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Original Article

Responses of seaweeds that use CO_2 as their sole inorganic carbon source to ocean acidification: differential effects of fluctuating pH but little benefit of CO_2 enrichment

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Laboratory studies that test the responses of coastal organisms to ocean acidification (OA) typically use constant pH regimes which do not reflect coastal systems, such as seaweed beds, where pH fluctuates on diel cycles. Seaweeds that use CO_2 as their sole inorganic carbon source (non-carbon dioxide concentrating mechanism species) are predicted to benefit from OA as concentrations of dissolved CO_2 increase, yet this prediction has rarely been tested, and no studies have tested the effect of pH fluctuations on non-CCM seaweeds. We conducted a laboratory experiment in which two ecologically dominant non-CCM red seaweeds (*Callophyllis lambertii* and *Plocamium dilatatum*) were exposed to four pH treatments: two static, pH_T 8.0 and 7.7 and two fluctuating, pH_T 8.0 ± 0.3 and 7.7 ± 0.3. Fluctuating pH reduced growth and net photosynthesis in *C. lambertii*, while *P. dilatatum* was unaffected. OA did not benefit *P. dilatatum*, while *C. lambertii* displayed elevated net photosynthetic rates. We provide evidence that carbon uptake strategy alone cannot be used as a predictor of seaweed responses to OA and highlight the importance of species-specific sensitivity to $[H^+]$. We also emphasize the importance of including realistic pH fluctuations in experimental studies on coastal organisms.

Keywords: carbon dioxide concentrating mechanisms (CCM), dissolved inorganic carbon, macroalgae, non-CCM, ocean acidification (OA), pH variability, physiology, seaweed

Introduction

Anthropogenic greenhouse gas emissions have increased atmospheric CO_2 concentrations from ~280 ppm since the 1850s to ~400 ppm in 2018, and they are projected to reach ~850 ppm by 2100 under the RCP (*Representative Concentration Pathway*) 6.0 scenario (IPCC, 2014; Hurd *et al.*, 2018). The oceans have absorbed ~30% of this emitted CO_2 causing a suite of chemical changes to the seawater carbonate system, termed ocean acidification (OA, Doney *et al.*, 2009). By the year 2100, pH is projected to decline by ~0.3 units from current levels (~pH 8), corresponding to a respective increase in the concentrations of H^+, CO_2, and HCO_3^- of ~100%, 200%, and 14% (Hurd *et al.*, 2009; IPCC, 2014). Substantial international research effort has been undertaken to examine how marine taxa will respond to OA (Gattuso *et al.*, 2015; Hurd *et al.*, 2018). However, as methods for investigating responses and our understanding evolve, further questions arise such as the relative importance of elevated CO_2 and H^+ in influencing the response of organisms to OA (van der Loos *et al.*, 2019).

The majority of experimental studies on the responses of coastal organisms to OA have maintained a constant pH regime for the duration of the experiment (Britton *et al.*, 2016; Wahl *et al.*, 2016; Hurd *et al.*, 2018). Such an approach is representative of the open ocean but does not reflect the dynamic nature of coastal systems where local biological (invertebrate and algal metabolism) and physical (e.g. upwelling events) factors alter carbonate chemistry (Hofmann *et al.*, 2011; Baumann and Smith, 2018). In seaweed-dominated systems, pH fluctuates on diel cycles as dissolved inorganic carbon (DIC) uptake by seaweeds causes an increase in daytime pH, and release of CO_2 overnight via respiration causes pH to decline (Delille *et al.*, 2009; Hofmann *et al.*, 2011; Cornwall *et al.*, 2013). Organisms in these habitats are subjected to highly variable pH and CO_2 concentrations that can be of a similar magnitude to the changes expected to occur due to OA (Hurd *et al.*, 2011; Hurd, 2015). Furthermore, it is likely that organisms will respond differently to OA when pH variability is superimposed on the projected pH in 2100 (Cornwall *et al.*, 2013; Roleda *et al.*, 2015; Britton *et al.*, 2016; Wahl *et al.*, 2016).

Seaweeds are important primary producers that support diverse and productive communities in temperate coastal waters through provision of habitat (Dayton, 1985; Steneck *et al.*, 2002; Hurd *et al.*, 2014), as a settlement substratum for invertebrate larvae (Roberts, 2001; Steneck *et al.*, 2002; Nelson, 2009), and as a food source for herbivores (Steneck *et al.*, 2002; Hurd *et al.*, 2014). Calcifying seaweeds (e.g. coralline algae) are thought to respond negatively to OA as their calcium carbonate skeletons are more costly to maintain under reduced pH (Fabricius *et al.*, 2015; McCoy and Kamenos, 2015; Cornwall *et al.*, 2017a). However, the response of non-calcifying (fleshy) seaweeds to OA has received far less attention (Koch *et al.*, 2013; Kroeker *et al.*, 2013; van der Loos *et al.*, 2019). The meta-analysis of Kroeker *et al.* (2013) suggests that fleshy seaweeds will respond positively to OA, as they will benefit from the projected increase in dissolved CO_2. However, a more recent analysis of the literature suggests that the response of fleshy seaweeds to elevated CO_2 is variable, and partly regulated by their carbon uptake mechanisms and/or their sensitivity to [H+] (van der Loos *et al.*, 2019). In addition, most of the studies used in the meta-analysis used constant pH conditions, which does not mimic the natural environment.

Fleshy seaweeds possess a range of carbon acquisition strategies, making predictions of how they will respond to OA difficult (Hepburn *et al.*, 2011; Cornwall *et al.*, 2017b; van der Loos *et al.*, 2019). Around 65% of seaweed species are able to utilize HCO_3^- (the most abundant form of DIC in seawater) via a carbon dioxide concentrating mechanism(s) (CCM), whereas ~35% rely solely on CO_2 which is taken up by passive diffusion (Raven *et al.*, 2005; Kübler and Dudgeon, 2015). To date, the majority (95%) of studies on fleshy seaweed responses to OA have focused on CCM species and generally have found positive responses or no effects (Cornwall *et al.*, 2012; Koch *et al.*, 2013; Kroeker *et al.*, 2013; van der Loos *et al.*, 2019). Non-CCM species are predicted to benefit from OA, with the additional CO_2 suggested to act as a "fertilizer" by increasing the availability of CO_2 at the site of RuBisCO (Hepburn *et al.*, 2011; Raven, 2011; Cornwall *et al.*, 2015; Kübler and Dudgeon, 2015). However, the response of non-CCM species to OA has been studied experimentally for only three species, with contrasting results: a positive response for one species and no effect for two species (Kübler *et al.*, 1999; Ho and Carpenter, 2017; van der Loos *et al.*, 2019). Expanding this knowledge is critical for understanding how seaweed assemblages may respond to a future higher CO_2 ocean because up to 90% of some seaweed communities can be comprised of non-CCM species (Cornwall *et al.*, 2015).

