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Ocean warming has greater and more consistent negative effects than ocean acidification on the growth and health of subtropical macroalgae

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ABSTRACT: Macroalgae are the major habitat-forming organisms in many coastal temperate and subtropical marine systems. Although climate change has been identified as a major threat to the persistence of macroalgal beds, the combined effects of ocean warming and ocean acidification on algal performance are poorly understood. Here we investigate the effects of increased temperature and acidification on the growth, calcification and nutritional content of 6 common subtropical macroalgae; *Sargassum linearifolium*, *Ulva* sp., *Amphiroa anceps*, *Corallina officinalis*, *Delisea pulchra* and *Laurencia decussata*. Algae were reared in a factorial cross of 3 temperatures (23°C [ambient], 26°C and 28°C) and 3 pH levels (8.1 [ambient], 7.8 and 7.6) for 2 wk. The highest (28°C) temperature decreased the growth of all 6 macroalgal species, irrespective of the pH levels. In contrast, the effect of decreased pH on growth was variable. The growth of *Ulva* sp. and *C. officinalis* increased, *L. decussata* decreased, while the remaining 3 species were unaffected. Interestingly, the differential responses of macroalgae to ocean acidification were unrelated to whether or not a species was a calcifying alga, or their carbon-uptake mechanism — 2 processes that are predicted to be sensitive to decreased pH. The growth of the calcifying algae (*C. officinalis* and *A. anceps*) was not affected by reduced pH but calcification of these 2 algae was reduced when exposed to a combination of reduced pH and elevated temperature. The 3 species capable of uptake of bicarbonate, *S. linearifolium*, *L. decussata* and *Ulva* sp., displayed positive, negative and neutral changes in growth, respectively, in response to reduced pH. The C:N ratio for 5 of the 6 species was unaffected by either pH or temperature. The consistent and predictable negative effects of temperature on the growth and calcification of subtropical macroalgae suggests that this stressor poses a greater threat to the persistence of subtropical macroalgal populations than ocean acidification under ongoing and future climate change.

KEY WORDS: Carbon-concentrating mechanisms · Primary production · Climate change · Coralline algae · Macroalgae

INTRODUCTION

Anthropogenic climate change is having a dramatic effect on marine ecosystems worldwide (Wernberg et al. 2016, Hughes et al. 2017). Increased sea surface temperatures have already affected the metabolism, behaviour, phenology and distribution of species across a range of taxa (Scheffer et al. 2001, Johansen et al. 2014, Byrne et al. 2017, Pecl et al. 2017). Ocean acidification (OA) — altered seawater

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chemistry as a result of uptake of atmospheric CO_2 emissions — is also predicted to alter the growth, calcification, reproduction and behaviour of many marine species (Langdon et al. 2000, Ross et al. 2011, Byrne et al. 2013, Watson et al. 2017). Ocean warming and acidification are both increasing at unprecedented rates (IPCC 2014), hence understanding their combined impacts on the performance and growth of marine organisms will be key to predicting future changes in marine communities (Byrne 2011, Przeslawski et al. 2015).

The effects of climate change on an ecosystem may be the most pronounced when they directly affect the health of the foundation species (Wernberg et al. 2012). For example, increasing sea surface temperatures have already lead to increased frequency of global coral bleaching events (Hughes et al. 2017, 2018), and the loss of habitat-forming kelp and macroalgae (Wernberg et al. 2016), and seagrasses (Orth et al. 2006). Macroalgae are foundation species in many shallow temperate and subtropical marine systems, providing habitat for a diversity of species (Steneck 2013). Any changes to the abundance and composition of macroalgae may have direct and/or indirect effects on ecosystem diversity and/or ecological processes that could cascade through the entire ecosystem (Wernberg et al. 2010). Ocean warming and OA have already been shown to alter the growth (Madsen & Sand-Jensen 1994, Zou 2005, Gao & Zheng 2010), competitive ability (Campbell et al. 2011, Diaz-Pulido et al. 2011, Hofmann et al. 2012) and resilience to storms (Wernberg et al. 2010) of many macroalgae.

Macroalgae are predicted to be particularly sensitive to increasing temperature as their rates of biochemical and physiological processes are largely determined by environmental temperature, similar to ectotherms (Davison 1991, Brown et al. 2004). Within a species' thermal tolerance range, the rates of physiological processes and traits (e.g. growth) typically increase with temperature until they reach a thermal optimum, after which rates rapidly decrease (Brown et al. 2004). The effect of temperature on organism performance will therefore depend on the species' thermal tolerance range and how close the organism's current environmental temperatures are to their thermal optima (Tewksbury et al. 2008). Changes in environmental temperature have already been shown to influence growth (Gutow et al. 2016), photosynthesis (Mertens et al. 2015), respiration (Carr & Bruno 2013), distribution (Wernberg et al. 2016), antibacterial chemical defence (Campbell et al. 2011) and competitive ability (Wernberg et al. 2010) across a range of individual macroalgal species. However, few studies have considered how these effects of temperature are influenced by ocean acidification.

The uptake of atmospheric CO_2 by the ocean is altering the carbonate chemistry of seawater, increasing the partial pressure of carbon dioxide (pCO_2), and bicarbonate (HCO_3^-) and hydrogen (H^+) ions and decreasing the concentration of carbonate minerals (predominately $CaCO_3$), a process coined ocean acidification or OA (Guinotte & Fabry 2008). While all macroalgae use carbon for photosynthesis, they differ in their sources and mechanisms for the uptake of carbon, and therefore may respond differently to OA (Hurd et al. 2011). Some species rely solely on passive uptake of CO_2 from surrounding seawater, while other species have carbon-concentrating mechanisms (CCMs) that facilitate the uptake of HCO_3^- for photosynthesis (Raven 1997). Species with CCMs can be further categorized into 3 groups based on their sources of inorganic carbon: (1) those that use HCO_3^- only, (2) those that use both HCO_3^- and CO_2, and (3) those that use CO_2 only for photosynthesis (Diaz-Pulido et al. 2016, Cornwall et al. 2017b). CCMs can be energetically costly; however, they make photosynthesis more efficient at the carbon fixation site (Raven 1997). The increased availability of CO_2 in seawater may benefit algae who utilize CO_2 as their main carbon source if they are carbon limited (Koch et al. 2013), and algae who utilize HCO_3^- only if they are able to switch to passive diffusion of CO_2 to offset the energetic costs of their CCM (Johnston & Raven 1990, Cornwall et al. 2017b). There are conflicting results within the growing body of literature investigating the response of productivity and growth of marine macroalgae to OA. Elevated pCO_2 has been shown to either increase (Falkenberg et al. 2014, Hofmann et al. 2015b), decrease (Hofmann et al. 2012, 2015a, Gutow et al. 2014), or have no effect on the growth or photosynthesis of marine macroalgae (Israel & Hophy 2002, Egilsdottir et al. 2013, Campbell et al. 2014). Recently, this variation in the response of macroalgae to OA has been linked to the macroalga's inorganic carbon physiology (Cornwall et al. 2017b), and suggests a more detailed categorization of dissolved inorganic carbon (DIC) affinity within species-specific CCMs may be beneficial in predicting macroalgal responses to OA (Cornwall et al. 2017b).

