Differential growth responses to water flow and reduced pH in tropical marine macroalgae

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A B S T R A C T

The physical environment plays a key role in facilitating the transfer of nutrients and dissolved gases to marine organisms and can alter the rate of delivery of dissolved inorganic carbon. For non-calcifying macroalgae, water motion can influence the physiological and ecological responses to various environmental changes such as ocean acidification (OA). We tested the effects of lowered pH under three different flow speeds while three dominant non-calcifying macroalgal species differing in their carbon-use and are commonly found in the back reefs of Moorea, French Polynesia. Relative growth rates (RGR) of two phaeophytes, Dictyota bartayresiana and Lobophora variegata (HCO3− users), and a rhodophyte, Amsia rhodantha (CO2 user) were measured to examine how the combined effects of OA and flow can affect algal growth. Growth rates were affected independently by pCO2 and flow treatments but there was no significant interactive effect. Additionally, growth rates among species varied within the different flow regimes. Of the three species, L. variegata had the overall greatest increase in RGR across all three flow speeds while A. rhodantha exhibited the greatest negative impact under elevated pCO2 at 0.1 cm·s−1. These differential responses among algal species demonstrate the importance of flow when examining responses to a changing environment, and if the responses of macroalgae differ based on their carbon-use strategies, it may provide advantages to some macroalgal species in a future, more acidic ocean.

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1. Introduction

The shift in benthic coral reef communities has been linked to a number of local drivers contributing to the decline in calcifying marine organisms and an increase in fleshy macroalgae (McCook, 1999). Additional to overfishing, nutrient runoff, hurricanes and cyclones, and coral diseases (Hughes et al., 2003), the global stressor of ocean acidification (OA) due to increasing atmospheric CO2 levels and the consequent reduction in seawater pH has negatively impacted calcifying organisms (Anthony et al., 2008; Comeau et al., 2016). Extensive research has focused on the responses of scleractinian corals and coralline algae to OA due to lowered carbonate saturation states and the reduction in calcification rates (Doney et al., 2009; Kleypas and Yates, 2009). Although these responses vary among species, calcifying organisms generally exhibit greater negative responses than non-calcifying organisms (i.e. fleshy macroalgae) (Kroeker et al., 2010). On the contrary, non-calcifying macroalgae have exhibited either negligible (Israel and Hophy, 2002; Fernández et al., 2015) or positive responses to higher CO2 levels (Connell and Russell, 2009; Diaz-Pulido et al., 2011; Porzio et al., 2011; Johnson et al., 2012), broadening interest to understand the varying responses of macroalgae to environmental changes.

The physical environment also plays a critical role influencing the physiological responses of benthic organisms. For marine macroalgae, water movement is a key factor influencing a number of physiological and biological processes: morphology (Hurd, 2000; Stewart and Carpenter, 2003), production and mass transfer (Ady and Steneck, 1985; Carpenter et al., 1991), photosynthesis and growth (Koch et al., 2013), some facilitating larval/spore dispersal (Lowe and Falter, 2015), light attenuation (Carpenter, 1985), and carbon acquisition (Hurd, 2000; Raven et al., 2005). The rate and mode of water motion (e.g. fast vs. slow; oscillatory vs. unidirectional) also contributes greatly to the variable effects of water motion on algal metabolism (Denny et al., 1985; Carpenter et al., 1991). An increase in water motion is beneficial for resource acquisition, ultimately increasing photosynthesis and growth, while a decrease in water motion (i.e. slow flow) can increase mass transfer limitation of gases and nutrients, potentially leading to negative effects on metabolic rates (Atkinson and Bilger, 1992; Hurd, 2000). However, decreased water motion also can ameliorate the negative effects of OA, particularly for calcifying organisms. This was
demonstrated by Cornwall et al. (2014) on coraline algae grown under slow flows yet maintaining net calcification at a lowered pH, illustrating how algae respond physiologically to buffer against OA. Conversely, OA conditions may benefit some non-calcareous algae and the increase in water motion may further enhance metabolic and growth rates by promoting mass flux of materials (Hurd, 2000; Stewart and Carpenter, 2003). In a reef-lagoon system that is primarily wave-driven (as seen in Moorea, French Polynesia), reef organisms such as macroalgae can experience natural fluctuations of high and low circulation in water motion in the back reef that can differentially affect the physiological adaptations among algae (Hench et al., 2008). Thus, the responses of fleshy macroalgae are likely modulated by other abiotic factors such as water flow, yet few studies have examined the interactive effects of water motion and OA.

