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Variable responses of temperate calcified and fleshy macroalgae to elevated $p\text{CO}_2$ and warming

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Anthropogenic carbon dioxide (CO_2) emissions simultaneously increase ocean temperatures and reduce ocean surface pH, a process termed ocean acidification (OA). OA is expected to negatively affect the growth and physiology of many calcified organisms, but the response of non-calcified (fleshy) organisms is less well understood. Rising temperatures and $p\text{CO}_2$ can enhance photosynthetic rates (within tolerance limits). Therefore, warming may interact with OA to alter biological responses of macroalgae in complicated ways. Beyond thresholds of physiological tolerance, however, rising temperatures could further exacerbate negative responses to OA. Many studies have investigated the effects of OA or warming independently of each other, but few studies have quantified the interactive effects of OA and warming on marine organisms. We conducted four short-term independent factorial CO_2 enrichment and warming experiments on six common species of calcified and fleshy macroalgae from southern California to investigate the independent and interactive effects of CO_2 and warming on growth, carbonic anhydrase (CA) enzyme activity, pigment concentrations, and photosynthetic efficiency. There was no effect of elevated $p\text{CO}_2$ on CA activity, pigment concentration, and photosynthetic efficiency in the macroalgal species studies. However, we found that calcareous algae suffered reduced growth rates under high $p\text{CO}_2$ conditions alone, although the magnitude of the effect varied by species. Fleshy algae had mixed responses of growth rates to high $p\text{CO}_2$, indicating that the effects of $p\text{CO}_2$ enrichment are inconsistent across species. The combined effects of elevated $p\text{CO}_2$ and warming had a significantly negative impact on growth for both fleshy and calcareous algae; calcareous algae experienced five times more weight loss than specimens in ambient control conditions and fleshy growth was reduced by 76%. Our results demonstrate the need to study the interactive effects of multiple stressors associated with global change on marine communities.

Keywords: carbon-concentrating mechanisms, carbon dioxide, carbonic anhydrase, global warming, multiple stressors, photosynthesis, seawater pH.

Introduction

The continued release of anthropogenic carbon dioxide (CO_2) from the burning of fossil fuels is a major driver of climate change, and is predicted to result in an increase in sea surface temperature and decreased pH in open ocean surface waters (IPCC, 2013). The oceanic uptake or absorption of anthropogenic CO_2 has already resulted in a drop of 0.1 pH units (and, consequently, a 16% decrease in aragonite and calcite saturation state) in open ocean surface water (Caldeira and Wickett, 2003; Feely *et al.*, 2012), a process termed ocean acidification (OA). Continued absorption of CO_2 by the

ocean is predicted to further reduce open ocean surface pH by 0.06–0.32 units by the end of the 21st century, through associated changes in carbonate chemistry, whereas atmospheric warming is predicted to result in an increase in sea surface temperature by 0.6–2.0°C (IPCC, 2013). Both OA and warming have the potential to negatively impact organisms' success and survival. Many OA studies to date have tested the effects of increased CO_2 in isolation (often with constant and stable carbonate chemistry parameters), reporting negative effects on survival, growth, calcification, and reproduction for many marine organisms (Hendriks *et al.*, 2010;

Kroeker *et al.*, 2010, 2013; McCoy and Kamenos, 2015). However, these studies have not necessarily been representative of the environmental conditions organisms experience *in situ*. Recent evidence indicates that natural variation in the carbonate system near shore is much more complex and difficult to predict than in the open ocean (Hoffmann *et al.*, 2011; Frieder *et al.*, 2012); this variation has only recently been incorporated into experimental designs (Cornwall *et al.*, 2013). Additionally, studies thus far predominantly test only a single stressor, and are therefore unable to capture synergistic effects of warming and elevated CO₂ [but see Anthony *et al.* (2008), Martin and Gattuso (2009), Byrne (2011), Diaz-Pulido *et al.* (2012), Johnson and Carpenter (2012), and Williams *et al.* (2014)], which may exacerbate or mitigate the effects of changing carbonate chemistry on species' performances. We addressed these issues by investigating the combined effects of increased CO₂ and warming on ecologically important temperate, fleshy, and calcified macroalgae.

Because of the necessary and diverse ecosystem services that different species of macroalgae provide, such as food, habitat, refugia, and settlement cues for invertebrate larvae, it is vital to understand their physiological tolerances and responses to environmental stressors (i.e. warming and OA), which in turn will determine population abundance and distribution. Calcified and fleshy algae may exhibit opposing responses to OA due to different physiological demands for particular carbon species. CO₂ enrichment has been shown to negatively affect the growth, calcification, and reproductive rates of at least some species of calcareous algae, as well as their physiological performance and competitive abilities (Gao *et al.*, 1993; Kleypas and Langdon, 2006; Diaz-Pulido *et al.*, 2007; Anthony *et al.*, 2008; Fabry *et al.*, 2008; Kuffner *et al.*, 2008; Martin and Gattuso, 2009; Price *et al.*, 2011; Johnson *et al.*, 2014a; Williams *et al.*, 2014). Fleshy macroalgae, in contrast, exhibit more variable responses to increased CO₂ (Beardall *et al.*, 1998; Cornwall *et al.*, 2012; Johnson *et al.*, 2014a), yet the physiological mechanisms underlying these responses are still largely unknown.

The lack of consistent responses among fleshy algae to CO₂ enriched seawater could be due, in part, to the capacity of different taxa to utilize different species of dissolved inorganic carbon, such as bicarbonate (HCO₃⁻), for photosynthesis. Macroalgae are able to uptake CO₂ directly via passive diffusion, but many species are unable to achieve maximum rates of photosynthesis using only CO₂ (Kübler *et al.*, 1999; Morison *et al.*, 2005). CO₂ is the least abundant species of inorganic carbon in seawater, it is highly diffusive, and it is thus difficult to concentrate at the site of photosynthesis. Some algae have evolved the use of carbon-concentrating mechanisms (CCMs), such as the enzyme carbonic anhydrase (CA), to convert bicarbonate (more easily concentrated in cellular tissue) to CO₂ (Sültemeyer, 1998). CA activity can be used as a proxy to assess whether marine algae employ internal and/or external CA as a CCM for photosynthesis. CA is energetically costly (Hepburn *et al.*, 2011), but pelagic phytoplankton have been shown to downregulate CA production under CO₂ enrichment because dissolved CO₂ is no longer limiting (Hopkinson *et al.*, 2011). Benthic algae may respond similarly, but this hypothesis has been tested on only a limited number of species (García-Sánchez *et al.*, 1994; Hofmann *et al.*, 2012a). Macroalgae that are able to downregulate CA activity may then be able to divert further energy into somatic or reproductive growth and/or photosynthetic machinery such as photosynthetic pigments.

In addition to CO₂, temperature-adaptive physiological variation and ecological interactions are instrumental in structuring

the biogeography and distribution of many marine species (Stillman, 2002; Compton *et al.*, 2007). Increases in temperature elevate the metabolic rates of many organisms living well within optimal temperature envelopes. In primary producers, photosynthetic rates gradually increase with temperature until an optimum is reached; if temperatures continue to rise and exceed thermal tolerance limits, photosynthetic rates will decline precipitously (Davison, 1991). Photosynthetic rates increase through the production of more photosynthetic pigments, or by increasing photosynthetic efficiency (Schreiber, 2004). Therefore, overall growth, productivity, and pigmentation of macroalgae are expected to be positively affected by temperature increases (Pereira *et al.*, 2006), within an optimal temperature envelope.