The response of photosynthetic organisms to fluctuating pH has been investigated for only 14 species with half of these studies on corals which either benefit or are unaffected by pH fluctuations (Dufault *et al.*, 2012; Comeau *et al.*, 2014; Camp *et al.*, 2016; Chan and Eggins, 2017; Cornwall *et al.*, 2018; Enochs *et al.*, 2018). Rates of growth and photosynthesis are generally reduced under fluctuating pH in coralline algae (Cornwall *et al.*, 2013; Johnson *et al.*, 2014; Roleda *et al.*, 2015), although calcification rates in *Hydrolithon reinboldii* are increased (Cornwall *et al* 2018) and unaffected in *Porolithon onkodes* (Johnson *et al.*, 2014). Growth and photosynthesis in coastal species of diatoms are unaffected by pH fluctuations whereas oceanic species are negatively affected (Li *et al.*, 2016). The effect of pH fluctuations on fleshy seaweed has been studied for only two species, both known to operate CCMs: the brown seaweed *Ecklonia radiata* (Britton *et al.*, 2016) and the red *Gracillaria lemaneiformis* (Qu *et al.*, 2017). *E. radiata* growth and photosynthetic rates were elevated in fluctuating pH under current pH levels, but under OA conditions growth was unaffected and photosynthetic rates were reduced. *G. lemaneiformis* displayed reduced rates of growth and photosynthesis in fluctuating pH at current pH levels and was unaffected by fluctuations under OA conditions. There have been no studies on the responses of non-CCM seaweeds to pH fluctuations but their reliance on CO_2 as their inorganic carbon source may make them susceptible to DIC limitation (assuming they are not light limited) during diel pH cycles because as pH increases during the day, only ~0.5% is present as CO_2 (pH_T 8.2). In the context of OA, pH variability is important as the effect of diel fluctuations in pH on a species can be different when the mean pH is at current day levels or the mean pH expected under OA (Johnson *et al.*, 2014; Britton *et al.*, 2016; Qu *et al.*, 2017).

In this study, we address the knowledge gap on the responses of non-CCM seaweeds to fluctuating pH and OA in a laboratory experiment using two highly abundant sympatric red seaweeds (*Callophyllis lambertii* and *Plocamium dilatatum*), known to be non-CCM species, from southeastern Australia (Cornwall *et al.*, 2015). We hypothesised that (i) diel fluctuating pH would cause reduced rates of both growth and photosynthesis under current ocean conditions due to lower concentrations of CO_2 during the day and this reduction would be ameliorated under future ocean conditions, and (ii) both species would have elevated growth and photosynthetic rates under future ocean conditions as the additional CO_2 would alleviate DIC limitation at today's concentrations in both static and fluctuating pH.

Methods

Seaweed collection

Approximately 50 mature individuals of both *Callophyllis lambertii* and *Plocamium dilatatum* were collected by divers using SCUBA at 8–10 m depth on 6 June 2017 at Coal Point, Bruny Island, Tasmania (43.335287°S, 147.324707°E). Individuals were placed in zip-lock bags with enough seawater to prevent desiccation and kept cool in an insulated container during transport to the laboratory 2 h away.

Pre-experimental treatment

To acclimate individuals to experimental conditions, they were cut to experimental size (~1.0 g). Each species was subsequently placed in separate 60 l containers with UV-sterilized seawater filtered to 1 μm under constant aeration at 14°C, which was refreshed daily. Light levels were 80 μmol photons m^{-2} s^{-1} (similar to light levels at the collection site, Supplementary Table S1) on a 12:12 light: dark cycle for 72 h.

Experimental culture conditions

Experiments were conducted in an automated culture system described in van der Loos *et al.* (2019), with minor modifications. The seawater in each of 48 culture chambers was replaced every 4 h with pH-adjusted seawater. Target pH_T was achieved using mass flow controllers (FMA5418A and FMA5402A, Omega Engineering, USA) to control the ratio of air and CO_2 in a mixed gas that was exposed to the incoming seawater from a single header tank using membrane contactors (3M™ Liqui-Cel™ MM-1 × 5.5 Series). The culture system was located in a walk-in temperature-controlled room set to experimental conditions of 14°C, with overhead lighting providing 80 μmol photons m^{-2} s^{-1} as this was similar to light levels measured at the collection site (Supplementary Table S1). Culture chamber seawater was maintained at 13.98°C ± 0.05. An automated spectrophotometric pH_T system (McGraw *et al.*, 2010) was integrated into a feedback system for the mass flow controllers to ensure incoming seawater was within 0.03 pH_T from the target value. Although pH_T was measured between 20.95°C and 23.44°C, values in this manuscript are reported at the experimental temperature (14°C). These calculations were conducted in CO_2 calc (Robbins *et al.*, 2010) using the dissociation constants of Mehrbach *et al.* (1973), refit by Dickson and Millero (1987) and the known alkalinity, temperature, and salinity of the seawater.

Experimental treatments

On day 1 of the 14-day experiment, 24 individuals of each species were added to individual culture chambers. Each individual chamber was exposed to one of four treatments ($n = 6$ replicates for each treatment and species combination): current static ($pH_T = 8.0$), current fluctuating ($pH_T = 8.0 \pm 0.3$), future static ($pH_T = 7.7$), and future fluctuating ($pH_T = 7.7 \pm 0.3$). The range of pH variability for the fluctuating treatments was informed by measurements of pH *in situ* (range of 0.3–0.5, D. Britton, unpublished data). Static treatments consisted of a constant pH_T over 24 h; pH_T in fluctuating treatments increased incrementally by 0.2 units every 4 h in the light and decreased incrementally by 0.2 units every 4 h in the dark.

To assess how the algae modified the chemical conditions of the seawater within the culture chambers during the 4-h water cycle, additional pH_T measurements were made within randomly selected culture chambers for each treatment 3 h 20 min (measurements of pH_T for each treatment: $N = 72$–160) after the seawater was replaced. Seawater samples for nutrient analysis were taken from the header tank ($n = 4$) and in a random subset of chambers for both species at the end of a water cycle (*C. lambertii*: $n = 12$ and *P. dilatatum*: $n = 16$). Samples were filtered with glass microfiber filters (pore size 1.6 μm, Whatman®) and frozen at −20°C in 12 ml polyethylene nutrient tubes. Samples were subsequently thawed and ammonium and nitrate concentrations were determined using a QuickChem® 8000 Automated Ion Analyser (LaChat Instruments).

