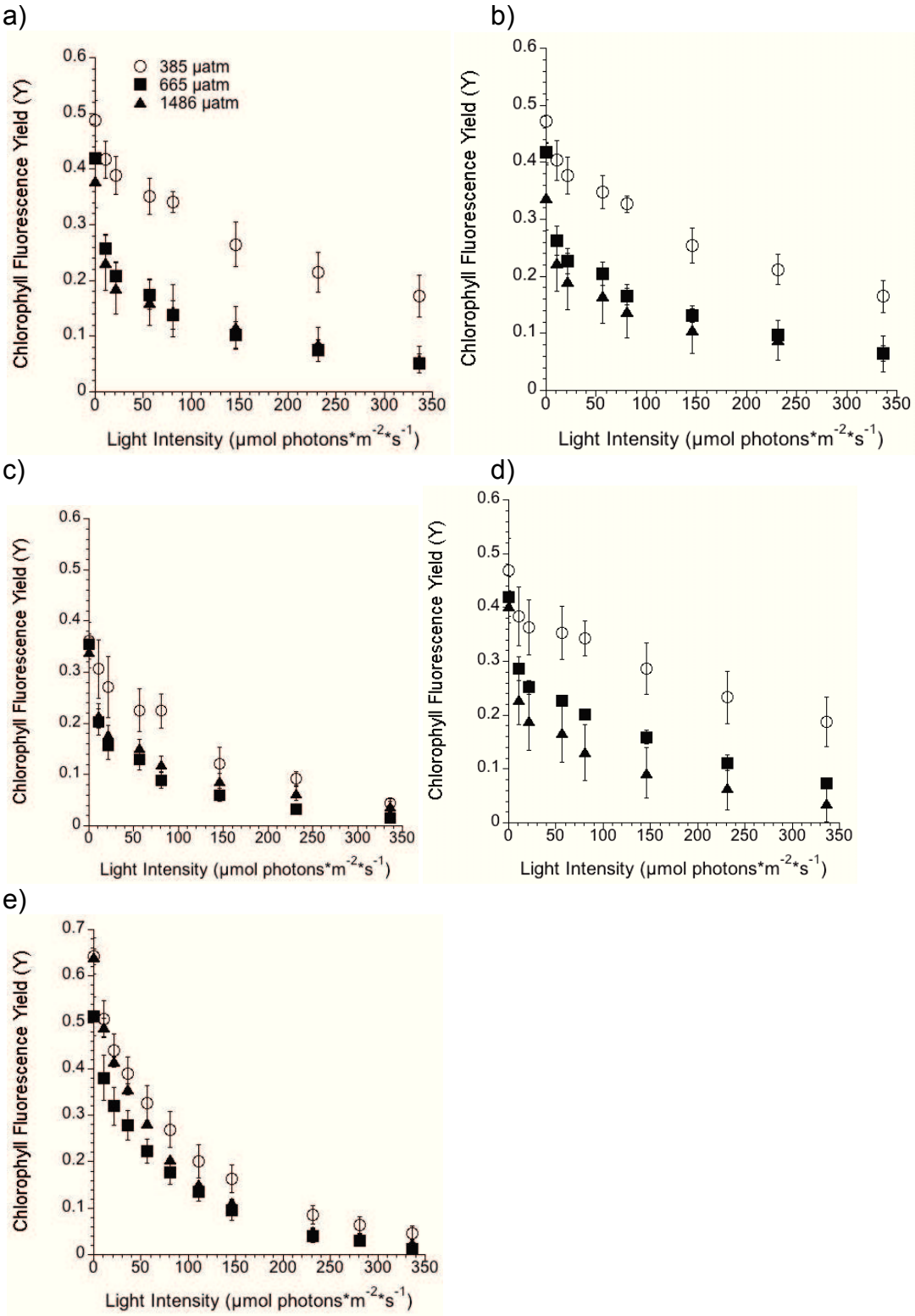

Figure 8. Mean chlorophyll fluorescence yield as a function of light intensity for four species investigated: a) *Corallina*, b) *Chondrus*, c) crustose calcifying algae, d) *D. incrassata* after 36 days of exposure to the CO_2 treatments and e) *Ulva linza*, which appeared late in the experiment

Percent cover, diversity and dominance

Due to the relatively large size of our mesocosms and long experimental period, we were able to detect changes in macroalgal community structure in response to elevated CO_2. Figure 9 shows the mean percent cover of all algal species present in the experimental communities. The total macroalgal cover increased over time in all treatments. Community diversity increased and dominance decreased over time (Tables 3 & 4), but neither was significantly affected by CO_2 treatment. When considering individual species, the percent cover of *Ulva linza*, which appeared in all treatments at the end of the experiment, was significantly negatively correlated to CO_2 concentration (Pearson's correlation = -0.381, p = 0.033, Figure 9). When the relative percent cover with respect to the calcifers (*Corallina* and crustose coralline algae) and the prevalent non-calcifying red alga *Chondrus crispus* are considered, a CO_2 effect is also present. Figure 10 shows, quantitatively and qualitatively, the percent change in cover over time for these three taxa. A mixed factorial ANOVA test indicated that there were significant main effects of time and CO_2 as well as an interaction between all three independent factors (Table 3). *Chondrus crispus* increased in all treatments over time, but the change in cover was greatest in the 1486 µatm treatment after 86 days. In contrast, the calcifying *Corallina* decreased in cover in the 1486 µatm treatment after just 36 days and in both elevated CO_2 treatments after 86 days, while the percent cover increased in the ambient treatment. The crustose coralline algae showed the highest decrease in cover at the highest CO_2 treatment after 86 days due to overgrowth by the noncalcifying species.

Table 4. Shanon-Weiner index of diversity(mean ±SE) and Simpson's dominance index (mean ±SE) for the macroalgal communities exposed to each CO_2 treatment at days 0, 36 and 86.

Day	CO_2 Level (µatm)	Shannon Wiener index of diversity	Simpsons Dominance index (1/D)
0	385	0.89(± 0.07)	0.88(± 0.02)
	665	0.82(± 0.04)	0.84(± 0.03)
	1486	0.85(± 0.08)	0.81(± 0.03)
36	385	0.87(± 0.09)	0.85(± 0.03)
	665	0.93(± 0.07)	0.81(± 0.04)
	1486	0.91(± 0.06)	0.78(± 0.03)
86	385	1.03(± 0.07)	0.73(± 0.03)
	665	1.09(± 0.06)	0.78(± 0.03)
	1486	1.09(± 0.08)	0.79(± 0.04)

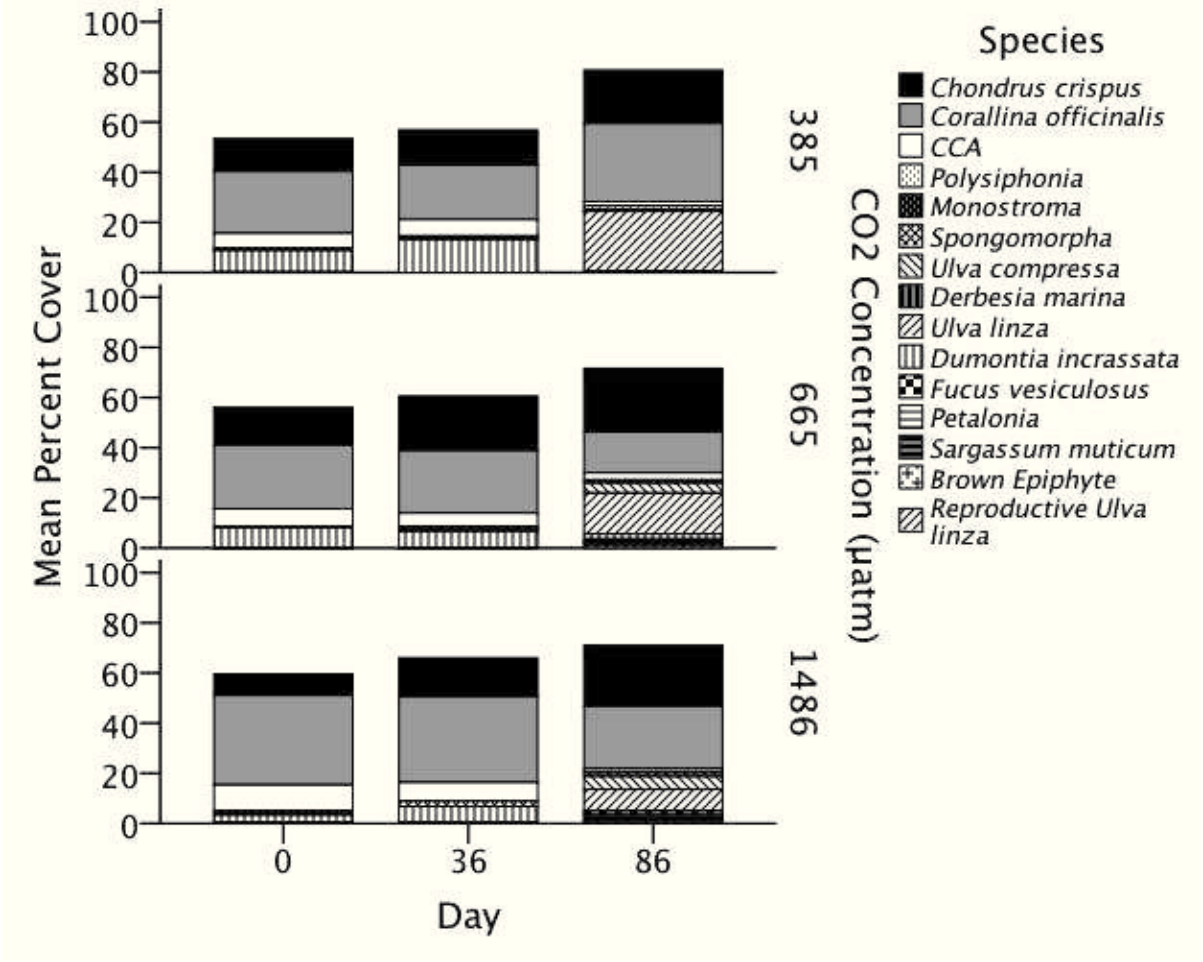

Figure 9. Mean percent cover of all species of algae present in the experimental communities at days 0, 36, and 86 for each CO_2 concentration.

a)

b)

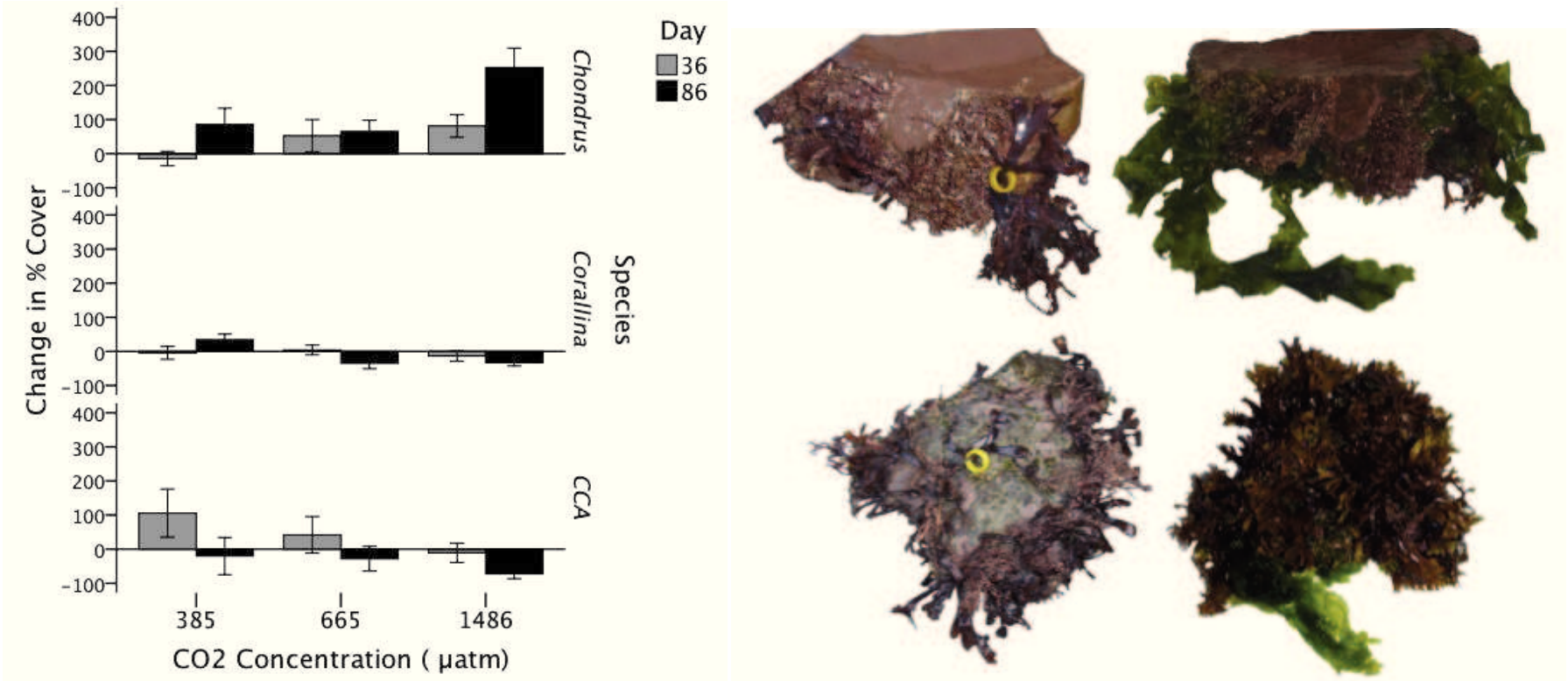

Figure 10. a) Mean percent change in cover with respect to initial after 36 and 86 days of exposure for *Chondrus*, *Corallina*, and crustose coralline algae. b) Examples of communities from the ambient and high CO_2 treatments at days 0 and 86 of the mesocosm experiment. In both communities, the presence of Ulva sp. appears after 86 days, but it is clear in the ambient CO_2 treatment that *Corallina* and *Chondrus* maintain their respective cover, while in the high CO_2 treatment, *Chondrus* encroaches on the space occupied by *Corallina* after 86 days.

Discussion

The physiological responses of the coexisting red macroalgae *Corallina* and *Chondrus* to elevated CO_2 are complex and shed some light on how competitive interactions may shift between calcifiers and noncalcifiers under future CO_2 conditions. The most striking responses are the decreased growth rates and inorganic carbon in the *Corallina* skeleton. Such responses in *Corallina* have been previously shown (Hofmann et al. 2012), but here we also present net calcification rates, which showed a parabolic relationship to CO_2 concentration (the highest rate was at 665 µatm CO_2). Such a calcification response has been reported for another coralline algae, *Neogoniolithon* sp. (Ries et al. 2009). The authors attributed the higher calcification rates at pCO_2 levels between 600 and 1100 to higher rates of photosynthesis providing more energy for pH regulation. In fact, evidence of carbon dioxide fertilization of photosynthesis in calcifying macroalgae is weak, as Gao and Zheng (2010) actually found a decrease in photosynthetic rates of *C. sessilis* at 1000 ppmv CO_2 compared to 380 ppmv, and Hofmann et al. (2012) and Cornwall et al. (2012) showed no significant differences in maximum photosynthetic rates of *Corallina* at CO_2 levels >1,300 µatm.

In our study, the highest maximum photosynthesis rate and calcification rate were measured in algae grown in the 665 µatm treatment, suggesting that moderately high CO_2 levels can stimulate photosynthesis and calcification to a limited extent. However, this stimulation of calcification rates does not necessarily result in a higher net deposition of $CaCO_3$, as skeletal inorganic carbon of *Corallina* decreased with increasing CO_2 concentration and increasing net calcification rate (above a threshold of 77% inorganic carbon). These results are consistent with Hofmann et al. (2012), who found that the area of deposited $CaCO_3$ between *Corallina* cells decreased under elevated CO_2. The reason for the descrepancy between calcification rates and skeletal inorganic carbon content could be higher dissolution rates in the dark. Martin and Gattuso (2009) found significantly higher dissolution rates of dead *Lithothamnion cabiochae* thalli under elevated CO_2 (700 ppm) compared to ambient levels (400 ppm), but found no significant difference in net calcification rates after 1 month of exposure to the experimental CO_2 treatments. Similar discrepancies between net calcification and dissolution rates have been found for some corals and mollusks

(Rodolfo-Metalpa et al. 2011). In the present study, the stimulation of calcification at moderate CO_2 concentrations allowed *Corallina* to maintain its inorganic skeleton despite (most likely) higher dissolution rates in the dark. However, there is a treshold CO_2 level beyond which calcification and photosynthesis are no longer able to maintain ambient deposition rates of $CaCO_3$, resulting in a less calcified skeleton. This threshold must lie somewhere between 1000 and 1400 µatm CO_2 for temperate coralline macroalgae, based on previously reported calcification rates of coralline algae under elevated CO_2 (Anthony et al. 2008, Ries et al. 2009, Martin and Gattuso 2009, Gao & Zheng 2010), the results of this study, and the evidence that photosynthetic rates and efficiency in *Corallina* spp. are decreased under CO_2 concentrations beyond 1000 µatm (Gao & Zheng 2010, Hofmann et al. 2012).

While *Corallina* is able to maintain a heavily calcified skeleton under moderately elevated CO_2, the energy cost of elevating calcification rates may still have an impact on the competitive success of this species. For example, growth rates and protein levels were both lower after 35 days of exposure to 665 µatm CO_2, while the calcification rate was elevated. In contrast, the noncalcifying *Chondrus* elevated its cellular protein and carbohydrate content after 35 days of exposure. In benthic calcifying animals, such an energy trade-off between net calcification rates and other physiological processes has been postulated (Findlay et al. 2011). The authors found that net calcification rates can be maintained or even elevated under high CO_2 conditions, but at costs such as increased metabolism or lower predation avoidance response. The presence of protective organic or tissue layers is also a significant factor affecting the responses of different calcifying organisms (Rodolfo-Metalpa et al. 2011). Therefore, an organisms' ability to cope with the changes associated with ocean acidification will depend on its ability to obtain additional resources needed to supply the high energy demands of maintaining calcification. These changes in energy allocation are likely to have contributed to the observed community shift seen in our study where the noncalcifying *Chondrus* increased in cover while the calcifying *Corallina* decreased in cover at both elevated CO_2 levels. The community shift observed in our study cannot be explained simply by changes in photosynthetic rates, as *Chondrus* showed only a marginal increase in oxygen production (nonsignificant at the 95% confidence interval level) at the highest CO_2 treatment after 35 days, and no change relative to the ambient level after 85 days. The

photosynthetic response of *Chondrus* is not surprising, as many authors have already shown that noncalcifying algae do not always respond to elevated CO_2 by increasing photosynthesis, and in fact some even decrease their photosynthetic rates (García-Sánchez et al. 1994, Mercado et al. 1999 Gordillo et al. 2001, Zou 2005). Therefore, nutrient availability is an important factor to consider when interpreting the responses of organisms to elevated CO_2.

Several of the responses measured in our study showed sensitivity to the interaction between exposure time and CO_2 concentration. In Figure 6, we show how protein, carbohydrate, and phycobiliprotein concentrations in *Chondrus* and *Corallina* change over time depending on CO_2 concentrations, resulting in groups that are separated by species, time and CO_2 concentration. Because time had such a large effect and nutrient concentrations changed over the duration of our study, our results suggest that seasonal fluctuations in inorganic nutrient supply also influence the effects of CO_2 on marine calcifying organisms. At the beginning of our experiment, nitrate concentrations were high but decreased steadily during the first half of the experiment (40 days), after which they slowly began to increase again but only to 40% of the initial level (data not shown). Furthermore, the water temperature constantly increased during the season and reached maximum levels at the end of the experiment. The combination of relatively low nitrate availability and high temperature could explain why the generally positive physiological responses to elevated CO_2 we saw in *Chondrus* after the first 35 days of the experiment were less clear at the end of the experiment, when nitrate availability was low. Gordillo et al. (2001) reported the signifigance of nitrogen availability in the response of the noncalcifying green alga *Ulva rigida* to elevated CO_2. Nitrogen limited algae exposed to elevated CO_2 showed increased growth rates but decreased net photosynthesis and soluble protein concentration. We saw a similar response in *Chondrus* after 85 days of exposure to elevated CO_2 when nitrate levels were much lower than during the first 35 days. The evidence from Gordillo et al. (2001) and the changes in physiological responses of *Chondrus* over time in our study support the notion that noncalcifying algae differ in their response to CO_2 depending on external energy availability. Therefore, in areas exposed to the combination of elevated CO_2 and eutrophication, changes in community structure between the coverage of calcifiers and noncalcifiers could be amplified. Such a response has already been shown in a

kelp understory, where turf algae expanded at the expense of calcifiers under elevated CO_2 and nutrient conditions (Russell et al. 2009).

