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Responses of calcifying algae (*Halimeda* spp.) to ocean acidification: implications for herbivores

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ABSTRACT: Ocean acidification (OA) can alter the development and physiology of many marine organisms. In addition to calcified invertebrates, studies documenting the responses of calcareous algae are critical because of their prominent role in habitat structure and carbonate production within coastal environments. While many studies report physiological responses, few have examined how OA might ultimately alter interactions with generalist herbivores via shifts in algal chemistry. This study describes a series of experiments that examine the influence of OA on the growth and herbivore defensive compounds of calcareous green algae (*Halimeda* spp.). One experiment was conducted in an open, outdoor seawater system with *H. opuntia*, while the other was conducted in an indoor, closed system with *H. incrassata* and *H. simulans*. Both experiments were conducted over similar ranges in pCO_2 (300 to 2400 µatm) and monitored shifts in calcification and herbivore defenses (calcium carbonate [$CaCO_3$] and terpenoid metabolite content). Feedings assays with common sea urchins (*Lytechinus variegatus* and *Diadema antillarum*) were further conducted to test the degree to which shifts in algal chemistry influence herbivore feeding preferences. Our results were variable among *Halimeda* spp., highlighting that OA-induced shifts in chemical composition are species-specific. OA reduced the $CaCO_3$ content (% dry wt) of *H. incrassata* yet had no effect on *H. opuntia* or *H. simulans*. Terpenoid metabolite concentrations were unaltered by pCO_2 for all species. Assays with sea urchins revealed that feeding significantly increased on diets of lower $CaCO_3$ and secondary metabolite content. Our work suggests that certain algal species may be relatively more susceptible to OA-induced shifts in chemical composition, and those shifts have the potential to weaken the efficacy of herbivore defenses. Future research on how OA influences marine plant–herbivore interactions will improve our broader understanding of how OA stands to alter community and ecosystem properties.

KEY WORDS: Climate change · Carbon dioxide · CO_2 · Calcification · Calcium carbonate · Secondary metabolites · *Halimeda opuntia* · *Halimeda incrassata* · *Halimeda simulans* · *Lytechinus variegatus* · *Diadema antillarum*

INTRODUCTION

Ocean acidification (OA) represents a long-term shift in seawater carbonate chemistry as atmospheric and oceanic concentrations of CO_2 rise. Anthropogenic activities have largely driven these changes, and since the Industrial Revolution, fossil fuel combustion and deforestation have increased atmospheric CO_2 concentrations by nearly 30% (Meehl et al. 2007). Current forecasts suggest that by the year 2100, CO_2 levels could rise from present values of 400 to nearly 1000 ppm under the most aggressive, albeit likely, emissions scenario (Zickfeld et al. 2013). The oceans have absorbed approximately one-third

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of these anthropogenic emissions (Sabine et al. 2004), resulting in a series of chemical reactions which increase seawater acidity and decrease the saturation states of calcium carbonate (Ω_{ca} and Ω_{ar}). Oceanic surface water pH has declined by 0.1 unit since the industrial period, and current projections portend an additional 0.3 to 0.5 unit reduction by the year 2100 (Caldeira & Wickett 2003, 2005). As has been widely reported, these shifts in carbonate equilibria can have consequences for calcified marine organisms via their ability to produce and maintain skeletons comprised of calcium carbonate ($CaCO_3$) (Orr et al. 2005, Kroeker et al. 2010).

Studies examining the responses of calcified green algae, particularly within the genus *Halimeda* (order Bryopsidales), have been on the rise (Ries et al. 2009, Price et al. 2011, Sinutok et al. 2011, 2012, Comeau et al. 2013, 2014, Hofmann et al. 2014). Calcified algae such as *Halimeda* play important roles in tropical and subtropical coastal environments via their production of $CaCO_3$ and contributions to biogenic sediments (Rees et al. 2007, van Tussenbroek & van Dijk 2007). Production rates of *Halimeda* can equal those of corals, approaching nearly 1.4 kg $CaCO_3$ m^{-2} yr^{-1} (Drew 1983, Payri 1988). Furthermore, within certain regions, *Halimeda* can form structurally complex mounds, serving as unique habitats for a variety of marine organisms (Rees et al. 2007). Thus, examining the responses and potentially broader consequences of OA on these prominent members of the coastal environment remains an important task.