While the impacts of elevated CO₂ and increased temperatures have been investigated individually, little is known about the potential interaction between CO₂ and rising ocean temperatures on the physiology of marine algae. Due to the potential positive effects of warming on photosynthesis, it has been suggested that increased temperatures may allow organisms to compensate for the negative effects of OA. However, the limited research available suggests these two stressors result in synergistic antagonistic impacts on calcifiers, such that warming exacerbates the effect of CO₂ enrichment (Anthony *et al.*, 2008; Gao *et al.*, 2012; Diaz-Pulido *et al.*, 2012). Relatively few multistressor studies exist to date for fleshy species [but see Connell and Russell, (2009)], but we predict that increased CO₂ and temperature would have a synergistic positive effect because both CO₂ and temperature have the potential to increase photosynthetic rates.

The response of organisms to environmental stressors, such as OA and warming, may be influenced by the extent of habitat specialization. Some species are ecological generalists and have wider tolerances for fluctuating temperature and/or *p*CO₂, and may be better equipped to tolerate and/or acclimate, and ultimately to survive, future global change (Zerebecki and Sorte, 2011). Most OA experiments have been conducted in recirculating or flow-through aquaria experiencing constant ambient control or elevated CO₂ conditions. However, recent studies have documented that pH and *p*CO₂ in the natural environment are highly dynamic, especially in shallow coastal systems. The variability in *p*CO₂ that exists over a diel or tidal cycle or during an upwelling event includes conditions equivalent to or surpassing those expected to occur by the turn of the century under OA (Hoffmann *et al.*, 2011). Therefore, some taxa, such as ecological generalists, may already have the potential to acclimate and cope with changing ocean chemistry. For example, Johnson *et al.* (2014b) found that *Porolithon onkodes*, a crustose coralline alga collected from a habitat experiencing more variable *p*CO₂, was able to calcify 42% more than individuals from habitats experiencing more constant conditions. This disparity indicates that organisms already existing in dynamic *p*CO₂ habitats may be acclimated to future OA. Given this variability in environmental conditions and species' response, it must be emphasized to capture natural variation in temperature and *p*CO₂ in experiments to enable more realistic predictions of ecosystem-level responses in the future.

In this study, we assessed the responses of six functionally different, common southern California macroalgae to increased *p*CO₂ and warming, by elevating temperature and *p*CO₂ above the existing natural variability of local coastal conditions. The species chosen were representative of potential varied vulnerabilities to OA and/or warming based on ecological habitat specialization, species origin (native vs. invasive species), and functional morphology

(calcified vs. fleshy, and articulated vs. encrusting). We measured several biological responses including growth rate, calcification rate, photosynthetic efficiency, pigment concentration, and CA activity. Two types of experiments were conducted: single manipulation (elevated $p\text{CO}_2$ only) and factorial design (elevated $p\text{CO}_2$ crossed with elevated temperature). In the $p\text{CO}_2$ -only experiments, we hypothesized that calcareous algae would experience decreased growth and calcification rates under naturally fluctuating, high $p\text{CO}_2$ conditions. If the fleshy algae downregulated CA activity, we predicted that they would be positively affected (increased growth, pigmentation, and photosynthetic efficiency) by the $p\text{CO}_2$ treatment. However, if CA enzyme activity was not downregulated, or was not present, we predicted that fleshy algae would not be affected by elevated $p\text{CO}_2$. We also predicted that the invasive species, or ecological generalist, would respond more positively to $p\text{CO}_2$ than the native counterpart. Calcified articulated species were predicted to respond more negatively to increased $p\text{CO}_2$ than encrusting species, due to higher surface area exposure to reduced carbonate saturation state seawater. In the factorial experiments, we expected all algae to have increased growth, production, photosynthetic efficiency, and higher pigment content under the warming treatment. We predicted that only the fleshy algae would have a synergistic positive effect with the combination of high $p\text{CO}_2$ and temperature, and that calcified algae would have mixed responses.

Material and methods

Study species and study system

All experiments were conducted in an experimental flow-through seawater system at the Scripps Institution of Oceanography (SIO) in La Jolla, California, from July 2012 to March 2013. Six commonly occurring calcified and fleshy macroalgae were chosen for four independent elevated $p\text{CO}_2$ or elevated $p\text{CO}_2 \times$ warming experiments that ranged in length from 17 to 31 d (Table 1). Two elevated $p\text{CO}_2$ experiments were conducted with paired species of algae comparing the effects of increased CO_2 on (i) native and non-native brown fleshy algae (*Dictyopteris undulata* and *Sargassum horneri*) and (ii) articulated and encrusting calcareous red algae (*Jania adhaerens* and *Lithothamnion californicum*). Elevated $p\text{CO}_2$ and warming experiments examined responses of single algal species to multiple stressors, including the fleshy red alga, *Plocamium cartilagineum*, and the calcified articulated red alga, *Corallina vancouveriensis*. Specimens were collected subtidally by snorkel (within 12 km of SIO) or from the low intertidal zone (<3.2 km from SIO), and were acclimated to laboratory conditions for a least 1 week in aerated, flow-through tanks. Water was sourced from the SIO pier, ~300 m

offshore at 3–4 m depth. Algal epiphytes were removed by hand using tweezers and specimens were dipped in insecticide (Garden Tech *Sevin* Concentrate Bug Killer: 4 l seawater: 20 ml *Sevin*) 2 d before the initiation of the experiment to remove herbivorous invertebrates that were found to have significant effects on biomass in preliminary trials. Carpenter (1986) showed that *Sevin* has no negative effects on algal biomass or productivity. Algal specimens were loosely attached to mesh stands and each placed in 1 l experimental aquaria.

Experimental conditions

The two single-manipulation $p\text{CO}_2$ experiments were conducted, with two levels of $p\text{CO}_2$ targeted at 400 and 900 μatm , with $n = 10$ replicates per treatment and species. For the two combined $p\text{CO}_2$ and warming experiments, factorial manipulations of $p\text{CO}_2$ and temperature were used, with target values for temperature (ambient = 15–17°C and elevated = +2°C above ambient) and $p\text{CO}_2$ at 400 and 900 μatm , with $n = 10$ replicates per treatment (Figure 1). All treatment levels were selected based on the IPCC (2013) Representative Concentration Pathway (RCP) 8.5 for conditions projected in 2100.

Aquaria were maintained under four full-spectrum 54 W Giesemann T-5 fluorescent bulbs (Supplementary Figure S1). The lights mimicked sunrise and sunset by gradually increasing or decreasing light levels over the course of an hour and were set to 10 h of daylight for experiments conducted in winter and 14 h of daylight in experiments conducted in summer (Table 1). Ambient and treatment aquaria alternated positions along the bench and their locations were rotated weekly to prevent lighting dissipation effects.