Seawater samples for DIC measurements were taken on day 13 in a random subset of culture chambers across both fluctuating static treatments ($n = 4$), at the approximate mean pH_T of the current and OA scenarios. Measurements were made when the pH_T of the fluctuating treatments were the same as the static treatments (pH_T 8.01 and 7.64 at the time of measurements). Samples were immediately poisoned with $HgCl_2$ and stored in darkness until later analysis. Measurements were made with a DIC analyser (Apollo SciTech DIC analyser model AS-C3) with an in-built CO_2 analyser (LI-COR LI-7000 CO_2/H_2O analyser). The CO_2 analyser was calibrated with a certified reference material provided by Andrew Dickson, Scripps Institute for Oceanography, San Diego, USA. A_T and DIC at the mean pH_T for each treatment was calculated in CO_2 calc (Robbins *et al.*, 2010) using the constants described above and the known DIC, pH_T, temperature, and salinity of the incoming seawater at pH 8.01 and 7.64).

Biotic responses

Relative growth rates (wet weight)

Individuals were blotted dry to remove surface water and weighed immediately prior to, and at the end, of the experiment. Relative growth rates (RGR) were calculated according to Kain (1982).

Photosynthetic and respiration rates

On day 13, oxygen evolution (i.e. net photosynthesis) was measured within each culture chamber at random over 2 h under the experimental irradiance; oxygen consumption (i.e. respiration) was measured for 1 h in the dark. All net photosynthesis measurements were made between 10: 00 and 16: 00 and respiration measurements between 20: 00 and 23: 00. Dissolved oxygen measurements were made with a portable oxygen meter and optical probe (Hach®, HQ40D Portable Multi Meter and LDO™ probe). Net photosynthetic rates and respiration rates were expressed as μmol O_2 g^{-1} h^{-1} released or consumed, respectively.

Pigment content, tissue C and N, and stable C isotopes

At the end of the experiment, tissue was cut (∼800 mg) and immediately frozen at −20°C, then freeze-dried the following day (Labconco® FreezeZone® 4.5). Samples were stored frozen at −20°C until analysis for chlorophyll *a*, phycocyanin, phycoerythrin, % C, % N, and stable C isotopes.

Chlorophyll *a* content was calculated by adding 20 mg of freeze-dried tissue to 4 ml of ethanol and incubating for 24 h at 4°C and shaken occasionally (Pritchard *et al.*, 2013). Following incubation samples were centrifuged at 15 000*g* for 20 min at 4°C (Pro-Research, Centurion Scientific Ltd) and the absorbance of aliquots was read at 649 and 665 nm with a UV-VIS spectrophotometer (Dynamica Scientific Ltd., Halo RB-10 UV-VIS ratio beam spectrophotometer). The concentration of chlorophyll *a* in extracts were calculated using the equations of Ritchie (2006) and expressed as mg g^{-1} dry weight.

For phycocyanin and phycoerythrin, 20 mg of freeze-dried algal tissue was ground with a mortar and pestle and incubated in 5 ml of 0.1 M phosphate buffer for 24 h at 4°C and shaken occasionally (Pritchard *et al.*, 2013). Following incubation, samples were centrifuged at 15 000*g* for 20 min. Aliquots were decanted, and absorbance was read at 564, 592, 455, 618, and 645 nm. The pellet was resuspended in 3 ml of the phosphate buffer and incubated for a further 24 h at 4°C. Samples were centrifuged at 15 000*g* for 20 min at 4°C and the absorbance of aliquots were read the same as in the first extraction. The concentration of phycocyanin and phycoxanthin in the extracts were calculated using the equations of Beer and Eshel (1985) and expressed as mg g^{-1} dry weight.

% C, % N, C: N, and $\delta^{13}C$ were determined using the methods outlined in Britton *et al.* 2016). Known amounts (ca. 5 mg) of freeze-dried samples were weighed into tin cups (Sercon, U.K.) and analysed using a NA1500 elemental analyser coupled to a Thermo Scientific Delta V Plus via a Conflo IV. Combustion and reduction were achieved at 1020°C and 650°C, respectively. Isotope values were normalized to the VPDB scale via a three-point calibration using certified reference material and both precision and accuracy were ±0.1% (1 SD). % C and % N composition for C: N values were calculated by comparison of mass spectrometer peak areas to those of standards with known concentrations.

Statistical analysis

All analyses were conducted in the statistical software R v. 3.5.0. (R Core Team, 2017). Two-way factorial ANOVAs were used to assess whether there were differences between treatments for all response variables with the fixed factors: "Type" (two levels: fluctuating and static) and "Era" (two levels: current and future) and the Type × Era interaction. When a significant interaction was detected (at $\alpha = 0.05$), separate one-way ANOVAs were used to determine differences between "Type" at each level of "Era." All model fits were inspected to ensure they conformed to the assumptions of the ANOVA model (homogeneity of variances and normality of residuals) using residual vs. fitted plots and normal Q–Q plots. When inspections of the model fit suggested the assumptions were not met, the Box-Cox method (Box and Cox, 1964) was used to determine an appropriate transformation (see Table 1). The Box-Cox method suggested drastic transformations for three response variables (C:N, %C, and $\delta^{13}C$ in *C. lambertii*) of Y^{-8}, Y^{8}, Y^{-20} respectively. As these transformations were extreme and did not change the interpretation of the model (i.e. no change to significance), we present the results from the untransformed data for these three responses. The ANOVA tables for the transformed data of C:N, %C, and $\delta^{13}C$ can be seen in Supplementary Table S2. Three individuals became necrotic within a week of starting the experiment (future fluctuating: 1 *P. dilatatum* individual, future fluctuating: 2 *C. lambertii* individuals), and were excluded from all analyses.

Results

Experimental culture conditions

pH_T levels were maintained close to target values. The mean pH_T (with standard error in parentheses) of each treatment was as follows: current static = 8.01 (±0.004); future static = 7.71 (± 0.009); current fluctuating = 8.10 (± 0.013); and future fluctuating = 7.59 (± 0.018). Note that fluctuating treatments have inherently more variability around the mean pH_T. A summary of the carbonate system parameters is shown in Table 2. The mean pH_T of seawater entering the culture chambers at the beginning of each 4-hourly cycle is shown in Figure 1. Seaweed metabolic processes (photosynthesis and respiration) had minimal impact on the pH_T within culture chambers by the end of the cycle (3 h 20 min after each seawater renewal). The average change in pH_T for each treatment at each water cycle is shown in Supplementary Table S3. Average concentrations of ammonium and nitrate at the beginning of each water cycle were $1 \pm 0.05\,\mu M$ and $3.01 \pm 0.07\,\mu M$, respectively. Final concentrations at the end of the water cycle were $1.13 \pm 0.04\,\mu M$ for ammonium and 1.04 ± 0.07 for nitrate.