While prior work has demonstrated a variety of organismal responses (photosynthesis, respiration, and calcification) to increased partial pressure of CO_2 (pCO_2), few studies have placed these physiological changes in the context of broader shifts in community and ecosystem processes. It is likely that OA will elicit changes in the marine environment that extend beyond physiology. For example, biotic interactions and community processes such as herbivory may be further modified by OA, potentially influencing algal assemblages. Studies of biological communities at natural CO_2 vents, which can serve as proxies for the broader impacts of OA, show significant changes in the species composition of herbivore and plant communities (Hall-Spencer et al. 2008, Fabricius et al. 2011). As such, experiments that elucidate the mechanisms driving OA-induced shifts in benthic and food web structure are needed (Hall-Spencer et al. 2008, Doney et al. 2009).

The chemical composition of algal biomass can strongly influence herbivory (Hay & Fenical 1988, Paul et al. 2001). However, attention toward how OA alters tissue content and biochemical properties (hereafter phytochemistry) has been lacking. Price et al. (2011) showed that thallus $CaCO_3$ content (% dry wt) was unaltered by OA in *H. opuntia*. However, for *H. taenicola*, strong declines in % $CaCO_3$ were detected. $CaCO_3$, while influencing herbivory by regulating tissue toughness and nutritional value, has also been suggested to act as an herbivore defense by interfering with herbivore feeding via a buffering effect on gut pH, thus affecting digestion by altering the effectiveness of key enzymes (Lobel 1981, Hay et al. 1994, Schupp & Paul 1994). Given that $CaCO_3$ content contributes to the structural integrity and herbivore defenses of *Halimeda* spp. (Littler et al. 1983, Lewis 1985, Paul & Hay 1986, Hay et al. 1994, Schupp & Paul 1994), these OA-induced shifts in phytochemistry may play a prominent role in the ability to resist mechanical damage and deter grazing.

Alternate phytochemical properties, such as concentrations of secondary metabolites, may also be influenced by OA and further act as an herbivore defense. *Halimeda* spp. produce several structurally similar diterpenoid metabolites, such as halimedatetraacetate and the epimers halimedatrial and epihalimedatrial (Paul & Fenical 1983, 1984, 1986), all serving as potent feeding deterrents toward a variety of reef fishes and urchins (Paul & Fenical 1986, Paul & Van Alstyne 1988). Given that the production and/or storage of these compounds is energetically costly (Coley et al. 1985, Hay & Fenical 1988), OA-induced shifts in photosynthetic rates (as demonstrated in Sinutok et al. 2011, 2012) may serve to alter secondary metabolite concentrations. Moreover, as $CaCO_3$ comprises a significant percentage of *Halimeda* mass, shifts in $CaCO_3$ content may also serve to indirectly alter concentrations of secondary metabolites. To date, few studies have examined how OA might alter the palatability of calcareous algae to herbivores despite repeated discussion of this topic (Price et al. 2011, Hofmann et al. 2012, 2014, Ragazzola et al. 2012). Given that herbivory is a key structuring agent in algal community composition (Lubchenco & Gaines 1981), both qualitative and quantitative distinctions in the production of phytochemical compounds is of primary importance to the broader ecology of reef habitats (Hay & Fenical 1988, Paul et al. 2001). This study examines the influence of OA on the phytochemical composition ($CaCO_3$ and terpene content) of calcareous green algae (*Halimeda* spp.) and further explores the implications for herbivory by 2 species of tropical sea urchins. We present a series of experiments that subjected 3 spe-

cies of calcareous green algae (*H. opuntia*, *H. incrassata*, and *H. simulans*) to a range of pCO_2 levels forecasted for the year 2100 and beyond. Pairwise-choice feeding assays with the sea urchins *Lytechinus variegatus* and *Diadema antillarum* were additionally conducted to examine how simulated OA-induced shifts in phytochemistry alter feeding preferences. Thus, we explicitly tested 2 main hypotheses: (1) that OA will strongly influence *Halimeda* phytochemical properties (via shifts in $CaCO_3$ content and terpene production); and (2) that across the magnitude of detected responses, these reductions will influence the feeding preferences of generalist herbivores in the marine environment.

MATERIALS AND METHODS

Expt 1: *Halimeda opuntia*

Algal collection

H. opuntia, a circumtropical alga found in seagrass meadows and on coral reefs, was collected by hand from nearshore locations along Key Largo, Florida, USA (25.02° N, 80.49° W) in spring 2011. Individual thalli (i.e. vegetative fragments) were collected at least 5 m apart and were transported within 24 h in aerated coolers to the Rosenstiel School of Marine and Atmospheric Science (RSMAS) at the University of Miami. Thalli were acclimated to ambient seawater conditions within a seawater system for 48 h prior to experimentation.