Each aquarium contained an individual algal specimen and was continuously supplied with flow-through filtered seawater (0.25 l filtered seawater min^{-1}). Constant seawater flow rates within each aquarium were maintained using Rain Bird pressure compensator modules placed within Rain Bird Xeri-Bird 8-Outlet Manifolds. This flow-through design prevented nutrient limitation or total alkalinity draw-down due to calcification in the long-term experimental setting, and allowed for natural temporal pH variability and high replication per treatment level.

$p\text{CO}_2$ treatments

Mass flow controllers (Omega FMA 5400/5500) were used to blend ambient air with pure CO_2 before bubbling gases into each aquarium to create experimental conditions. Filtered ambient air, originating in a non-oil-based Ingersoll air compressor, was sent

Table 1. Details of each of the four experiments performed in this study (two elevated $p\text{CO}_2$ only and two elevated $p\text{CO}_2$ and warming experiments on southern CA macroalgae).

Exp #	Experiment type	Dates	# Days	Species 1	Cal/ non-cal	Native/ invasive	Species 2	Cal/ non-cal	Native/ invasive	Collection depth
1	OA	3 July 2012–19 July 2012	17	<i>Dictyopteris undulata</i>	Non-cal	Native	<i>Sargassum horneri</i>	Non-cal	Invasive	2–3 meters
2	OA	26 July 2012–22 August 2012	27	<i>Jania adhaerens</i>	Cal	Native	<i>Lithothamnion californicum</i>	Cal	Native	Intertidal
3	OA + warming	28 November 2012–19 December 2012	21	<i>Plocamium cartilagineum</i>	Non-cal	Native	–	–	–	Intertidal
4	OA + warming	5 February 2013–8 March 2013	31	<i>Corallina vancouveriensis</i>	Cal	Native	–	–	–	Intertidal

Cal, calcified; Non-cal, non-calcified/fleshy.

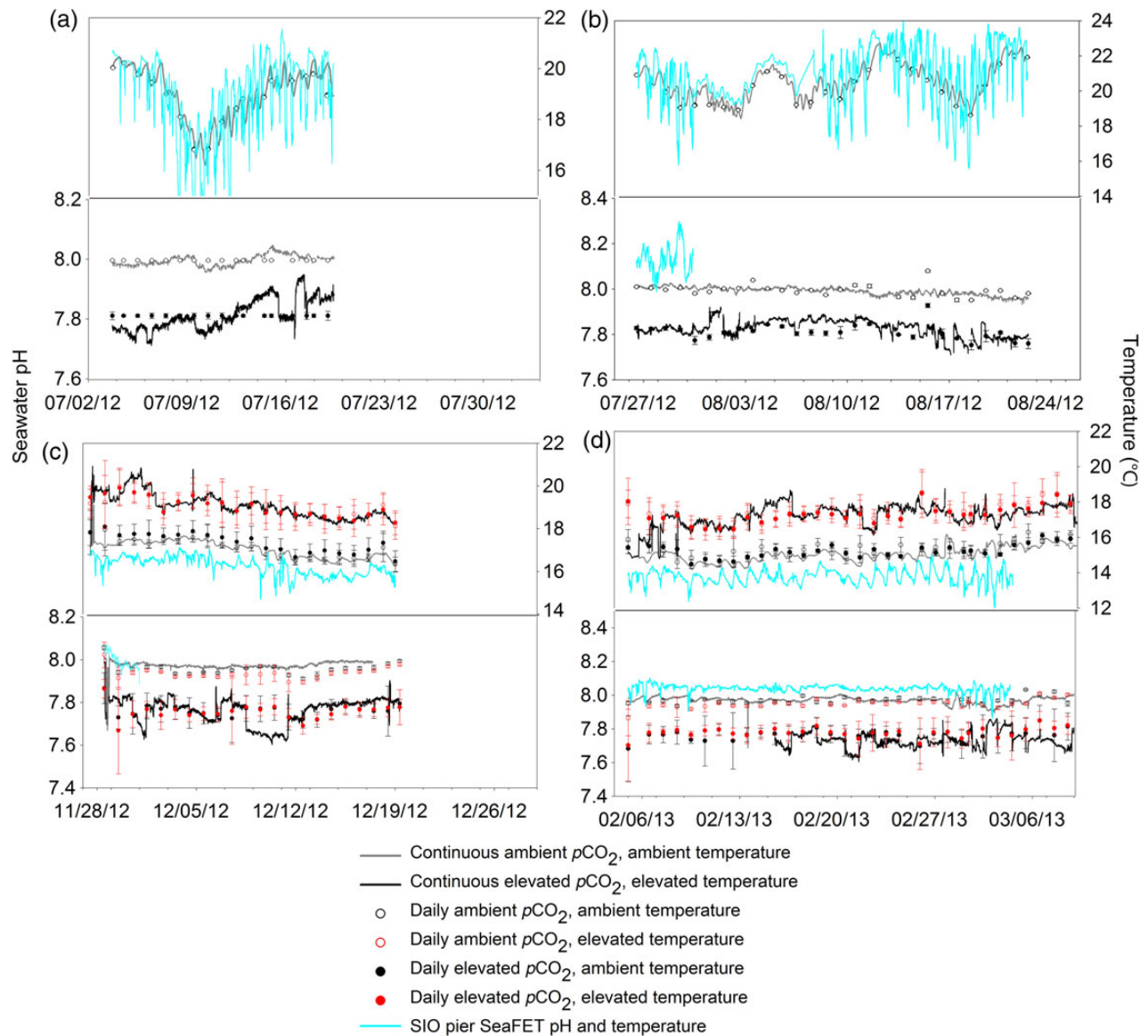


Figure 1. [colour version] Daily mean (\pm SE) and continuous (logged every 15 min) temperature and pH for all experiments. Solid grey lines are ambient CO_2 , ambient temperature treatments, and solid black lines are elevated CO_2 , elevated temperature treatments collected continuously from Honeywell Non-Glass pH Electrode Durafets that were kept in control (without alga sample) aquaria. The solid blue lines are continuous *in situ* pH measurements collected by a SeaFET off the SIO pier. The points are daily means (\pm SE) from a HACH (HQ40d meter and PCH201 glass electrode pH probe) that were calibrated daily with certified Tris buffer from the Dickson laboratory at the SIO. (a) $p\text{CO}_2$ only (exp. 1, Table 1); average ambient pH: 8.02 ± 0.006 ; average elevated CO_2 pH: 7.80 ± 0.011 ; average ambient temperature: $18.92 \pm 0.172^\circ\text{C}$. (b) $p\text{CO}_2$ only (exp. 2, Table 1); average ambient pH: 8.00 ± 0.014 ; average elevated CO_2 pH: 7.81 ± 0.009 ; average ambient temperature: $20.20 \pm 0.137^\circ\text{C}$. (c) $p\text{CO}_2$ and warming (exp. 3, Table 1); average ambient pH: 7.96 ± 0.007 ; average elevated CO_2 pH: 7.75 ± 0.025 ; average ambient temperature: $17.21 \pm 0.072^\circ\text{C}$, average elevated temperature: $19.02 \pm 0.069^\circ\text{C}$. (d) $p\text{CO}_2$ and warming (exp. 4, Table 1); average ambient pH: 7.97 ± 0.017 ; average elevated CO_2 pH: 7.81 ± 0.032 ; average ambient temperature: $15.27 \pm 0.049^\circ\text{C}$; average elevated temperature: $17.35 \pm 0.064^\circ\text{C}$.

directly to the ambient treatment aquaria or through a desiccant (DRIERITE Laboratory Air and Gas Drying) unit before being blended with pure CO_2 . A CO_2 gas analyser (LI-COR 820) was used to manually set and log the concentration of CO_2 in the CO_2 -air blend that entered aquaria. Blank control aquaria ($n = 3$ per treatment), which did not contain living samples, were maintained to quantify potential impacts the organisms may have had on seawater carbonate chemistry, though unlikely given the flow-through nature of the system. Ambient pH conditions were set to represent field conditions at the site of seawater intake. The desired decreased

pH levels and saturation states were created by constantly bubbling a CO_2 -air blend into individual treatment aquaria (1-l glass aquaria) at a rate sufficient to lower the seawater pH (pH_{SW}) by 0.2 ± 0.05 (Figure 1) from ambient given that the aquaria water residence time was ~ 4 min.