The results of our mesocosm study indicate that elevated surface seawater CO_2 levels reachable within the next 100-200 years could change the structure of temperate intertidal macroalgae communities containing dominant calcifying species, such as *Corallina*. The observed changes in community structure would have important ecological implications. *Corallina officinalis* often grows in areas with strong currents and wave action, and serves as a habitat and buffer for meiofauna and substrate for other algae (Dommasnes 1968). With the removal or weakening of this species, many other algae would lose their habitats and substratum for growth, while changes in carbohydrate content of the different macroalgal species would change the nutritional content and palatability of the algae for grazers.

The rapid increase in CO_2 concentrations applied to experimental treatments in our study and in almost all ocean acidification studies is often a target of criticism because it is considered unrealistic. However, the changes in community structure we observed in this study are consistent with observations of others that have investigated competitiion between calcifiers and noncalcifiers that have been exposed to naturally different CO_2 concentrations for a long period (at least decades). For example, Porzio et al. (2011) reported a significant reduction in cover of calcitic macroalgae and the dominance of a few noncalcifying species and Martin et al. (2008) reported a lower abundance of calcifying seagrass epiphytes in areas with naturally elevated CO_2 concentrations resulting from submerged volcanic vents. These observations are similar to the results we found, in that both high Mg-calcite depositing taxa (*Corallina* and crustose coralline algae) decreased in cover in both elevated CO_2 treatments, while *Chondrus* cover increased the most in the highest CO_2 treatment. Our study also adds to the strong body of evidence suggesting that crustose coralline algae are particularly susceptible to ocean acidification (Anthony 2008, Jokiel et al. 2008, Kuffner et al. 2008, Martin et al. 2008, Büdenbender et al. 2011).

In conclusion, our study has added to the limited pool of long term and competition-based experiments between multiple species under expected future CO_2, and

contributes to increasing evidence that elevated surface seawater CO_2 will affect community composition of temperate rocky shore macroalgae communities by altering the competitive relationship between calcifiers and noncalcifiers.

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Chapter 4

Physiological responses of the calcifying chlorophyte *Halimeda opuntia* to elevated inorganic nutrients and carbon dioxide

Physiological responses of the calcifying chlorophyte *Halimeda opuntia* to elevated inorganic nutrients and carbon dioxide

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Abstract

As atmospheric carbon dioxide emmssions increase, the gas dissolves into surface oceans and decreases the surface pH and aragonite saturation state. As a result, calcifying organisms are expected to suffer under future ocean conditions, but their physiological responses will depend on their nutrient status. Because many coral reefs experience high inorganic nutrient loads from anthropogenic activity, reef organisms in localized areas will have to cope with elevated carbon dioxide and inorganic nutrients. *Halimeda opuntia* is a calcifying chlorophyte alga that contributes to tropical coral reef accretion and is a dominant primary producer on coral reefs. Therefore, we investigated the physiological response of *Halimeda opuntia* to elevated carbon dioxde and inorganic nutrients in order to determine how the alga will respond to these anthropogenically-induced environmental factors. We found a significant affect of CO_2 on inorganic carbon tissue content, and an interactive effect of CO_2 and inorganic nutrients on nitrate reductase activity. Calcification was not significantly affected by CO_2 or inorganic nutrients. A diatom bloom occurred under elevated CO_2 and inorganic nutrient conditions, suggesting these two factors in combination could affect the competitive interactions between *H. opuntia* and its noncalcifying epiphytes. The effect of elevated CO_2 and inorganic nutrients on energy allocation in *H. opuntia* is discussed.

Keywords

Halimeda, calcification, ocean acidification, eutrophication, photosynthesis

Introduction

Since the industrial revolution, the sea surface waters have dropped 0.1 units pH and are expected to drop a further 0.3 to 0.5 units by 2100 (Feely et al. 2008; Caldeira & Wickett 2003; Orr 2005). Adding dissolved CO_2 to seawater increases the concentrations of bicarbonate (HCO_3^{2-}) and hydrogen ions (H^+), which leads to a decrease in pH, carbonate ions (CO_3^{2-}), and subsequently aragonite saturation state (Fabry et al., 2008). By the end of this century the atmospheric CO_2 partial pressure in the atmosphere could reach up to 1000 ppm (Intergovernmental Panel on Climate Change; IPCC). Such atmospheric changes are expected to lead to a decline in CO_3^{2-} ions of about 60 % in surface ocean waters (Brewer 1997; Feely et al. 2004). These changes in ocean water chemistry could have significant consequences for calcifying organisms, which use CO_3^{2-} ions to build their calcified shells and skeletons.

Many calcifiers have been shown to be sensitive to changes in the carbonate saturation state, as this leads to increased difficulties in the formation of biogenic calcium carbonate (Orr et al., 2005). For coral reefs, an ecosystem consisting heavily of calcifying organisms, the effect of ocean acidification is anticipated to be negative (Langdon et al. 2000; 2003; Leclercq et al. 2000; Guinotte et al. 2003; Albright et al. 2008; 2010; Jokiel et al. 2008; Andersson et al. 2009). However, many tropical calcifying species have shown mixed responses to ocean acidification scenarios (Andersson et al. 2009; Ries 2009; Fabricius et al. 2011; McCulloch et al. 2012; Rodolofo-Metalpa et al. 2011). This wide variety of responses of calcifying organisms is likely due to differences in experimental design, temperature, light intensities, seawater chemistry, and nutrient regimes or food availability (Kleypas et al. 2006; Hurd et al. 2009), as well as differences in population responses. It is therefore important to conduct experiments that combine ocean acidification scenarios with other factors such as temperatue, light, and nutrient or food availability, because changes in these parameters are occuring simulatneously with changes in seawater carbonate chemistry. Multiple stressors likely have cumulative or interactive impacts that cause more complex but meaningful responses at the physiological and ecological level than any single stressor would have (Guinotte & Fabry 2008).

The effects of the combination of elevated temperature and CO_2 on calcifying organisms have already been investigated widely, particularly in coral reef research (Reynaud et al. 2003; 2004; McNeil et al. 2004; Hoegh-Guldberg et al. 2007; Manzello 2010; Sinutok et al. 2011; McCulloch et al. 2012), but the combination of anthropogenically elevated inorganic nutrients (eutrophication) and CO_2 is not as strongly investigated (Renegar & Riegl 2005; Kleypas et al 2006; Nelson 2009; Russell et al. 2009; Chauvin et al. 2011). Nevertheless, this combination of factors is important to investigate, since the responses of calcifiers to elevated CO_2 can be strongly influenced by nutrient or food availability (Russell et al. 2009; Holcomb et al. 2010; Chauvin et al. 2011; Findlay et al. 2011; Matthiessen et al. 2012). Therefore, the response of calcifiers to elevated CO_2 will likely differ regionally based on local nutrient regimes, which has implications for management decisions (Russell et al. 2009).

Eutrophication in coral reefs is a particularly important topic, as elevated inorganic nutrients can cause noncalcifying macroalgae to outcompete calcifying algae and corals when herbivory is low (Done 1992; Hughes 1994a; Lapointe 1997; Jompa & McCook 2002; Burkepile & Haye 2006). In addition to preventing new coral settlement and decreasing light availability, these phase shifts lead to changes in macroalgae composition where larger fleshy algae are more abundant than the calcifying algae, which act as cement for coral reefs (crustose coralline algae) and produce $CaCO_3$ sand (Littler 1972; Hillis-Colinvaux 1980; Drew 1983; Littler & Littler 1984; Davies & Marshall 1985; Drew & Abel 1988; Littler et al. 1988; Diaz-Pulido et al. 2007; Rees *at al.*, 2007; Hughes 1994; Rees et al. 2007).

The aragonite-despositing members of the green algal genus *Halimeda* (Caulerpales, Halimedaceae) have been shown to be suceptible to ocean acidification (Robbins et al. 2009; Price et al. 2011; Sinutok et al. 2011) and eutrophication alone (Delgado and Lapointe 1994), but to date, no studies have looked at the combined effects of elevated CO_2 and inorganic nutrients on these algae. Accumation of dead *Halimeda* spp. skeletons produce bank-like mounds (bioherms) containing high amounts of carbonate sediment (Littler et al. 1988; Rees et al. 2007). *Halimeda* is therefore an important contributor to carbonate sediments (Hillis-Colinvaux 1980; Drew 1983; Marshall & Davies 1985; Drew & Abel 1988; Diaz-Pulido et al. 2007; Rees *at al.*,

2007). Estimates suggest modern *Halimeda* bioherms accumulate globally 0.15 to 0.4 Gt $CaCO_3$ * $year^{-1}$, which is a major part of the annual coral reef carbonate production (Milliman 1993; Hillis 1997).

Halimeda opuntia is a common reef species that can be found on Curaçao in high abundances at shallow depth (van den Hoek et al. 1972; 1975; Kuenen & Debrot 1995). An increase in nutrients in the coastal waters of Curaçao is already taking place in Willemstat due to sewage and groundwater discharge influencing the coral reef ecosystem (Gast et al. 1999). Therefore, the aim of this study was to find out if an important reef-dwelling calcifying macroalgae, *Halimeda opuntia*, is negatively impacted by ocean acidification scenarios, and if the combination of eutrophication with elevated CO_2 will amplify or alleviate any negative effects from elevated CO_2. In addition to investigation the response of photosynthesis, calcification, and tissue nutrient content of *H. opuntia* to elevated CO_2 and inorganic nutrients, we also measured enzyme activity of two key enzymes (carbonic anhydrase and nitrate reductase) involved in inorganic carbon and nitrogen uptake and assimilation.

Materials and Methods

Culture conditions

Halimeda opuntia fragments were collected from a Caribbean reef off the coast of Curaçao in March 2011. The algae were maintained in a large mesocosm tank for 2 months in the Marine Aquaculture Facility (MAREE) at the Leibniz-Zentrum für Marine Tropenökologie in Bremen, Germany. Ambient abiot conditions consisted of 27°C, a salinity of 33 psu, 8.00 pH units, and 100 µmol photons m^{-2} s^{-1} on a 12:12 hour light/dark cycle. *Halimeda opuntia* thalli were divided into 4 pieces at least 1 week prior to the experiment.

Experimental design

The experiment was conducted using a randomized 2x2 factorial design to test the effects of nutrient (nitrate and phosphate) and CO_2 addition on *H. opuntia* physiology. 16 aquaria (20 liters) were randomly assigned 1 of 4 treatmens: no CO_2 and no nutrient addition (-CO_2-NP), just CO_2 addition (+CO_2-NP), just nutrient addition (-CO_2+NP), and CO_2 and nutrient addition (+CO_2+NP). One algal thallus was placed in each aquarium. Nutrient enrichment was achieved by adding 100 µM KNO_3 and

10µM KH_2PO_4. Carbon dioxide addition was controlled using the IKS Aquastar system (Iks Computer Systeme GmbH, Karlsbad, Germany) which monitored CO_2 bubbling via solenoid valves that were programed to open and close when the pH deviated from pH 7.75 (±0.05). All aquaria were constantly aerated with ambient air. Artificial seawater was prepared with reverse osmosis water and Red Sea Reef Salt to a salinity of 33 psu. Light conditions during the experiment were 50 µmol photons m^{-2} s^{-1}. The carbonate system of the seawater was determined by using pH and total alkalinity as two of the three carbonate system parameters. The remining parameters (pCO_2, HCO_3^-, CO_3^{2-}) were calculated using CO2calc (Robbins et al. 2010), using the dissociation constants for CO_2 and $KHSO_4$ from Mehrbach et al. (1973) refit by Dickson & Millero (1987) and Dickson (1990), respectively. Table 1 shows the mean seawater chemistry conditions for each treatment during the experiment.

Growth, photochemistry and pigment content

The fresh weight of each algal thallus was measured weekly by blotting the algae dry with paper towels until no change in weight was detectable. Relative growth rates (RGR) were calculated as RGR = $\ln(FW_t/FW_{to})/t$ *100 where FW_t and $FW_{t0}$are the fresh weight at time t (days) and time 0, respectivley. Photosynthetic parameters, including maximum quantum yield (F_v/F_m), relative electron transport rate (rETR) and nonphotochemical quenching (NPQ) were measured weekly via Pulse Amplitue Modulated (PAM) Fluorometry with a DIVING-PAM (Heinz Walz GmbH, Effeltrich, Germany). Photosynthesis-Irradiance curves were obtained using dark adapted algae (10 min dark adaptation) exposed to 1 min light steps between saturation pulses. The photochemical parameters I_k, $rETR_{max}$, and α (light saturation point, maximum relative electron transport rate, and phochemical efficiency, respectively) were calculated from a nonlinear regression analysis using the model from Eilers and Peeters (1988)

Pigment content was analyzed via High Performance Liquid Chromotography (HPLC). Halimeda tissue samples (200-400 mg) were frozen in liquid nitrogen and stored at -80°C until preparation for pigment extraction. The tissue samples were ground into a powder using a Mikro-Dismembrator (B. Braun Biotech International, Goettingen, Germany) for 1 minute at 2000 rpm. The pigments were extracted from the algal powder in 1 ml of 90% acetone at 7°C for 24 hours. The extracts were then

centrifuged (5 min, 4°C, 19,000 *g*) and filtered (0.45 µm Minisart syringe filters) and pigments were separated according to Jeffrey et al. (1997) on a Spherisorb ODS-2 column (250 x 4.6 mm, particle size 5 µm) with 3 solvents (A: 80:20 methanol:0.5 M ammonia acetate pH 7.2, B: 90:10 acetonitrile in water, C: ethyl acetate) using a LaChrom Elite HPLC (VWR Hitachi, Tokyo, Japan). Pigment concentrations were calculated based on DHI pigment standards (DHI Laboratory Products, Hoegholm, Denmark).

Calcification and tissue carbon and nitrogen content

Calcification rates were calculated using the alkalinity anomaly technique, which is based on the assumption that every mole of precipitated $CaCO_3$ reduces the total alkalinity by 2 equivalents. *Halimeda opuntia* fragments were incubated in 100 ml glass Schott bottles and the change in alkalinity from time 0 to 4 hours after the incubation was used to estimate calcification based on the equation $G = 0.5p\ (TA_0\text{-}TA_4)*V/FW/t$ where G is the calcification rate in mmol $CaCO_3$ •g FW^{-1} •hr^{-1}, TA_0 is the initial total alkalinity of the seawater (µM), TA_4 is the total alkalinity of the seawater after the 4 hour incubation, V is the volume of the incubation chamber (L), FW is the fresh weight of the algal thallus (g), and t is the period of incubation (hours).

The total carbon and nitrogen content in *H. opuntia* thalli was measured using a EuroEA 3000 Elemental Analyzer (Eurovector, Milan, Italy). *Halimeda* tissue was dried at 60°C for 48 hours and ground to a powder with a mortar and pestle. Separate tissue samples were also analyzed for organic carbon by acidification of the inorganic fraction with 1 N HCl (100 µl per 3 mg tissue).

Enzyme activity (external carbonic anhydrase and *in situ* nitrate reductase)

External carbonic anhydrase activity was measured according to Haglund et al. (1992). Fragments of *H. opuntia* thalli (50 – 200 mg dry weight) were placed in a glass test tube, to which 3 ml of assay buffer (50 mM Tris, pH 8.5, 25 mM dithiothreitol, 25 mM isoascorbic acid, 5 mM EDTA) and 2 ml of ice-cold CO_2-saturated water were added to start the reaction. The time it took for the pH to drop 0.4 units (within the pH range of 8.1-7.1) was recorded. Enzyme activity was calculated as $(t_b/t_s\ -1)/DW$, where t_b and t_s are the time in seconds it took for the pH

to drop 0.4 units in the blank (no algae) and the sample (algal thallus), respectively, and DW is dry weight of the algal sample after drying at 60°C for 48 hours.

Nitrate Reductase activity was measured *in situ* according to the method by Corzo and Niell (1991). The assay buffer (0.1 M Phosphate buffer containing 0.5 mM EDTA, 0.1% EDTA, 30 mM KNO_3, 10 µM glucose) was bubbled with N_2 gas for 5-10 minutes prior to the assay. *Halimeda opuntia* fragments were added to 5 ml amber vials containing 3 ml of assay buffer and each assay was bubbled again with N_2 gas for 1 minute. The algae was incubated in the dark at 30°C for 30 minutes. The reaction was stopped by removing 1 ml of the assay volume and adding 200 µl of 4% sulfanilimide in 3N HCl, and 300 µl of 0.1% n-(1-napthyl) ethylenediamine dihydrochloride. The absorbance of each sample was measured at 540 nm after a 15-minute period to allow for complete color change. Nitrate reductase activity was normalized to chlorophyll *a* content in the incubated tissue. Following the enzyme assay, the algae fragments were incubated in 4 ml of 100% dimethylflouride at 4°C in the dark. The extracted chlorophyll concentration was measured spectrophotometrically according to Porra et al. (1989).

Statistical analysis

All response variables measured at the end of the experiment were analyzed using a multivariate analysis of variance (MANOVA) with CO_2 and nutrients as the independent factors. ANOVA assumptions were met by transforming the data when they did not satisfy tests for normal distribution.

Results

Seawater chemistry, growth, photochemistry and pigment content

The mean seawater chemistry parameters, including pH, pCO_2 and HCO_3^-, are shown in Table 1. Relative growth rates (% day^{-1}) were very low throughout the experiment (Figure 1a), most likely due to high turnover of old calcified material that was lost from the algal thalli during the course of the experiment. Neverthless, we were able to observe new segments being produced throughout the experiment, indicating that the algae were growing despite losing old segments. There was a clear effect of nutrients on growth rates (Table 2), as the nutrient limited algae increased growth rates over time, whereas the nutrient enriched algae decreased

growth rates over time, regardless of CO_2 concentration. The negative impact of high inorganic nutrient concentrations on growth was most likely due to the competitive effect of explosive diatom growth, which occurred in all aquaria with added nutrients.

Table 1. Seawater chemistry for the 20 treatment tanks containing *H. opuntia*. Values are weekly means (±SE, n = 4).

Experimental Treatment	Temperature (°C)	Salinity	NO_2^- (μM)	NH_4^+ (μM)	PO_4^{3-} (μM)	pH (NBS)	Total Alkalinity μmol kg SW^{-1}	pCO_2 (μatm)	HCO_3^- (mol kg SW^{-1})	CO_3^{2-} (μmol kg SW^{-1})	$\Omega_{calcite}$	$\Omega_{aragonite}$
-CO_2-NP	25.4 ± 0.06	33.3 ± 0.06	16.9 ± 0.04	4.0 ± 0.3	0.0 ± 0.0	8.126 ± 0.005	3186.7 ± 122.9	415 ± 22	2366 ± 100	352 ± 12	8.6 ± 12	5.7 ± 0.2
-CO_2+NP	25.4 ± 0.07	33.4 ± 0.08	114 ± 3.0	8.0 ± 1.7	9.6 ± 0.4	8.363 ± 0.016	3713.3 ± 86.3	352 ± 23	2561 ± 72	501 ± 20	12.2 ± 0.5	8.0 ± 0.3
+CO_2-NP	25.4 ± 0.07	33.5 ± 0.08	17.0 ± 0.1	7.0 ± 1.3	0.0 ± 0.01	7.736 ± 0.004	4467.5 ± 464.1	1705 ± 165	3934 ± 413	239 ± 27	5.8 ± 0.6	3.8 ± 0.4
+CO_2+NP	25.3 ± 0.06	33.7 ± 0.07	116 ± 1.7	10.2 ± 1.7	10.0 ± 0.2	7.716 ± 0.009	4073.6 ± 94.8	1643 ± 41	3596 ± 81	208 ± 8	5.1 ± 0.2	3.3 ± 0.1

Table 2. Results from MANOVA tests, including F-ratios, degrees of freedom (in parentheses) and p-values significant at the 95% confidence level.