Biotic responses

Relative growth rates (wet weight)

RGRs of *C. lambertii* were 59% higher on average under static pH relative to fluctuating pH in both current and future ocean conditions (Figure 2a, Table 1). There were no significant differences in the RGR of *P. dilatatum* between treatments (Figure 2a, Table 1).

Photosynthetic and respiration rates

Net photosynthetic rates were 56% higher for *C. lambertii* cultured under static pH relative to fluctuating pH treatments (pooled means of current and future ocean conditions, interaction non-significant, Figure 2b, Table 1). *C. lambertii* also had 41% higher net photosynthetic rates when cultured under future ocean conditions relative to current conditions (pooled means of static and fluctuating treatments, interaction non-significant, Figure 2b, Table 1). No differences in the net photosynthetic rates of *P. dilatatum* were observed between treatments (Figure 2b, Table 1). Respiration rates were 470% higher on average in *C. lambertii* and 194% higher on average in *P. dilatatum* grown under future ocean conditions relative to current conditions regardless of whether pH was static or fluctuating (Figure 2c, Table 1).

Pigment content, tissue C and N, and stable C isotopes

No significant differences in chlorophyll *a* content was observed between treatments for either species (Table 1). Phycocyanin content was, on average, 37% higher for *P. dilatatum* cultured under current ocean conditions relative to future conditions, regardless of whether pH was static or fluctuating (Table 1) whereas phycocyanin content did not vary significantly between treatments for *C. lambertii* (Table 1). Phycoerythrin was undetectable in both species.

C: N ratios were 7% higher on average for *C. lambertii* cultured under static pH relative to fluctuating pH (Figure 3a, Table 1), while *P. dilatatum* had 6% higher C: N ratios on average under fluctuating pH relative to static pH (Figure 3a, Table 1). There was a significant interaction between "Type" and "Era" for % C (Figure 3b, Table 1) and % N in *C. lambertii* (Figure 3c, Table 1). The interaction for % C was caused by % C being lower in individuals cultured under fluctuating pH, but only under future ocean conditions. The interaction for % N was driven by % N content being higher in individuals cultured under fluctuating pH in current ocean conditions while no differences were detected under future ocean conditions. The % C of *P. dilatatum* were not significantly different between treatments (Figure 3b, Table 1) while the % N values were 0.16% (actual value) higher in individuals cultured under static pH relative to individuals cultured under fluctuating pH (Figure 3c, Table 1). $\delta^{13}C$ values were unaffected by treatment in *C. lambertii*, while *P. dilatatum* had on average more negative $\delta^{13}C$ values under future ocean conditions (Table 1). Both species had $\delta^{13}C$ values lower than −30 (*C. lambertii*: -35.49 ± 0.14 on average across treatments, *P. dilatatum*: -32.66 ± 0.19 on average across treatments) indicating that both species were non-CCM species.

Discussion

Experimental treatments simulating diel fluctuations in the seawater carbonate system (i.e. pH/H^+, CO_2, HCO_3^-) and OA

Table 1. Analysis of variance (ANOVA) table for all response variables.

Species	Response	Differences	*p*-value			Transformation
			Type	Era	Type * era	
C. lambertii	RGR	S > FL	**0.007**	0.24	0.45	
	Net PS	S > FL, F > C	**0.007**	**0.041**	0.086	
	Respiration	F > C	0.44	**0.0008**	0.37	$Y^{0.4}$
	C: N	S > FL	**0.035**	0.34	0.18	
	% N	**CF > CS**, FF = FS	0.07	0.19	**0.008**	
	% C	CF = CS, **FF < FS**	0.28	0.55	**0.009**	
	δ^{13}C	–	0.13	0.25	0.29	
	Chlorophyll	–	0.69	0.075	0.52	$\log_e Y$
	Phycocyanin	–	0.79	0.7	0.13	$Y^{-0.6}$
P. dilatatum	RGR	–	0.96	0.1	0.58	
	Net PS	–	0.29	0.45	0.22	
	Respiration	F > C	0.089	**0.013**	0.99	
	C: N	FL > S	**0.022**	0.53	0.25	
	% C	–	0.94	0.18	0.67	
	% N	S > FL	**0.043**	0.14	0.17	
	δ^{13}C	F < C	0.75	**0.0007**	0.1	
	Chlorophyll	–	0.3	0.99	0.3	
	Phycocyanin	C > F	0.76	**0.046**	0.09	Y^{-1}

The table displays *p*-values, transformations, and the nature of differences for both *C. lambertii* and *P. dilatatum*. Significant effects ($\alpha = 0.05$) have *p*-values displayed in bold. For differences in main effects "Type" (two levels: static and fluctuating pH) and "Era" (two levels: current and future), acronyms are displayed: F, future; C, current; S, static; and FL, fluctuating. For significant interactions, differences between "Type" are displayed in bold when differences were significant at $\alpha = 0.05$ using separate one-way ANOVAs at each level of "Era." Treatment acronyms: CF, current fluctuating; CS, current static; FF, future fluctuating; FS, future static. Degrees of freedom = 1 for main effects and the interaction and 17–18 for residuals.

Table 2. Carbonate system parameters at the mean pH_T of each treatment.

Treatment	pH_T(14°C)	Salinity (PSU)	DIC(μmol kg^{-1})	A_T (μmol kg^{-1})	CO_2(μmol kg^{-1})	[H^+](mol l^{-1})
Current static	8.01	36	2169	2375	17.15	9.77×10^{-9}
Current fluctuating	8.11	36	2119	2375	13.18	7.76×10^{-9}
Future static	7.71	36	2338	2422	38.13	1.95×10^{-8}
Future fluctuating	7.59	36	2378	2422	51.32	2.57×10^{-8}

pH_T and salinity were measured while A_T, DIC at mean treatment pH_T, CO_2, and [H^+] were calculated at the mean pH_T of each treatment using the programme CO_2 calc (Robbins *et al.*, 2010) from the DIC samples measured at pH_T 8.01 and 7.64 and the known salinity, pH_T and temperature of the seawater.