Temperature treatments

Ambient temperatures were set according to field conditions at the site of seawater intake. Elevated temperatures of $2 \pm 0.5^\circ\text{C}$ in the experimental aquaria (Figure 1) were created by placing the aquaria

inside a large water bath that was heated using aquarium heaters (Hydro Thermal Submersible 400 W). Six water baths were used with three ambient and three with the elevated temperature treatment. The position of aquaria bubbled with high CO₂ (*n* = 4 per water bath) and that of aquaria bubbled with ambient air (*n* = 4 per water bath) were alternated within each bath.

In situ pH and temperature data acquisition

An autonomous Ion Sensitive Field Effect Transistor (ISFET) Honeywell Durafet pH sensor (hereafter called SeaFET; Martz *et al.*, 2010) was deployed next to the SIO seawater intake pipe, allowing for continued monitoring of ambient field conditions (Figure 1). Measurements were taken every 15 min, and discrete samples for total alkalinity (A_T) and dissolved inorganic carbon (DIC) were collected weekly from alongside the sensors for calibration and quality control. The water samples were analysed in the Dickson laboratory at SIO according to standard operating procedures (SOPs; Dickson *et al.*, 2007). The sensor was serviced monthly to remove biofouling organisms that may have affected sensor measurements.

Monitoring experimental conditions

Temperature and pH_{sw} were measured daily at midday (13:00 PST ± 2 h) in all aquaria using a hand-held pH meter (HACH HQ40d Portable pH, Conductivity, Dissolved Oxygen, ORP and ISE Multi-Parameter meter). The glass electrode pH probes (HACH, PCH201) were calibrated daily against certified Tris buffer from the Dickson laboratory at SIO to account for probe error and drift. Minor adjustments were made to bubbling rates in individual aquaria if the experimental pH did not lie within the desired window of 0.2 ± 0.05 pH units below ambient. Temperature and pH_{sw} were also continuously logged (Honeywell Durafet Non-Glass pH Electrodes) every 15 min in one blank control ambient air, ambient temperature, and one CO₂ enriched, elevated temperature aquaria (if a warming treatment was used; Figure 1).

In addition to temperature, light intensities were continuously logged every 15 min (Onset HOBO® Pendant UA-002-64) in the control aquaria without algae. Light intensities, measured in lux, were converted to available photosynthetically active radiation (PAR) using the following conversion: 1 μmol photon m⁻² s⁻¹ = 51.2 lux (Valiela, 1984; Supplementary Figure S1). These conversions were validated by additional PAR measurements made in the water baths, using a LICOR 4π quantum sensor.

To quantify the effects of treatments on water chemistry, water samples were collected from all control aquaria (without algae; *n* = 6 for pCO₂-only experiments and *n* = 4 for factorial pCO₂ and warming experiments) and one aquaria per treatment per species at two time points or more during each experiment. The samples were collected and analysed in the Dickson laboratory at the SIO according to the SOP (Dickson *et al.*, 2007). Total DIC was determined using a Single Operator Multi-parameter Metabolic Analyser (SOMMA) and an UIC Model 5011 CO₂ coulometer (SOP 2). A_T was determined by open cell acid titration using a Metrohm Dosimat Model 665 and Thermo Scientific Ross potentiometric pH probe and meter (SOP 3b). Salinity was determined using a Mettler Toledo Model DE45 density meter. Seawater DIC parameters (HCO₃⁻, CO₃²⁻, CO₂, and pCO₂), pH_{sw}, and saturation state of carbonate minerals (Ω-calcite and Ω-aragonite) were calculated based on measured DIC and A_T using the computer program CO2SYS (version 14; Pierrot *et al.*, 2006) and stoichiometric dissociation constants defined by Mehrbach *et al.* (1973) and refit by Dickson and Millero (1987) (Table 2).

Table 2. Mean seawater chemistry for experimental aquaria ± SE, including both blank control aquaria and aquaria with macroalgae; discrete samples were averaged across treatments.

Exp.	Treatment	Daily mean temperatures	# Discrete samples	Salinity	A _T (μmol kg ⁻¹)	DIC _T (μmol kg ⁻¹)	pH _{sw}	HCO ₃ ⁻ (μmol kg ⁻¹)	CO ₃ ²⁻ (μmol kg ⁻¹)	CO ₂ (μmol kg ⁻¹)	pCO ₂ (μatm)	Calcite	Aragonite
1	Ambient air	18.91 ± 0.24	10	33.54 ± 0.002	2239 ± 3.115	2012 ± 1.024	8.02 ± 0.006	1834 ± 2.350	164 ± 2.100	13.8 ± 0.193	408 ± 5.786	4.0 ± 0.051	2.6 ± 0.033
1	High CO ₂	18.94 ± 0.24	10	33.55 ± 0.002	2234 ± 0.525	2105 ± 4.434	7.80 ± 0.012	1975 ± 6.144	105 ± 2.467	25.2 ± 0.753	744 ± 22.831	2.5 ± 0.060	1.6 ± 0.039
2	Ambient air	20.20 ± 0.19	14	33.57 ± 0.005	2241 ± 6.820	2010 ± 3.649	8.00 ± 0.014	1828 ± 7.357	168 ± 5.375	14.1 ± 0.513	444 ± 16.975	4.1 ± 0.131	2.6 ± 0.085
2	High CO ₂	20.22 ± 0.19	14	33.57 ± 0.006	2235 ± 0.904	2090 ± 3.513	7.81 ± 0.009	1952 ± 5.044	115 ± 2.081	23.2 ± 0.505	731 ± 17.104	2.8 ± 0.050	1.8 ± 0.033
3	Ambient air	17.09 ± 0.10	4	33.49 ± 0.016	2237 ± 3.921	2049 ± 1.567	7.96 ± 0.002	1893 ± 0.706	139 ± 1.683	16.9 ± 0.154	479 ± 1.785	3.4 ± 0.041	2.2 ± 0.027
3	Ambient air high temperature	19.03 ± 0.09	3	33.52 ± 0.009	2238 ± 4.254	2048 ± 2.045	7.93 ± 0.004	1889 ± 2.307	141 ± 2.174	17.2 ± 0.230	519 ± 4.550	3.4 ± 0.053	2.2 ± 0.036
3	High CO ₂	17.34 ± 0.09	4	33.51 ± 0.012	2236 ± 3.577	2143 ± 8.204	7.72 ± 0.019	2026 ± 9.597	85 ± 3.113	31.8 ± 1.513	901 ± 45.261	2.1 ± 0.075	1.3 ± 0.048
3	High CO ₂ high temperature	19.01 ± 0.10	5	33.51 ± 0.007	2235 ± 2.778	2130 ± 5.451	7.72 ± 0.013	2008 ± 6.534	92 ± 2.237	29.8 ± 0.916	896 ± 29.997	2.2 ± 0.054	1.4 ± 0.035
4	Ambient air	15.32 ± 0.07	3	33.47 ± 0.014	2230 ± 2.395	2041 ± 7.033	8.00 ± 0.008	1886 ± 9.472	139 ± 2.894	16.3 ± 0.444	436 ± 9.255	3.3 ± 0.070	2.1 ± 0.046
4	Ambient air high temperature	17.41 ± 0.09	4	33.43 ± 0.043	2228 ± 3.407	2036 ± 6.424	7.97 ± 0.002	1878 ± 8.182	141 ± 4.183	16.4 ± 0.285	466 ± 71.366	3.4 ± 0.052	2.2 ± 0.036
4	High CO ₂	15.22 ± 0.06	5	33.46 ± 0.008	2227 ± 2.372	2095 ± 19.414	7.85 ± 0.046	1964 ± 27.370	106 ± 10.683	24.3 ± 2.722	650 ± 71.366	2.6 ± 0.258	1.6 ± 0.165
4	High CO ₂ high temperature	17.29 ± 0.08	4	33.47 ± 0.013	2222 ± 4.508	2114 ± 9.289	7.76 ± 0.030	1993 ± 12.994	93 ± 6.206	28.7 ± 2.371	817 ± 62.913	2.2 ± 6.206	1.4 ± 2.371