OA is also predicted to negatively impact calcified algae due to reductions in the carbonates available to build calcareous structures and increases of H^+ ions that may cause dissolution of existing tissues (Büdenbender et al. 2011, Diaz-Pulido et al. 2011, 2012,

Johnson & Carpenter 2012, Kamya et al. 2017). To add further complexity to this problem, photosynthesis and calcification have been shown to be positively linked (Digby 1977, Hofmann & Bischof 2014). The fixation of CO_2 during photosynthesis increases the pH of surrounding seawater, thereby increasing the calcite saturation state to favour $CaCO_3$ deposition in the tissues (Digby 1977). In addition, CO_2 is a by-product of calcification, thereby stimulating photosynthesis (McConnaughey 1991). Lastly, there is also evidence that coralline algae may be able to use HCO_3^- and CO_2 for calcification, leading to potential shifts between calcification and net dissolution as CO_2 and HCO_3^- concentrations increase under OA conditions, as both processes will be affected by these changes. Until recently, coralline algae have been thought to be particularly vulnerable to dissolution under OA conditions (Kuffner et al. 2008) as they deposit the highly soluble high-Mg calcite within their intercellular spaces and surface of the cell (Littler 1976). However, recent evidence suggests that at least some calcified algae may be able to regulate their internal and surface pH to buffer against the effects of OA (crustose coralline algae: Hofmann et al. 2016, Cornwall et al. 2017a).

To date, the majority of research investigating the effects of OA on coralline algae have reported reduced growth (e.g. Ragazzola et al. 2012, James et al. 2014), calcification (e.g. Gao & Zheng 2010, Kamenos et al. 2013) and increased dissolution (e.g. Reyes-Nivia et al. 2014) of individuals, and reductions in overall abundance (e.g. Kuffner et al. 2008, Ordoñez et al. 2014) and recruitment (e.g. Porzio et al. 2013). However, a few studies have reported potential dissolution while rates of growth, calcification and/or photosynthesis have remained unaffected or even increased in response to OA (e.g. Egilsdottir et al. 2013, Kamenos et al. 2013, Noisette et al. 2013), suggesting that calcification and dissolution may be most sensitive to OA. Therefore, calcification and dissolution may be the most sensitive of the aforementioned physiological processes. In tropical crustose coralline algae, changes in relative concentrations of aragonite versus dolomite within the tissues have been reported in response to OA (Diaz-Pulido et al. 2014). Further, the magnitude of responses of coralline algae to OA can also be influenced by diffusive boundary layers (Cornwall et al. 2014) and the rate of declining pH (Kamenos et al. 2013). Due to the complex interaction between photosynthesis, calcification and dissolution in response to OA and its interaction with other environmental variables, the response of these processes to a changing ocean is still poorly understood.

Relatively few studies have examined the potential interaction between increasing temperature and OA on marine macroalgae across a range of co-occurring species (Wernberg et al. 2012). This is important, as these 2 factors are occurring simultaneously (IPCC 2014). Therefore, understanding the combined effects between ocean warming and acidification on macroalgae may provide insight as to how these 2 factors may impact the composition of foundation species in a community (Kroeker et al. 2013b, Olabarria et al. 2013). Increasing temperature and OA may also interact to affect the palatability of algal tissue to herbivores, and given that the abundance of primary producers is very strongly regulated by herbivory in marine systems (Poore et al. 2012); any changes to the tissue traits are likely to affect the strength of top-down control of algal abundance. The objective of this study was to investigate the effects of simultaneous OA and warming on the growth, calcification, carbon metabolism and tissue quality (C:N ratio and calcification) of 6 co-occurring subtropical macroalgae from a climate change 'hotspot' in south-eastern Australia. Sea surface temperatures in this region are predicted to increase by >4°C, which is the upper range of predicted warming of 2.6 to 4.8°C for the worlds oceans for 2100 (Hobday & Lough 2011, IPCC 2014).

MATERIALS AND METHODS

Study species and collection sites

Six common co-occurring macroalgal species were collected by hand from shallow (<2 m) subtidal coastal habitats in the autumn (May–June) of 2011 from the Coffs Harbour region, central New South Wales, Australia. Five macroalgal species (*Sargassum linearifolium*: leathery phaeophyte; *Ulva* sp.: foliose chlorophyte; *Laurencia decussata*: non-calcified branching rhodophyte; and *Corallina officinalis* and *Amphiroa anceps*: both geniculate coralline rhodophytes) were collected from Charlesworth Bay (30° 16′ 03.0″ S, 153° 08′ 16.19″ E), and the sixth species (*Delisea pulchra*: non-calcifying branching rhodophyte) was collected from the south side of Muttonbird Island, Coffs Harbour, New South Wales, Australia (30° 18′ 19.7″ S, 153° 08′ 53.7″ E). Ambient seawater pH for this region averaged 8.07 ± 0.002 SE for the duration of the study. Average coastal sea surface temperatures in the region range from 20 to 26°C, and was 22.23 °C (0.004 SE) at the time of the study (IMOS 2018).

Experimental treatments and sample preparation

Algae were placed in individual aquaria within 30 min of collection, rinsed with filtered seawater and cleaned of visible fauna and epiphytes. Algae were cut into similar lengths of 2 to 4 cm composed largely of meristematic tissue, weighed by blotting the sample dry, and placed in individual 100 ml containers with 40 ml fresh filtered seawater. Algae were negatively buoyant and sank to the bottom of the containers. The large size of *S. linearifolium*, *D. pulchra* and *L. decussata* allowed 9 pieces of meristematic tissue to be sourced from each thallus. This allowed the same individual 'clone' to be replicated across all treatments. For the remaining 3 species, each replicate was taken from a separate individual thallus. Average ± SE initial wet weights of experimental thalli were: 0.024 ± 0.001 g for *S. linearifolium*, 0.0299 ± 0.0009 g for *Ulva* sp., 0.046 ± 0.002 g for *A. anceps*, 0.046 ± 0.002 g for *C. officinalis*, 0.055 ± 0.005 g for *D. pulchra* and 0.040 ± 0.002 g for *L. decussata* across all pH and temperature treatment combinations. Each sample was randomly allocated to one of 9 pH and temperature treatments.