differentially affected two ecologically dominant, temperate non-CCM seaweeds. We hypothesized that both species would have reduced growth and photosynthetic rates in fluctuating pH under current ocean conditions and that these reductions would be ameliorated under future ocean pH. These hypotheses were unsupported, with *C. lambertii* having reduced growth and photosynthetic rates in fluctuating pH regardless of the mean pH (current or future) while those of *P. dilatatum* were unaffected by pH fluctuations. We also hypothesized that elevated CO_2 under OA scenarios would act as a "fertilizer" to benefit both species. There was weak evidence to support this hypothesis for *C. lambertii*, which displayed elevated net photosynthetic rates under the future scenario but no effect on growth. However, there was no evidence of a fertilization effect for *P. dilatatum* in any OA treatment. Both species are obligate CO_2-using species and the differential responses indicate that factors other than their passive inorganic carbon uptake strategy (cf. Hepburn *et al.*, 2011) are responsible for their response to OA: This finding supports that of van der Loos *et al.* (2019) who suggest that inorganic carbon uptake strategy alone is not sufficient to predict the responses of fleshy seaweeds to OA. We detected interactive effects of mean pH and pH fluctuations, highlighting the need to incorporate fluctuating pH into experimental treatments when the study organism occurs in naturally fluctuating pH environments such as seaweed beds. Failure to include relevant pH fluctuations that are representative of the natural environment as part of experimental treatments may result in false conclusions being drawn when assessing the response of coastal species to OA.

Responses to fluctuating pH

The contrasting responses of these functionally similar, sympatric seaweeds to fluctuating pH supports the trend in research outcomes demonstrating species-specific responses of photosynthetic organisms to pH fluctuations. To date, the effect of fluctuating pH on photosynthetic organisms has been tested for 16 species and the results have been varied: Table 3 displays the growth, photosynthetic, respiration, and calcification responses of photosynthetic organisms to fluctuating pH under both current and future ocean pH (see references therein). While there are some general trends such as corals either benefitting or being unaffected and coralline algae being negatively affected (Table 3, but see Cornwall *et al.*, 2018), responses are typically species specific and can even vary within a species, e.g. *H. reinboldii* responds

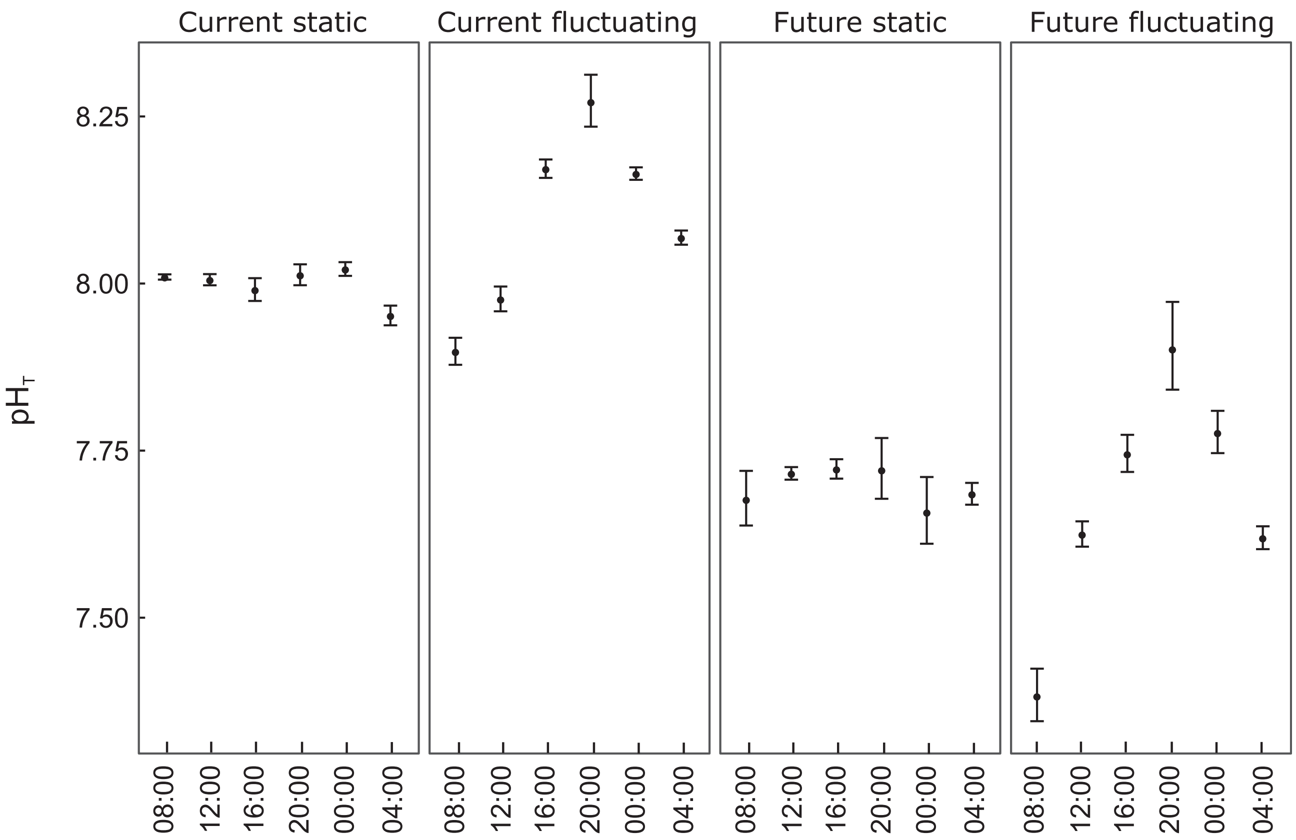

Figure 1. Average pH_T of seawater for each treatment following a seawater refresh of the culture chambers (4-h intervals), error bars are standard error (measurements of pH_T for each treatment: $N = 144–153$).

differently depending on the collection habitat (Cornwall *et al.*, 2018). Furthermore, as in this study, commonalities between species (e.g. functional group or carbon uptake strategy) do not appear to explain the differential responses to fluctuations: Four species of fleshy seaweeds have been tested (including the two in this study) with positive, negative, and neutral responses observed (Table 3).

We predicted that both species in this study would be negatively affected by fluctuating pH under current ocean conditions due to DIC limitation during periods of high pH in daylight and that this negative effect would be alleviated under future ocean conditions due to increased DIC availability. While this hypothesis held true for *C. lambertii* under current ocean conditions, it was also negatively affected by fluctuating pH in the OA scenario. This suggests that DIC limitation was unlikely to be the driver of the negative response to fluctuating pH. Furthermore, the symmetrical nature of the pH fluctuations in this study (see Figure 1) resulted in the total DIC in daylight being similar to that of night time (Supplementary Table S4). Additionally, total DIC in static and fluctuating treatments were nearly identical (when compared at current and future mean pH) and only the variability in DIC was different (Supplementary Table S4). Therefore, DIC was higher in the fluctuating treatments during the morning and decreased throughout the day. Seaweeds are known to have diurnal cycles in photosynthetic rates independent of light levels (e.g. Mishkind *et al.*, 1979), and differences in the natural circadian rhythms of these species could have contributed to the observed responses in net photosynthesis. For example, if *C. lambertii* had elevated photosynthetic rates toward the end of the day, then the reduced DIC availability at this point could have contributed to the negative effect of fluctuating pH. In contrast, *P. dilatatum* may have maximal photosynthetic rates around mid-day and hence there would have been no differences in DIC availability at this time between static and fluctuating treatments. This may explain why *P. dilatatum* was relatively unaffected by fluctuating pH compared to *C. lambertii*, but any interactive effects of circadian rhythms in photosynthesis and pH fluctuations require testing.