Seawater pH (pH_{sw}) and DIC parameters (pCO₂, HCO₃⁻, and CO₃²⁻) were calculated from measured values of total DIC (DIC_T) and A_T with the computer program CO2SYS (version 14; Pierrot *et al.*, 2006). Mean temperatures calculated from daily means across all aquaria within a treatment.

Response variables

Algal growth rates were calculated as a change in wet weight for fleshy algae and a change in buoyant weight (Davies, 1989) for calcareous algae. The fleshy algae were spun in a salad spinner, then gently blotted dry with paper towels before being weighed. Samples were weighed immediately before and after the experiments, and growth rates were calculated as the difference between final and initial, standardized by initial weight and by the duration of the experiment ($\text{g g}^{-1} \text{d}^{-1}$).

Dark-adapted yield, a proxy for photosynthetic efficiency, was measured using a submersible pulse amplitude modulation (PAM) fluorometer (DIVING-PAM, Walz, Germany). The ratio of variable to maximal fluorescence in a darkened sample is correlated with the quantum yield of photosynthesis and is a convenient measure of the maximum potential quantum yield (Björkman and Demmig, 1987; Jones et al., 1999). The maximum quantum yield was determined by dark adapting the algae for 1 h at the end of each experiment. After dark adaption, F_v/F_m was measured using a saturating pulse with the diving PAM.

Photosynthetic pigments were assessed by collecting a wet weight tissue sample ($<0.1 \text{ g}$) from each specimen at the end of each experiment. Samples were submerged in 1.0 ml of dimethylformamide and stored in the dark at 0°C for at least 24 h. The resulting liquid was transferred to glass cuvettes and absorbance at wavelengths: 480, 510, 630, 643, 664 and 750 nm was measured using a spectrophotometer (HP 8453) to quantify chlorophyll *a* and carotenoid content. Pigment concentrations were calculated based on Jeffrey and Humphrey (1975) and normalized to each subsample's wet weight. Phycobilin pigment content was assessed for red algae from two experiments (Supplementary Table S3) following the protocol developed by Rosenberg and Ramus (1982) in phosphate buffer and was normalized to subsample wet weight.

CA activity was assessed for all samples from two experiments using the potentiometric technique described by Giordano and Maberly (1989). Immediately following the end of each experiment, 1.0 g tissue (wet weight) was removed from each algal specimen; *P. cartilagineum* samples were flash frozen in liquid nitrogen before being placed in -80°C freezer, and *D. undulata* and *S. horneri* samples were placed directly in a -80°C freezer, until they could be processed. Preliminary trials demonstrated no difference in CA activity expression between the two freezing methods (data not shown). Total CA was extracted on ice (at 5°C) by grinding the algal samples with acid-washed sand and 5 ml of Tris-borate buffer. Extracted enzymes were separated from ground algal contents by centrifuge [as per Giordano and Maberly (1989)]. The supernatant was aliquoted into two portions: one to measure enzymatic activity and one as a control (boiled at 100°C for 10 min to denature the enzyme). The reaction was initiated by adding 1.5 ml of distilled deionized water, saturated with CO_2 using dry ice, to 0.1 ml of the supernatant stirred in a glass reaction chamber kept at 5°C with 15 ml of phosphate buffer ($\text{pH } 8.36 \pm 0.02$). Hand-held pH meters (HQ40d HACH) with glass electrode pH probes (PHC201 HACH) were used to record the time taken for the pH to fall from 8.2 to 7.8. Probes were three-point calibrated daily to NBS buffers. The relative rate of pH decline, which was assumed to be linear, was compared between treatment samples and boiled controls. CA activity units were standardized by the fresh weight of the alga.

Statistical analysis

Mean values (\pm standard error) were calculated for growth rates, F_v/F_m , pigment analysis, and CA activity for each species and

treatment combination. All statistical analyses were completed with the program JMP (version 10). The assumption of normality was validated using the Shapiro–Wilk test and Normal Quantile Plots of residuals. For the elevated $p\text{CO}_2$ -only experiments where data were normal, a two-tailed *t*-test was used to test the null hypothesis that elevated $p\text{CO}_2$ had no effect on the response variables. For the factorial experiments of CO_2 addition and warming (both fixed factors with two-levels), a two-way analysis of variance (ANOVA) was used to examine differences among treatments. *Post hoc* contrasts were used to compare relative responses among subsets of treatments.

Results

By bubbling a CO_2 blend into treatment aquaria, we successfully maintained the two distinct treatments, ambient and elevated $p\text{CO}_2$, while simultaneously incorporating natural pH variability (Figure 1 and Table 2).

Response of native vs. invasive fleshy species to increased $p\text{CO}_2$

The invasive fleshy brown alga *S. horneri* was not affected by the high CO_2 treatment relative to the controls ($t_{18} = 0.57$, $p = 0.57$; Figure 2a); however, the native fleshy brown alga species *D. undulata* experienced elevated growth rates when exposed to high CO_2 ($t_{18} = 2.22$, $p = 0.03$; Figure 2a). Photosynthetic performance as measured by CA (Figure 3 and Supplementary Table S1), pigment concentrations (chl *a*, carotenoids, and phycobilins) (Supplementary Tables S3 and S4), and F_v/F_m (Figure 4 and Table 2) were not affected by the $p\text{CO}_2$ treatments (Supplementary Tables S1–S4). CA activity, irrespective of the CO_2 treatment, was detected in *D. undulata* and *S. horneri* relative to controls containing the denatured enzyme suggesting the use of CA as a CCM (Figure 3 and Supplementary Table S1).