OA is altering the partitioning of dissolved inorganic carbon (DIC), increasing CO₂ and HCO₃⁻ while decreasing CO₂³⁻ concentrations (Andersson and Mackenzie, 2011; Veron, 2011). The alteration in DIC speciation can cause responses in calcifying and non-calcifying organisms to shift, thereby potentially affecting their metabolism, growth rates, and relative abundances. Macroalgae depend on the supply of inorganic carbon (Ci) for photosynthesis and growth, and their capability to assimilate Ci either via diffusion (for CO₂) or active uptake of HCO₃⁻ using carbon-concentrating mechanisms (CCMs) depends on the availability of CO₂ present (Beardall et al., 1998; Giordano et al., 2005). In many cases, CO₂ alone is insufficient for maximum photosynthesis (Giordano et al., 2005; Hurst et al., 2009), therefore the increase in concentrations of CO₂ is predicted to have various (positive) effects on carbon assimilation, photosynthesis, and growth for both CO₂ and HCO₃⁻ users. For obligate CO₂ users lacking CCMs, increase atmospheric CO₂ dissolution in seawater can alleviate carbon limitation and mitigate the potential disadvantages in their competitive ability with other non-calcareous algae. For HCO₃⁻ users, OA may not enhance photosynthetic rates as expected for CO₂ users (Zou, 2005; Hurd et al., 2009), but they may downregulate their CCM activity, thus reducing energetic investment for CO₂ active uptake (Raven and Hurd, 2012). Their ability to readily convert HCO₃⁻ to CO₂ is achieved by either using extracellular carbonic anhydrase (CA) or having CA acting intracellularly (Hurd et al., 2009; Fernández et al., 2014; Rautenberger et al., 2015). This evolutionary development has provided macroalgae with CCMs a more energetically costly, but beneficial advantage, eliminating the risk of carbon limitation under present pCO₂ in most environments (Giordano et al., 2005). These responses are species-specific (Kram et al., 2015), and the majority of studies have focused on OA effects in isolation (e.g. Cornwall et al., 2012; Fernández et al., 2015; Kim et al., 2016; Ober et al., 2016). Furthermore, the response and level of sensitivity to OA may vary across taxa due to the different carbon uptake strategies (i.e. CO₂-only user or facultative HCO₃⁻-user: Table 1) in fleshy macroalgae (Kroeker et al., 2010; Cornwall et al., 2012).

The present study focused on three dominant non-calciying macroalgal species: Lobophora variegata (Phaeophyta), Dictyota bartayresiana (Phaeophyta), and Amanias rhodantha (Rhodophyta) commonly found in the back reef of Moorea, French Polynesia. Additionally, the three species have different carbon uptake strategies. L. variegata is known to be a HCO₃⁻ user (Koch et al., 2013) while a pH drift analysis (Maberly, 1990) was conducted prior to treatment to determine the Ci assimilation of D. bartayresiana and A. rhodantha. This technique measures the change in pH when carbon is assimilated through photosynthesis, and when the pH is raised above 9, generally indicates the alga utilizing HCO₃⁻ (Axelsson and Uusitalo, 1988; Maberly, 1990; Diaz-Pulido et al., 2016). The pH drift experiment revealed D. bartayresiana as a HCO₃⁻ user (raising pH > 9) and A. rhodantha as a CO₂ user (carbon saturated at a pH of 8.51) (R. Carpenter unpub. data). We examined the combined effects of unidirectional flow speeds and lowered pH on macroalgal growth rates and whether their responses varied based on their DIC use. We tested the following hypotheses: (i) algal growth rates would increase with increasing flow speed, (ii) A. rhodantha (CO₂ user) would benefit under elevated pCO₂ by having higher growth rates than D. bartayresiana and L. variegata, (iii) the growth rates of HCO₃⁻ users would not vary between current and elevated pCO₂ conditions, and (iv) the interactive effect of flow and OA would result in higher growth rates for A. rhodantha than the other two species. The relative growth rates were measured as a proxy of fitness to assess how the interaction of OA and flow can affect algal biomass.

2. Methods

2.1. Study site and collection

This study was conducted in Moorea, French Polynesia in June–July 2014. Three dominant non-calciying species, Lobophora variegata, Dictyota bartayresiana, and Amanias rhodantha of similar biomass were collected on the back reef of the north shore at a depth < 3 m (Fig. 1). L. variegata exists in three morphologies, a ruffled form, a decumbent form, and an encrusting form (Coen and Tanner, 1989). The decumbent and encrusting forms were commonly seen in Moorea and therefore, the decumbent morphology was used, as it was most similar to the other two fleshy macroalgal species. These species were chosen from preliminary field surveys on overall abundance of macroalgae on the back reef of the north shore in 2014 (unpub. data). Additionally, they represented some of the common species in the back reef of Moorea for benthic algal cover of 2014 (Carpenter, 2015), with majority of the algal cover being non-calciying macroalgae. The most abundant non-calciying species were: Amanias rhodantha (3%), Dictyota bartayresiana (8%), Lobophora variegata (-1%), Chnoospora implexa (-1%), Sargassum pacificum (-1%), Turbinaria ornata (10%), and algal turf (43%).