Response variable	CO_2 Effect	Inorganic Nutrient Effect	Interactive Effect (CO_2 x DIN)
Nitrogen (mmol gDW-1)	F(1, 10) = 25.8, p = 0.048	-	-
C_{Inorg} (% DW)	F(1, 9) = 23.7, p = 0.001	-	-
C_{org}	F(1, 10) = 4.7, p = 0.055	-	-
Calcification (μmol CaCO3 gFW^{-1} hr^{-1})	-	-	-
RGR (% day-1)	-	F(1, 9) = 9.5, p = 0.012	-
I_k	-	-	-
$rETR_{max}$	-	-	-
alpha	-	-	-
Chl b (μg μg Chl a^{-1})	-	-	-
Betacarotene (μg μg Chl a^{-1})	-	-	-
Fucoxanthin:Chl b ratio	-	F(1, 10) = 5.0, p = 0.049	F(1, 10) = 6.7, p = 0.028
NRA (% of control)	-	F(1, 10) = 5.7, p = 0.038	F(1, 10) = 7.2, p = 0.023
eCAA (% of control)	-	-	-

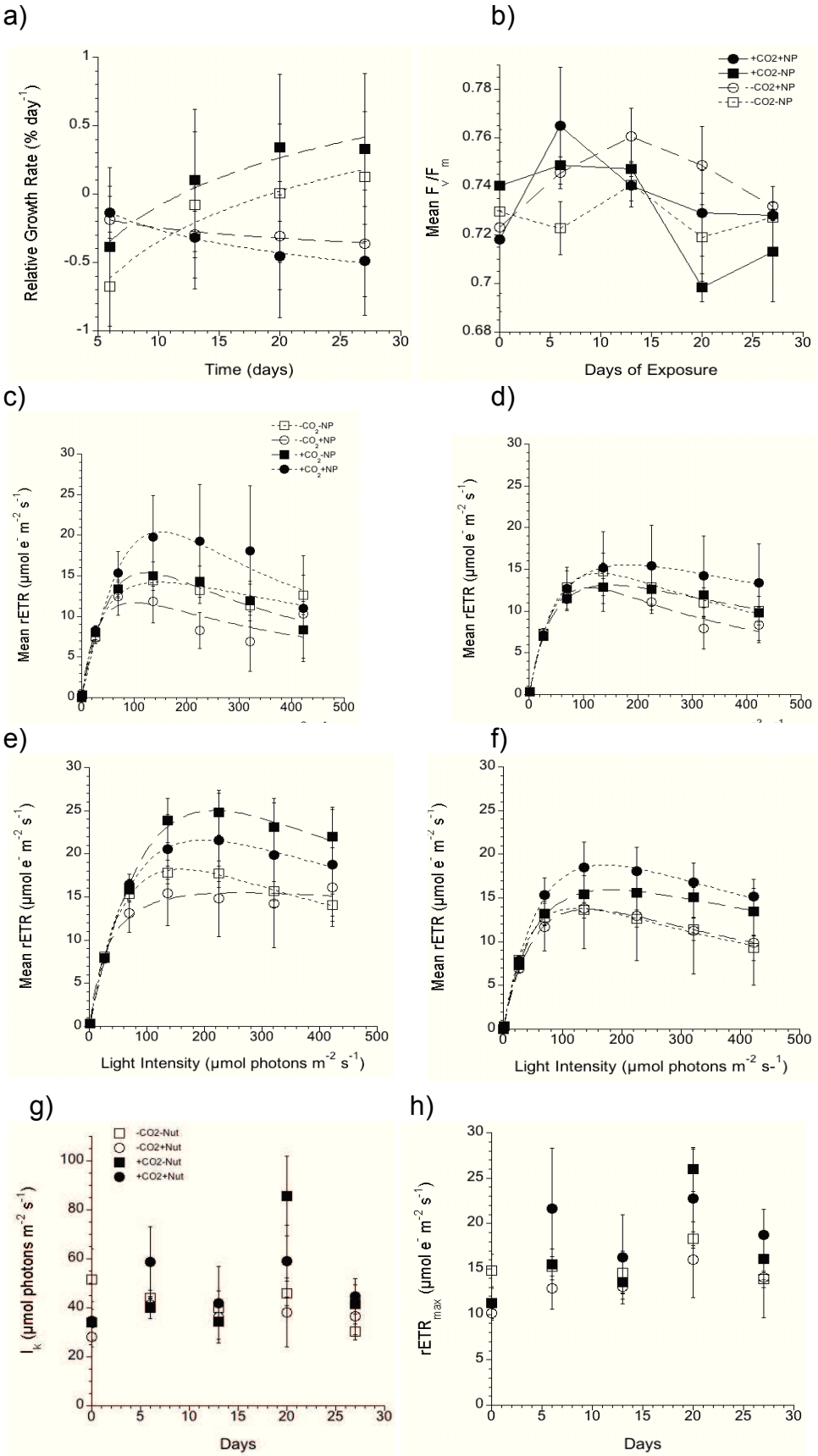

Figure 1 a) Mean (±SE, n = 4) relative growth rate, b) maximum quantum yield of photosystem II (F_v/F_m), c-f) rETR versus irradiance after 1 (c), 2 (d), 3 (e), and 4 (f) weeks of exposure to the experimental treatments, g) light saturation point (I_k) and h) maximum relative electron transport rate ($rETR_{max}$) of *H. opuntia* over time for each treatment.

Maximum photochemical quantum yield (F_v/F_m) of *Halimeda opuntia* ranged from 0.70 - 0.76, but was not significantly affected by inorganic nutrients or CO_2 (Table 2, Figure 1b). Light curves with *H. opuntia* rETR as a function of light intensity are shown for each week at each CO_2 and nutrient treatment in Figure 1 (c-f). After 4 weeks, rETRmax, alpha, and Ik were also not significantly affected by CO_2 or nutrients, although after 20 days there was a general trend that *H. opuntia* grown under elevated CO_2 had higher $rETR_{max}$ and I_k values regardless of nutrient concentration compared to ambient CO_2-grown algae (Figure 1g-h).

There was no significant effect of CO_2 or inorganic nutrients on pigment concentrations in *H. opuntia* (Tables 2 & 3, Figure 2). However, due to the presence of diatom epiphytes that remained after brushing the algae prior to analysis, there was a detectable interactive effect of elevated CO_2 and inorganic nutrients on the ratio of chlorophyll *c2*:*b*, as well as a main effect of nutrients on the fucoxanthin:chlorophyll *b* ratio (Table 2 & 3). Both pigment ratios were highest in the $+CO_2$+NP treatment and lowest in tihe $+CO_2$-NP treatment. Nutrients had no effect under ambient CO_2 conditions, indicating that the diatoms were able to take advantage of the elevated CO_2 and inorganic nutrients.

Table 3. Mean (±SE) concentration of chloropyll *b*, siphonoxanthin and siphonein, and chlorophyll *a*:*b* ratios of *H. opuntia*. The chlorophyll *c*:*b* and fucoxanthin:chlorophyll *b* ratios are also shown to indicate the relative amount of diatom epiphytes.

Experimental Treatment	Chlorophyll b (μg μg Chl a^{-1})	Chlorophyll a:b Ratio	Siphonoxanthin (area μg FW^{-1})	Siphonein (area μg FW^{-1})	Chlorophyll c:b Ratio	Fucoxanthin:Chlorophyll b Ratio
$-CO_2$-NP	0.66 ± 0.02	1.51 ± 0.05	4.02 ± 1.7	6.20 ± 1.9	0.007 (± 0.003)	0.040 (±0.015)
$-CO_2$+NP	0.65 ± 0.02	1.54 ± 0.06	4.79 ± 1.2	6.73 ± 1.8	0.008 (± 0.003)	0.036 (±0.017)
$+CO_2$-NP	0.69 ± 0.03	1.47 ± 0.05	6.33 ± 2.3	8.19 ± 2.9	0.003 (± 0.002)	0.009 (±0.007)
$+CO_2$+NP	0.63 ± 0.01	1.60 ± 0.04	5.01 ± 1.9	5.93 ± 2.5	0.017 (± 0.004)	0.034 (±0.016)

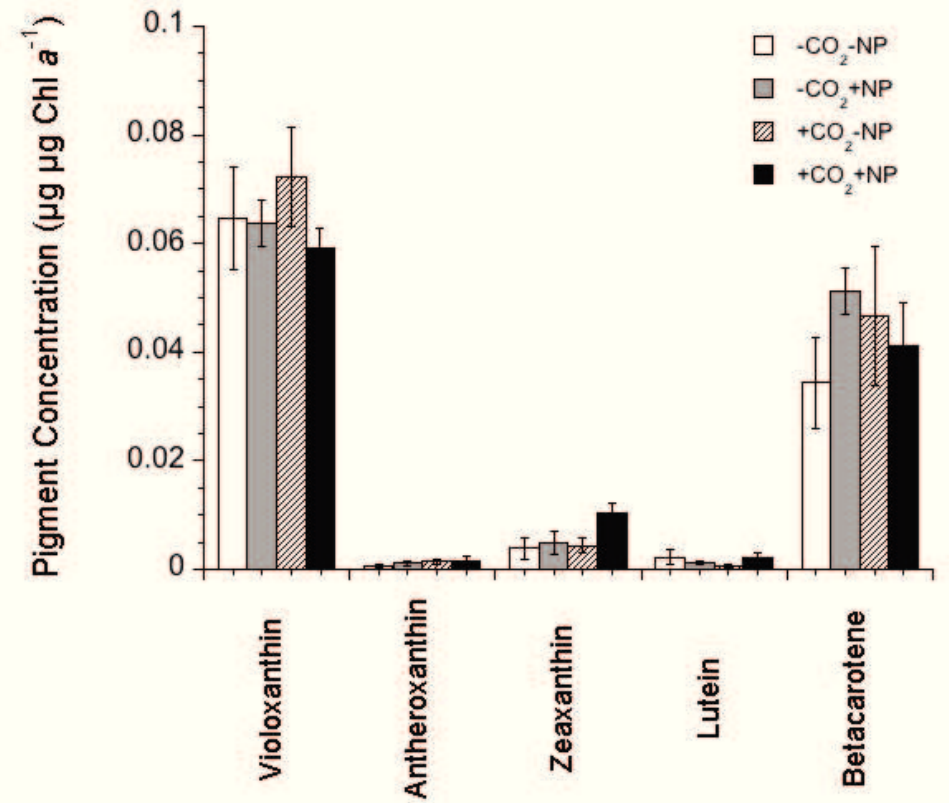

Figure 2 Mean accessory and protective pigment concentrations in *H. opuntia* tissue after 4 weeks of exposure.

Calcification and tissue carbon and nitrogen content

Calcification rates were not significantly affected by CO_2 or inorganic nutrients alone, but the meabn calcification rates increased with increasing aragonite saturation states (Tables 2 & 3). The relationship between calcification rates and $rETR_{max}$ was affected by the experimental treatments (Figure 3), as algae grown under elevated CO_2 condtions generally had higher $rETR_{max}$ values but lower calcification rates.

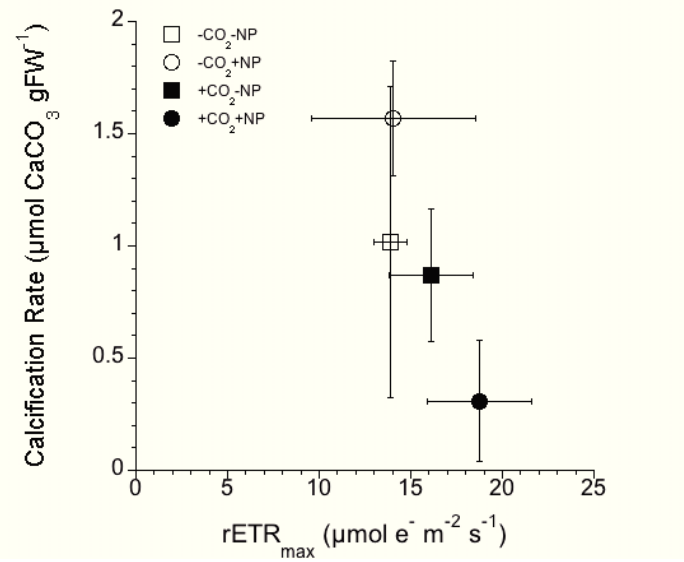

Figure 3 Relationship between mean calcification rates and mean $rETR_{max}$ values after 4 weeks of exposure for each treatment.

The percentage of algal tissue made up of $CaCO_3$, was significantly affected by CO_2 but not nutrients (Tables 2 & 4) and this subsequently affected the total C:N and inorganic:organic carbon ratios. The percentage of tissue made of $CaCO_3$ was highest in algae grown under normal CO_2 conditions and lowest in the tissue of algae grown under elevated CO_2 conditions (Table 4). Organic carbon and nitrogen content were higher in the elvated CO_2 treatments regardless of nutrient level (Table 4), and regardless of experimental treatment, there was a significant correlation between organic carbon and nitrogen (pearson correlation = 0.944, p = 0.000; Figure 4).

Table 4. Calcification rates, $CaCO_3$, organic carbon (C_{org}) and nitrogen (N) tissue content and organic carbon to nitrogen molar ratios of *H. opuntia* after 4 weeks of exposure to the experimental treatments. Unshared superscripts indicate significant differences ($p < 0.05$).

Treatment	Calcification Rate (μmol $CaCO_3$ g FW^{-1} hr^{-1})	$CaCO_3$ (% DW)	C_{org} (% DW)	N (% DW)	C_{org}:N
$-CO_2$-NP	0.32 ± 0.09^a	90.9 ± 0.8^a	5.5 ± 0.5	0.57 ± 0.08^a	32.5 ± 2.7
$-CO_2$+NP	1.57 ± 0.26^b	90.1 ± 1.2^a	5.7 ± 0.2	0.57 ± 0.04^a	33.7 ± 0.69
$+CO_2$-NP	0.87 ± 0.30^a	82.1 ± 1.2^b	7.4 ± 0.8	0.80 ± 0.09^b	30.8 ± 1.6
$+CO_2$+NP	0.31 ± 0.27^a	84.2 ± 2.4^b	6.1 ± 0.5	0.67 ± 0.05^b	31.6 ± 1.9

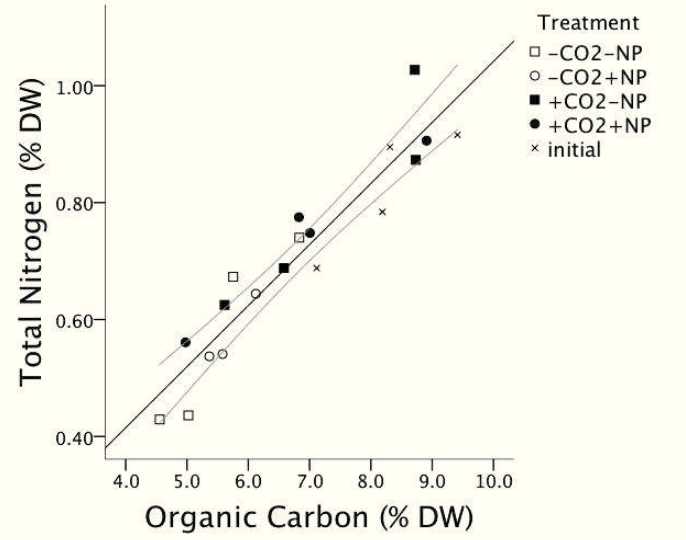

Figure 4 Relationship between nitrogen (% dw) and organic carbon (% dw) content of *H. opuntia* in all treatments after 4 weeks exposure as well as initial samples. There was a significant correlation between nitrogen and organic carbon (pearson correlation = 0.944, p = 0.000). Linear correlation shown with 95% confidence intervals

Enzyme activity (external carbonic anhydrase and *in situ* nitrate reductase)

External carbonic anhydrase activity (eCAA) and *in situ* nitrate reductase activity (NRA) were significantly affected by the treatment conditions, as there was a significant interaction between CO_2 and inorganic nutrients as well as a main effect of nutrients (Table 2). The effect of nutrients on eCAA was significant only under elevated CO_2 conditions, as the eCAA of algae grown under elevated CO_2 without nutrient enrichment was 192% (±29) that of the eCAA in algae grown under control conditions (Figure 5). On the other hand, *in situ* NRA decreased under elevated CO_2, and increased under elevated CO_2 plus inorganic nutrients, in relation to the control treatment (-CO_2-NP).

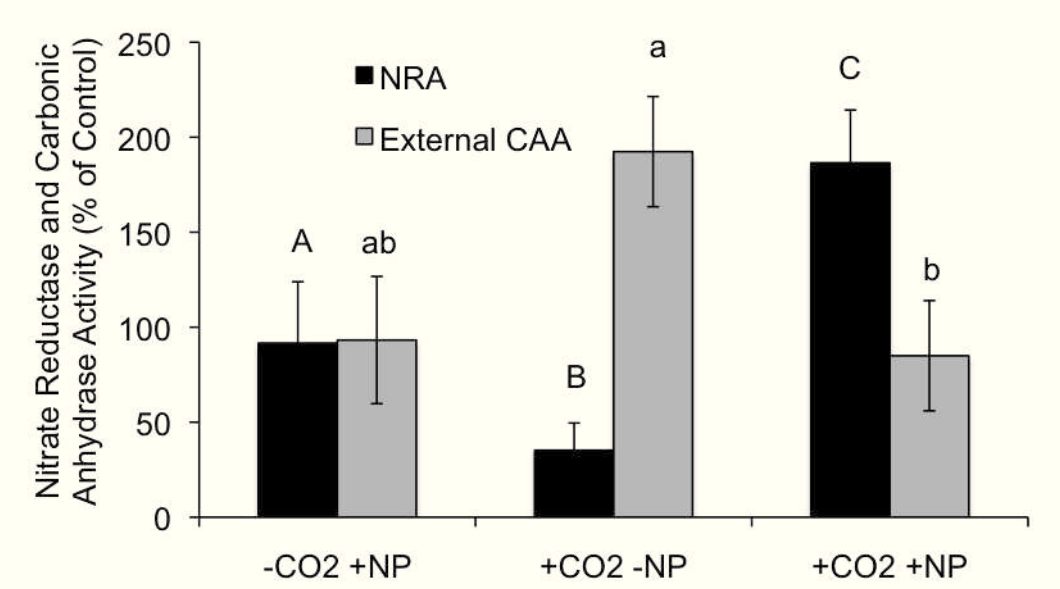

Figure 5 Mean nitrate reductase activity and external carbonic anhydrase activity, expressed as percent of the control mean ($-CO_2$-NP)

Discussion

The results from our inorganic nutrient and CO_2 enrichment experiment show that some physiological responses of *Halimeda opuntia* are sensitive to only CO_2, while others are affected by an interaction between the two factors. In general, photochemistry and tissue carbon and nitrogen content (%$CaCO_3$, total C:N and inorganic:organic carbon ratios) were influenced by CO_2, while there was a main effect of inorganic nutrients on growth and pigment concentration. Calcification, pigment concentration, and enzyme activities were affected by an interaction between elevated CO_2 and inorganic nutrients.

The increase in $rETR_{max}$ under elevated CO_2 alone that we observed at the end of our experiment contradicts the results of Price et al. (2011), who found that elevated CO_2 (pH 7.7, pCO2 946 ppm) decreased $rETR_{max}$. However, their experiment lasted only 2 weeks, and our significant CO_2 effect was only visible after 2 weeks. This discrepancy provides an example of how time is an important factor to consider in ocean acidification experiments. Furthermore, their experiment was conducted under high natural light intensities and a higher temperature (28°C), which when combined with elevated CO_2, may have had a photoinhibitive effect rather than stimulative effect, as found in our study under lower light and temperature conditions.

In our experiment, growth rates were affected by inorganic nutrients, but in general they were not a good indication of fitness due to the high rate of segment shedding by *H. opuntia*. However, the growth rates were clearly higher in the treatments without nutrient addition regardless of CO_2 concentration, which was most likely due to the high diatom growth in the treatments with elevated CO_2. Based on the fucoxanthin/chlorophyll c_2b ratios and visible observations, the explosion of diatoms in the +CO_2+NP treatment indicated that these organisms were able to exploit the excess inorganic carbon, nitrogen and phosphorous levels to increase their growth. An increase in diatom abundance, photosynthesis and growth under elevated CO_2 has previously been reported by (Johnson et al. 2011; Wu et al. 2010). Our results suggest that elevated inorganic nutrients in combination with elevated CO_2 could alter the competitive interactions between *H. opuntia* and its diatom epiphytes by giving the diatoms a competitive advantage.