Response of articulated vs. encrusting calcified algae to increased $p\text{CO}_2$

Both of the coralline algae, *J. adhaerens* (articulated) and *L. californicum* (encrusting), showed a negative growth response to elevated $p\text{CO}_2$, but these trends were not statistically significant (*J. adhaerens*: $t_{18} = 1.58$, $p = 0.13$; *L. californicum*: $t_{18} = 0.37$, $p = 0.71$; Figure 2b). F_v/F_m (Figure 4) for both species and pigment concentrations for *J. adhaerens* (chl *a* and phycobilins) were not affected by the $p\text{CO}_2$ treatments (Supplementary Tables S2, S3, and S5).

Response of fleshy and calcified macroalgae to increased $p\text{CO}_2$ and warming

Plocamium cartilagineum grew over the course of the factorial experiment in all treatments, aside from the warming-only treatment where it lost biomass. However, exposure to elevated $p\text{CO}_2$ mitigated the negative temperature effect so that, on average, the warmed specimens gained 170% more mass when simultaneously exposed to elevated $p\text{CO}_2$ (Figure 2c). Thus, there was a significant interaction between the increased $p\text{CO}_2$ and warming treatments on the growth rates of *P. cartilagineum* (two-way ANOVA, $p\text{CO}_2 \times \text{Temp}$: $F_{1,36} = 6.96$, $p = 0.012$; Figure 2c). Elevated $p\text{CO}_2$ significantly affected overall growth rates, but to different magnitudes and in opposing directions depending on temperature exposure. In ambient temperature conditions, elevated $p\text{CO}_2$ depressed growth of *P. cartilagineum* by 45% on average compared with the untreated specimens; however, this difference was not significant due to high variability between replicates. CA (Figure 3 and Supplementary Table S1),

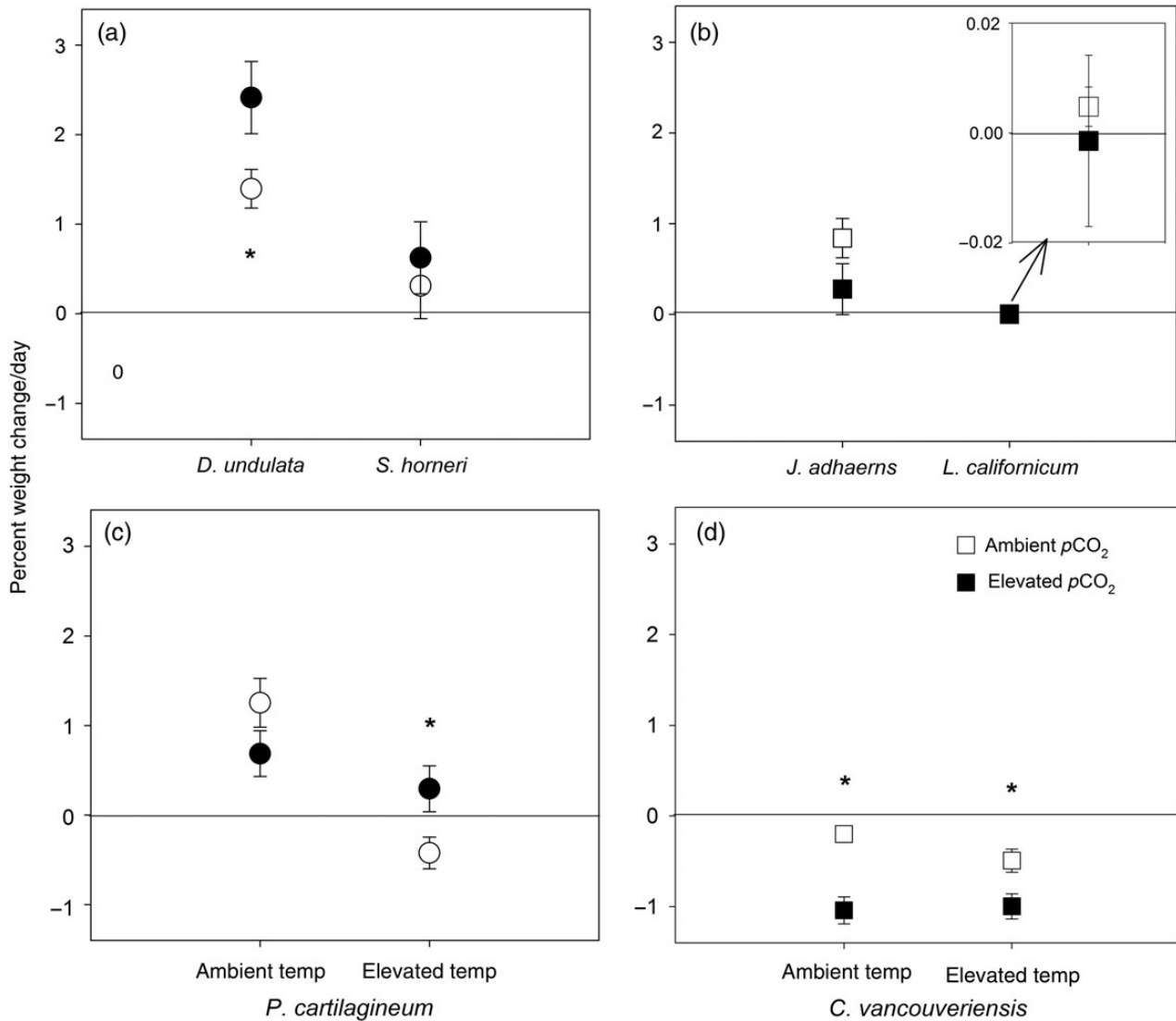


Figure 2. Results from increased $p\text{CO}_2$ experiments showing mean percent weight change/d (\pm SE) on (a) the brown, fleshy invasive *S. horneri* vs. the brown, native species *D. undulata* and (b) the red, calcified articulated *J. adhaerens* vs. non-articulated *L. californicum*. $p\text{CO}_2 \times$ warming experiments on (c) the red, fleshy *P. cartilagineum* and (d) the red, calcified *C. vancouveriensis*. Circles designate fleshy species and squares designate calcified species. Open shapes indicate ambient air treatment and closed shapes indicate elevated $p\text{CO}_2$ treatment. A significant difference between treatments is denoted by * $p < 0.05$.

pigment concentrations (chl *a* and phycobilins; Supplementary Tables S3 and S6), and F_v/F_m (Figure 4 and Supplementary Table S2) were not affected by any treatment, or combination thereof. Activity levels of CA were found to be positive for *P. cartilagineum*, relative to denatured enzyme controls, suggesting that the enzyme is active and used as a CCM by this alga (Figure 3 and Supplementary Table S1).

Corallina vancouveriensis, an articulated coralline alga, experienced negative growth rates due to bleaching and tissue fragmentation across all treatments (Figure 2d). Despite this overall loss, there were detectable significant effects of $p\text{CO}_2$ on growth and calcification (two-way ANOVA, $p\text{CO}_2$: $F_{1,36} = 30.42$, $p < 0.01$; Temp: $F_{1,36} = 1.03$, $p = 0.31$; $p\text{CO}_2 \times$ Temp: $F_{1,36} = 1.91$, $p = 0.17$). Growth rates for *C. vancouveriensis* were significantly reduced by 420% when exposed to increased CO_2 . The combination of increased CO_2 and temperature relative to control ambient conditions also had a negative effect on growth, although those two treatments did not

differ significantly from each other. *Corallina vancouveriensis* was not affected by increased temperature alone. F_v/F_m (Figure 4 and Supplementary Table S2) and pigment concentrations (chl *a*; Supplementary Tables S3 and S7) were not affected by any treatment, or combination thereof.