New algal samples were collected weekly (n = 40 per species), 2 days prior to each flow/pCO₂ experiment and brought to the Richard B. Gump South Pacific Research Station located in Cook’s Bay, Moorea. Algae were placed in a flow-through seawater table, cleaned of epiphytes, then each algal individual was placed into a nylon monofilament mesh bag with 1.3-cm openings (Fig. 2C and D). Mesh bags were used to retain as much algal biomass as possible and minimize the effects of

Table 1

<table>
<thead>
<tr>
<th>Carbon use strategy</th>
<th>Disadvantages</th>
<th>Predictions to OA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂-only user (Non-CCM species)</td>
<td>• CO₂ concentration in seawater is low.</td>
<td>• Enhance photosynthesis</td>
<td>1, 3, 6, 8, 9</td>
</tr>
<tr>
<td></td>
<td>• CO₂ diffusion rates are slow in seawater.</td>
<td>• Increase growth</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Likely to be carbon-limited</td>
<td>• Alter/improve competitive ability with other fleshy algae</td>
<td></td>
</tr>
<tr>
<td>Facultative HCO₃⁻/CO₂ user (CCM present)</td>
<td>• CCMs are energetically costly.</td>
<td>• Down-regulate active CO₂ uptake may lead to enhanced growth</td>
<td>2, 4, 5, 7, 8, 10, 11, 12</td>
</tr>
<tr>
<td></td>
<td>• May be restricted to high light habitats</td>
<td>• Expected to exhibit little (positive) or neutral responses in photosynthesis compared to CO₂ users</td>
<td></td>
</tr>
</tbody>
</table>

References: (1) Cornwall et al. (2012); (2) Cornwall et al. (2015); (3) Diaz-Pulido et al. (2016); (4) Fernández et al. (2015); (5) Giordano et al. (2005); (6) Hepburn et al. (2011); (7) Hurd et al. (2009); (8) Kübler et al. (1999); (9) Rautenberger et al. (2015); (10) Raven (1991); (11) Raven et al. (2014); (12) Zou (2005).
fragmentation. They were made large enough to reduce clumping effects by cutting nylon monofilament mesh into 21 cm × 10 cm sheets, folded in half and the ends were tied in opposite directions with monofilament line to inflate the mesh bags and allow space for each thallus to grow. Each bag was attached to a 5-cm² PVC plate (0.6-cm thick) acting as a weight. Light (photosynthetically active radiation, PAR) measurements were taken inside and outside the mesh bags using the Walz pulse amplitude modulated fluorometer (PAM) 2π cosine light sensor calibrated to a LI-COR 2π quantum sensor (LI 192SA), giving PAR experienced by the algae. Individuals were kept in a flow-through seawater table for 2 days during preparation before being transferred to their respective treatments (n = 10 individuals per flume).

2.2. Experimental conditions

The experiment was conducted in four outdoor flumes with a working section of 0.3 × 0.3 × 5.0 m for each flume, together containing 700 L of seawater (Fig. 2A and B). Seawater was pumped from Cook’s Bay at 12-m depth and filtered through a sand filter (nominal filter size ~100 μm). The seawater was dispensed directly into the upstream end of each flume at 5 L·min⁻¹, and passed through flow straighteners (20-cm long, 3-cm diameter stacked PVC pipes) positioned at the each end of the flume, creating unidirectional flow. Seawater was re-circulated back to a 125-cm return section using a W. Lim WAVE II 375W pump. Weekly light levels were measured between 11:30–13:00 h for overall light conditions using a LiCor 4π quantum sensor (LI-193) and a LI-1400 m. A 4π sensor was used to account for light reflecting off the sides and bottoms of the flumes. The flumes experienced outdoor light attenuated with fiberglass covers to match the intensity to levels of the back reef (mean PAR ~900 μmol photons m⁻²·s⁻¹). Light measurements were taken 15 cm above the flume bottom at three points along each flume: upstream, middle, and downstream and were averaged to account for variability in light exposure along the flume.

Three different flow speeds were used: 8.5 cm·s⁻¹ representing an average flow speed in the back reef <3-m depth (Hench et al., 2008), 5 cm·s⁻¹, and <1 cm·s⁻¹ treatment. Flow treatments were created in three 1-week incubations where all four flumes experienced a set flow speed for 7 days. Due to logistical limitations, pCO₂ treatments were

![Fig. 1. Photographs of the three species of macroalgae found in <3 m depth in the back reef of the north shore of Moorea, French Polynesia.](image)

![Fig. 2. Photograph of (A) the four outdoor flumes; each flume with a working section of 0.3 × 0.3 × 5.0 m in which pCO₂ and flow speed treatments were established. (B) Individual flume with macroalgal species. Examples of algae; (C) D. bartayresiana and (D) A. rhodantha placed in nylon monofilament mesh bags.](image)
maintained in the same flumes for each flow treatment, with two flumes at ambient pCO₂ and the other two at elevated pCO₂ (see below). At <1 cm s⁻¹ flow, the incoming fresh seawater naturally created slight water movement (0.1 cm s⁻¹), but to prevent additional water movement, the main pumps were turned off during the treatment. Flow rates were measured on day 1 and 4 of the treatments for consistent velocities throughout the experiment using a Nortek Vectrino Acoustic Doppler Velocimeter (ADV), positioned 15 cm above the flume bottom. Algal individuals were moved randomly every 2 d to reduce positional effects and after 7 days, the flumes were drained and rinsed with freshwater before the next flow speed treatment began with newly collected individuals.