Algae with elevated inorganic nutrients had the highest calcification rates when grown under ambient CO_2 conditions, but in contrast to previous work (Price et al., 2011; Sinutok et al. 2011), we did not observe lower calcification rates in *H. opuntia* exposed to elevated CO_2 compared to algae grown under ambient conditions. This is likely due to the high alkalnity and $\Omega_{aragonite}$ of the artificial seawater as well as higher maximum electron transport rates. Borowitzka & Larkum (1976) also found that calcification rates of *H. tuna* were constant between pH 7.0 - 8.5, and even observed an increase in calcification after short term exposure to pH 6.5 and attributed it to higher photosynthetic rates. Despite similar calcification rates among the different CO_2 treatments, the amount of $CaCO_3$ in the algal skeleton and the inorganic:organic carbon ratios were lower in the elevated CO_2 treatments compared to ambient CO_2 conditions. These results suggest that net calcification under elevated CO_2 conditions in the light are not able to compensate for high dissolution rates that may be occuring in the dark due to respiration and low aragonite saturation states. Indeed, it is becoming more clear in ocean acidification research that high dissolution rates, rather than low calcification rates, are the real threat to calcifying organisms under elevated CO_2 (Roleda 2011; Ries 2011; Rodolfo-Metalpa et al. 2011). Nevertheless, there have been previous reports of poorly calcified *Halimeda* spp. living on Australian coral reefes, indicating that some species can still survive without having heavily calcified skeletons (James et al, 1999). Such a decrease of inorganic carbon in the skeleton could make the alga more palatable and an easier and more nutritional target for grazers, but a possible compensation for having softer skeleton would be to increase anti-herbivore metabolites, which have been shown to be prevalent in *Halimeda* spp. (Paul & Fenical, 1983; 1984). Organic carbon concentrations were slightly elevated in both elevated CO_2 treatments, which could have been a result of higher concentrations of anti-herbivore metabolites. In addition to having potential affects on grazers, the changes in skeletal composition that were observed in this study may have important implications for coral reef accretion, as *Halimeda* spp. are important reef building organisms due to the contribution of their dead skeletons to coral sands (Drew 1983). If their skeletons have lower inorganic content under elevated CO_2 conditions, the relative contribution of *Halimeda* spp. skeletons to tropical carbonate sands would decrease under predicted future ocean conditions.

We recognize that the results for our experiment strongly contradict those of Price et al. (2011), who found that *H. opuntia* decreased calcification rates and increased shedding rates, but did not show changes in skeletal $CaCO_3$ composition under elevated CO_2. In addition to the confounding effect of differences in experimental temperature, light, and seawater chemistry conditions, it is also possible that different populations of *H. opuntia* respond differently to elevated CO_2. It has already been established that different *Halimeda* spp. respond differently to increasing CO_2 (Price et al. 2011; Sinutok et al. 2011), but future studies should also investigate if there are population differences.

The ability of *H. opuntia* to maintain normal calcification rates under elevated CO_2 is likely to have high energetic costs. Although the organic carbon to nitrogen balance did not change in our experiment, the enzyme activities showed that there was an opposite effect of high and low inorganic nutrients on eCAA and NRA under elevated CO_2. The decrease in NRA and increase in eCAA under just elevated CO_2 conditions suggest that *H. opuntia* may have allocated more energy to maintaining its calcification rate by increasing eCAA and downregulating NRA. Recent evidence suggests that HCO_3^- rather than CO_3^{2-} is the substrate for calcification (Ries 2011; Jokiel 2011; Roleda et al. 2012), and Borowitzka & Larkum (1976) reported that *Halimeda* spp. uses respiratory CO_2 as a substrate for photosynthesis. Therefore upregulation of eCAA is required to regulate the interconversion between CO_2 and HCO_3^-. In higher plants and fresh water algae, a decrease in NRA in response to elevated CO_2 has been reported in some species (Purvis et al. 1974; Hocking & Meyer 1991; Geiger et al. 1999; Xia & Gao 2005). Stitt & Krapp (1999) suggest that elevated CO_2 could increase the preference for ammonium over nitrate due to the decrease in photorespiration and associated recycling of ammonium that occurs under elevated CO_2. This process results in the formation of glutamine and subsequent repression of NRA. Such a response would save energy due to the lower energy requirements of ammonium assimilation compared to nitrate (Losado & Guerro 1979; Syrett 1981) and would allow the alga to allocate more energy to producing carbonic anhydrase for regulating inorganic carbon and maintaining calcification. Under the combination of elevated inorganic nutrients and CO_2, the stimulation of NRA is consistent with the results from Gordillo et al. (2001), who

found a similar response in the noncalcifying chlorophyte *Ulva rigida*. Furthermore, Gordillo et al (2006) reported decreases in eCAA in some arctic seaweeds under nutrient enrichment and suggested that energy investment into carbon assimilation was sacrified at the expense of nitrate assimilation and macromolecule production. This could explain why we didn't see elevated eCAA in *H. opuntia* grown in the $+CO_2$+NP treatment.

Our experiment with elevated CO_2 and inorganic nutrients demonstrates that the response of *H. opuntia* to future surface ocean conditions will be dependent on localized nutrient conditions. When nutrients are sufficient, *H. opuntia* is able to alter its enzymatic activity and subsequently carbon and nitrogen assimilation while maintaining healthy calcification rates under elevated CO_2. However, when nutrients are saturating, as under eutrophic conditions, noncalcifying organisms may benefit at the expense of calcifiers. Although we did not test this hypothesis in our study, the high diatom growth in our $+CO_2$+NP treatment indicated that hint to such an effect. Russell et al. (2009) did test this hypothesis, and reported that the combination of elevated CO_2 and inorganic nutrients altered subtidal rocky habitats by decreasing the competitiveness of crustose coralline algae compared to algal turfs. It will be necessary to conduct further studies to determine how *Halimeda* spp. will compete with noncalcifiers under elevated CO_2 and inorganic nutrients, and how the presence/absence of grazers also affects their competition.

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Chapter 5

The effect of elevated CO_2 and inorganic nutrients on competition between the calcifying chlorophyte *Halimeda opuntia* and its noncalcifying epiphyte *Dictyota* sp.

The effect of elevated CO_2 and inorganic nutrients on competition between the calcifying chlorophyte *Halimeda opuntia* and its noncalcifying epiphyte *Dictyota* sp.

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Abstract

Ocean acidification studies in the past decade have greatly improved our knowledge of how calcifying organisms respond to increased surface ocean CO_2 levels, and it has become evident that for many organisms, nutrient availability is an important factor that influences their physiological response and their competition with other species. Therefore, we simulated the combination of ocean acidification with eutrophication (nitrate and phosphate enrichment) to investigate the physiological responses of a calcifying chlorophyte macroalga (*Halimeda opuntia*) and its common noncalcifying epiphyte (*Dictyota* sp.) as well as their competitive interactions. We found significant main effects of inorganic nutrient enrichment and CO2 on almost all responses measured, as well as significant interactive effects on growth, photosynthesis and changes in percent cover of each species. However, in contrast to previous studies, we did not observe a decrease in calcification rates of *H. opuntia* under elevated CO_2. *Dictyota* sp. is an opportunistic species that was able to take advantage of nutrient enrichment by significantly increasing in cover and biomass, and this effect was greatest at elevated CO_2 levels. Our results suggest that *H. opuntia* will not suffer greatly under ocean acidification conditions in areas where inorganic nutrients are low. However, without top-down grazer control, *Dictyota* sp.

has a competitive advantage over *H. opuntia* in eutrophied areas, and this effect is amplified at elevated CO_2 concentrations.

Introduction

In recent decades, the increasing CO_2 concentrations in surface ocean waters (ocean acidification) as a result of anthropogenic CO_2 input into the atmostphere has been a widely studied field, and therefore the amount of information on the physiological responses of calcifying organisms to ocean acidification has greatly increased. Although calcifying marine organisms show a variety of responses to increasing CO_2 concentrations (Langer et al. 2006; Ries 2009; Fabricius et al. 2011; Hurd et al. 2011), a clear trend that has been seen in many studies is that the food or nutrient availability of an organism is an important factor that infuences its response to increasing CO_2 (Renegar & Riegl 2005; Russell et al. 2009; Holcomb et al. 2010; Chauvin et al. 2011; Findlay et al. 2011; Matthiessen et al. 2012). Furthermore, as already demonstrated in subtidal rocky habitats by Russell et al. (2009), the global impact of increasing surface ocean CO_2 concentrations will differ at regional levels, depending on other abiotic factors such as temperature. In tropical environments, excess nutrient availability, or eutrophication, is a serious problem that has been shown to cause phase shifts on coral reefs by increasing the competitiveness of fleshy macroalgae at the expense of corals, especially when herbivory is low (Done 1992; Hughes 1994; Miller & Hay 1996; Lapointe 1997; McCook 1999; McCook et al. 2001; Jompa & McCook 2002; Burkepile & Haye 2006). Such a phase shift can result in lower coral recruitment due to decreased light availability, lack of available substrate and/or chemical inhibition of settlement (Birkeland 1997; Edmunds & Carpenter 2001; McCook et al 2001 and references therein; Kuffner et al. 2006; Hughes et al. 2007; Diaz-Pulido et al. 2010). We therefore expect that calcifying organisms on tropical reefs with strong local anthropogenic eutrophication pressure will be more susceptible to the negative effects of ocean acidification than those living in oligotrophic reefs.

While some corals have shown high sensitivity to ocean acidification (Langdon et al. 2000; 2003; Leclercq et al. 2000; Albright et al. 2008; 2010; Jokiel et al. 2008; Andersson et al. 2009), others have shown the ability to withstand decreasing pH and aragonite saturation states associated with increasing CO_2 levels due to

protective tissue layers, by controling the pH within their calcifying fluid, or simply by living without a calcified skeleton (Reynaud et al. 2003; Fine and Tchernov 2007; Krief et al. 2010; Fabricius et al. 2011; Rodolfo-Metalpa et al. 2011; McCulloch et al. 2012). Furthermore, calcifying macroalgae that are important for $CaCO_3$ sand production, such as *Halimeda* spp., have shown sensitivity to ocean acidification (Robbins et al. 2009; Price et al. 2011; Sinutok et al. 2011), but can maintain and even increase calcification rates under moderate CO_2 levels (Ries 2009).

McConnaughey and Whelan (1997) reported that the mechanism of calcification serves as a proton source for nutrient and HCO_3^- uptake in calcifying marine primary producers. As such, the authors proposed that a calcified skeleton is an adaptive advantage over noncalcifiers under oligotrophic conditions. However, under eutrophied conditions, this advantage seems to be negligible when herbivory does not control the fleshy algae population, because noncalcifying macroalgae are often more stimulated by nutrient enrichment than calcifying species (Zabala & Ballesteros 1989; Delgado & Lapointe 1994; Lapointe et al. 1997). It is therefore important to investigate how calcifying and noncalcifying macroalgae will compete under future CO_2 conditions in combination with local factors such as nutrient regimes.

The calcifying chlorophyte macroalgae in the genus *Halimeda* are important coral reef sediment producing organisms whose dead skeletons produce bank-like mounds (bioherms) containing high amounts of carbonate sediment (Littler et al. 1988; Rees et al. 2007). *Halimeda* spp. are therefore important contributors to carbonate sediments (Hillis-Colinvaux 1980; Drew 1983; Marshall & Davies 1988; Drew & Abel 1988; Diaz-Pulido et al. 2007; Rees at al. 2007). Estimates suggest modern *Halimeda* bioherms accumulate globally 0.15 to 0.4 Gt $CaCO_3$ $year^{-1}$, which is a major part of the annual coral reef carbonate production (Milliman 1993; Hillis 1997; Rees et al. 2007).

The calcification mechanism of *Halimeda* spp. has been well documented (Borowitzka & Larkum 1976a; 1976b; 1976c; 1977; 1987). Despite the isolated site of calcification within the intracellular (utricular) spaces of these algae with respect to the outer seawater, several species have been shown to be sensitive to low pH (Robbins et al. 2009; Price et al. 2011; Sinutok et al. 2011). While these studies did

not report the nutrient levels at which their experiments were conducted, their reported sensitivity to ocean acidification could be amplified by eutrophication in natural conditions due to competition from noncalcifying opportunistic macroalgae.
The noncalcifying phaeophyte algae in the genus *Dictyota* are common competitors with *Halimeda* sp. and stimulated by inorganic nutrient enrichment (Lapointe et al 1987; Delgado & Lapointe 1994; Lapointe et al. 1997). *Dictyota* spp. also produce phlorotannins that are protective agents against herbivores, making them strongly competitive under eutrophic conditions, even when herbivory is high (Targett et al. 1992; Stachowicz & Hay 1999). Furthermore, many noncalcifying macroalgae show stimulated photosynthesis and growth under elevated CO_2 conditions (Gao et al. 1991; 1993; Kübler et al. 1999; Gordillo et al. 2001; Zou 2005; Suárez-Álvarez et al. 2011). Therefore, we expected that combined CO_2 and inorganic nutrient enrichment would have amplified beneficial effects for *Dictyota* sp. at the expense of the calcifying competitor *Halimeda opuntia*. We therefore tested how these two abiotic factors affect the photosynthesis, growth, calcification (for *H. opuntia*) and competitive interactions of these two important coral reef dwelling macroalgae to determine how they will respond under future CO_2 conditions depending on local nutrient regimes.

Materials and Methods

Experimental Design

The macroalgae used in this experiment were collected in Willemstad, Curaçao (former Netherlands Antilles) at 5 m depth in January 2012 and maintained in a recirculating artificial seawater system at the Leibniz Center for Tropical Marine Ecology in Bremen, Germany. The algae were maintained at 25°C, salinity 33, and 150 µmol photons m^{-2} s^{-1} light intensity on a 12:12 light:dark cycle until the beginning of the experiment in March 2012.

Some coral reefs in Curaçao are exposed to eutrophication due to high sewage discharge, industrial waste, rain runoff, and groundwater seepage (Gast 1998). Healthy reef conditions in Curaçao have a seawater nitrate concentration of approximately 0.5 µM, while eutrophied reefs have ten times that amount (up to 5 µM

DIN) and harbor water DIN concentrations reach up to 40 µM. Phosphate concentrations on healthy reefs in Curaçao are usually below 0.05 µM, while eutrophied reefs experience up to 0.3 µM (Gast 1998). Such low concentrations of inorganic nutrients on healthy reefs are due to rapid recycling of the nutrients, but it is generally thought that higher concentrations of inorganic nutrients are available from the sediment, particulate organic matter and nitrogen-fixing bacteria, as the high productivity rates on coral reefs could not be supported by such low nutrient concentrations (Webb et al. 1975; Wiebe et al. 1975; Froelich 1983; Mwashote & Jumba 2002; Rasheed et al. 2002). Therefore, we chose relatively high concentrations of nitrate and phosphate for our eutrophied treatment in order to ensure that the algae used in our experiment were nutrient replete. The concentration of inorganic nutrients in our unenriched seawater were as low as we could reach using milli-Q treated distilled water with added Red Sea Reef salt.

Our experiment consisted of four CO_2 levels (200, 390, 700, 900 µatm CO_2) and two inorganic nutrient levels (nitrate and phosphate enriched: 50 µM NO_3^{2-}, 5 µM PO_4^{3-} or unenriched: 1.4 µM NO_3^{2-}, 0.09, µM PO_4^{3-}). A combination of the two independent factors (CO_2 and inorganic nutrients) resulted in 8 treatments, and we had five replicate algal thalli in separate flasks for each treatment. The experimental units were one liter glass round bottom flasks that were continuously bubbled with pre-mixed air containing the CO_2 concentration of interest using a computerized 5-channel gas mixing system (HTK Hamburg GmbH, Hamburg, Germany). Inorganic nutrients were added separately to each flask, and distilled water was added to the flasks that did not receive nutrient enrichment. Reservoir tanks for each CO_2 treatment were continuously bubbled with the pre-mixed gas, and this water was used to change the water in each flask three times per week.

The experimented was conducted twice: once with the 200, 700 and 900 µatm CO_2 treatments with and without nutrients, and a second time with the 390 and 900 µatm CO_2 treatments (each with and without nutrients). The results from the separate 900 µatm CO_2 treatments were compared to ensure that the different dates of the experiment did not have an effect on the algal response. Each experiment lasted four weeks, during which time growth, calcification and chlorophyll fluorescence were measured weekly, while O_2 evolution was measured at the end of the four weeks.

Photographs were taken at the beginning and end to assess changes in community composition.

Prior to the experiment, fragments of *H. opuntia* (3-4 g FW) were cleaned of epiphytes except for *Dictyota* and given three days to acclimate to the experimental set-up in artificial seawater bubbled with ambient air before the experimental treatments were applied. The initial *Dictyota* cover was limited to 10% of the *H. opuntia* thalli.

Seawater chemistry was monitored regularly throughout the experiment. The pH, salinity and temperature in every flask were measured daily, and 50 ml samples were taken from the reservoir tanks weekly for alkalinity measurements.

Growth and Calcification

The fresh weight of the communities (containing minimal *Dictyota* tissue) was weighed at the beginning of the experiment and the fresh weight of *H. opuntia* was measured after four weeks when all *Dictyota* was removed. The initial fresh weight of *Dictyota* was assumed to be neglible relative to *H. opuntia*, and therefore we could calculate relative growth rates (RGR) of *H. opuntia* according to the equation RGR = $\ln(FW_t/FW_i)/t \times 100$, where FW_i was the initial fresh weight of *H. opuntia* containing very few *Dictyota* sp. thalli and FW_t was the fresh weight of *Halimeda* after t = 4 weeks once the *Dictyota* sp. was removed. The fresh weight of any shed segments was also weighed each week and subtracted from the initial weight in the calculation of RGR. The segment shedding rate was calculated as % FW_i day^{-1}, where $\%FW_i$ was the fresh weight of the total segments lost after four weeks as a percentage of the initial fresh weight.

The growth rate of *Dictyota* sp. was calculated by weighing all algal material removed from the *H. opuntia* thalli after four weeks. We assumed an initial weight of zero, and calculated the growth rate (mg day^{-1}) by dividing the final weight by the duration of the experiment (in days).

Calcification of *H. opuntia* was measured using the buoyant weight technique {Davies 1989} and the alkalinity anomaly technique (Chisholm & Gattuso 1991), allowed us to couple photosynthesis to calcification during closed incubations. For buoyant weight measurements, the algal communities (*H. opuntia* + *Dictyota*) were placed in a basket suspended in seawater below a balance and the buoyant weight was measured. Calcification (mg mg^{-1} $CaCO_3$ day^{-1}) was calculated as the change in buoyant weight over time standardized to the initial buoyant weight. The buoyant weight of any shed segments was also measured and subtracted from the initial weight in the calculation of calcification rates. The alkalinity anomaly technique was used to determine calcification rates of *H. opuntia* at the end of four weeks after all epiphytic *Dictyota* sp. was removed. Closed incubations under dark and light conditions were conducted, and alkalinity samples were taken at the beginning and end of each incubation. Net calcification (G; µmol $CaCO_3$ gFW^{-1} hr^{-1}) was calculated as the change in alkalinity (ΔA_T) relative to the incubation volume (V), seawater density (ρ) the algal fresh weight (FW) and the incubation time ($G = -0.5\rho \times \Delta A_T \times V / FW / t$).

Chlorophyll Fluorescence and Photosynthesis

Chlorophyll fluorescence of the macroalgal communities was measured using an Imaging Pulse Amplitude Modulated Chlorophyll Fluorometer (MAXI version Imaging-PAM *M*-Series, Heinz Walz GmbH, Effeltrich, Germany). The maximum photochemical quantum yield of photosystem II (F_v/F_m) was measured after 5 minutes of adaptation in the dark. Light curves were conducted in order to calculate electron transport rates (ETR). The time it took for *H. opuntia* and *Dictyota* sp. to recover to steady state (F_0) after a saturation pulse was measured to determine the appropriate time interval for each light step of the light curve. We determined that one minute light intervals were enough for complete recovery of the ground state chlorophyll fluroescence and therefore used one-minute light steps ranging from 0-500 µmol photons m^{-2} s^{-1}. The light intensities at each step were calibrated with a US-SQS spherical micro quantum sensor (Heinz Walz GmbH, Effeltrich, Germany). Electron ransport rates were calculated according to the equation $ETR = A \times 0.5 \times \Phi_{PSII} \times E$ for *Halimeda opuntia* and $ETR = A \times 0.8 \times \Phi_{PSII} \times E$ for *Dictyota* sp. where 0.5 and 0.8 were the fraction of absorbed light directed to PSII for green and brown

algae, respectively (Grzymski et al. 1997; Figueroa et al. 2003), A was the mean absorbed quanta calculated as the integrated spectral absorptance from 400-700 nm using an integrating sphere, Φ_{PSII} was the quantum yield of photosystem II (PSII) charge separations, and E was the irradiance (µmol photons m^{-2} s^{-1}) at each light step. Absorptance was measeared using an integrating sphere connected to a Shimadzu UV 2401 PC UV-Vis recording spectrophotometer and calculated according to the formula A = 1- T - R, where T and R were transmittance and reflectance of the algal thallus, respectively. Five samples from different thalli were taken at the beginning of the experiment to estimate the mean absorptance for *H. opuntia*. The ETR of *Dictyota* was estimated using the absorptance of *Dictyota dichotoma* from Frost-Christensen and Sand-Jensen (1992). The actual absorptance values for each individual thallus could not be measured because we did not destructively sample during the experiment. Therefore the ETR values are still an estimation, but more accurate than rETR.