Discussion

We investigated the effects of CO_2 enrichment alone and the independent and combined effects of CO_2 enrichment and warming on common species of temperate marine algae. We expected the fleshy algae to respond positively or not at all to increases in CO_2 depending on their ability to use CA as a CCM. Our results showed that while one of three fleshy taxa did in fact respond positively to CO_2 elevation, we were not able to correlate these responses to CA activity (or lack thereof). One of three calcareous algae species was negatively affected by elevated CO_2 , as hypothesized, whereas the other two

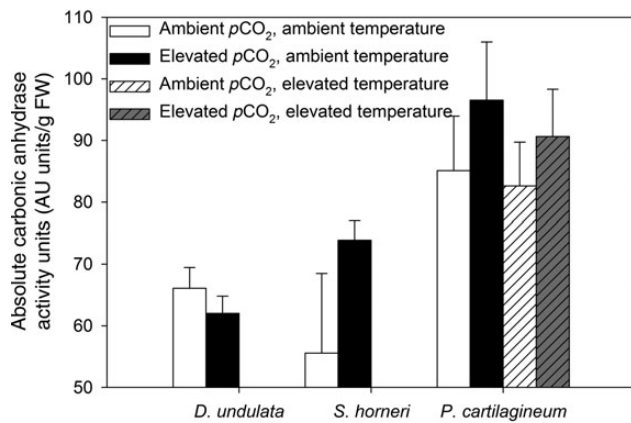


Figure 3. Mean (\pm SE) CA activity units for three non-calcified species of macroalgae (*D. undulata*, *S. horneri*, and *P. cartilagineum*). See Supplementary Table S1 for statistical results.

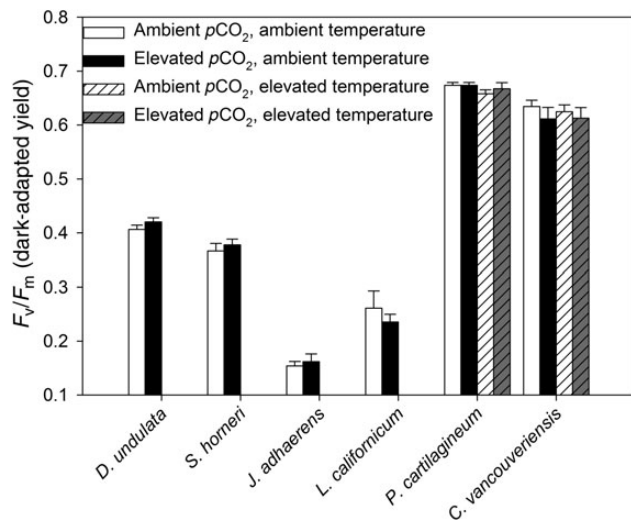


Figure 4. Mean (\pm SE) F_v/F_m (dark-adapted yield) for six common southern California fleshy and calcified macroalgae. Chlorophyll fluorescence, a proxy for photosynthetic efficiency, was measured using a pulse amplitude-modulated fluorometer. See Supplementary Table S2 for statistical results.

calcareous taxa showed no response. Both fleshy and calcified macroalgae were expected to be positively affected by increased temperature; however, we found negative effects on fleshy *P. cartilagineum* and no effect on calcareous *C. vancouveriensis*. For *P. cartilagineum*, we found a significant interaction between elevated $p\text{CO}_2$ and temperature while a significant interaction was absent from the *C. vancouveriensis* experiment, suggesting that the responses of benthic marine communities to global change are likely to be complex.

The increase of CO_2 and/or temperature did not affect F_v/F_m or pigment concentrations for any of the calcified and fleshy macroalgae examined (Supplementary Tables S2–S7). Similar studies have likewise found no effect of elevated CO_2 on these physiological response variables. Cornwall *et al.* (2013) exposed a temperate calcifying coralline, *Arthrocardia corymbosa*, to elevated CO_2 conditions for 40 d, and found that while algal growth rates were significantly reduced, there was no effect of increased CO_2 on F_v/F_m or pigment concentrations. Hofmann *et al.* (2012b) also found that

F_v/F_m was not affected by increased CO_2 in the temperate calcified macroalgae *Corallina officinalis*, although it also exhibited significantly reduced growth rates. Despite these results, one would still expect to see some form of enrichment of photosynthesis as more CO_2 is made available; perhaps measuring photosynthesis directly via oxygen evolution would be more appropriate in future studies. Given the diversity in algal taxonomy, morphology, and physiology, it is not surprising that the responses of different species to elevated CO_2 and/or warming are largely species-specific (Price *et al.*, 2011; Comeau *et al.*, 2014, Johnson *et al.*, 2014a).

Increased $p\text{CO}_2$ impacts on invasive vs. native species

We compared the response of a native and an invasive brown alga to elevated $p\text{CO}_2$ conditions, and found that the native *D. undulata* may grow faster under future $p\text{CO}_2$ conditions than the non-indigenous *S. horneri*. This suggests that *D. undulata* may be more successful than *S. horneri* under future OA conditions. Though *S. horneri* can invade new habitats under current conditions (Miller *et al.*, 2007), its competitive ability under future OA conditions may be reduced. Many studies have shown that elevated $p\text{CO}_2$ positively affects terrestrial invasive species; however, few marine examples exist. Nagel *et al.* (2004) found that an invasive desert grass had a significant reduction in the energetic cost of above-ground biomass construction under CO_2 enrichment, which its native counterpart did not exhibit. Mateos-Naranjo *et al.* (2010) reported that an invasive *Spartina* (saltmarsh grass) exhibited increased growth under elevated atmospheric CO_2 . While individual species growth rates differ in our study, the invasive, brown fleshy alga *S. horneri* was not significantly affected by elevated $p\text{CO}_2$, whereas the native, brown fleshy *D. undulata* was significantly positively affected, suggesting differential effects on competitive interactions with future OA.

Increased $p\text{CO}_2$ impacts on encrusting vs. articulated coralline algae

Calcareous algae face a paradox under elevated $p\text{CO}_2$ conditions. While there is increased CO_2 available for photosynthesis, the corresponding decrease in carbonate saturation state may limit their ability to calcify. Here, CO_2 enrichment consistently had a negative effect on growth, although the magnitude of the response was species-specific and differed greatly between the articulated and crustose coralline algal species. McCoy and Ragazzola (2014) argued that increased $p\text{CO}_2$ may be more stressful for species of calcified algae with thicker cell walls and crusts, because they contain larger quantities of skeletal calcium carbonate (CaCO_3) per unit biomass of photosynthetic tissue. Our results indicated that *C. vancouveriensis*, a densely branched species with a high surface area to biomass ratio, exhibited the largest negative response, suggesting that the amount of tissue exposed to reduced carbonate saturation state water may contribute to the strong negative responses observed. Furthermore, the effects of OA may not be as negative to coralline algae photosynthesis as early research indicated. While it is widely accepted that net calcification rates are expected to decline, our study and others (Hofmann *et al.*, 2012b; Cornwall *et al.*, 2013) have yet to find a relationship between declining net calcification and photosystem functions, such as F_v/F_m and pigment concentration.