2.3. Carbonate chemistry

pCO₂ was regulated using a pH-stat aquacontroller (Neptune Systems) that bubbled in either pure CO₂ or CO₂-free air. Two flumes were regulated at ~400 μatm (ambient pCO₂ level) and the other two flumes at ~1000 μatm (targeted high pCO₂ level), the expected atmospheric pCO₂ level projected in 2100 under a “business as usual” scenario from the IPCC (2013) Representative Concentration Pathways (RCP) 8.5 (van Vuuren et al., 2011).

Flumes were maintained at a mean temperature of 27.1 ± 0.5 °C using chillers (DA-500B Arctica, JBJ, USA), mimicking the back reef temperature conditions (~27.5 °C) during May–July 2014 (Leichter, 2015). Temperature and pH were recorded using a hand-held pH meter (Orion 3-stars) fitted with a Mettler DG 115-SC pH probe, which was calibrated every other day against certified TRIS buffer (A. Dickson, San Diego, USA). pH (total scale) levels also were measured weekly using m-cresol dye (Dickson et al., 2007), providing results that were within ±0.03 pH units of those obtained with the portable pH meter. Total alkalinity (A₇) was measured every 2 days using an automatic titrator (T50, Mettler-Toledo) using 50-mL seawater samples collected the day of sampling following the standard procedure of Dickson et al. (2007). Titrations of certified reference materials (CRM) provided by A.G. Dickson (Batch 130) were used to determine the accuracy and precision of the titrated samples, yielding A₇ values within 9 μmol·kg⁻¹ of the certified value. The R package seacarb (Lavigne and Gattuso, 2013) was used to calculate the carbonate system parameters with salinity, temperature, A₇, and pH₇.

2.4. Growth measurements

To determine growth rates, initial and final wet weight was measured for each individual. Algae in their mesh bags were spun in a salad spinner for 10 s and immediately weighed (±0.001 g). The relative growth rate is a common measure of change in weight over time using initial and final weights. To calculate the relative growth rate (RGR), the following equation adapted from Yong et al. (2013) was used:

\[
\text{RGR (\%day}^{-1} \text{)} = \log \left( \frac{W_f}{W_i} \right) \times \frac{100}{t}
\]

Growth is expressed as percent change per day, where Wᵢ and Wᵢ are the final and initial wet weights, respectively, and t is the total time in days the algae were subjected to a treatment.

2.5. Statistical analyses

Physical conditions in the flumes were analyzed using a two-way partially-nested analysis of variance (ANOVA), with pCO₂ and flow as fixed effects and flume as a random factor nested within the interaction of pCO₂ and flow treatment. All effects were tested using α = 0.05, however to increase statistical power, the flume was dropped from the statistical model when not significant under a more conservative criterion of α = 0.25 (Quinn and Keough, 2002). Growth rates of the algal species were analyzed using a three-way partially-nested ANOVA with the between-plot factors (pCO₂ and flow) as fixed factors, replicate flumes as the random plot nested within the interaction of pCO₂ and flow treatment, and algal species as a fixed, within-plot factor (see electronic Supplementary material, S1). Relative growth rates of each species were used as the dependent variable to test for treatment effects after 7 days. Tukey’s post hoc tests were used to analyze differences between species and treatments (flow and pCO₂). All analyses were performed using RStudio software (R Foundation for Statistical Computing), and assumptions of normality and equality of variance were evaluated through graphical analyses of residuals.

3. Results

Mean PAR in the four flumes during the incubations was 843 ± 19 μmol photons m⁻²·s⁻¹ and 1040 ± 66 μmol photons m⁻²·s⁻¹ in the ambient treatments and 872 ± 14 μmol photons m⁻²·s⁻¹ and 808 ± 14 μmol photons m⁻²·s⁻¹ in the elevated pCO₂ treatments (±SE, n = 18). Weekly light levels did not differ among flumes (one-way ANOVA, F₆,₈ = 1.495, p = 0.288). Light inside and outside the mesh bags was comparable (~190 μmol photons m⁻²·s⁻¹), indicating no shading effect from the mesh. Physical parameters varied consistently among treatments (Table 2).

Total alkalinity (A₇) did not vary significantly between pCO₂ (F₁,₈ = 1.235, p = 0.390) or flow (F₁,₈ = 1.987, p = 0.218) treatments, or within flumes among pCO₂ and flow treatments (F₆,₇₂ = 1.391, p = 0.230). A₇ averaged 2316 ± 3, 2320 ± 3, and 2317 ± 2 μmol·kg⁻¹ at 0.1, 5, and 8.5 cm·s⁻¹ flow speed (±SE, n = 8), respectively, for the ambient pCO₂ and 2309 ± 1, 2319 ± 4, and 2319 ± 2 μmol·kg⁻¹ at 0.1, 5, and 8.5 cm·s⁻¹ flow speed (±SE, n = 8) for elevated pCO₂. Temperature was consistent across all flumes, and did not differ between pCO₂ (F₁,₈ = 0.045, p = 0.839) or flow (F₂,₆ = 2.100, p = 0.204).