A factorial cross of 3 temperatures (23°C [ambient], 26°C and 28°C) and 3 pH levels (8.1 [ambient], 7.8, and 7.6) was used, yielding 9 experimental treatments. These temperature treatments are commensurate with projections for warming (2.6 to 4.8°C) and acidification (0.3 to 0.5 pH units) of southeastern Australian waters by the year 2100 (Hobday & Lough 2011, IPCC 2014). Each algal species was subjected to increased temperature and acidification in all combinations of treatments with 5 replicates per treatment. The algae were gradually introduced to increased temperature and pH treatments over a 2 d period (temperature: 2.5°C per day; pH: 0.25 pH units per day) and then held at the target treatment levels for 2 wk.

The experimental pH of flowthrough seawater was regulated using a CO_2-injection system and temperature was regulated with 300 watt bar heaters (Aqua One) within header tanks (60 l for CO_2, which fed to smaller, 30 l temperature header tanks), which then fed experimental seawater at a flow rate of ~0.13 ml s^{-1} to individual rearing containers (for full details see the Supplement at www.int-res.com/articles/suppl/m595p055_supp.pdf). The system was illuminated at 30 µmol photons m^{-2} s^{-1} supplied by 3 twin 36-watt 'cool white' fluorescent lights for a 16 h night:8 h day photoperiod. The experimental light level was equivalent to midday light measurements at 1–2 m water depth at the Charlesworth Bay collection site — 29.8 (±1.92 SE, n = 10) µmol photons m^{-2} s^{-1}.

Temperature, pH (NIST scale) and salinity were measured daily in all seawater treatments using a portable multi-probe (Hach HQD) by randomly choosing 5 individual rearing containers per pH and temperature treatment per day. The pH probe was calibrated daily using high-precision buffers (pH_{NIST} 4.0, 7.0 and 10 at 20°C, ProScitec). Total pH scale (pH_T) of the experimental seawater was determined using seawater TRIS buffer using the millivolt scale (Dickson 1993). For measurements of total alkalinity, water samples (100 ml) were collected daily and filtered with a 0.45 µm syringe filter, and preserved using saturated mercuric chloride ($HgCl_2$) solution. Total alkalinity was measured by potentiometric titration using an automatic titrater at 24.17 °C (±0.25 SE) (Metrohm 888 Titrando), calculated using the Gran method and compared with Dickson total alkalinity standards. Partial pressure of CO_2 (pCO_2) and calcite saturation states (ΩCa) were calculated using the CO2SYS MS Excel Macro (Pierrot et al. 2006; Table S1 in the Supplement) using the dissociation constants of Merbach (1973) as refitted by Dickson & Millero (1987) from measures of salinity, temperature, pH_T (total scale) and total alkalinity.

Over the 2 wk experimental period, the pH treatments averaged 8.074 (±0.002 SE, n = 225), 7.786 (±0.002 SE, n = 225) and 7.599 (±0.003 SE, n = 225) pH_{NIST}. Temperature treatments averaged 22.82°C (±0.040 SE, n = 225), 26.0°C (±0.047 SE, n = 225) and 28.39°C (±0.086 SE, n = 225). Carbonate chemistry parameters for the treatments are presented in Table S1 in the Supplement.

Algal growth

Algal growth as a relative growth rate (RGR) was quantified as the increase in wet mass as a percentage of the initial mass of each alga. Each alga was weighed every other day for the 2 wk experimental period by gently blotting dry with paper towel and recording the wet mass to the nearest microgram (µg). Individual thalli of each species were of similar length and weight at the beginning of the experiment. Any indications of stress, in particular bleaching of algal tissue, were noted. Growth of *S. linearifolium*, *C. officinalis*, *D. pulchra* and *L. decussata* were estimated as the change in mass over 2 wk, while growth of *Ulva* sp. and *A. anceps* was estimated after 9 d as *Ulva* sp. spawned on the tenth day, and the majority of individuals of *A. anceps* died in the pH 8.1/29°C treatment on the 11th day.

CCMs and pH drift

The ability of the algae to use bicarbonate for photosynthesis was determined by a pH drift experiment (Hepburn et al. 2011). In a pH drift experiment, macroalgae in a closed system will alter DIC concentrations during photosynthesis, and thereby alter the alkalinity of the seawater. If the macroalgae raise the pH above 9 where there is very little CO_2, this is indicative that the algae use HCO_3^- only for photosynthesis (Cornwall et al. 2012). If the macroalgae are unable to raise the pH above 9, this indicates that CO_2 is a carbon source for photosynthesis, and can be indicative of CO_2-only users or CO_2 + HCO_3^- users (Diaz-Pulido et al. 2016). For the drift experiment, fresh algae were collected by hand from the same collection sites. Only non-coralline algae were used in this analysis as calcification and photosynthesis are linked, and these 2 processes both together and independently alter seawater alkalinity and DIC concentrations. Therefore, a pH drift analysis is inappropriate for determining carbon-use strategies in calcifying algae. Nine replicates (~0.8 g wet weight) of each alga (excluding *A. anceps* and *C. officinalis*) were placed in individual 40 ml airtight glass vials filled with seawater of ambient pH 8.1. To ensure that photosynthesis was not light limited, the vials were placed in random order directly in front of a fluorescent light of 120 µmol photons m^{-2} s^{-1} for 24 h (Hepburn et al. 2011). A pilot study determined 24 h as the optimal time to measure maximal pH. Initial and final pH of the seawater was measured using a portable pH meter (Hach HQD Portable Multiprobe ± 0.02 pH_{NIST} from factory calibration). After 24 h, the algae were removed and the seawater was left to sit in the open containers for a further 24 h, after which the pH was recorded. Treatments in which the pH of the water returned to, or approached, ambient conditions (pH_{NIST} = ~8.1) after 24 h (i.e. the seawater had re-equilibrated with the atmosphere) indicated that algal exudates or processes other than photosynthesis did not interfere with changes to seawater pH (Hepburn et al. 2011).

Tissue quality and carbon:nitrogen ratio

At the end of the growth experiment, the algal tissue was freeze dried in preparation for elemental analyses. The coralline algae (*C. officinalis* and *A. anceps*) were treated with 2 ml of 1.0 M HCl to remove carbonates, rinsed with distilled water and then re-dried. Total carbon (%) and nitrogen (%) content were then measured using an Environmental Analytic Isotope Ratio Mass Spectrophotometer (IMRS; Flash EA 112 and Delta V Plus) connected by an Interface at 1020°C (Conflo IV). Precision estimates using laboratory STD AT2 were better than 0.2‰ for both nitrogen and carbon. As *Ulva* sp. released spores in the 26°C and 28°C temperature treatments, it was excluded from analysis for carbon and nitrogen.