The chlorophyll fluroescence parameters ETR_{max} (maximum relative electron transport rate), E_k (light saturation point) and alpha (electron transport efficiency) were calculated by nonlinear curve fit analysis of the ETR versus irradiance curves based on the model by Eilers and Peeters (1988).

Photosynthetic rates were calculated from gross photosynthesis and respiration measurements. Oxygen evolution was measured after four weeks for the total community and for *H. opuntia* alone once *Dictyota* sp. was removed. Oxygen evolution rates for *Dictyota* were calculated by subtracting *H. opuntia* from the community rates. Oxygen evolution was measured during 20-minute incubations in the light and dark using a four channel Firesting O_2 fiber optic oxygen meter with contactless oxygen sensor spots (Pyro Science GmbH, Aachen, Germany). The sensor spots were submerged in the seawater and attached to the flasks using non-toxic silicone glue. The incubations were conducted within the one-liter experimental flasks, which were sealed with a stopper and stirred with a magnetic stir bar (bubbling was stopped during this period). Due to Pyroscience equipment limitations, oxygen evolution was only measured in the second experiment when the ambient and highest CO_2 treatments were used. However, oxygen evolution was measured in

all treatments during both experiments using a separate fiber optic sensor set-up, but these results are not presented here, as they will be presented in a later paper.

Community Composition

Photographs of the macroalgal communities were taken at the beginning and end of the experiment for analysis of percent cover of each species. The images were analyzed using the Coral Point Count with excel extensions (CPCE) software program {Kohler and Gill 2006}. A 10 x 10 point grid was placed over each communitiy and the species present at each point was recorded. The percent cover of each species was calculated based on the total number of points containing algae. The change in percent cover was calculated as $(PC_t-PC_i)/PC_i$ x 100, where PC_i was intial percent cover and PC_t was the percent cover after four weeks.

Statistical Analysis

Statistical analysis of the response variables was conducted using factorial analysis of variance (ANOVA) tests with CO_2, nutrients, and when appropriate, species as independent factors. When a response variable was measured over time, a repeated measures mixed factorial ANOVA was conducted, with time treated as a repeated measures factor. The chlorophyll fluorescence parameters calculated from nonlinear curve fitting of the ETR versus irradiance curves were analyzed using a multivariate analysis of variance (MANOVA). Pairwise comparisons were made using Tukey's HSD pot-hoc test. When the data did not meet the assumption of normality, they were log transformed. If transformation was not possible, a nonparametric Kruskal-Wallis test was conducted.

Results

Seawater chmistry, growth and calcification

The mean seawater chemistry parameters of the reservoir tanks are shown in Table 1. The saturation state for aragonite and calcite was greater than one in all treatments despite elevated CO_2 and decreased pH in two of the treatments relative

to the ambient conditions. The growth rate of *H. opuntia* was significantly affected by a significant interaction between CO_2 and inorganic nutrients ($F(3, 32) = 4.696$, $p = 0.008$) as well as by a main effect of both factors ($F(1, 32) = 18.7$, $p =$; $F(3, 32) = 6.55$, $p = 0.002$). Relative growth rates were higher in algae grown with inorganic nutrient enrichment compared to unenriched algae in all CO_2 treatments except for the 200 µatm treatment, where the algae were likely carbon limited. The segment shedding rate was not significantly affected by the experimental treatments (Kruskal-Wallis $H(9) = 3.85$, $p = 0.921$; Figure 1).

Table 1. Mean (± SE) seawater chemistry parameters of the reservoir tanks treated with CO_2 only (no inorganic nutrients added).

CO_2 Treatment	Temperature (°C)	pH_{total}	A_T^* (µmol kg SW^{-1})	pCO_2 (µatm)	HCO_3^- (µmol kg SW^{-1})	CO_3^{2-} (µmol kg SW^{-1})	CO_2 (µmol kg SW^{-1})	Ω_{Ca}^*	Ω_{Ar}^*
200	25.05 ± 0.1	8.38 ± 0.13	2215 ± 385	210 ± 120	1408 ± 417	319 ± 28	6.0 ± 3.5	7.78 ± 0.67	5.11 ± 0.44
390	24.96 ± 0.1	8.00 ± 0.02	1994 ± 121	392 ± 15	1577 ± 90	166 ± 15	11.2 ± 0.43	4.05 ± 0.37	2.66 ± 0.24
700	25.05 ± 0.1	7.94 ± 0.03	2919 ± 175	701 ± 54	2407 ± 146	218 ± 18	20 ± 1.6	5.32 ± 0.44	3.50 ± 0.29
900	24.86 ± 0.1	7.79 ± 0.03	2617 ± 190	917 ± 90	2257 ± 164	151 ± 16	26.3 ± 2.5	3.68 ± 0.40	2.41 ± 0.26

*Abbreviations: A_T = total alkalinity, Ω = saturation state of calcite (Ca) and aragonite (Ar).

Table 2. ANOVA and MANOVA results

Response variable	CO_2	DIN	CO_2 x DIN	Species	Species x DIN	Species x CO2	Species x DIN X CO_2	Time x CO_2	Time x DIN
Relative Growth Rate	F(3, 32) = 5.409, p = 0.002	F(1, 32) = 16.831, p = 2.6E-4	F(3, 32) = 4.4, p = 0.010	n.a.	n.a.	n.a.	n.a.	n.a	n.a
Growth Rate	F(3, 31) = 13.9, p = 6.3E-6	F(1, 31) = 11.5, p = 0.002	F(3, 31) = 5.4, p = 0.004	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Calcification	F(3, 31) = 3.3, p = 0.034	-	-	n.a.	n.a.	n.a.	n.a.	F(6, 62) = 4.0. p = 0.002	F(2, 62) = 3.2, p = 0.047
F_v/F_m	-	-	-	F(1, 59) = 5.5, p = 0.022	F(1, 59) = 4.3, p = 0.042	-	-	n.a.	n.a.
Net Photosynthesis	-	-	-	F(1, 24) = 13.7, p = 0.001	-	-	F(1, 24) = 4.1, p = 0.054	n.a.	n.a.
ETR_{max}	F(3, 59) = 5.4, p = 0.002	F(1, 59) = 31.9, p = 4.9E-7	-	F(1, 59) = 31.1, p = 6.0E-7	F(1, 59) = 4.8, p = 0.033	-	-	n.a.	n.a.
E_k	F(3, 59) = 2.8, p = 0.048	F(1, 59) = 13.5, p = 0.001	-	F(1, 59) = 19.4, p = 4.6E-5	-	-	-	n.a.	n.a.
alpha	-	F(1, 59) = 8.9, p = 0.004	-	F(1, 59) = 4.6, p = 0.037	-	-	-	n.a.	n.a.
% Change in Cover	-	-	-	F(1, 64) = 39.6, p = 3.2E-8	F(1, 64) = 9.2, p = 0.003	F(1, 64) = 5.6, p = 0.002	F(3, 64) = 7.8, p = 1.6E-4	n.a.	n.a.

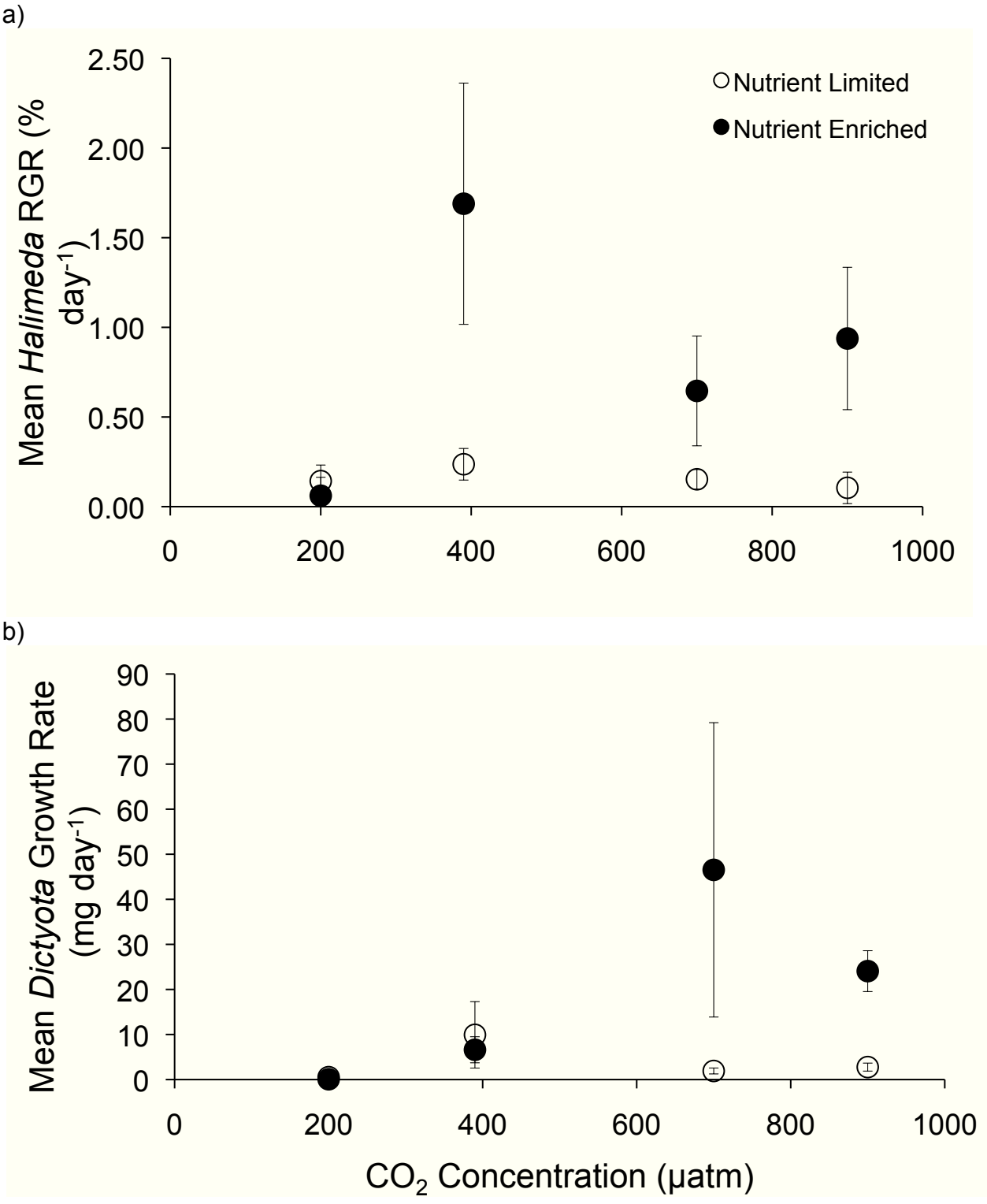
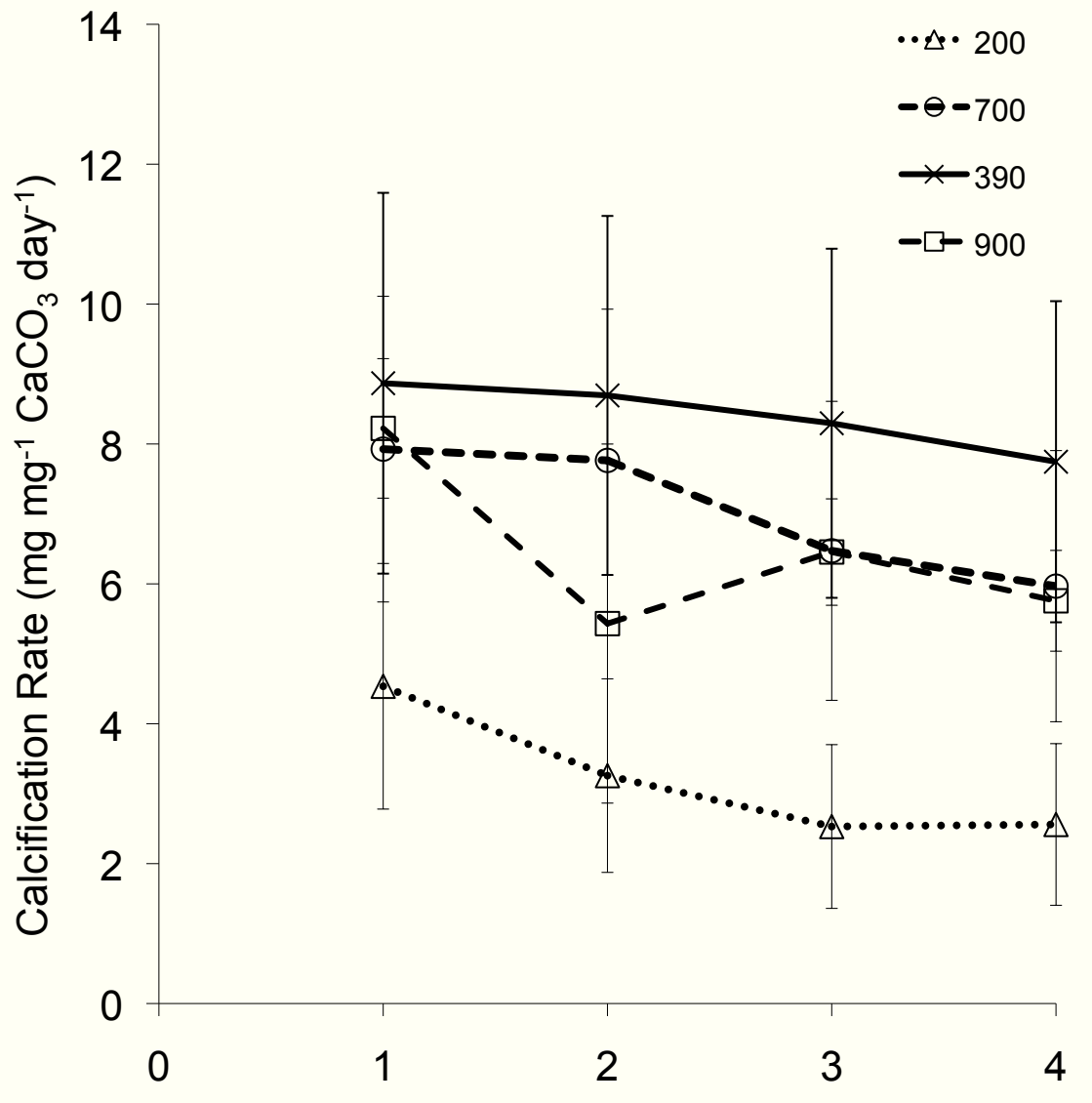


Figure 1. a) Mean (±SE, N = 5) relative growth rate of *H. opuntia* and b) mean growth rate (± SE) of *Dictyota* sp. as a function of CO_2 under nitrate and phosphate limited (open circles) and saturated (filled circles) conditions

Net calcification rates of *H. opuntia* measured by buoyant weight were affected by a main effect of CO_2 and interactions between time and CO_2 and time and inorganic nutrients (Table 2, Figure 2). Calcification rates were lowest at 200 µatm CO_2, and

these values were significantly different from the calcifation rates at 900 µatm CO_2, regardless of nutrient concentration. Without nutrient enrichment, the calcification rates of *H. opuntia* at all CO_2 levels decreased over time. Under inorganic nutrient enrichment, calcification rates increased over time at ambient CO_2 conditions and remained stable at the elevated CO_2 conditions. There was no significant difference between the calcification rates at ambient CO_2 and elevated CO_2.

a)



b)

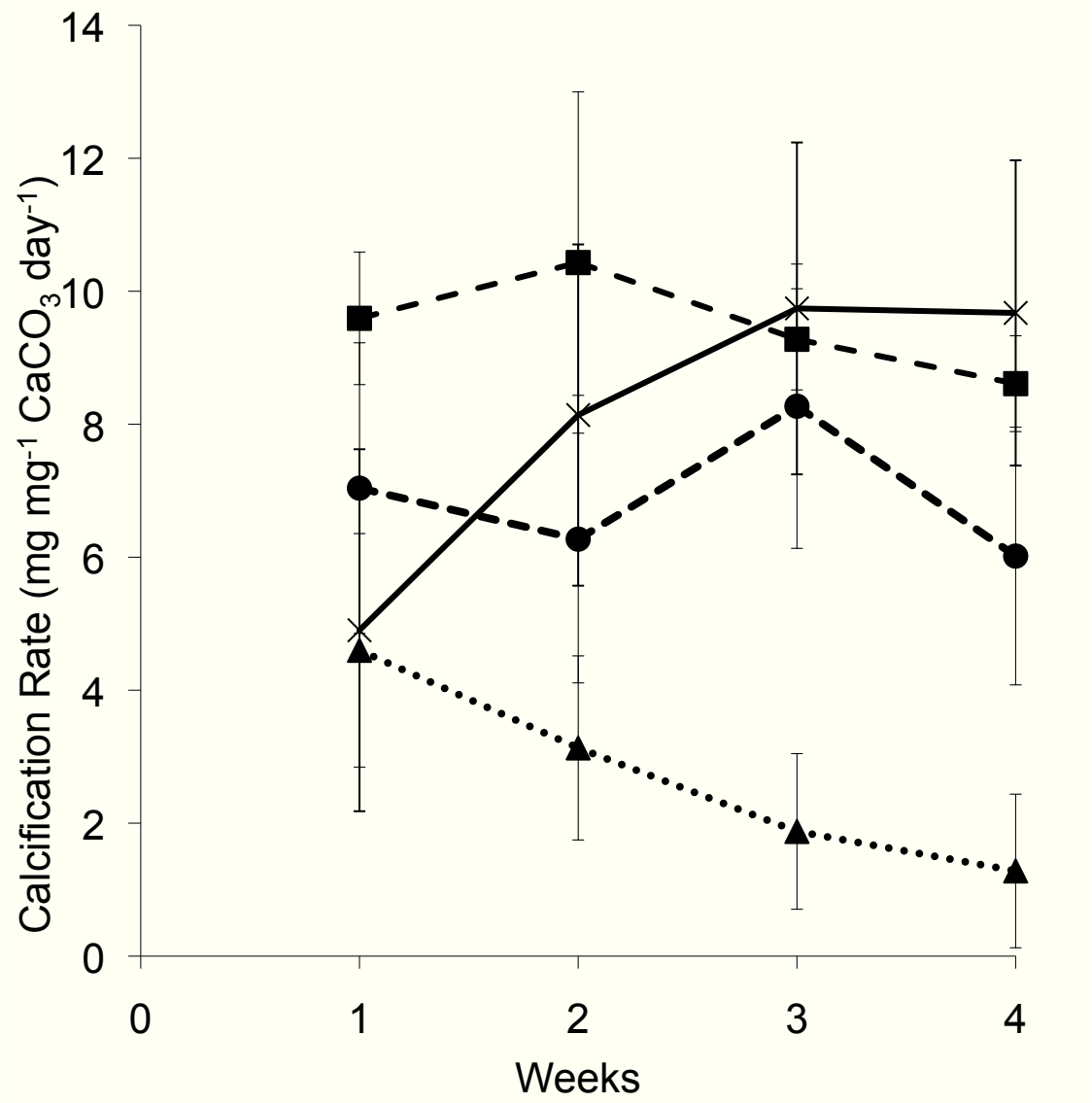


Figure 2. Mean (± SE, N = 5) net calcification rates of *H. opuntia* over time for each CO_2 treatment without (a) and with (b) nitrate and phosphate enrichment. Calcification rates are based on buoyant weight measurements standardized by initial buoyant weight.

Net calcification rates of *H. opuntia* under dark and light conditions measured at the end of the experiment after all *Dictyota* sp. was removed are shown in Figure 3. In the dark, only *H. opuntia* enriched with nutrients showed net dissolution at all CO_2 levels, but this value did not differ greatly from zero. In the light, *H. opuntia* grown without inorganic nutrient enrichment had the highest variability in calcification rates at the highest and lowest CO_2 levels, but the means did not differ between CO_2 treatments. Finally, calcification rates were highest in *H. opuntia* grown under nitrate and phosphate enriched conditions and showed a parabolic response to CO_2 concentrations, with the highest mean and the highest variability at 700 µatm CO_2.

Note that these calcification rates are likely underestimated if nitrate uptake rates by the algae were high. Nitrate uptake is accompanied by an increase in total alkalinity, which would result in an underestimation of calcification rates.