Fluctuations in pH are actually fluctuations in H^+ concentration ($[H^+]$). Species-specific sensitivity to elevated $[H^+]$ has recently been suggested as a predictor of the response of algae to OA (Bach *et al.*, 2013; van der Loos *et al.*, 2019) and this may explain, in part, the differential responses of *C. lambertii* and *P. dilatatum* to fluctuating pH. Seaweed must maintain intracellular pH within relatively narrow limits with intracellular pH of photosynthetic organisms varying ~0.1 pH units per unit decrease in external pH (Raven, 2011, 2013) and this likely comes at an energetic cost. As such, energetic demands may be increased in highly variable $[H^+]$ conditions such as those in seaweed beds, which were simulated in this experiment, and these demands may differ between species. Reductions in seawater pH can impair the ability of some phytoplankton to remove excess H^+ from the cell (Taylor *et al.*, 2012), and it is possible that this may occur in some seaweed species. Whether or not variability in $[H^+]$ would have similar effects to continually high $[H^+]$ remains unclear however, as no study has tested the effect of variable $[H^+]$

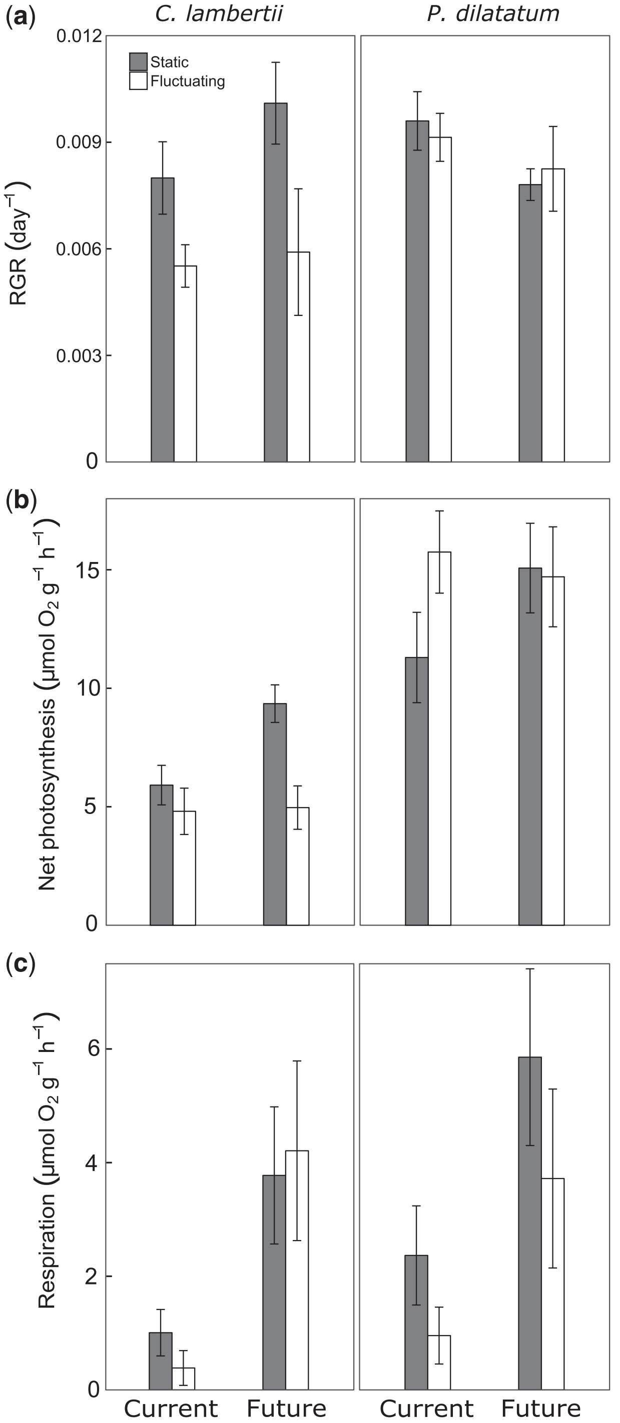

Figure 2. Responses of *C. lambertii* (left) and *P. dilatatum* (right) to current and future ocean pH after 14 days in culture. Shaded bars refer to treatments where pH was static and white bars refer to treatments where pH was fluctuating. Response variables and significant differences are as follows: (a) RGR (day^{-1}), *C. lambertii*: static > fluctuating ($p = 0.007$), *P. dilatatum*: no significant differences. (b) Net photosynthesis (µmol O^2 g^{-1} h^{-1}), *C. lambertii*: Static > fluctuating ($p = 0.007$), future > current ($p = 0.041$), *P. dilatatum*: no significant differences. (c) Respiration (µmol O^2 g^{-1} h^{-1}), *C. lambertii*: future > current ($p = 0.0008$), *P. dilatatum*: future > current ($p = 0.013$). Data are presented as means ± standard error, $n = 4$–6. All rates are calculated on a wet weight basis.