Calcification rate

Calcification rates of the coralline algae, *C. officinalis* and *A. anceps*, were estimated using the 'alkalinity anomaly technique' (Chisholm & Gattuso 1991). Fresh thalli were collected and incubated in pH and temperature treatments for 2 wk as described above. Following the incubation period, approximately 0.2 g of each algal species were placed in individual 40 ml airtight containers containing experimental seawater and no air (3 pH × 3 temperature treatments) with 3 replicates per treatment, for each species. Small amounts of dissolution occur in the dark when respiration occurs instead of photosynthesis. Therefore, closed containers were left for 24 h in water baths to regulate temperature and under 16 h night:8 h day of 20–30 µmol photons m^{-2} s^{-1} light to allow for the measurement of a daily calcification rate. Initial and final measurements of total alkalinity of the experimental seawater were measured as described above. The production of calcium carbonate by the thalli was calculated as:

$$\Delta CaCO_3 = (T_{Ai} - T_{Af}) \times 0.5 \times 1000 \times V \times T^{-1} \times W^{-1}$$

where, $\Delta CaCO_3$ is the rate of $CaCO_3$ production (µmol h^{-1} g^{-1}), T_{Ai} and T_{Af} are the initial and final total alkalinity (mmol l^{-1}) respectively, V is the volume of seawater in the closed containers (l), T is time (h) and W is wet weight of the algae (g).

Statistical analyses

For *A. anceps*, *C. officinalis* and *Ulva* sp., the percentage growth, calcification rates (if applicable), pH drift and carbon to nitrogen ratio (C:N) were contrasted among treatments by analyses of variance (ANOVA) with pH and temperature as fixed factorial factors. For *S. linearifolium*, *D. pulchra* and *L. decussata*, the same response variables were analysed with linear mixed effects models with temperature

and pH as fixed factorial factors and genetic clones (i.e. replicates from the same plant) as a random blocking factor using the 'nlme' package (Pinheiro et al. 2017). The proportion of variance explained by random effects were calculated for each species by approximating fitted values for fixed effects and extracting variance components for random effects and the residuals. Linear mixed models were only used for 3 of the 5 species because clones were not used for *Ulva* sp., *A. anceps* or *C. officinalis* (and thus there was no random effect of clone). Statistical tests were conducted in the R environment (R Development Core Team 2016). If replicates among treatments were unbalanced, a restricted maximum likelihood framework was used (as opposed to maximum likelihood) for mixed effects tests. Marginal sums of squares (type III SS) were used to partition model components when treatments were unbalanced for linear models with only fixed effects (i.e. *C. officinalis*, *A. anceps* and *Ulva* sp.). For all tests, model residuals were examined for homogeneity of variance and normality using diagnostic plots (e.g. residual vs. fitted plot and *Q*–*Q* plot, respectively), and were transformed accordingly if needed (see Tables 1–3 for specific transformations). When significant differences among treatments were detected, post hoc Tukey pairwise tests among means were conducted with the 'glht' function in the 'multcomp' package (Hothorn et al. 2008). When an interaction between treatments was significant, multiple comparisons were conducted using the 'multcompView' package.

RESULTS

Algal growth

All algae increased in mass over the 2 wk experimental period across all treatments. The RGR of *Amphiroa anceps* was the least variable (average RGR ± SE range: 9.31% ± 1.71 to 45.31% ± 4.52) and relative growth of *Sargassum linearifolium* was the most variable (25.82% ± 19.46 to 232.21% ± 82.93) across all pH and temperature treatments (Fig. 1). Algal growth was significantly lower in the 28°C temperature treatment than the ambient (23°C) treatment for all algal species, with these declines consistent among pH treatments (Fig. 1, Table 1). Overall, the reductions in growth

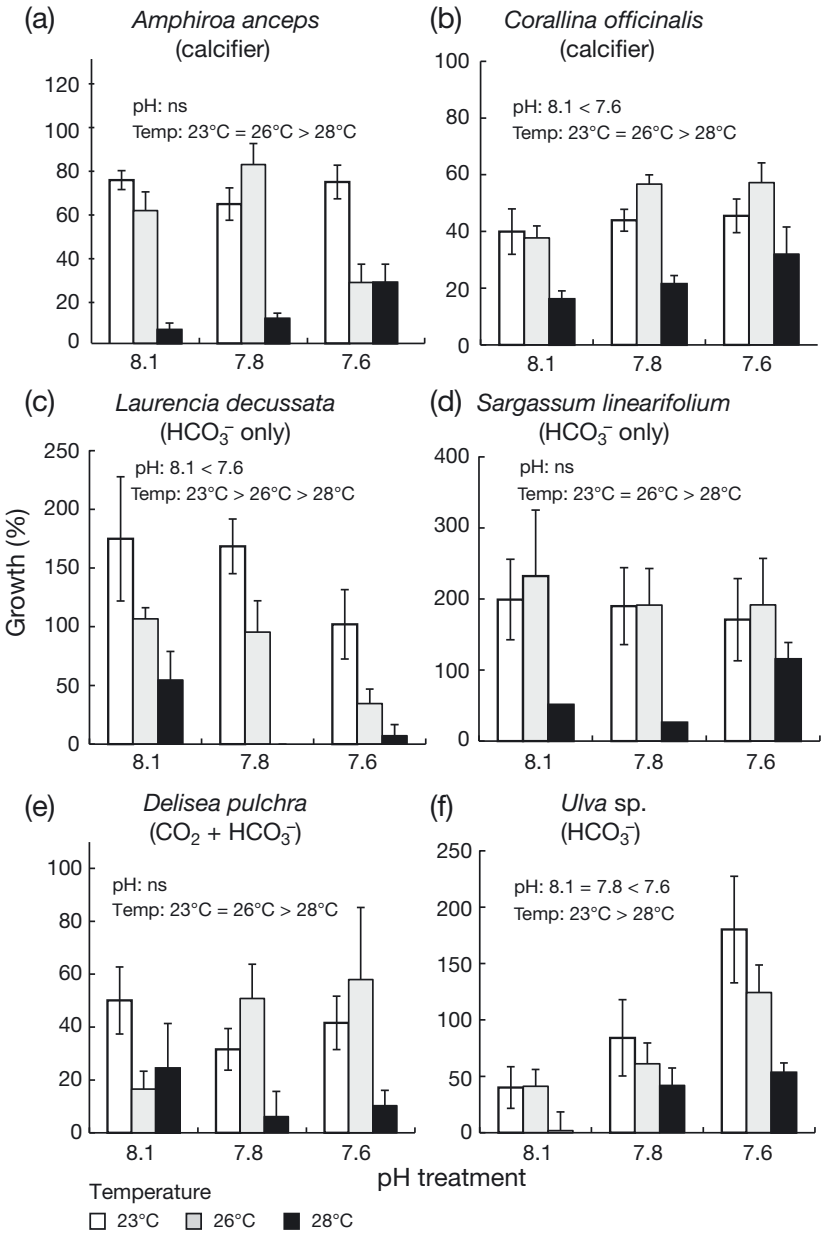

Fig. 1. Percent growth of all algal species in each of the 9 combinations of pH (8.1, 7.8 and 7.6) and temperature (23°C, 26°C and 28°C) after 2 wk (with the exception of *Ulva* sp., after 8 d, and *A. anceps*, after 9 d). Data are means ± SE, n = 14 or 15. Negative growth in the pH 7.8/28°C treatment was excluded. Note that the scale of the axes differ among species