Increased $p\text{CO}_2$ impacts on fleshy vs. calcareous algae

Overall, fleshy algae responded more positively to elevated CO_2 than calcareous algae. One of the three fleshy species used in experiments had a significantly positive response to increased CO_2 , whereas one

of the three calcified species had a significantly negative response; no calcified algae were positively affected, and no fleshy algae were negatively affected by increased CO₂. This is in agreement with the most findings thus far in the literature [reviewed in Kroeker *et al.* (2010, 2013), Hendriks *et al.* (2010), and Johnson *et al.* (2014a)]. CO₂ enrichment resulted in significantly increased growth rates for the fleshy *D. undulata*, while the other two fleshy species were not affected. Other studies have found that other species of fleshy algae exhibited increased growth rates under elevated CO₂ (Zou, 2005; Connell and Russell, 2009; Russell *et al.*, 2009). However, until we understand the physiological mechanisms behind these responses to increased CO₂, predicting species' responses to OA will be challenging.

Synergistic interactions of increased pCO₂ and warming

We hypothesized that increased temperature would positively affect both fleshy and calcareous algae, but that fleshy algae would have a synergistically positive response to increased temperature and CO₂. Interestingly, for the fleshy red alga *P. cartilagineum*, the effects of CO₂ on growth rate were only positive under increased temperatures, suggesting that the increase in temperature offsets the negative effects of increased CO₂. Growth rates were negative overall in response to increased temperature, despite the antagonistic interaction with exposure to elevated pCO₂. During the course of the experiment (November–December 2012), the average ambient seawater temperature was 17.21 ± 0.07°C (± SE), whereas the average elevated temperature treatment was 19 ± 0.06°C (Figure. 1). However, for 2011 and 2012, the maximum temperature recorded off the SIO Pier in the summer (June–September) was 22.48 and 23.94°C, respectively (SCCOOS, publically available data). The resulting loss of biomass is surprising, therefore, since the experimentally elevated temperature was well within the natural temperature range. The negative response may have been in part a result of the shock from the rapid introduction of algal specimens into warm experimental conditions, rather than gradual acclimation as they would experience seasonally. These algae were collected from the intertidal however, where they likely experience large daily fluctuations in temperature and pH. The loss in biomass therefore may not have been a negative reaction after all, but could have been a result of increased reproductive fragmentation. A study completed in Stillwater Cove, Carmel Bay, California, found that *P. cartilagineum* produces spores throughout the entire year, with slight peaks in spring and summer (Downing, 1995), and that *P. cartilagineum* utilizes vegetative fragmentation as a form of reproduction. Since the experimental temperature increases simulated summer conditions, the alga may have increased vegetative fragmentation, causing the high temperature treatment to lose biomass. All algal fragments found during the experiment were collected; however, because of the flow-through nature of the experimental design, it was not always possible to identify the fragments' origin.

The calcareous species *C. vancouverensis* was significantly negatively affected by elevated CO₂, regardless of temperature. Similar negative effects were found by Hofmann *et al.* (2012a, b) who exposed *C. officinalis* to elevated CO₂ levels. In each study, they found *C. officinalis* to have decreased growth rates and hypothesized that it may become less competitive under future CO₂ levels. Diaz-Pulido *et al.* (2012) also found similar negative results of elevated CO₂ on a tropical coralline alga, with rates of advanced partial mortality (% dead white, and green areas colonized by endolithic algae) increasing from <1 to 9% under high CO₂. Unlike the results presented here, they found that a 3°C increase in temperature

exacerbated the effects of CO₂ and partial mortality increased to 15%. The temperature treatment did not affect *C. vancouverensis* in this study.

CCMs as a CO₂ relief mechanism for fleshy species

We did not find significant effects of elevated pCO₂ on CA activity; however, this may be an artifact of the methods used to measure the enzymatic activity. It may also be that, given the differential response of fleshy algal growth to CO₂ relative to calcifier growth, CCMs such as CA may be mechanisms underlying different responses. CCM activity levels may be responsive to other environmental factors not tested in these experiments. For example, Cornwall *et al.* (2015) suggests that the activity levels of CCMs of some species may be more flexible to light levels, rather than CO₂. Additionally, downregulation of any active CCM component, which uptakes bicarbonate, directly may explain changes in growth rates, pigmentation, etc., as CA enzymes are just one example of a CCM.

Although the treatment effects were not significant, CA activity was found in the fleshy algae, *D. undulata* and *S. horneri*. There have been no other studies investigating CA activity in either of these species. Thomas and Tregunna (1968), however, reported that *Sargassum muticum* does not directly use CO₂ for photosynthesis, thus concluding that it must use bicarbonate ions for photosynthesis. If *S. horneri* also relies on bicarbonate, as opposed to CO₂ for photosynthesis, this species may not downregulate CA activity, preventing it from taking advantage of the more readily available CO₂ for growth. If this is the case, then future OA conditions may limit the success of invasive *S. horneri* when compared with native species in similar subtidal habitat such as *D. undulata*.

Although *P. cartilagineum* growth rates were not affected by an increase in CO₂ alone, high levels of CA were found. While isotopic evidence from Raven *et al.* (2005) suggested that *P. cartilagineum* relies on diffusive uptake of CO₂ rather than CCMs, Mercado *et al.* (2009) show that in fact several of these algal species may actually have higher CA activity than species where evidence generally points towards the presence of CCMs. The lack of response to increased CO₂, but the detectable CA enzyme activity units, suggests that *P. cartilagineum* may not have been carbon-limited in our experimental setting. If so, then *P. cartilagineum* may be less competitive against other fleshy species in the future, such as *D. undulata*, which was able to utilize increased CO₂ in our experiments.

Conclusions and implications

The data presented here provide additional support for the hypothesis that the responses of marine algae to increased pCO₂ will be species-specific, but in general more negative for calcifying vs. fleshy taxa. We provide data on a small subset of the diverse assemblage of macroalgae common on temperate shores that contribute to the growing body of information on the likely future effects of OA. More information is still needed on the responses of species to more gradual and thus more realistic increases in pCO₂ to allow for acclimatization or adaptation. Additionally, more long-term experiments [multiple months; as in Martin and Gattuso (2009) and Form and Riebesell (2012)] are needed as lengthened experiments may reveal impacts on organisms that are missed in shorter duration experiments (Dupont *et al.*, 2010). Finally, the interactive effects of multiple stressors, including additional local and global impacts, are needed to improve our predictive capacity of future change. It must be emphasized that a variety of response variables be examined to identify additional effects of OA, warming, and their interaction to be able to extrapolate these effects to an ecosystem level.

Macroalgae are ecosystem engineers, providing habitat, refugia, and energy as a food source to countless organisms in coastal habitats. Understanding how climate change will affect them will give researchers a better idea of how coastal ecosystems may change in the coming centuries.

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Supplementary data

Supplementary material is available at the *ICES/JMS* online version of the manuscript.

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