Mean pCO₂ levels for the ambient treatments were maintained at 397 ± 8, 373 ± 14, and 404 ± 9 μatm at 0.1, 5, and 8.5 cm·s⁻¹ flow speeds, respectively (±SE, n = 14). For the elevated pCO₂ treatments, values were maintained at 990 ± 47, 998 ± 45, and 1065 ± 35 μatm at 0.1, 5, and 8.5 cm·s⁻¹ flow speeds, respectively (±SE, n = 14). pCO₂ differed between treatments (F₁,₆ = 865.06, p < 0.001) but not between flow treatments (F₂,₆ = 2.674, p = 0.148) or among flumes within the interacting treatments (F₆,₇₂ = 0.682, p = 0.664). Similarly, pH differed between ambient and elevated pCO₂ treatments (F₁,₆ = 1411.03, p < 0.001), but not between flow treatments (F₂,₆ = 3.571, p = 0.095), or between flumes within treatments (F₆,₇₂ = 0.644, p = 0.695).

There was no significant effect of nesting flume within treatments, (F₁,₁₂₃₄ = 1.168, p = 0.304), therefore flumes were pooled within each pCO₂ by flow treatment and individuals were used as replicates (n = 20). Three-way ANOVA (Table 3) revealed that algal growth rates were significantly different between pCO₂ (F₁,₃₄₂ = 4.384, p = 0.037) and flow treatments (F₂,₃₄₂ = 161.998, p < 0.001), but the interaction between the two was not significant (p = 0.183). There were differences in growth among species (F₂,₃₄₂ = 87.473, p < 0.001) and planned comparisons revealed that L. variegate had overall higher growth rates than both D. bartayresiana and A. rhodantha, but there was no difference in growth rates between D. bartayresiana and A. rhodantha. RGR also varied by species within each flow treatment (F₂,₃₄₂ = 29.12, p < 0.001; Fig. 3). Growth of L. variegate and A. rhodantha were affected by all three flow treatments, having the greatest increase at 5 cm·s⁻¹. Under ambient and elevated pCO₂, growth of L. variegate increased 3.27 ± 0.26% day⁻¹ and 2.64 ± 0.35% day⁻¹, respectively (n = 20), and for A. rhodantha, increased by 1.36 ± 0.31% day⁻¹ and 1.26 ± 0.16% day⁻¹, respectively (n = 20). However, under no flow, there was a negative effect on A. rhodantha, reducing growth by −1.66 ± 0.20% day⁻¹ (n = 20) at ambient conditions, which was the greatest reduction among the three species (Fig. 3C). D.
barятарисана only differed in RGR between the mid flow and no flow treatments, having a 0.62 ± 0.30% day⁻¹ (n = 20) increase in biomass at 5 cm·s⁻¹ flow at ambient pCO₂. Under no water motion, there were reduced growth rates under both CO₂ treatments, with a greater reduction at elevated pCO₂ (−0.80 ± 0.15% day⁻¹, n = 20, Fig. 3A). However, there was neither a species-within-CO₂ treatment (F2,342 = 0.540, p = 0.707) effect, nor a species within interacting flow and pCO₂ effect (F6,342 = 0.540, p = 0.707).

4. Discussion

This study examined the interactive effects of CO₂ enriched seawater with water motion on the growth rates of three dominant non-calcifying macroalgal species. Macroalgal growth rates were independently affected by pCO₂ and water flow, and the growth rates were reduced from 5 to 8.5 cm·s⁻¹ for all three species, contradicting our first hypothesis that increasing flow speed would increase growth rates. Additionally, growth rates of A. rhodantha were not higher under OA nor were the growth rates among species affected by the interaction of pCO₂ and flow. However, within flow treatments, the magnitude of growth differed by species. L. variegata had a positive RGR at 0.1 cm·s⁻¹ and double and five-fold the growth rate of A. rhodantha and D. bartayresiana at 5 cm·s⁻¹, respectively. As the increase in dissolved CO₂ continues, the role of OA will be a critical component in predicting responses in non-calcareous macroalgae. Coupled with water flow, it can provide a better understanding of how physical parameters can modulate physiological processes of benthic reef organisms.

Under current CO₂ conditions, HCO₃⁻ is the predominant DIC species present compared to CO₂, 92% and 1%, respectively (Hurd et al., 2009). The current large HCO₃⁻ pool provides an advantage to algae with CCMs that have become efficient at CO₂ fixation compared to obligate CO₂ users (Fernández et al., 2015). However, the relative proportions of DIC in seawater will shift under OA, with an expected increase of 194% in dissolved CO₂ and a 14% increase in HCO₃⁻ (Royal Society, 2005). This increase in dissolved CO₂ is hypothesized to benefit obligate CO₂ users, potentially enhancing metabolic processes and growth (Cornwall et al., 2012). There was an overall CO₂ effect on macroalgal

Table 2
Mean carbonate chemistry for each flow speed treatment (0.1 cm·s⁻¹, 5 cm·s⁻¹, 8.5 cm·s⁻¹) at ambient and high pCO₂ treatments. The partial pressure of CO₂ (pCO₂) was calculated from salinity (PSU), pH₇ (total scale), total alkalinity (A₅), and temperature. SE of temperature and salinity were ~0.2. Values are mean ± SE (n = 14 except A₅).