Table 1. Analyses of the growth (%) of each algal species in the pH and temperature (3 pH × 3 temperature treatments with 5 replicates each). For *Ulva* sp. *Amphiroa anceps* and *Corallina officinalis*, growth was analysed with analyses of variance with temperature and pH as fixed factorial factors. For *Sargassum linearifolium*, *Delisea pulchra* and *Laurencia decussata*, growth was analysed with linear mixed models with temperature and pH as fixed factorial factors and individual thallus as a random factor. Significant treatment effects ($p \leq 0.05$) are in bold. Note that *S. linearifolium* is missing one observation due to a death in the pH 8.1/28°C treatment (Den df = 31)

		Sargassum linearifolium[a]		*Delisea pulchra*[a]		*Laurencia decussata*[a]	
Source	df	*F*	p	*F*	p	*F*	p
pH	2, 32	1.78	0.19	0.35	0.7	4.28	**0.02**
Temperature	2, 32	8.72	**<0.01**	6.75	**<0.01**	21.5	**<0.01**
pH × Temperature	4	1.54	0.22	1.82	0.15	1.44	0.24

		Ulva sp.			*Amphiroa anceps*			*Corallina officinalis*		
Source	df	MS	*F*	p	MS	*F*	p	MS	*F*	p
pH	2, 36	1.92	7.22	**<0.01**	4.62	0.04	0.96	730.33	4.38	**0.02**
Temperature	2, 36	0.91	3.44	**0.04**	3976.71	34.31	**<0.01**	2991.7	17.93	**<0.01**
pH × Temperature	4, 36	0.13	0.49	0.74	230.93	1.99	0.12	126.59	0.76	0.56

[a]Species with growth data 4th-square root transformed

ranged from a 30% decline in *Corallina officinalis* to a 95% decline in *Ulva* sp. between the 23°C and 28°C treatments (Fig. 1). Bleaching of the tissues of *Delisea pulchra*, *A. anceps* and *Laurencia decussata* were observed in the 28°C treatments.

In contrast to temperature, the effects of pH on algal growth varied among species (Fig. 1, Table 1). The relative growth rates of *Ulva* sp. and *C. officinalis* increased with decreasing pH, while the relative growth rate of *L. decussata* decreased (Fig. 1b,c,f). There was no detectable effect of pH on the growth of *S. linearifolium*, *A. anceps* and *D. pulchra* (Fig. 1, Table 1). For the 3 algal species in which individual clones were used across treatments, the within-clone variability (i.e. the random effect) for algal growth was low (*S. linearifolium*: 7% of model variation; *D. pulchra* and *L. decussata*: <1% of model variation).

Tissue quality and carbon:nitrogen ratio

Carbon:nitrogen ratios for each macroalgal species were relatively insensitive to changes in pH and temperature (Fig. 2, Table 2). The only exception was the higher C:N ratio of *S. linearifolium* in the 28°C treatment compared with the 23°C treatment (Fig. 2d, Table 2). This change was driven by an increase in carbon content of *S. linearifolium* with temperature ($F_{2,30}$ = 11.80, p = 0.0002, Tukey's post hoc: 23°C < 26°C = 28°C; Table S2 in the Supplement); no changes in the nitrogen content of *S. linearifolium* were detected (Figs. S2 & S3, Tables S3 & S4 in the Supplement). In addition, increasing temperature led to increases in the absolute nitrogen and carbon content of *A. anceps* and *C. officinalis* (Figs. S1 & S2 in the Supplement), resulting in no change to the C:N ratio (Fig. 2). The within-clone variability (i.e. the random effect) associated with C:N ratios was low for *D. pulchra* and *L. decussata* (<1% of model variation), but high for *S. linearifolium* (64% of model variation).

CCMs and pH drift

The pH compensation points of *S. linearifolium*, *Ulva* sp. and *L. decussata* were all >9.0, indicating that these species use HCO_3^- as their main carbon source. The pH compensation point of *D. pulchra* was <9.0, indicating that it does not directly take up HCO_3^- and uses CO_2 for photosynthesis (Fig. 3, $F_{5,51}$ = 63.95, p < 0.01).

Calcification rate

The effect of water temperature on the calcification rates of both *A. anceps* and *C. officinalis* was not consistent among pH treatments (a significant Temperature × pH interaction, Table 3). For *A. anceps*, calcification rate was similar across all temperatures within the highest (8.1) pH treatment, but decreased significantly with temperature within the 2 lower pH (7.8 and 7.6) treatments (Fig. 4a). For *C. officinalis*, calcification rates decreased with increasing temperature