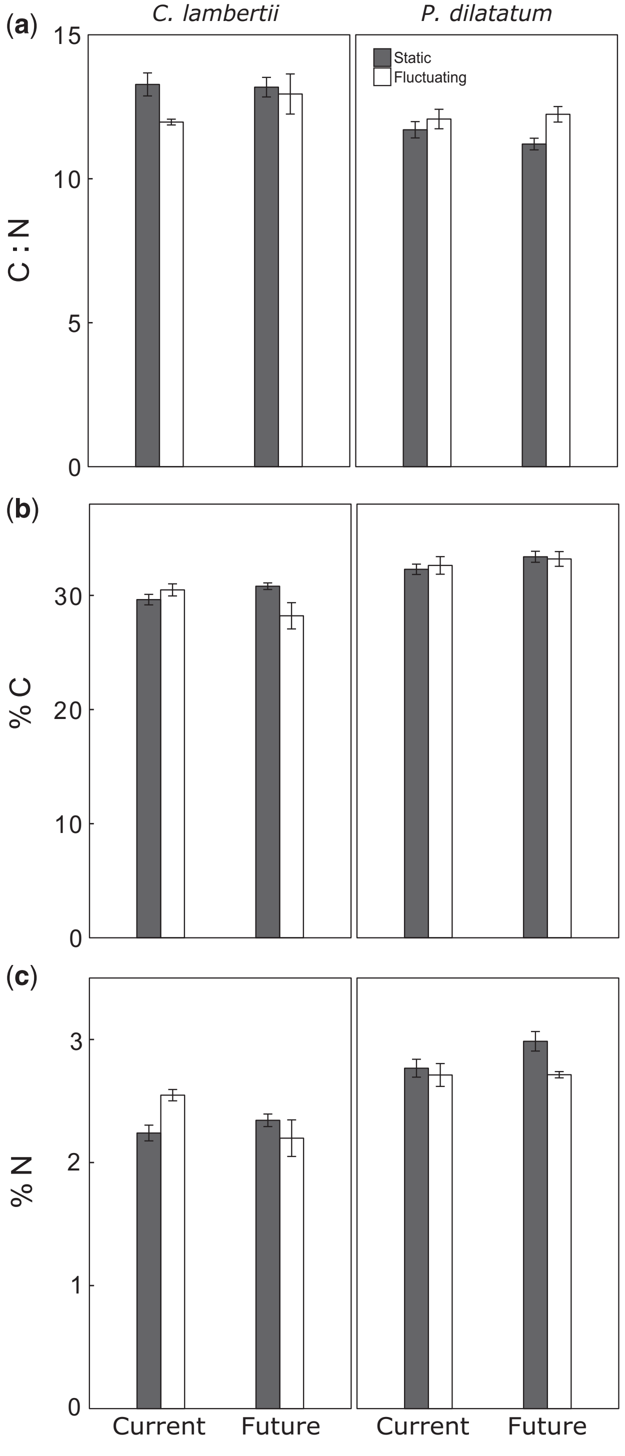

Figure 3. Responses of *C. lambertii* (left) and *P. dilatatum* (right) to current and future ocean pH after 14 days in culture. Shaded bars refer to treatments where pH was static and white bars refer to treatments where pH was fluctuating. Response variables and significant differences are as follows: (a) C: N ratios, *C. lambertii*: static > fluctuating ($p = 0.035$), *P. dilatatum*: fluctuating > static ($p = 0.022$). (b) % C, *C. lambertii*: interaction ($p = 0.009$), *P. dilatatum*: no significant differences. (c) % N, *C. lambertii*: interaction ($p = 0.008$), *P. dilatatum*: static > fluctuating ($p = 0.043$). Data are presented as means ± standard error, $n = 4$–6.

Table 3. Published responses of photosynthetic organisms to diel fluctuations in pH under ambient and OA conditions.

Organism	Effect of fluctuating pH								Study
	Ambient				OA				
	G	PS	R	C	G	PS	R	C	
Coralline (*A. corymbosa*)	↓	–			↓	–			Cornwall *et al.* (2013)
Coralline (*A. corymbosa* recruits)	↓				↓				Roleda *et al.* (2015)
Coralline (*P. onkodes*)		↓		–		–		–	Johnson *et al.* (2014)
Coralline (*H. reinboldii*)		–	–	↑/–		–	–	–	Cornwall *et al.* (2018)
Kelp (*E. radiata*)	↑	↑			–	↓			Britton *et al.* (2016)
Red seaweed (*G. lemaneiformis*)	↓	↓			–	–			Qu *et al.* (2017)
Red algae (*C. lambertii*)	↓	↓	–		↓	↓	–		This study
Red algae (*P. dilatatum*)	–	–	–		–	–	–		This study
Diatom (*T. oceanica*)	–	↓	↑		↓	–	–		Li *et al.* (2016)
Diatom (*T. weissflogii*)	–	–	–		–	–	–		Li *et al.* (2016)
Coral (*S. caliendrum*)	↑				↑				Dufault *et al.* (2012)
Coral (*A. hyacinthus*)				–				↑	Comeau *et al.* (2014)
Coral (*A. cervicornis*)				↑				–	Enochs *et al.* (2018)
Coral (*A. formosa*)				↑					Chan and Eggins (2017)
Coral (*A. palmata*)		–	–	–		–	–	–	Camp *et al.* (2016)
Coral (*A. astreoides*)		–	–	–		–	–	–	Camp *et al.* (2016)
Coral (*Goniopora* sp.)		–	–	–		–	–	–	Cornwall *et al.* (2018)

All responses are relative to static pH at the mean pH of the respective scenario (i.e. ~pH 8.0 and 7.7). G, growth; PS, photosynthesis; R, respiration; and C, calcification. "–" refers to no effect and blank spaces indicate the variable was not measured or not tested by the authors. The calcification response of *H. reinboldii* under ambient pH from Cornwall *et al.* (2018) was dependent on the location individuals were collected from. Individuals collected a site with high pH variability responded positively to fluctuating pH, while those from a site with low pH variability were unaffected.

independent of variable DIC. Furthermore, the mechanisms of intracellular pH regulation in seaweeds has only been examined in a few species (Raven and Smith, 1980; Kirst and Bisson, 1982; Gibbon and Kropf, 1993). Studies also employ different experimental methods to achieve fluctuations (see discussion below) and measure different response variables (see Table 3). As such, it is difficult to elucidate what changes in the seawater carbonate system are affecting species responses. Further research to separate the effects of variability in DIC and $[H^+]$ and elucidate physiological, biochemical, and molecular mechanisms are required if we are to understand why photosynthetic organisms display such varied and unpredictable responses to pH fluctuations. Doing so will allow researchers to understand why different responses to OA are often observed when using static pH treatments compared to the more environmentally relevant fluctuating pH treatments (e.g. Johnson *et al.*, 2014; Britton *et al.*, 2016).

Comparing studies that use different methods to fluctuate pH

For seaweeds, the method used to create diel fluctuations in pH in the laboratory may affect the response of species to the variability in pH. In this study, we used a "six-step method" to create diel fluctuations in which the pH was altered six times over 24 h. For seaweeds, most studies have used a simple regime consisting of high pH during the day and low pH during the night (hereafter day/night regime, Table 3). Exceptions are Cornwall *et al.* (2018) who used an intermediate change in pH at mid-day (i.e. four steps over 24 h, hereafter four-step method) and Qu *et al.* (2017) who achieved diel fluctuations in pH by increasing the density of cultivated seaweed. For corals and diatoms, a range of methods have been employed including continual changes (Li *et al.*, 2016; Enochs *et al.*, 2018), the day/night regime (Dufault *et al.*, 2012; Comeau *et al.*, 2014), the four-step method (Cornwall *et al.*, 2018), and pumping water into culture chambers directly from mesocosms where biological activity alters carbonate chemistry (Camp *et al.*, 2016; Chan and Eggins, 2017).