<table>
<thead>
<tr>
<th>Flow speeds</th>
<th>Treatment</th>
<th>T (°C)</th>
<th>Sal PSU</th>
<th>pH₇</th>
<th>pCO₂ (μmol·kg⁻¹)</th>
<th>A₅ (μmol·kg⁻¹)</th>
<th>HCO₃⁻ (μmol·kg⁻¹)</th>
<th>CO₂ (μmol·kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 cm·s⁻¹</td>
<td>Ambient CO₂</td>
<td>27.2</td>
<td>35.4</td>
<td>8.05 ± 0.01</td>
<td>397.44 ± 7</td>
<td>2316.17 ± 7</td>
<td>1751.19 ± 7</td>
<td>10.65 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>High CO₂</td>
<td>27.3</td>
<td>34.9</td>
<td>7.71 ± 0.02</td>
<td>989.84 ± 47</td>
<td>2309.03 ± 1(8)</td>
<td>2006.99 ± 11</td>
<td>26.47 ± 1.2</td>
</tr>
<tr>
<td>5 cm·s⁻¹</td>
<td>Ambient CO₂</td>
<td>27.3</td>
<td>36.0</td>
<td>8.07 ± 0.01</td>
<td>372.91 ± 14</td>
<td>2320.14 ± 3(8)</td>
<td>1718.27 ± 14</td>
<td>9.93 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>High CO₂</td>
<td>27.2</td>
<td>35.9</td>
<td>7.71 ± 0.02</td>
<td>998.30 ± 45</td>
<td>2319.22 ± 4(8)</td>
<td>2012.21 ± 11</td>
<td>26.62 ± 1</td>
</tr>
<tr>
<td>8.5 cm·s⁻¹</td>
<td>Ambient CO₂</td>
<td>27.0</td>
<td>35.8</td>
<td>8.04 ± 0.01</td>
<td>403.53 ± 10</td>
<td>2316.50 ± 2(8)</td>
<td>1753.28 ± 9</td>
<td>10.82 ± 0.3</td>
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<tr>
<td></td>
<td>High CO₂</td>
<td>26.9</td>
<td>35.8</td>
<td>7.68 ± 0.01</td>
<td>1065.21 ± 35</td>
<td>2318.91 ± 2(8)</td>
<td>2033.00 ± 8</td>
<td>28.64 ± 1</td>
</tr>
</tbody>
</table>

Table 3
Results of the three-way ANOVA testing the effects of CO₂ and water flow on the growth rates among the three species, D. bartayresiana, L. variegata, and A. rhodantha. df = degrees of freedom, SS = sum of squares.

<table>
<thead>
<tr>
<th>Effect</th>
<th>df</th>
<th>SS</th>
<th>F values</th>
<th>p values</th>
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<tr>
<td>pCO₂</td>
<td>1</td>
<td>19.6</td>
<td>4.377</td>
<td>0.037</td>
</tr>
<tr>
<td>Flow</td>
<td>2</td>
<td>1450.9</td>
<td>162.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Species</td>
<td>2</td>
<td>783.4</td>
<td>87.471</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>pCO₂ × Flow</td>
<td>2</td>
<td>15.3</td>
<td>1.798</td>
<td>0.183</td>
</tr>
<tr>
<td>Species × pCO₂</td>
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<td>2.222</td>
<td>0.110</td>
</tr>
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<td>Species × Flow</td>
<td>4</td>
<td>521.7</td>
<td>29.125</td>
<td>&lt;0.001</td>
</tr>
<tr>
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<td>Residuals</td>
<td>342</td>
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Fig. 3. Relative growth rates (RGR) of Dictyota bartayresiana, Lobophora variegata, and Amansia rhodantha subjected to three flow speeds (0.1 cm·s⁻¹, 5 cm·s⁻¹, and 8.5 cm·s⁻¹) and two pCO₂ levels. White bars represent RGR measured in ambient pCO₂ conditions (~1000 μatm), black bars represent RGR measured in elevated pCO₂ conditions (~200 atm). Bars represent mean ± standard error (n = 20).
growth rates that seemed to be driven by differences in growth rates of L. variegata, but growth rates for both HCO$_3^-$ users were unaffected under OA, supporting our third hypothesis. While the two species utilizing HCO$_3^-$ differed in their growth responses, it was attributed to flow and not elevated CO$_2$. Fernández et al. (2015) found similar results that OA did not alter the photosynthetic and growth rates of a HCO$_3^-$ using temperate alga Macrocystis pyrifera. These results were expected, particularly for HCO$_3^-$ users, under the notion that they are carbon saturated, and would be minimally affected under OA. However, contrary to our second hypothesis, it did not differ among species within CO$_2$ treatments, suggesting that OA does not enhance or affect growth rates of these algal species regardless of their differences in carbon acquisition.