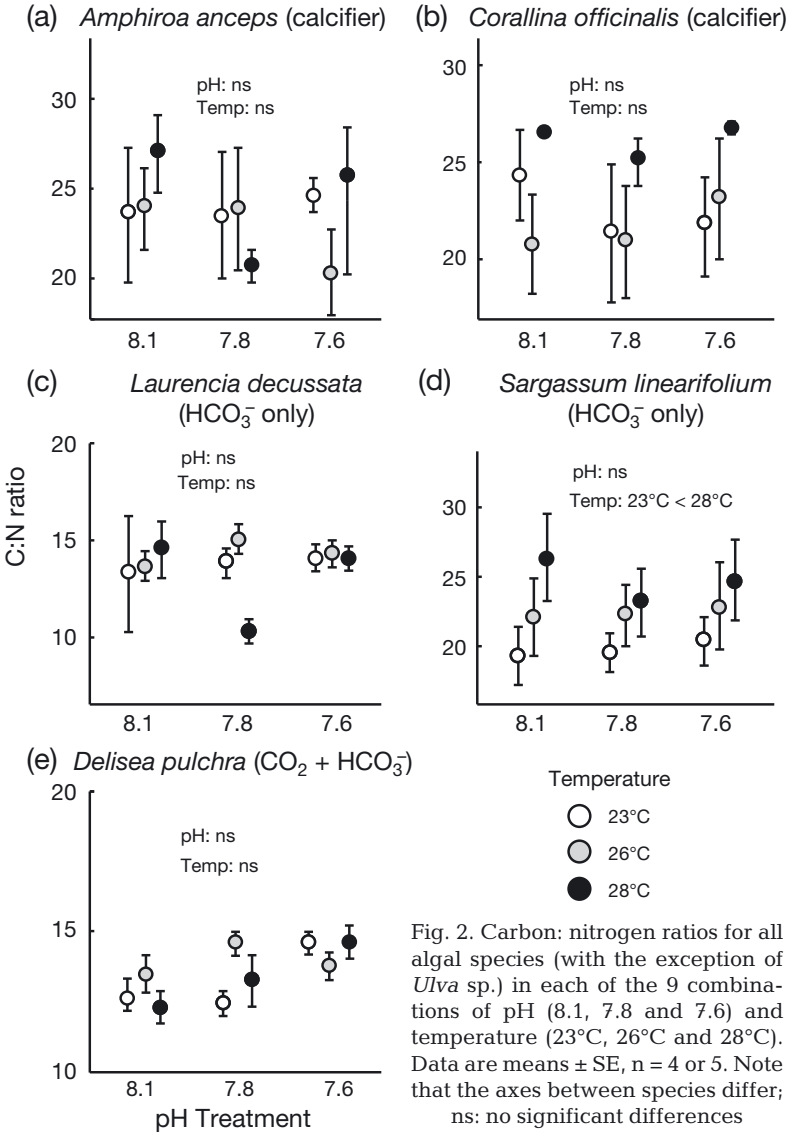

Fig. 2. Carbon: nitrogen ratios for all algal species (with the exception of *Ulva* sp.) in each of the 9 combinations of pH (8.1, 7.8 and 7.6) and temperature (23°C, 26°C and 28°C). Data are means ± SE, n = 4 or 5. Note that the axes between species differ; ns: no significant differences

within the pH 8.1 treatment, but did not differ among temperatures in the pH 7.8 and 7.6 treatments (Fig. 4b, Table 3). Calcification rates of *A. anceps* were negative at 28°C in the pH 7.8 and pH 7.6 treatments, and at 26°C in the pH 7.6 treatment, indicating dissolution of calcite. Similarly calcification rates of *C. officinalis* were negative at 28°C in the pH 7.6 (Fig. 4b).

DISCUSSION

Increasing sea surface temperatures and OA are widely viewed as 2 of the major threats to habitat-forming marine species; however, the relative impact of these stressors on macroalgae in the subtropics is largely unknown. We showed that increasing water temperature suppressed the growth of 6 species of macroalgae in the subtropics and that these effects were independent of OA (i.e. decreasing pH). In contrast, the effects of increasing pCO_2 varied among species, with growth increasing in *Ulva* sp. and *Corallina officinalis*, decreasing in *Laurencia decussata*, and not changing in *Sargassum linearifolium*, *Amphiroa anceps* and *Delisea pulchra.* Interestingly, the differential responses of macroalgae to decreasing pH were unrelated to the presence of calcification in the algal tissues, or their mechanism of carbon uptake, 2 processes that are predicted to be sensitive to decreased pH (Hurd et al. 2009, Diaz-Pulido et al. 2016, Cornwall et al. 2017a). Calcium carbonate began dissolving from calcified macroalgae at low pH (7.6), but growth was either unaffected or enhanced at this pH level. These findings illustrate the complexities of the responses of marine macroalgae, both among species and between physiological processes, to changing environmental conditions.

Rearing macroalgae at 28°C (i.e. 5°C above ambient) for 2 wk reduced growth by 30–95% compared with conspecifics reared at ambient temperatures. This decline in growth with increasing temperature is consistent with previous experimental studies that show significant reductions in biomass (5–35% reductions) of most subtropical macroalgae in response to short-term (2–3 wk) exposure to elevated (3–4°C above ambient) temperatures (Poore et al. 2016, Provost et al. 2017). Such short-term increases in temperature are typical of marine heatwaves, although +5°C is extreme. For example, the 2016 El Niño led to seawater temperatures off of Muttonbird Island in the Coffs Harbour region being 1.8–3.5°C above the long-term average for 20 d in late February and early March 2016 (thermistor data provided by the NSW Department of Primary Industries). Warming of 2–4°C above long-term averages off the Western Australian coast caused an ecosystem shift from habitat-forming kelps to a tropical/subtropical system dominated by algal turf communities and corals (Wernberg et al. 2016). The differential effects of elevated temperatures on macroalgae reported in previous laboratory and field studies and the present study (i.e. lethal vs. sublethal) are most likely attrib-

Table 2. Analyses of the C:N ratio of each algal species (with the exception of *Ulva* sp.) for the pH and temperature treatments (3 pH × 3 temperature treatments with 4 or 5 replicates each) (details of predictor variables as in Table 1). Significant treatment effects ($p \leq 0.05$) are in bold font and associated post hoc Tukey's tests are presented. Note that the residual df differs slightly among species due to a loss of replicates during stable isotope processing (34 for *Corallina officinalis*, 34 for *Delisea pulchra* and 35 for *Laurencia decussata*)

		Sargassum linearifolium[a]		*Delisea pulchra*		*Laurencia decussata*	
Source	df	*F*	p	*F*	p	*F*	p
pH	2,30	0.03	0.97	2.30	0.12	1.28	0.30
Temperature	2,30	4.48	**0.02**	1.13	0.34	1.44	0.26
pH × Temperature	4,30	0.25	0.91	0.69	0.61	2.19	0.10

		Amphiroa anceps			*Corallina officinalis*[b]		
Source	df	MS	*F*	p	SS	*F*	p
pH	2	17.06	0.44	0.65	18.9	0.32	0.73
Temperature	2	13.55	0.35	0.71	158.5	2.65	0.08
pH × Temperature	4	28.59	0.73	0.58	31.2	0.26	0.90
Residual	36	39.08			1015.7		

[a]C:N data for *S. linearifolium* was transformed ($x^{-1.5}$)
[b]Marginal sums of squares (Type III SS) were used to partition model components for *C. officinalis*

uted to the duration of exposure to elevated temperatures (i.e. 10 wk heatwave vs. 2 wk experimental period). Importantly, short-term marine heatwaves are predicted to increase in severity and frequency as greenhouse gas emissions continue to increase (Harley et al. 2006), with future suppression of growth and survivorship of macroalgae within their current ranges, and shifts in their geographic distributions likely. Understanding the impacts and developing strategies to mitigate the effects of ocean warming on macroalgae is a matter of urgency.

The responses of the 6 macroalgal species to OA were variable, and did not conform to expectations based on the presence of calcification in the algal tissues or the presence of a CCM. Macroalgal taxa that have a CCM are predicted to either benefit or be unaltered by increased pCO_2 depending on the metabolic costs of running a CCM (e.g. Zou 2005, Wu et al. 2008, Hepburn et al. 2011, Raven et al. 2011). While the growth of 2 of the HCO_3^--using species (*S. linearifolium* and *Ulva* sp.) conformed to expectations and is consistent with previous studies (Axelsson et al. 1995, Wu et al. 2008), the reduced growth of *L. decussata* (the third HCO_3^- user) under low pH was counter to expectations. Carbonic anhydrase (CA) is an important enzyme for CCMs, converting HCO_3^- and H^+ ions to CO_2 and H_2O for CO_2 to then be used for photosynthesis. It has been suggested that in some instances, the production of CA can be suppressed if the algae are grown in a high pCO_2 environment, rendering the algae less efficient at carbon uptake (Gao et al. 1993). However, in other instances, such as in coralline species, CA activity can be stimulated in a high pCO_2 environment as found in *C. officinalis* (Hofmann et al. 2013). It has also been shown that CA assists in calcification of coralline species and may facilitate synergistic interactions between photosynthesis, calcification and growth (Hofmann & Bischof 2014). Therefore, a more detailed understanding of the type(s) of CCMs and their energetic costs, how they relate to calcification processes, and the plasticity in response to changing pCO_2 is needed to predict the likely responses of individual species to OA.