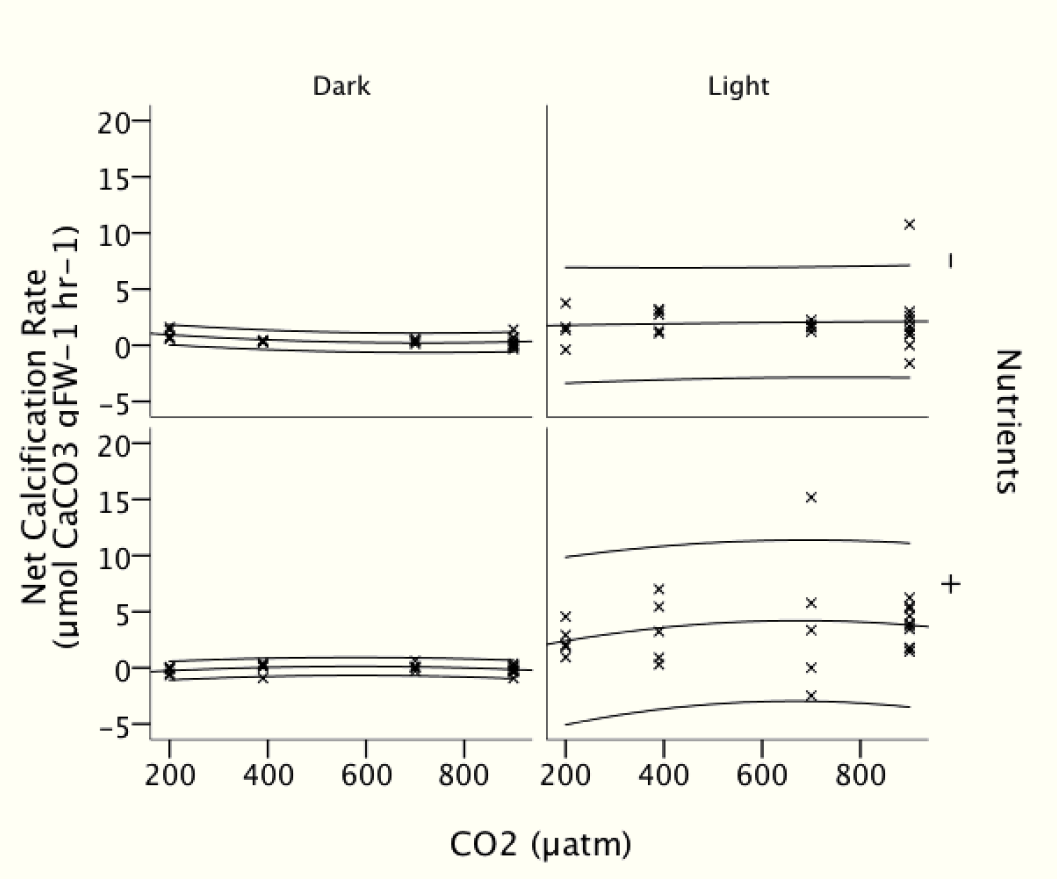


Figure 3. Net calcification rates of *H. opuntia* as a function of CO_2 in dark (left) and light (right) conditions with (top) and without (bottom) inorganic nutrient enrichment measured using the alkalinity anomaly technique. Lines represent 95% confidence intervals.

Chlorophyll Fluroescence and Photosynthesis

The maximum photochemical quantum yield of photosystem II (F_v/F_m) was affected by a significant interaction between species and inorganic nutrients, but not CO_2 (Table 2, Figure 4). *Halimeda opuntia* had higher F_v/F_m values than *Dictyota* under unenriched conditions, but not under nutrient enriched conditions. *Dictyota* had the lowest F_v/F_m ratios under unenriched conditions at the two elevated CO_2 concentrations, while nutrients did not have a strong effect of *H. opuntia* F_v/F_m values, except at the lowest CO_2 concentration, where enriched conditions decreased the F_v/F_m.

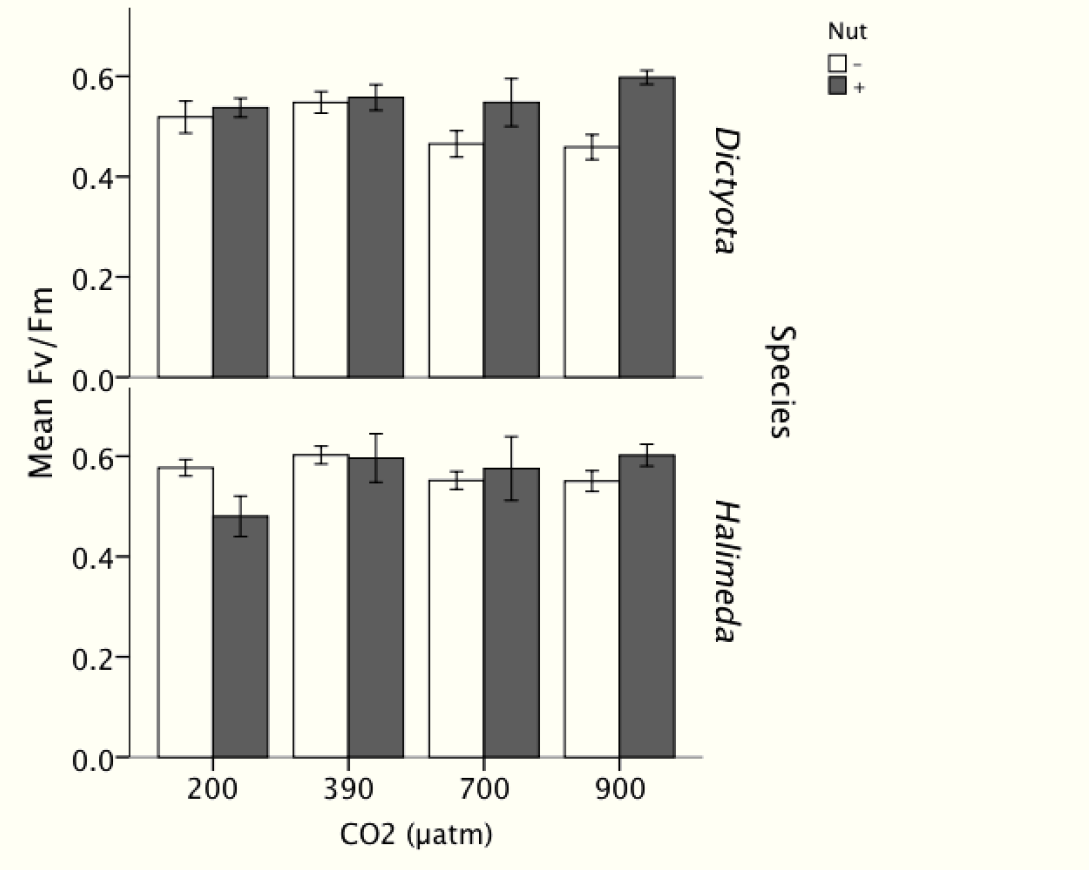


Figure 4. Mean F_v/F_m (±SE, N = 5) for *H. opuntia* and *Dictyota* sp. after four weeks of exposure to the four CO_2 experiments with (dark bars) and without (white bars) inorganic nutrient enrichment.

The chlorophyll fluroescence parameters ETR_{max} and E_k were significantly affected by a main effect of CO_2, nutrients, and species based on a multivariate analysis of variance (Tables 2 & 3, Figure 5). Alpha was significantly affected by inorganic nutrients and differed significantly between species. There was also an interactive effect of species and inorganic nutrients in ETR_{max}.Both species showed a decreasing trend with increasing CO_2 under unenriched conditions, while *H. opuntia* was stimulated at all CO_2 conditions other than ambient with nutrient enrichment, and *Dictyota* sp. had significantly higher ETR_{max} and E_k values under elevated CO_2 and nutrient enrichment. On the other hand, *H. opuntia* ETR_{max} values were not strongly affected by nutrient enrichment, but the light saturation points were, as *H. opuntia* grown under nutrient enrichment had higher E_k values than those under unenriched conditions. CO_2 did not have a significant effect on alpha, but under all CO_2 conditions except for ambient, nutrient enriched algae had higher alpha values than unenriched algae.

Table 3. Mean (± SE) chlorophyll flluroescence parameters based on the nonlinear curve fit analysis of the ETR vs. irradiance curves for *H. opuntia* and *Dictyota* sp. exposed to four CO_2 concentrations with (+) and without (-) nutrient enrichement.

Treatment	*H. opuntia*			*Dictyota sp.*		
	ETR_{max}	E_k	alpha	ETR_{max}	E_k	alpha
200-	12.3 ± 0.63	71.0 ± 5.5	0.17 ± 0.01	22.2 ± 5.3	107.4 ± 16.0	0.20 ± 0.03
200+	13.2 ± 3.5	87.6 ± 24.7	0.16 ± 0.01	37.6 ± 10.9	168.8 ± 50.0	0.23 ± 0.04
390-	11.0 ± 1.5	74.5 ± 13.4	0.19 ± 0.02	15.3 ± 3.2	87.5 ± 14.4	0.19 ± 0.05
390+	13.1 ± 1.1	76.3 ± 15	0.19 ± 0.02	26.1 ± 3.7	147.6 ± 13.8	0.18 ± 0.03
700-	9.4 ± 1.2	68.7 ± 4.9	0.14 ± 0.01	14.5 ± 2.3	114.4 ± 10.7	0.13 ± 0.02
700+	15.8 ± 2.7	90.5 ± 22.1	0.20 ± 0.04	32.1 ± 7.4	141.1 ± 17.0	0.22 ± 0.04
900-	8.13 ± 0.93	69.9 ± 2.9	0.12 ± 0.02	8.9 ± 2.5	61.1 ± 9.3	0.14 ± 0.03
900+	11.2 ± 1.8	76.5 ± 5.7	0.14 ± 0.02	25.7 ± 3.7	111.2 ± 19.6	0.24 ± 0.01

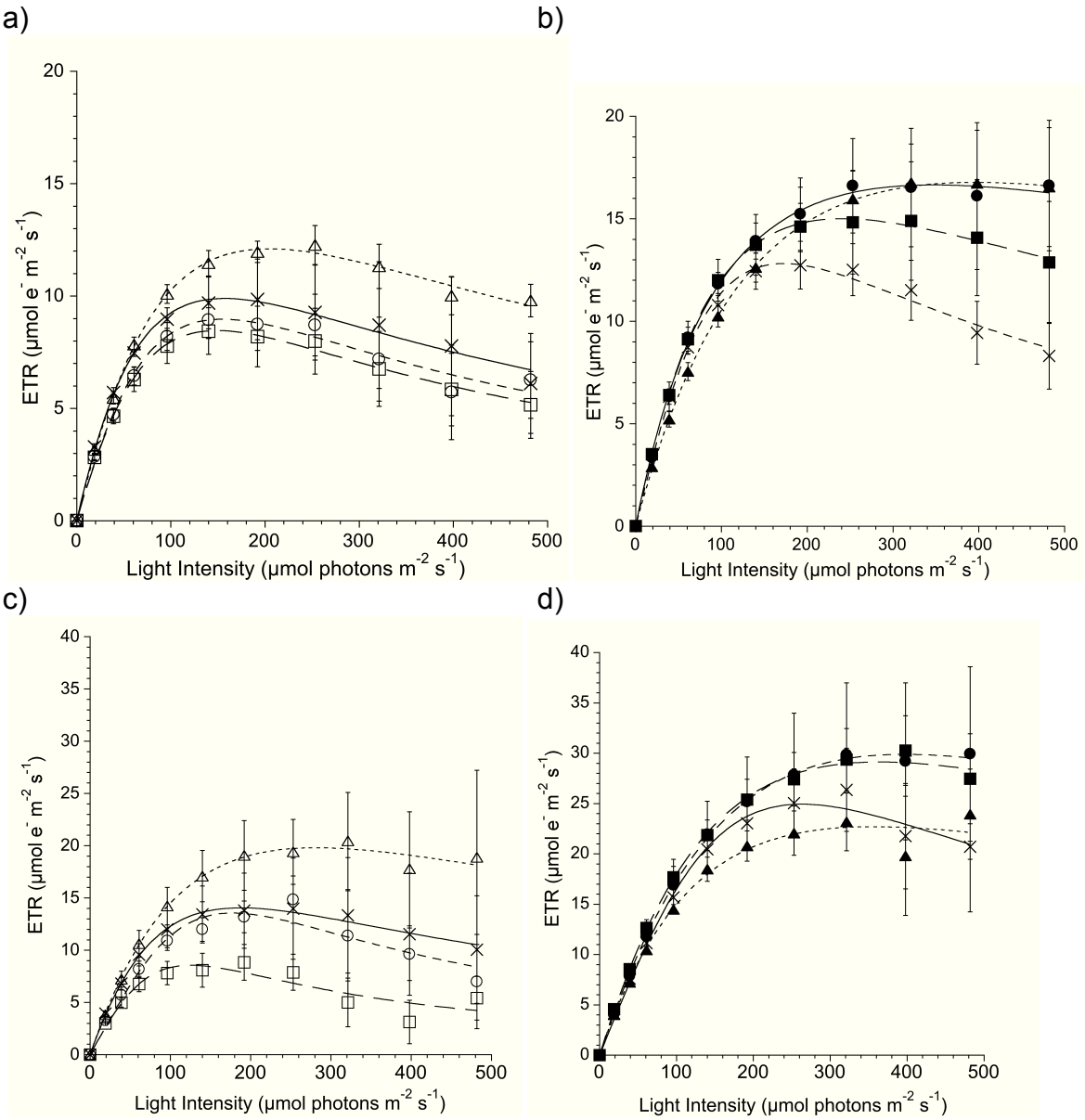


Figure 5. Mean (± SE, N = 5) electron tranpsort rates of *H. opuntia* (a-b) and *Dictyota* sp. (c-d) exposed to the experimental CO_2 treatments under nutrient depleted (a & c) and enriched (b & d) conditions. Trianges: 200, crosses: 390, circles: 700, squares: 900 µatm CO2; open: unenriched, filled: nutrient enriched.

Net photosynthetic rates were affected by a significant interaction between CO_2, inorganic nutrients, and species (Table 2, Figure 6). *Dictyota* sp. had higher net photosynthesis rates than *H. opuntia* under all treatment conditions, and CO_2 had an opposite effect on photosynthesis depending on the nutrient condition. Under unenriched conditions, *Dictyota* sp. had higher photosynthetic rates at 900 µatm CO_2, while it had higher photosynthetic rates under ambient CO_2 conditions when enriched with nitrate and phosphate. Photosynthetic rates of *H. opuntia* were not affected by CO_2, but were higher under inorganic nutrient enrichment than under unenriched conditions.

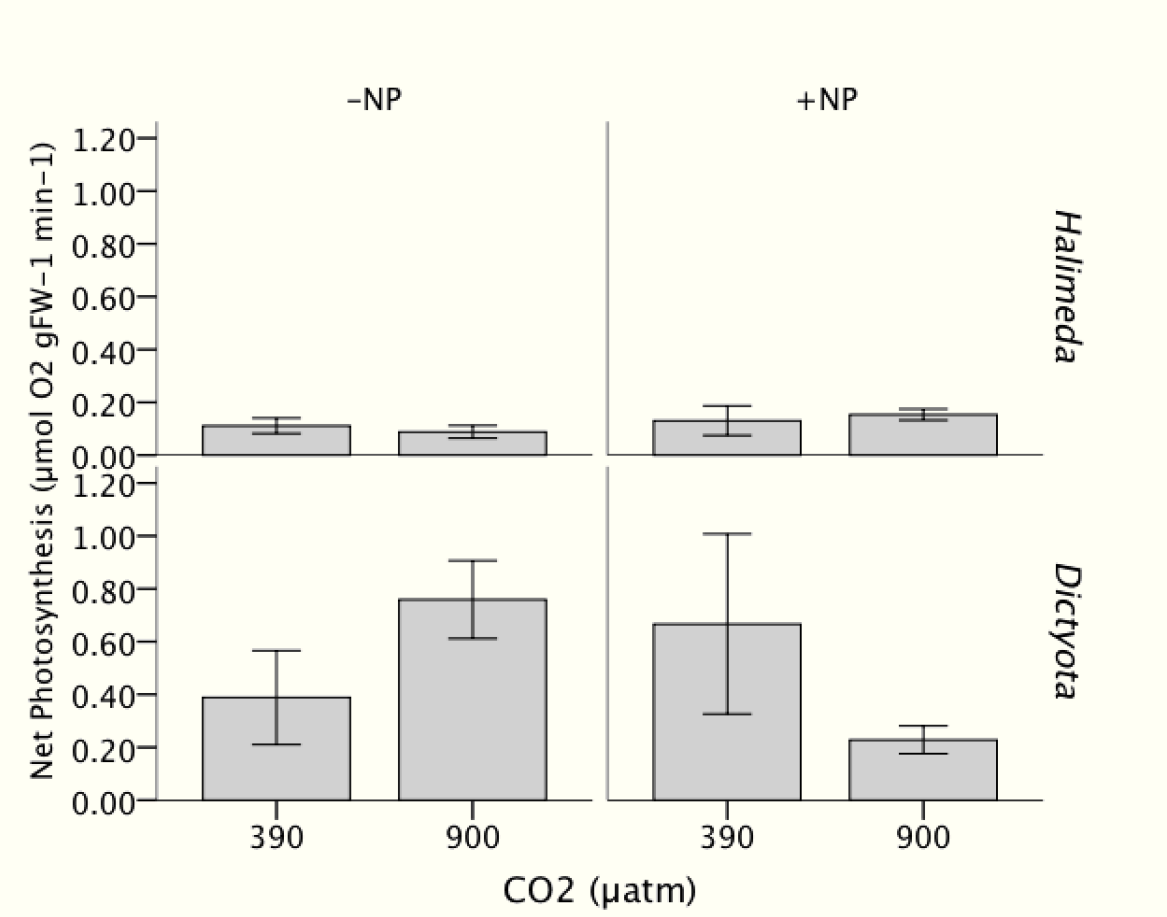
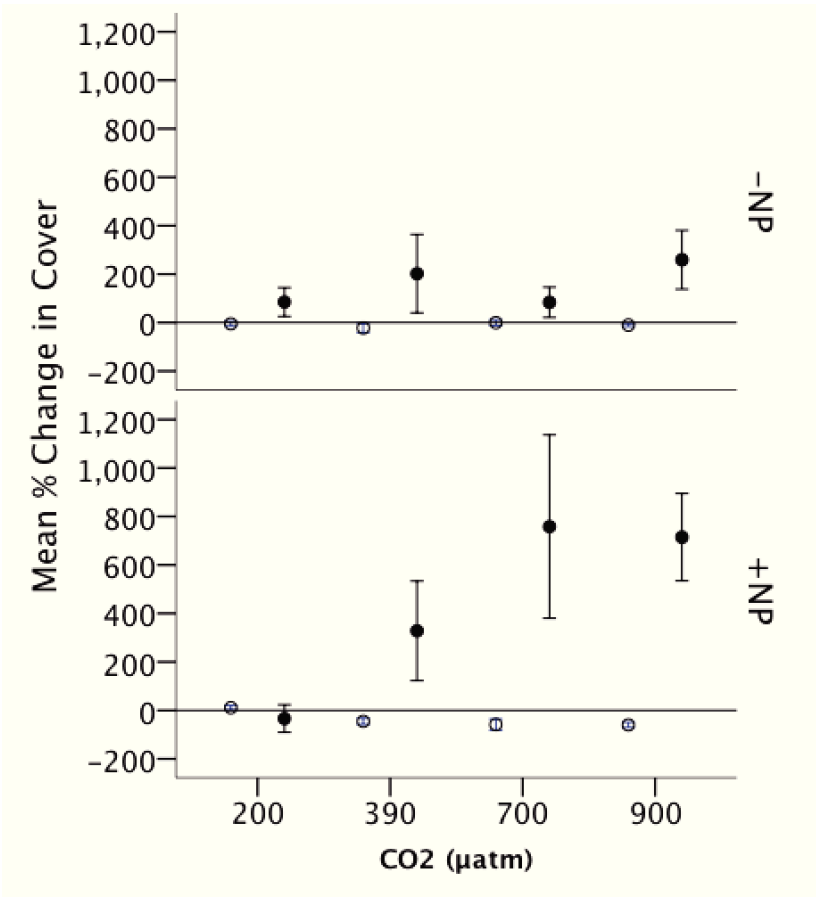


Figure 6. Mean (±SE, N = 4) net photosynthetic rates for *H. opuntia* (top) and *Dictyota* sp. under ambient and 900 µatm CO_2 and two inorganic nutrients conditions (unenriched and enriched).