A significant difference was found among species within flow treatments in the present study. Growth rates were lowest at 0.1 and 8.5 cm·s$^{-1}$ for all three species. At 0.1 cm·s$^{-1}$, there may be insufficient uptake of nutrients and reduced gas exchange, negatively affecting physiological responses and lowering growth rates (Hurd, 2000). D. bartaryresiana and A. rhodantha both experienced negative growth rates at 0.1 cm·s$^{-1}$ while L. variegata had positive growth rates, suggesting a lower sensitivity to reduced flow compared to the other two species. This might be explained by differences in boundary layer dynamics and morphology between the species that can be affected by fluctuations in water motion. One such boundary layer is the diffusion boundary layer (DBL) where concentration gradients form through the diffusion of materials from the bulk fluid to the thallus surface where they are taken up (Wheeler, 1980; Tansk et al., 2015). The DBL thickness varies inversely with water velocity (Carpenter and Williams, 2007; Raven and Hurd, 2012), so under slower flow speeds, a thicker DBL should be present, increasing mass transfer limitation and leading to a reduction in macroalgal primary productivity (Hurd, 2000; Cornwall et al., 2014). Although the DBL thickness was not measured in this study, reduced flow likely affected the DBL negatively affecting growth rates of all three species. Additionally, the level of sensitivity to reduced flow can vary due to the physiological adaptations influenced by the hydrodynamic environments in which macroalgae grow (Hurd, 2000). Water motion can affect the morphology and surface area of an organism, which can be a determining factor for diffusion of gases (Stewart and Carpenter, 2003). Algae tend to have more flexible morphologies with narrower and more streamlined blades (Hurd and Pilditch, 2011) in high-flow habitats to decrease vulnerability to dislodgement but have lower surface area/volume ratios (SA/V) (Koehl and Alberte, 1988). In low-flow habitats, algae have wider blade morphologies with higher SA to maximize nutrient uptake despite increasing area on which drag forces can act (Stewart and Carpenter, 2003; Hurd and Pilditch, 2011). L. variegata typically can be found in crevices or lower surfaces of coral boulders (Coen and Tanner, 1989) with lower flow speeds, which may explain why L. variegata, having wide decumbent blades and higher SA, had positive growth rates when subject to no water flow and an overall higher RGR across all three flow treatments.

Contrary to our hypothesis that increasing flow speed will increase growth rates, all three species exhibited minimal growth under the highest flow treatment. Instead, we found the highest growth rate at 5 cm·s$^{-1}$, which may indicate a potential threshold to enhance the flux of CO$_2$, O$_2$, and nutrients for growth. Two possible explanations for why growth rates were not enhanced at the highest flow are: first, both D. bartaryresiana and A. rhodantha have flexible thalli with undulated blades. Increase flow speeds can cause the thalli to collapse and self-shade from drag forces (Hurd, 2000; Raven and Hurd, 2012), reducing photosynthetic activity and nutrient acquisition (Hay, 1981). In addition, high flow also could result in fragmentation or loss of biomass from shear stress. Second, although increased flow speeds can enhance growth rates (Carpenter et al., 1991), it also can result in negative effects on the thalli (e.g. fragmentation) or decrease the ability to uptake and retain CO$_2$ and/or HCO$_3^-$. CO$_2$ is an important substrate for photosynthesis and under high flow speeds, the thickness of DBL decreases. But in turn, could also increase the potential efflux of CO$_2$ out of the cell, as the algae are unable to retain the rapidly diffusing ions across the cell membrane (Hurd, 2000).

The reduced growth rates of both D. bartaryresiana and A. rhodantha from 5 to 8.5 cm·s$^{-1}$ may be an artifact of thalli clumping and potential self-shade due to their flexible morphologies. L. variegata also displayed results similar to the other species under the highest flow speed despite being morphologically different. The decumbent form of L. variegata has convoluted blades and lobed margins (Enríquez and Rodríguez-Román, 2006) and when grown, form flat, semi-circular blades (Coen and Tanner, 1989). Thus, a potential contributing factor to the lowered growth rates of this species under high flow may be related to the removal of the external CA from shear stress rather than thalli clumping, as individual blades of L. variegata were used during the treatment. The dislodgement of the extracellular CA could compromise the efficiency to readily convert HCO$_3^-$ to CO$_2$ (Hurd, 2000). Although speculative, this idea was supported by Enriquez and Rodriguez-Roman (2006) who tested the decrease in photochemical efficiency of L. variegata under higher flows and after adding a CA inhibitor, resulted in no change in photosynthetic efficiency, indicating the loss of the external CA. Collectively, the potential effects of thalli clumping, external CA dislodgement, and changes in the flux of substrates (CO$_2$, O$_2$, and nutrients) under higher flow speeds can result in adverse effects on metabolism and growth that outweigh the benefits.