OA is expected to be particularly deleterious for calcifying species such as *C. officinalis* and *A. anceps* with the reduced carbonate saturation state of increased pCO_2 making it more energetically costly for calcareous species to calcify and subsequently grow (Diaz-Pulido et al. 2014). Species from the order Corallinales are the only calcifying macroalgae to deposit the highly soluble high-Mg calcite in both the cell surface and in the cell walls (Littler 1976), which makes them most vulnerable to dissolution in OA conditions (Hofmann & Bischof 2014). OA had a negative effect on calcification in both calcifying species of this study, but the effects on the growth of these species differed. Reduced calcification in response to increased pCO_2 has been reported across a wide range of coralline algae (Kroeker et al. 2010, Hofmann & Bischof 2014) and is exacerbated by increased temperature with warming further reducing calcification and causing dissolution (Martin & Gat-

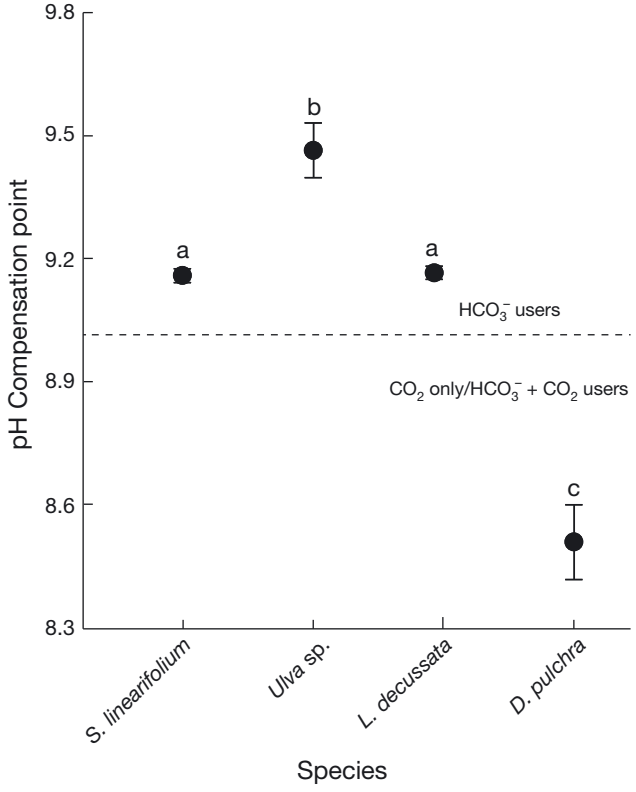

Fig. 3. pH compensation points for non-calcifying algal species maintained in a closed system for 24 h. Data are means ± SE, n = 9. Treatments that share a letter do not differ in post hoc tests

tuso 2009), as found here. In our study, the absolute growth (change in mass) was positive, albeit small, across all treatments; ranging from 0.0034 g (±0.0008 SE) in the pH 8.1 and 28°C treatment to 0.0137 g (±0.0025 SE) in the pH 7.6 and 26 °C treatment in *A. anceps* and 0.0082 g (±0.0012 SE) in the pH 8.1 and 28°C treatment to 0.0322 g (±0.0022 SE) in the pH 7.8 and 26°C treatment in *C. officinalis* (Fig. S3 in the Supplement). Interestingly, the treatments that yielded the highest growth rates (i.e. increase in mass) of the calcifying species also resulted in the highest rates of calcium dissolution (Fig. 4, Fig. S3), suggesting that organic matter accumulation was greater than the rate of dissolution (Hofmann & Bischof 2014). Previous studies investigating the effects of OA on *Corallina* have reported mixed results, with some reporting declines in both mass and calcification (*C. officinalis*: Hofmann et al. 2012; *C. sessilis*: Gao & Zheng 2010), while others have reported no change to growth and photosynthesis, but increased dissolution (*C. officinalis*: Yildiz et al. 2013; *C. elongata*: Egilsdottir et al. 2013). The different responses of these species is difficult to resolve and requires further investigation, but could relate to differences in experimental protocols and environmental conditions (e.g. light) among studies, or species-specific differences in the ratio of $CaCO_3$ and other organic matter (Hofmann & Bischof 2014). For example, absence of growth could be attributed to a reduction in $CaCO_3$ caused by dissolution (as growth is mostly determined by change in mass) or the increasing energetic demand in maintaining calcification under reduced calcite saturation states (Yildiz et al. 2013). This is possible as photosynthesis and calcification have been shown to not only be positively linked, but also temperature-dependant processes leading to the potential for growth and calcification even if dissolution is present (Digby 1977, Hofmann & Bischof 2014).

Although OA reduced calcification of both coralline species in our study, it is important to recognise that some coralline algae have been shown to be able to calcify, even under moderate OA conditions (pH 7.7–7.8) predicted for the year 2100 (Kamenos et al. 2013, 2016). This may be due to the diffusion boundary layer that can form in some species as a result of flow and metabolic processes, and subsequently buffers the corrosive effect of decreased pH (Cornwall et al. 2014). Alternatively, some coralline algae are able to increase the pH in the microenvironment between their surface and the seawater (Hofmann et al. 2016) and at the site of calcification

Table 3. Analyses of variance for the effects of pH and temperature on calcification rates (µmol g^{-1} d^{-1}) of *Corallina officinalis* and *Amphiroa anceps* (3 pH × 3 temperature treatments with 3 replicates each). Significant treatment effects ($p \leq 0.05$) are in bold. Contrasts between main effects were explored with relation to interactions among treatment levels (see Fig. 2)

		Corallina officinalis			*Amphiroa anceps*		
Source	df	MS	*F*	p	MS	*F*	p
pH	2	4.24×10^{-6}	16.15	**<0.01**	1.06×10^{-7}	22.24	**<0.01**
Temperature	2	6.76×10^{-6}	25.77	**<0.01**	9.49×10^{-6}	19.83	**<0.01**
pH × Temperature	4	9.81×10^{-5}	3.74	**0.02**	2.42×10^{-6}	5.05	**<0.01**
Residual	18	2.62×10^{-5}			4.79×10^{-5}		