Community Composition

The percent change in cover from initial to week four of the experiment was significantly different between the two algae, and there was a significant interaction between all independent factors (species, inorganic nutrients, and CO_2; Table 2, Figure 7a). When the algae were enriched with nutrients, *Dictyota* sp. increased cover in all CO_2 treatments except the lowest, while *H. opuntia* showed the exact opposite trend. Under unenriched conditions, the cover of *H. opuntia* did not change with respect to CO_2, while *Dictyota* increased the most in the ambient and highest CO_2 treatments. The ratio of the percentage of the community biomass of *Dictyota* sp. to *H. opuntia* was significantly positively correlated to CO_2 under inorganic nutrient enrichment, but there was no significant effect of CO_2 under unenriched conditions (Spearman's correlation coefficient = 0.809, p (two-tailed) = 9.6E-7, Figure 7b). The highest CO_2 treatments had the highest ratios of *Dictyota*:*Halimeda* biomass under nutrient enriched conditions, while under unenriched conditions, *Dictyota* sp. made up only a small percent of the biomass compared to *H. opuntia* under all CO_2 conditions. Figure 8 shows qualitatively how the *Dictyota* sp. cover increased dramatically under nutrient enriched conditions, and that this effect was stronger under elevated CO_2 compared to ambient CO_2 conditions.

a)



b)

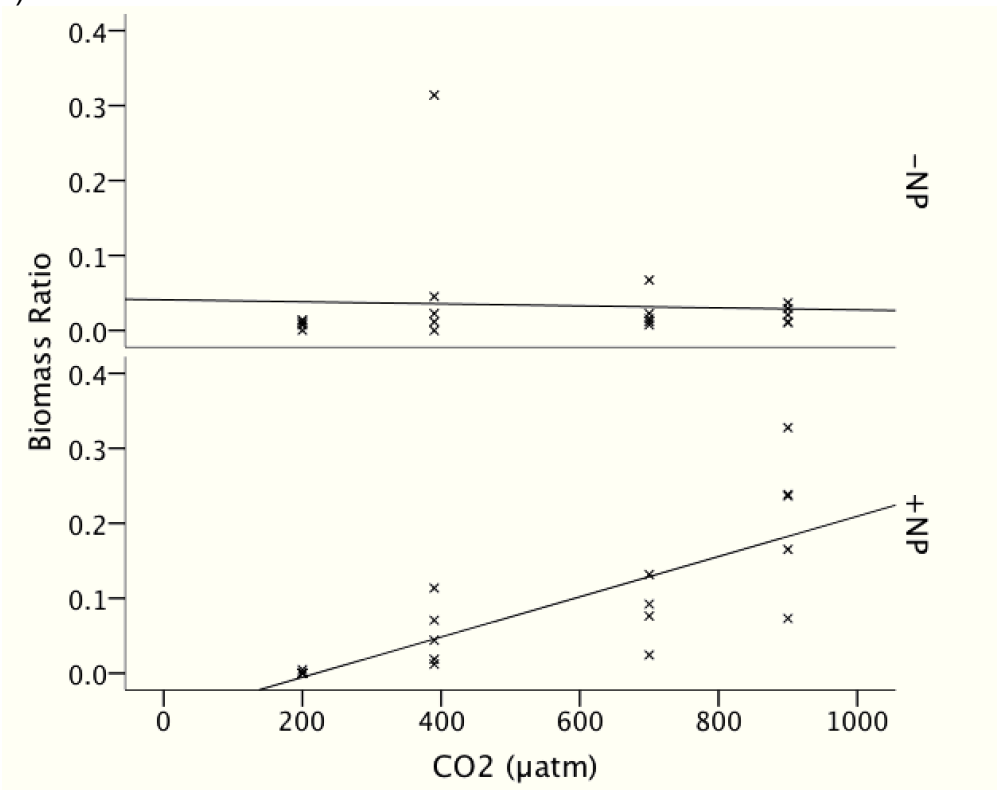


Figure 7 a) Mean (± SE, N = 5) a) percent change in cover after four weeks of exposure to the experimental treatments for *Halimeda opuntia* (open circles) and *Dictyota* sp. (closed circles) at all CO_2 treatments with (top panel) and without

(bottom panel) inorganic nutrient enrichment. b) The percentage of community biomass of *Dictyota* sp. to *H. opuntia* as a function of CO_2 after four weeks.

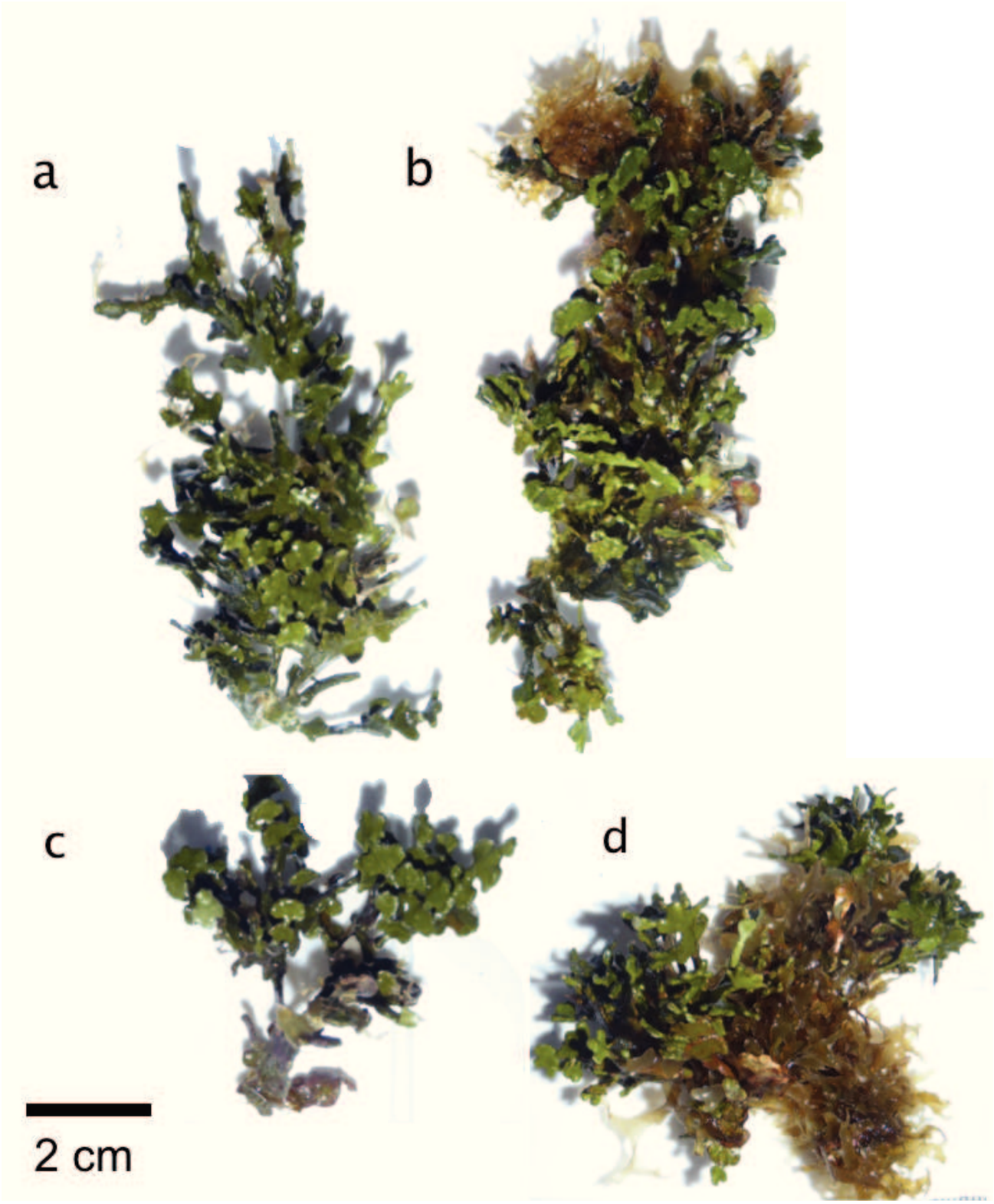


Figure 8. Images of *H. opuntia* after four weeks of exposure to 390 and 900 µatm CO_2 without and with inorganic nutrient enrichment: a) 390-NP b) 390+NP, c) 900-NP, d) 900+NP. The cover of Dictyota in the treatments with nutrient enrichment is much higher than without nutrient enrichment, and is even more pronounced at 900 µatm CO_2 than at ambient CO_2.

Discussion

The interactive effect of increasing CO_2 and inorganic nutrients will be an important factor determining the health and competitive interactions in future tropical macroalgal communities. We have shown that the physiology and competitive interactions between a calcifier (*H. opuntia*) and noncalcifier (*Dictyota* sp.) are affected by both CO_2 and nutrient enrichment. In contrast to previous studies (Price et al. 2001; Sinutok et al. 2011), we observed an increase in maximum ETR and slighty elevated net calcification rates at moderately elevated CO_2 level. Our results suggest that under oligotrophic conditions, CO_2 alone is not greatly stressing *H. opuntia*, and our observed parabolic trend of net calcification rates as a function of CO_2 are complementary to those found by Ries (2009). The differences between our study and previous results (Price et al. 2001; Sinutok et al. 2011) could be due to differences in population responses, or nutrient, light and temperature differences between experiments.

In our study, *Dictyota* was the better competitor under elevated CO_2 and inorganic nutrient enrichment. *Dictyota* sp. had the highest growth rates, ETR_{max}, and increase in percent cover at CO_2 concentrations above ambient when nutrients were high. However, at ambient CO_2, *H. opuntia* growth increased drastically with nutrient enrichment, while *Dictyota* sp. did not. These results indicate that under ambient CO_2, *H. opuntia* was still competitive with *Dictyota*, but the combination of elevated CO_2 and nutrients made *Dictyota* sp. more competitive than *H. opuntia*. The lower growth rates of *H. opuntia* at elevated CO_2 levels were only observed under nutrient enriched conditions when *Dictyota* sp. growth rates were highest. Therefore, our results suggest that reduced *H. opuntia* growth was not a direct result of elevated CO_2, but rather an indirect effect due to the higher growth rate and shading by *Dictyota*. Beach et al. (2003) found that *H. tuna* heavily epiphytized with *Dictyota* sp. had slower growth rates than unepiphytized algae. The authors attributed this effect to shading, but also found that *Dictyota* chemically affected *H. incrassata*, as the alga had higher respiration rates when grown without epiphytes and exposed to *Dictyota*-conditioned water.

The higher growth rate of *Dictyota* sp. under nutrient enrichment at 900 µatm CO_2 was accompanied by a lower net photosynthesis (and higher respiration rates, data

not shown) rate compared to the ambient CO_2 condition, which would explain a decrease in C:N ratios at 900 compared to 700 µatm CO_2 (Appendix). Gordillo et al. (2001) reported a similar phenomenon for *Ulva rigida* cultivated under CO_2 and nitrate enrichment. The authors suggested that photosynthesis was already saturated at the ambient CO_2 level, and therefore the alga reduced its organic carbon release to provide the extra carbon needed to increase growth rates. *Dictyota* sp. produces phlorotannins, which are strongly carbon-based compounds that have been shown to deter herbivory (Hay et al. 1994; Steinberg 1984; 1986; 1988; Targett et al. 1986; Targett & Arnold, 1998; Stachowicz & Hay 1999). Although nitrogen enrichment has been shown to decrease phlorotannin production in brown algae (Arnold et al. 1995), perhaps this relationship changes under moderately elevated CO_2 and nutrients, as phlorotannin production is directly correlated to C:N ratios, and *Dictyota* sp. had the highest C:N ratio at 700 µatm CO_2 plus inorganic nutrients. Swanson & Fox (2007) observed elevated phlorotannin production in kelp grown under elevated CO_2. Retaining more of these secondary metabolites combined with faster growth rates would give *Dictyota* sp. a competitive advantage over grazers under moderately elevated CO_2 and inorganic nutrient conditions. However, whether or not macroalgae – both calcifying and noncalcifying - are changing levels of phlorotannins under elevated CO_2 and nutrients was not measured in our experiment, but it will an important direction for future research, especially because grazers exert strong top-down control on noncalcifying macroalgae in areas with high inorganic nutrient loads (i.e. Littler & Litter 1984; Carpenter 1986; Steneck 1988; Lapointe et al. 1997; Thacker 2001; Belliveau & Paul 2002).

The observed strong influence of inorganic nutrients on the relationship between *Dictyota* sp. and *H. opuntia* is consistent with field observations of these two taxa (Delgado & Lapointe 1994: Lapointe 1997). Delgado & Lapointe (1994) reported that nutrient enrichment enhanced the productivity of felshy macroalgae more than calcareous algae, and predicted that eutrophication could decrease carbonate accretion on tropical coasts. Such changes in copmetitive interactions between corals and fleshy algae are also well documented (Done 1992; Hughes 1994; Miller & Hay 1996; Lapointe 1997; McCook 1999; McCook et al. 2001; Jompa & McCook 2002; Burkepile & Haye 2006). Our results suggest that the addition of CO_2 may

exacerbate the effect of eutrophication on competitive relationships between calcifiers and noncalcifiers.

In conclusion, our results suggest that *H. opuntia* will show mild changes under ocean acidification conditions in areas where inorganic nutrients are low. However, without top-down grazer control, *Dictyota* sp. has a competitive advantage over *H. opuntia* in eutrophied areas, and this effect is amplified at elevated CO_2 concentrations that are likely to occur by the end of this century.

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General Discussion

Differential sensitivity of macroalgae to ocean acidification

The studies carried out during this thesis revealed that there are differences in the physiological responses of calcifying macroalgae to elevated CO_2, but similar patterns of competitive interactions between calcifiers and noncalcifiers occur under elevated CO_2 regardless of species and latitude. The noncalcifying macroalgae investigated in this study generally showed higher photosynthetic rates, growth rates and percent cover under elevated CO_2 than under ambient CO_2 conditions when inorganic nutrients were replete. These positive responses to CO_2 are in agreement with previous studies that have showed stimulated growth and photosynthesis in noncalcifying macroalgae (Gao et al. 1991: 1993; Küber et al 1999; Gordillo et al. 2001; Zou 2005). The stimulation of *C. crispus* photosynthesis, carbohydrate, protein content and percent cover by elevated CO_2 is most likely due to the carbon uptake mechanism in this alga. Although it uses carbonic anhydrase to dehydrate HCO_3^- into CO_2, it does not have an active HCO_3^- uptake mechanism and therefore must rely on diffusive CO_2 entry into the cell (Smith & Bidwell 1989). Therefore, higher CO_2 concentrations benefit this alga by increasing the diffusion gradient outside the cell relative to the inside. *Dictyota* sp. showed similar positive responses to elevated CO_2, but this species is able to use HCO_3^- as a substrate for photosynthesis (Raven & Osmond 1992). Mercado et al. (1998) reported that the CA activity in *Dictyota dichotoma* had a low affinity for organic carbon compared to other macroalgae. If *Dictyota* sp. has a similar low-inorganic carbon affinity CA, then that would explain why this alga was stimulated by elevated CO_2.

While the noncalcifying algae were generally stimulated by elevated CO_2, the calcifying species investigated in this study showed species-specific responses to elevated CO_2. *Corallina officinalis*, a calcifying red alga that deposits high-Mg calcite extracellulary, was the most sensitive species to ocean acidification conditions examined in this thesis. The alga had slower growth rates, photosynthetic rates, and deposited less $CaCO_3$ between the cells under elevated CO_2 conditions. However, its calcification rates were elevated at moderate CO_2 concentrations, while the inorganic carbon content of the skeleton ($CaCO_3$) was negatively correlated to CO_2 concentration. The most obvious question still remaining is why the deposition of $CaCO_3$ decreased despite increased calcification rates. Ries (2011) found that one

species of coralline algae can decrease the Mg/Ca ratio of its skeleton in response to elevated CO_2, most likely as an attempt to decrease the solubility of the skeleton, thereby preventing more rapid dissolution. Recent studies have suggested that increased dissolution rates, rather than decreased calcification rates, are the real threat to calcifying organisms under ocean acidification conditions (Ries 2009; Rodolfo-Metalpa 2011; Roleda et al. 2012). The combination of decreasing skeletal solubility by decreasing the Mg/Ca ratio and speeding up calcification rates may be an attempt by the alga to accomodate for high dissolution that may be occuring under low pH conditions. The elevated carbonic anhydrase activity observed in *C. officinalis* under high CO_2 conditions may also be a physiological response by the alga to maintain a balance between calcification and dissolution. More on the potential importance of carbonic anhydrase in calcification is discussed below.

Changes in skeletal structure as a result of elevated CO_2 have also been reported in *H. opuntia* (Robbins et al. 2009). The aragonite crystals in *H. opuntia* were reportedly thinner and denser under elevated compared to ambient CO_2 conditions. The authors attributed this to a higher turnover rate of the crystals, i.e. crystallization was terminated and initiated more frequently. This response implies that the calcification and dissolution processes were affected by CO_2. I found slightly elevated net calcification rates of *H. opuntia* at 700 µatm CO_2 when the algae were nutrient enriched, which was again complementary to the results reported by Ries (2009), but we found no evidence of increased dissolution rates because net calcification rates in the dark were not affected by CO_2. However, it is possible that the incubation period was not sufficient enough to document such a change.

The photosynthetic responses of the two calcifying species investigated also differed with respect to elevated CO_2. The ETR in both species was stimulated by elevated CO_2, but *Corallina officinalis* photosynthetic oxygen evolution decreased in contrast to *H. opuntia*, whose photosynthetic rates increased at 900 µatm CO_2 relative to ambient conditions. While the latter response only occurred under nutrient enriched conditions, *C. officinalis* was not nutrient limited during the experiment, so the results are comparable. The differences in photosynthetic responses could be related to the efficiency of carbon concentrating mechanisms, and/or light intensity and absorption by the algae. In both species, the carbonic anhydrase activity increased under

elevated CO_2. Assuming that most of this increased activity was directed toward maintaining or increasing calcification rates to offset higher dissolution rates, perhaps photosynthesis in *C. officinalis* suffered by not receiving as much benefit from CA. Another possibility is that because the skeleton of *C. officinalis* had less inorganic carbon under elevated CO_2 conditions, the light scattering properties of the skeleton were affected and therefore also the amount and quality of light that reached the light harvesting complexes. The scattering properties of calcified skeletons have been shown to be very important for light harvesting by the photosymbiont algae in symbiotic corals (Enriquez et al. 2005; Téran et al. 2010). With a less calcified skeleton, perhaps the light harvesting complexes of *C. officinalis* were receiving less light than under normal conditions, and therefore maximum photosynthetic rates were lower. However, this does not explain why *H. opuntia* had higher photosynthetic rates under elevated CO_2, as its CA activity was also stimulated and it skeleton had less inorganic carbon under elevated CO_2. A possible explanation is that the CA enyzme in *H. opuntia* is more efficient, or simply that the higher temperatures allowed for higher activity and therefore better efficiency. General differences in carbon concentrating mechanisms between the two species, including differences in ion transport mechanisms, inorganic carbon substrate, and location of enzymes between the two species, and their response to elevated CO_2 are also possible explanations and were beyond the scope of this work, but should nevertheless be further explored. I also only measured photosynthetic rates of *H. opuntia* at a single light intensity, so the shape and parameters of the photosynthesis-irradiance curve are unknown, including the maximum photosynthetic rates. It's possible that the maximum photosynthetic rate shows a different pattern than the photosynthetic rate at the light intensity we measured.

The role of carbonic anhydrase in macroalgal calcification

The ability of organisms to control the pH and inorganic carbon speciation at the site of calcification is a key to being able to control calcification (Borowitzka 1976b; Ries 2009; Krief et al. 2010; Findlay 2011; Hurd et al. 2011; Rodolfo-Metalpa 2011; McCulloch et al. 2012; Roleda 2012). Organisms that have protective organic layers or tissue covering their calcified skeleton are somewhat protected from dissolution (Ries et al. 2009; Rodolfo-Metalpa 2011), but biological control over the process of

calcificiation will be important for organisms that don't have protective organic layers or tissues. One hypothesis for biological control over calcification in corals is that organisms regulate their cellular proton exchange via proton pumps (Ries 2011; Jokiel 2011a; Jokiel 2011b). However, these hypotheses tend to ignore the importance of enzyme activity in regulating inorganic carbon speciation. Carbonic anhydrase has been shown to play a role in the calcification process of many organisms, particularly corals, by regulating the internal and external cellular speciation of dissolved inorganic carbon (Kingsley and Watabe 1987; Niemer et al. 1994; Al-Horani et al. 2003; Rahman et al. 2007; Tambutté et al. 2007). Both species investigated in this thesis showed increased external CA activity when exposed to elevated CO_2. Although CA is recognized as being important for calcification, it is still not clear exactly if and how it functions during calcification in macroalgae.