Interestingly, L. variegata showed markedly higher RGR at 5 cm·s$^{-1}$ compared to the other two more flexible fleshy species, and between the two HCO$_3^-$ users, there were different patterns of RGR despite being from the same family (Dictyotaceae). D. bartaryresiana showed greater sensitivity to changes in water motion and exhibited lower growth rates than L. variegata for all three flow speeds. This is consistent with other studies looking at physiological performance in macroalgae with CCMs like Enriquez and Rodríguez-Román (2006), who demonstrated lower sensitivity to decreased water motion in L. variegata compared to a seagrass species with more strap-like morphology. They also found in their study the photosynthetic optima for L. variegata was under intermediate flow speed while photosynthesis declined with increasing velocity. In addition, both L. variegata and D. bartaryresiana have CCMs but the role of CCM can differ, as certain species can have different mechanisms to assimilate HCO$_3^-$ (Fernández et al., 2014). Yet it was still expected that the two HCO$_3^-$ users would exhibit somewhat similar responses. The growth rate of D. bartaryresiana under high flow was negative, suggesting fragmentation (unpublished preliminary studies) since both L. variegata and A. rhodantha exhibited rates that were positive under high flow. This was also seen by Renken et al. (2010) reporting a high rate of fragmentation of Dictyota pulchella due to its susceptibility to dislodgement from wave exposure. However, between the two morphologically similar species, D. bartaryresiana and A. rhodantha still showed similar growth responses to water motion. This highlights the importance of morphological adaptations among species that can affect physiological performance in a physical environment.

Our fourth hypothesis that interaction between water flow and OA would result in higher growth rates of A. rhodantha than the other two species was not supported. This contradicts the prediction that CO$_2$ users are expected to benefit with increasing CO$_2$ concentrations compared to HCO$_3^-$ users. Growth rates for D. bartaryresiana may have been underestimated, but L. variegata still displayed overall higher RGR than A. rhodantha. Under OA conditions, it is possible that algae with CCMs can down-regulate their CCM activity, allocating more energy towards growth (Giordano et al., 2005). Furthermore, L. variegata blades have larger surface area that potentially could result in increased light capture, offering an advantage to having flat, decumbent blades than flexible, undulated blades regardless of flow velocities. Interestingly, the red alga did exhibit the strongest negative effect on growth rates among the three species when combined with no water motion. This suggests high sensitivity when limited by carbon and mass-transfer.
Still, A. rhodantha was unresponsive to CO₂, indicating that this particular species is not affected by elevated CO₂ as previously predicted for an obligate CO₂ user. Cornwall et al. (2012) found similar results examining photosynthetic responses of five macroalgal species based on their carbon use under OA conditions, and found that the obligate CO₂ users did not benefit under OA conditions as predicted in their study. Although they did not measure the same response variables, differences in photosynthetic rates would be predicted to affect algal growth rates. This was also seen in a red alga Pyropia haitanensis where growth rates were not affected when exposed to elevated CO₂ and temperature (Liu and Zou, 2015). However, other studies (e.g. Kübler et al., 1999) demonstrated that increasing CO₂ concentrations increased growth rates for obligate CO₂ species. Further research is required to examine the level of sensitivity present to CO₂ and water motion. This argues for more, and longer-term experiments to test the effects of OA with variable environmental conditions (e.g. light or flow) to elucidate the underlying mechanisms influencing macroalgal responses.

As an increasing number of studies are focusing on understanding the underlying mechanisms involved in organismal responses to global threats, it is important to examine multiple environmental and physical factors simultaneously to better understand the variability in algal responses. For coral reefs, the natural heterogeneous environment can affect species distribution and assemblage, influencing the responses of reef organisms. Furthermore, these responses may differ depending on the type of reef habitat (e.g. back reef vs. outer reef) the algae are found in (Hurd, 2000). For example, two separate studies on reef communities of different habitats showed that net calcification in both the back reef and outer reef habitats were negatively affected by OA, but water flow had a greater effect on the back reef communities (Comeau et al., 2014) than the outer reef communities (Comeau et al., 2016). While these studies examined the calcification rates of reef communities, it highlights how water flow can affect different species assemblages physiologically in various habitats. For the present study, the three commonly abundant non-calciﬁcating macroalgal species found in the back reef exhibited a stronger response to water ﬂow than OA. As many benthic organisms are exposed to a dynamic flow environment, it may result in species-speciﬁc responses due to different physiological adaptations to ﬂow conditions (Cornwall et al., 2014). Differing responses of macroalgae to water ﬂow and OA might alter competitive outcomes between species, potentially changing community composition. This study demonstrates how prevalent water ﬂow is in modulating macroalgal responses and the importance in considering flow into future experimental designs. Additionally, the variability in responses of non-calcareous macroalgae utilizing different DIC species is species-speciﬁc when many environmental factors are involved. This is in line with a number of studies showing variable directional responses in growth rates of macroalgae (e.g. Fernández et al., 2014). Predicting the role of OA on algal physiology related to their DIC use is complex. The high variability of responses among species is due, in part, to the lack of understanding of the underlying mechanisms influencing physiological responses. While studies suggest that OA may enhance metabolic processes, studies on the inﬂuence and activity of CCMs are lacking (Raven et al., 2011; Johnson et al., 2014). If macroalgal responses are affected by their ability to utilize different DIC species, it may have profound influence on species interactions that might determine future competitive interactions among benthic organisms and drive the trajectories of the community structure of future coral reefs.

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