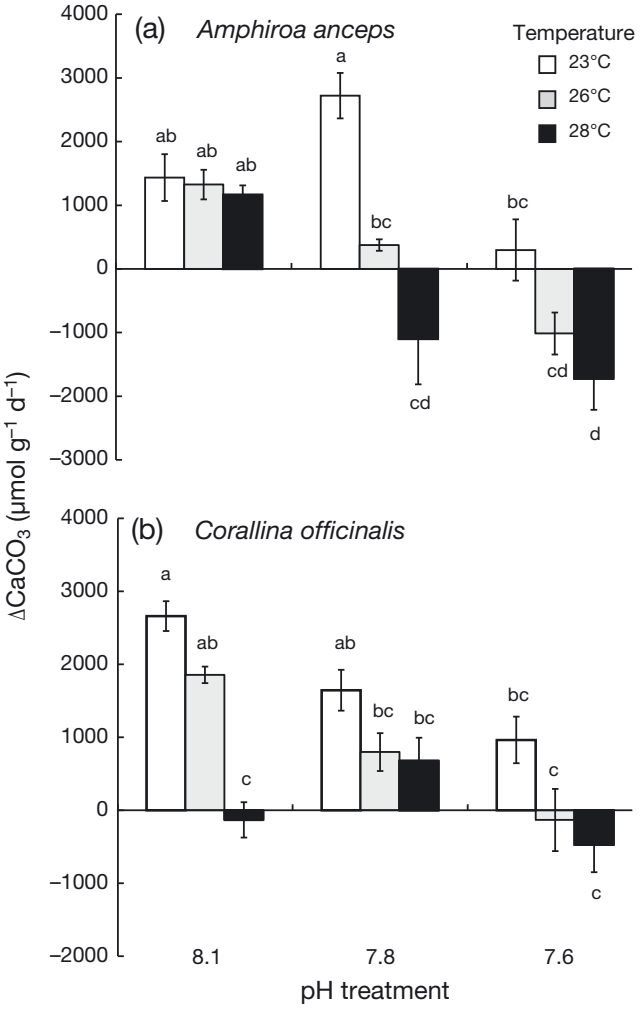

Fig. 4. Calcium carbonate ($CaCO_3$) production of (a) *Amphiroa anceps* and (b) *Corallina officinalis* in each of the 9 combinations of pH (8.1, 7.8 and 7.6) and temperature (23°C, 26°C and 28°C) in a closed system over 24 h. Data are means ± SE, n = 3. Treatments that share a letter do not differ in post hoc tests. Note that the scales of the axes between species differ

within the calcifying fluid (Cornwall et al. 2017a). These processes appear to be context, species and mechanism specific (Roleda et al. 2012, Cornwall et al. 2017a), but may explain why growth and calcification for both study species were decoupled. The variability in growth and calcification responses of both calcifying species to acidification is consistent with other studies (e.g. Hofmann et al. 2012, Comeau et al. 2013). Calcification is a key algal trait that allows persistence of coralline algae in heavily grazed systems and so reduced calcification is likely to have important consequences in a changing environment (Büdenbender et al. 2011, Hofmann et al. 2012).

Understanding how other changes to plant tissue quality, such as increases in the C:N ratio, potentially making algae less palatable to herbivores, will also be important for predicting the ecosystem wide impacts of a changing ocean. The C:N ratio of most algae in this study was unaffected by both temperature and OA. The only exception was *S. linearifolium* in which the C:N ratio increased with temperature. However, the fixed effects of the analysis did not account for much of the variation. The lack of change to the C:N ratio in these 6 species may be due to the short duration of this study, and quantifying potential longer-term changes to the nutritional quality will be important for understanding the likely outcomes of plant–herbivore interactions. Many other studies have reported no change to %C, %N or the C:N ratio in response to increased temperatures (e.g. Simonson et al. 2015) or a combination of pH and temperature (e.g. Poore et al. 2013, Mensch et al. 2016). However, Brown et al. (2014) reported an increase in the C:N ratio of *Macrocystis pyrifera* in response to an increase in temperature (+3°C) and OA (1500 µatm) after a 4 wk experimental period. As herbivores strongly determine algal abundance in coastal systems (Poore et al. 2012), it is important to consider how changes to both herbivore grazing rates and algal palatability may be affected by climate change (Kroeker et al. 2010). The control of primary producers by herbivores is predicted to be stronger with increased temperature (O'Connor 2009), and consumption rates have been shown to decrease (Siikavuopio et al. 2007, Falkenberg et al. 2014), increase (Cummings et al. 2011) or be unaffected (Gooding et al. 2009) by increasing pCO_2. Few studies have investigated whether these changes are best explained by effects on the consumers or producers.

Predicting the effects of ocean warming and acidification is also complicated by potential interactions between these 2 stressors (e.g. Wernberg et al. 2012, Koch et al. 2013, Kroeker et al. 2013a, Przeslawski et al. 2015). Our results suggest that although pH and temperature acted independently on macroalgal growth, the effects of pH on the calcification rate of the 2 coralline algae did vary with temperature. As calcification scales positively with temperature (Martin et al. 2006), increasing temperature may buffer the negative effects of OA (Byrne et al. 2013). However, it seems that for calcareous algae, warming exacerbates the negative effects of pH on calcification (Anthony et al. 2008, Diaz-Pulido et al. 2012, Sinutok et al. 2012, this study). This might be because warming increases the rate of dissolution of algal $CaCO_3$ (e.g. Reyes-Nivia et al. 2013). Coralline algae

are composed of high-Mg calcite, a highly soluble form of $CaCO_3$, which is predicted to become undersaturated in the ocean worldwide as a result of predicted warming for the year 2100 (Kuffner et al. 2008, Martin & Gattuso 2009). This is important for calcareous algae because of the role $CaCO_3$ plays in their ecology. Coralline algae are critical to coastal ecosystems as they provide structure, habitat, food, induce larval settlement and metamorphosis of a variety of different species, and play key roles in carbonate deposition (Nelson 2009). As coralline algae also range from polar to tropical habitats and from deep to shallow zones, the synergistic interaction between pH and temperature on calcification seen in this study could be detrimental to ecosystems worldwide (Nelson 2009).

In summary, increasing ocean temperatures appear to pose a greater threat than OA to the growth and persistence of subtropical macroalgal assemblages. Elevated water temperatures had consistent negative effects on macroalgal growth, while the effects of OA were species specific. Counter to expectations, changes in macroalgal growth in response to reduced pH were not related to the presence of calcium carbonate in the algal tissues, or inorganic carbon physiology. The results of this study further reinforce the point that macroalgal responses to climate change are species, process and mechanism specific, making it difficult to draw generalities in predicting likely future trajectories. Clearly, further research is needed to understand how marine macrophytes and other marine habitat-forming organisms will be affected by ocean warming and acidification, and how these changes may influence trophic interactions (O'Connor 2009, Kroeker et al. 2013a, Poore et al. 2013) and the structure of communities (Asnaghi et al. 2013, Ferrari et al. 2015). Given the fundamental role that primary producers play in marine food webs and nutrient cycling, the development of an accurate prediction model for future climate change impacts is critical.

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