Carbonic anhydrase is an important enzyme in algal carbon concentrating mechanisms because it catalyzes the conversion of CO_2 to HCO_3^- and vice versa. As *Halimeda* spp. use respiratory CO_2 in calcification (Borowitzka & Larkum 1976; Lee & Carpenter 2001), they probably use external carbonic anhydrase (eCA) to convert CO_2 to HCO_3^-, which then dissociates to CO_3^{2-} and can be deposited as $CaCO_3$. The protons produced may be exchanged for Ca^{2+} ions via a Ca^{2+}/H^+ exchanger (Tambutté et al 2007; McConnaughey & Falk 1991). On the other hand, coralline algae use HCO_3^- as a substrate for calcification (Digby 1977a; 1977b). In this case, eCA may act as a buffer for removing protons by converting some of the HCO_3^- into CO_2 (Tambutté et al 2007), which can then be used for photosynthesis (Figure 1). Such a process is consistent with the hypothesis that calcification can stimulate photosynthesis in some algae (McConnaughey 1991; McConnaughey & Whelan 1997).

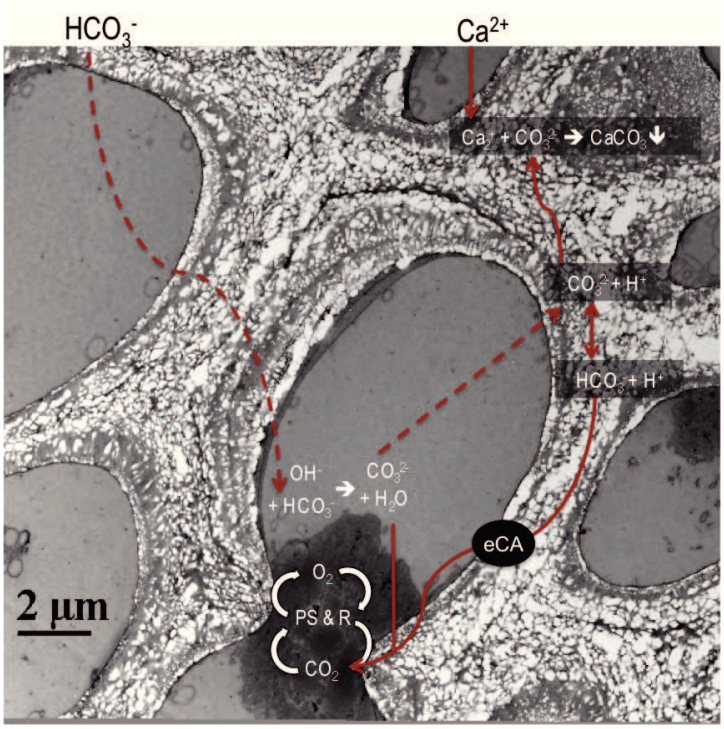

Figure 1. Potential calcification mechanism in *Corallina officinalis* as shown in the introduction, but with the addition of the external carbonic anhydrase enzyme shown as a catalyst for the production of CO_2 from HCO_3^- which is then used for photosynthesis.

The effect of ocean acidification on nutrient assimilation

The dynamics of the carbon/nitrogen balance in macroalgae is complex. Noncalcifying macroalgae have shown a variety of responses to elevated CO_2 with respect to nutrient uptake and assimilation. Some species show increases in protein concentrations and nutrient uptake, while others only show increases in carbohydrate content and even decreases in protein (Gao et al. 1991: 1993; García-Sánchez et al. 1994; Andria et al. 1999; Mercado et al. 1999; Gordillo et al. 2001; Zou et al. 2005; Suárez-Álvarez et al. 2011). There is therefore no typical response of macroalgae to CO_2 with respect to nutrient uptake and assimilation. Of course the internal nutrient status of cells is highly dependent on external inorganic nutrient conditions, and therefore there are synergistic effects of these two abiotic factors on the nutrient status of the cell, which is discussed below. Here I will focus only on the direct effect of CO_2 on nutrient uptake and assimilation.

In *C. officinalis*, phosphate and nitrate uptake rates decreased as a function of increasing CO_2 concentration. In contrast, nitrate reductase activity was higher in

elevated CO_2 treated algae than in algae grown under ambient conditions, but it was highest in the medium CO_2 treatment (665 µatm CO_2). As nitrate reductase is the enzyme responsible for reducing nitrate to nitrite, which is then further reduced to ammonium, it seems counter-intuitive that the enzyme responsible for nitrate assimilation increased in activity while nitrate uptake rates declined. Gordillo et al. (2001) and Mercado et al. (1999) also observed CO_2-stimulated nitrate reductase activity in *Ulva rigida* and *Porphyra leucosticta*, respectively, despite lower photosynthetic rates and lower soluble protein content in *U. rigida* and growth inhibition in *P. leucosticta*. Gordillo et al. (2001) attributed the stimulation of NRA to a direct effect of elevated CO_2 on the synthesis of the enzyme, rather than an indirect effect of the metabolic response. Magnusson et al. (1996) also reported inhibition of nitrate uptake in *Ulva lactuca* under elevated CO_2, and speculated that glutamine synthase may have been inhibited. They also suggested that reduction of nitrate was not inhibited, but rather amination was reduced, which is consistent with our results showing increases in nitrate reductase activity. The metabolic response of *U. lactuca* was attributed to CO_2 inhibition of the carbon concentrating mechanisms, either via low internal pH or high CO_2-feedback inhibition of a process related to the amination process. The complexity of macroalgal responses to elevated CO_2 with respect to inorganic carbon and nitrogen uptake and assimilation illustrates that the overall response of macroalgae to CO_2 is difficult to understand, much less predict, and much more research is needed to determine how these metabolic processes are regulated by CO_2.

Synergistic effects

As stated above, the changes in metabolic processes in macroalgae in response to elevated CO_2 are difficult to understand, and part of the reason is due to the tight coupling of carbon and nitrogen metabolism, which are of course both affected by CO_2 and inorganic nutrients. Although CO_2 alone affects the carbon and nitrogen metabolism of macroalgae, the response is also dependent on inorganic nutrient history and availability. In *H. opuntia*, we observed strong synergistic effects of CO_2 and inorganic nutrients on almost all response variables measured. Particularly strongly affected was the activity of the enzymes carbonic anhydrase and nitrate reductase, as well as growth and photosynthesis. In general, inorganic nutrient

enrichment stimulated algal growth and photosynthesis, and its combination with CO_2 resulted in an amplified increase in these parameters, with the exception of growth in *H. opuntia*, which was inhibited by overgrowth of *Dictyota* sp. under elevated CO_2 and inorganic nutrients combined. Activity of the enzymes investigated showed quite unexpected results, especially for *H. opuntia*, as nitrate reductase was inhibited by CO_2 alone, but was stimulated by the combination of CO_2 and inorganic nutrients in comparison to algae grown under control conditions. Carbon dioxide is therefore affecting nutrient assimilation, but exaclty how it is affected depends on the concentration and types (nitrate, ammonium and/or phosphate) of inorganic nutrients available.

Inorganic nutrient availability is an important factor to consider when investigating macroalgae metabolic responses to elevated CO_2. However, there are also several additional abiotic factors that could be important players in our journey to understanding and predicting the responses of individual species and communities to future environmental conditions. For example, rising temperature due to global warming is a large concern and has received a lot of attention in recent decades with respect to marine biological research, especially in coral reef environments (Reynaud et al. 2003; McNeil et al. 2004; Langdon & Atkinson 2005; Silverman et al 2007; Rodolfo-Metalpa et al 2010; 2011). Particularly relevant to this thesis are the results reported by Sinutok et al. (2011) who found that elevated temperature amplified the negative effects of CO_2 on *H. opuntia*. Among non-calcifying species, Magnusson et al. (1996) reported sensitivity of nitrate metabolism in *U. lactuca* to temperature, as the inhibitory effect of CO_2 to nitrate uptake depended on temperature. Therefore energy balance and metabolism in macroalgae will depend on both factors. They also investigated the responses of algae that had been cultured under different light conditions, and again found different responses.

Light is another important factor to consider when investigating macroalgal physiology – both quantity and quality. Gao & Zheng (2009) found that UV exposure amplified the negative effects of CO_2 on growth, photosynthesis and calcification in the coralline alga *C. sessilis*. Because eutrophication and high suspended sediment loads often occur together due to land runoff, and ocean acidification can alter the influence of light on macroalgal communities (Russel et al. 2011), these factors are

also likely to be interacting in marine environments in ways which are difficult to manipulate in the lab. It is therefore important to mention here that the work done in this thesis was focused on identifying individual species responses under controled conditions, and then expanding experimental work to include communities in order to simulate field conditions, such as with the mesocosms that were used on Sylt. However, such controlled conditions can never exactly replicate field conditions. One important factor in mesocosm experiments that is usually lacking is tidal amplitude and simulated wave action. Without these factors, which are extremely dynamic in intertidal and reef communities, observed results may not represent the actual response of communities in the field. For example, Hurd et al. (2011) reported that wave exposure is an important factor to consider in ocean acidification research because of the effect it has on the boundary layer between the seawater and the surface of an algal thallus. As boundary layer thickness dictates the diffusion rate and concentration gradients of molecules at the cell surface, and can affect the ability of organisms to biologically control the conditions of their boundary layer, changes in this element could have profound influences on the response of macroalgae to ocean acidification. It could also have implications for different responses between temperate and tropical macroalgal communities, as the tidal amplitude and wave action is often more extreme in temperate rocky intertidal environments than in tropical reefs or lagoons. Therefore more field-manipulated experimental work must be done in order to better predict community responses to elevated CO_2 and other changing environmental factors. Nevertheless, the experimental work form this thesis produced interesting results with respect to competitive interactions between macroalgae and overall community responses to elevated CO_2, which I discuss below.

The effect of ocean acidification on macroalgal communities

In the studies conducted for this thesis, both a temperate intertidal a tropical lagoon community showed similar responses to ocean acidifcation with respect to the relationship between calcifiers and noncalcifiers. In general, the calcifying species were overgrown and eventually shaded by the noncalcifying species when CO_2 concentrations were elevated. The implications of these results are that calcifying algae could suffer under future ocean conditions if CO_2 levels continue to rise due to

overgrowth and outcompetition by noncalcifying species. Our results are consistent with other mesocosm and field experiments that have reported outcompetition of calcifiers by noncalcifiers under manipulated conditions, or the lack of calcifying species at vent sites were CO_2 levels are naturally high (Kuffner et al. 2006; 2008; Hall-Spencer et al. 2008; Jokiel et al. 2008; Diaz-Pulido et al. 2011; Porzio et al. 2011). The addition of inorganic nutrients amplified this affect in the tropical community of *H. opuntia* and *Dictyota* sp., as *Dictyota* sp. growth and cover was highest under elevated CO_2 and inorganic nutrients, while the opposite was true for *H. opuntia*. Therefore, calcifiers may be especially at risk in eutrophied environments.

Although the community response to CO_2 was dramatic, the absence of grazers in these studies is a factor that must be recognized. Grazers exert strong control over noncalcifying macroalgae cover in temperate and tropical macroalgal communities (Leighton 1966; Lubchenco 1978; Steneck 1988; Bell 1991; Tegner & Dayton 1991; Geertz-Hansen et al 1993; Belliveau & Paul 2002; Burkepile & Hay 2006; Masterson et al. 2008). Therefore, it is still to be determined if grazers would control the overgrowth of noncalcifying algae with respect to the calcifying algae under elevated CO_2 conditions. Changes in the nutritional content and palatability of the algae will also be important for grazers. The inorganic C:N ratios of *H. opuntia* and *Dictyota* sp. were higher under elevated CO_2, but this response depended on nutrient availability. Under nutrient enriched conditions, *Dictyota* had higher C:N ratios at elevated CO_2 concentrations, but *H. opuntia* did not. Therefore, the nutritional status of the algae with respect to grazers will depend on both CO_2 and nutrient availability. Futhermore, the palatability of calcifying algae may increase due to lower skeletal $CaCO_3$, which could result in higher grazing rates. On one hand, grazers could control the overgrowth of noncalcifying algae with respect to calcifiers, but if the calcifiers become more palatable and are more heavily grazed, then the shift from calcifiers to noncalcifiers would be even more dramatic. Finally, another factor that could affect grazing behavior on macroalgae is the production of herbivore-deterrent secondary metabolites by macroalgae. *Halimeda opuntia* produces the diterpenoids halimedatrial and halimedatetraacetate whose volatility is triggered by grazing damage or thallus injury (Paul & Fenical 1983; 1984; 1986; Paul & Van Alstyne 1988; 1992) and *Dictyota* produces a variety of diterpenes that have been shown to deter grazing (Steinberg & Paul 1990; Pereira et al. 2000; Vallim et al. 2005 for review;

Paula et al. 2011). Whether or not production of these compounds changes with CO_2 and inorganic nutrient availability is also still to be determined. All of these unknowns are areas that should be explored in future research concerning ocean acidification, eutrophication, and macroalgae-macroalgae and macroalgae-grazer interactions under changing environmental conditions.

Seasonal differences between temperate and tropical environments could determine how macroalgal communities will respond to future CO_2 conditions. Seasonal fluctuations in temperature, light, and nutrient availability are much stronger in temperate environments than in tropical environments. I found a seasonal effect of temperature and nutrient availbility on enzyme activity in *C. officinalis* during the seasonal fluctuations between spring and summer. However, the differences between winter and summer are even greater, and it is still to be determined how macroalgal communities will respond depending on seasonality. In temperate and arctic waters, the saturation state of $CaCO_3$ is naturally lower in winter than in summer due to the ability of cold water to absorb more CO_2 than warm water (Feely et al. 2004; Orr et al. 2005). Therefore, lower $CaCO_3$ saturation states combined with low temperature and light limitation in winter could be more stressful for calcifying algae than summer conditions (Tyrell et al. 2008). On the other hand, tropical macroalgal communities have less dramatic seasonal fluctuations, which may allow them to better cope with ocean acidification than temperate communities. Nevertheless, under eutrophied conditions, tropical macroalgal communities will most likely exhibit a shift in dominance from calcifiers to noncalcifiers under future CO_2 concentrations.

Conclusions

This thesis has demonstrated that calcifying macroalgae are generally more sensitive to ocean acidification than noncalcifying macroalgae. Furthermore, many physiological processes, including photosynthesis, growth and nutrient uptake and assimilation are affected by elevated CO_2 in both calcifying and noncalcifying algae. Therefore, the consequences of CO_2 on these organisms are complex, and could have important implications for grazers and overall nutrient cycles due to changes in stochiometric composition of the algae. The interaction of seasonal changes with

elevated CO_2 futher complicates the response of macroalgae to ocean acidification, but it is still to be determined how temperate macroalgae will respond under winter conditions. In addition to the physiological responses of macroalgae to elevated CO_2, I observed significant changes in macroalgal communities, in which noncalcifying algae overgrew and outcompeted their calcifying counterparts. In situations where inorganic nutrients were in excess, this trend was amplified in a tropical community. I therefore conclude that ocean acidification is a relevant threat to macroalgal communities, in both temperate and tropical environments, and that eutrophication will amplify the negative effects of elevated CO_2 on these communities.

Outlook

As mentioned above, there are still many questions to be answered when it comes to investigating the performance and competition of macroalgae under elevated carbon dioxde concentrations that are expected to occur by the end of the century. The combined effects of elevated CO_2 with increasing temperature, changes in light intensity and quality, tidal regimes, and inorganic nutrients will be important topics to investigate. Combining controlled laboratory experiments with manipulated field experiments will also be an important step to increase our understanding how these communities will respond to their changing environment. Furthermore, the studies conducted for this thesis focused only on adult stages of the life history of these organisms. Investigating the reproductive success, recruitment and survival of macroalgae under changing abiotic conditions is also crucial to understanding how they will fare under future marine conditions. Finally, their adaptive capabilities must be investigated, in order to determine if the responses of individuals are representative of future responses, or if acclimation of individuals will eventually allow for adaptation of species to cope with their rapidly changing environment. The balance between the rate of adaptation and the rate of environmental change is an important factor that will control whether or not adaptation will occur.

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Appendix

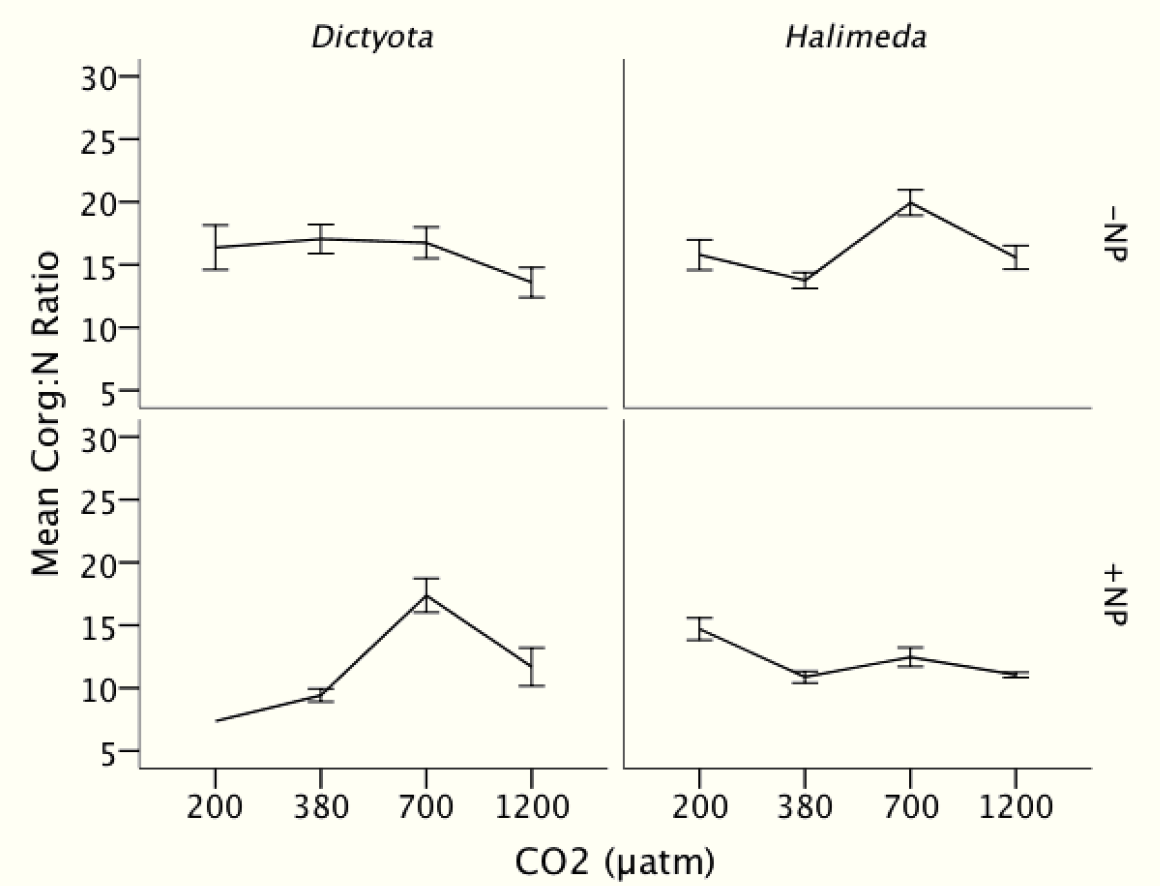

Figure 1. Organic carbon to nitrogen ratios for *H. opuntia* and *Dictyota* sp. after four weeks of exposure to the experimental CO_2 treatments without (top panels) and with (bottom panels) inorganic nutrient enrichment. These data were not included in the thesis manuscripts because they are still preliminary as some measurements from the *Dictyota* samples must be reanalyzed.

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Anschrift

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ERKLÄRUNG

Hiermit erkläre ich, dass ich die Doktorarbeit mit dem Titel

Macroalgal performance and competition under elevated CO_2

selbstständig verfasst und geschrieben habe und außer den angegebenen Quellen keine weiteren Hilfsmittel verwendet habe.

Ebenfalls erkläre ich hiermit, dass es sich bei den von mir abgegebenen Arbeiten um drei identische Exemplare handelt.

……
Unterschrift