While all methods listed above mimic natural fluctuations to some extent, there are inherent differences in the chemistry of each method and comparisons between studies with different methods may be limited. For example, the day/night regime results in substantially lower DIC and CO_2: HCO_3^- ratios during the day compared to at night and abrupt changes in pH occur between day and night. Conversely, the four- and six-step methods have far less abrupt changes in pH and if fluctuations are symmetrical, nearly identical DIC availability and CO_2: HCO_3^- ratios between day and night. This distinction is important because photosynthesis by non-CCM species may become more limited by CO_2 availability while photosynthesis is occurring in the day/night regime relative to the four- or six-step methods, whereas CCM species grown under a day/night regime may be forced to utilize the more energetically expensive HCO_3^-. For both uptake strategies, the biological responses recorded may be an artefact of the methodology rather than the phenomenon being tested. Furthermore, the relative energetic costs associated with two abrupt changes in $[H^+]$ over 24 h vs. six smaller changes or continual changes remain unknown but are likely to differ. Currently, it is difficult to determine whether different methods of achieving diel fluctuations in pH are having substantial impacts on responses due to confounding factors such as type of organism studied (e.g. calcified vs. fleshy seaweeds are likely to respond differently), and the response variables measured (see Table 3). However, as pH fluctuations do affect the outcomes of experiments, studies should aim to mimic the gradual changes in pH that occur naturally in the field as closely as possible.

Responses to OA

We predicted that elevated CO_2 would have a "fertilization" effect and increase the growth and photosynthetic rates of both species. *P. dilatatum* displayed no evidence of an increase in either photosynthesis or growth, while *C. lambertii* showed an increase only in photosynthetic rates. While modelling and theoretical work suggests that non-CCM species should benefit from elevated CO_2 concentrations (Hepburn *et al.*, 2011; Raven, 2011; Cornwall *et al.*, 2015; Kübler and Dudgeon, 2015), experimental work suggests that this may not be the case. Of the five non-CCM species tested (including the two in this study), only one has shown an increase in growth rates (Kübler *et al.*, 1999) while the other four species have shown no responses (Ho and Carpenter, 2017; van der Loos *et al.*, 2019; this study). Photosynthetic rates of non-CMM species have not typically been higher under elevated CO_2 (Cornwall *et al.*, 2012; van der Loos *et al.*, 2019; *P. dilatatum*—this study), with the exception of *C. lambertii* in this study. Thus, on current evidence, a lack of a CCM is not a good indicator that a species will benefit from OA.

Species-specific differences in the amount, maximal RuBisCO activity (V_{max}), and affinity of RuBisCO for CO_2 (K_m) may exist between non-CCM species (Israel and Hophy, 2002; van der Loos *et al.*, 2019) and this could explain why *C. lambertii* had increased photosynthetic rates under future ocean conditions while *P. dilatatum* did not. For example, if the V_{max} of *C. lambertii* is higher than what can be reached under current CO_2 levels, if it is able to increase the amount of RuBisCO or it has a low K_m, then additional CO_2 may lead to elevated photosynthetic rates. In contrast, if *P. dilatatum* has a V_{max} that is reached at current CO_2 levels, is unable to increase the amount of RuBisCO or has a high K_m value it may be unable increase photosynthetic rates under elevated CO_2. Further work examining the species-specific RuBisCO kinetics of non-CCM species are required if we hope to predict the response of fleshy seaweed species and communities to OA.

Elevated $[H^+]$ may also act antagonistically to offset any benefit of elevated DIC. To date, there has only been one study that has assessed the response of seaweed to elevated $[H^+]$ in the context of OA (Roleda *et al.*, 2012): they found a significant reduction in germination of *Macrocystis pyrifera* meiospores when seawater pH was reduced without a simultaneous increase in DIC, whereas, the addition of DIC to seawater with lowered pH ameliorated this reduction. Sensitivity to $[H^+]$ was proposed as an important factor to consider by van der Loos *et al.* (2019) and this could explain the lack of elevated growth rates for both these species under elevated DIC. Respiration rates were elevated for both species under OA conditions and this increase in metabolic rate was not allocated toward growth or storage as % N, % C, and pigment content did not increase in either species and phycocyanin content was slightly reduced under OA conditions in *P. dilatatum*. This raises the possibility that energy was being allocated toward maintaining intracellular pH (Raven, 2011, 2013) in both species and the increased energy arising from elevated photosynthesis in *C. lambertii* was also allocated to regulation, leading to a lack of an increase in growth rates. However, as we did not explicitly test the mechanisms driving the observed responses, these suggestions are speculative and require experimental testing. Future studies that attempt to separate the relative importance of elevated DIC vs. elevated $[H^+]$ are required if we are to predict how species will respond to OA. There is likely a complex interaction between the potential benefit of elevated DIC and the potential negative effects of elevated $[H^+]$ and the relative importance of these factors may be species specific (van der Loos *et al.*, 2019).

Interactive effects of pH fluctuations and OA: important implications for interpretations of species and community responses to OA

We detected significant interactions between fluctuating pH and OA for both % N and % C content in *C. lambertii* and weak, non-significant evidence of an interaction for net photosynthesis in *C. lambertii* and phycocyanin content in *P. dilatatum*. The presence of interactive effects of pH fluctuations and OA has important implications for interpretations of how coastal species and communities will respond to OA. Coastal species that exist in variable pH environments such as those within seaweed beds and coral reefs may respond differently to OA when pH is either static or fluctuating as demonstrated in this study and in Table 3. For example, if we conducted our study using only static pH and compared the net photosynthetic response of *C. lambertii* between current pH and OA pH we would infer that this species would benefit from OA using a simple one-way ANOVA (see Supplementary Table S5). However, if we conducted our study using only fluctuating pH, we would infer that the photosynthetic rates of this species would be unaffected by OA (see Supplementary Table S5). This simple example, along with the contrasting responses of multiple species to pH fluctuations under current or future ocean pH shown in Table 3, clearly demonstrates that research on species that occupy environments with inherent pH variability must incorporate this variability into experimental treatments where possible or we risk making misleading interpretations.

Author's contributions

D.B., C.N.M., and C.L.H. designed the study. D.B. carried out the experiment. C.M.M. designed and built the automated culture system. C.M.M. and A.T.R. assisted in post-experiment data and sample processing. D.B., C.N.M., and C.L.H. undertook the data analysis. All authors contributed to the drafting of the manuscript.

Supplementary data

Supplementary material is available at the *ICESJMS* online version of the manuscript.

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