CO_2 enrichment: outdoor open system (RSMAS)

The first experiment ran from April 5 to June 1, 2011 (58 d), at the South Florida Coral Reefs and Climate Change Laboratory. This facility contains a series of experimental seawater tanks located in a greenhouse structure that permits exposure to natural sunlight yet protection from wind and rain. To test the effects of elevated pCO_2 on the growth and phytochemistry of *H. opuntia*, 5 distinct pCO_2 levels were distributed across 10 separate fiberglass tanks (0.6 m wide × 0.54 m long × 0.22 m deep; each containing approximately 50 l of seawater). Each tank was provided with a continuous supply of high-quality natural seawater from a pumping system that draws from an inlet located in nearby Bearcut Passage, Biscayne Bay, FL. The water was filtered to remove particles larger than 10 µm and supplied to the tanks at a rate of ~30 ml min^{-1}. This rate exceeded daily evaporation rates and further met algal demand for dissolved inorganic carbon (DIC) and total alkalinity (TA) by a sufficient margin. Thus, salinity and carbonate chemistry within the control tanks were maintained at levels similar to the natural environment. The salinity was measured with a YSI model 50 temperature/salinity meter calibrated weekly against a 50.0 mS standard. Salinity ranged from 32.0 to 36.7 and averaged 34.1 ± 1 (SD). Seawater temperature in the tanks was held at 27°C using Omega Engineering CN7533 digital temperature controllers. Photosynthetically available radiance (PAR, measured with a LI-COR 192 PAR sensor with a cosine collector) averaged 331 µmol photons m^{-2} s^{-1} at noon. Each of the 10 tanks was randomly assigned to 1 of 5 pCO_2 levels (2 replicate tanks per treatment): 317, 611, 914, 1548, and 2379 µatm (Table 1). The CO_2 levels were achieved by bubbling seawater (using venturi injectors in 200 l sump tanks) with either ambient air or ambient air that had been mixed with pure CO_2 gas to a specified level. Each sump tank continuously exchanged seawater with the experimental 50 l tank where the algae were held. Mass flow controllers (Sierra Instruments 810C) set the treatment gas concentrations by controlling the mixing ratio of ambient air and pure CO_2. While the sump tanks are covered, the experimental tanks are uncovered (so the test organisms can receive natural light) and hence open to exchange gas with the atmosphere. For this reason, it was necessary to empirically adjust the composition of the CO_2-air mixture until the desired CO_2 level in the experimental tanks was reached. An equilibrator/LI-COR CO_2 analyzer that permitted real-time readout of the CO_2 partial pressure (xCO_2) was used to visualize tank pCO_2 while the adjustments were being made. Despite being an open system, consistent CO_2 levels were maintained over many weeks and generally held to within ±3 to 6%. An advantage of this mode of CO_2 control is that while it poises the CO_2 at the desired level, it allows the test organisms to impose natural diel variability to the carbonate chemistry. The equilibrator/LI-COR system revealed that pCO_2 varied from a maximum at dawn to a minimum at dusk, with an amplitude that averaged 12% of the mean level. The resulting day–night swing in the saturation state of aragonite (Ω_{ar}) was 0.95 to 0.65 in the highest CO_2 treatment and 1.12 to 0.90 in the second-highest treatment. Given the existence of this diel variability, all water samples for carbonate chemistry were collected at noon, when photosynthesis and calcification rates were highest and all chemical param-

Table 1. Summary of measured and calculated seawater carbonate parameters (means ±1 SE) from repeated measurements in each tank during the course of each experiment. Carbonate parameters were calculated from separate pH (n = 14) and total alkalinity (TA) (n = 14) measurements in each tank in Expt 1 and separate pH (n = 16) and TA (n = 6) measurements in each tank in Expt 2. All calculations were conducted with the program CO2SYS using the dissociation constants of Mehrbach et al. (1973), as refit by Dickson & Millero (1987). Ω_{ar}= saturation state of aragonite, Ω_{ca} = saturation state of calcite, $CO_{2(aq)}$ = aqueous CO_2, CO_3^{2-} = carbonate ion, HCO_3^- = bicarbonate, pCO_2 = partial pressure of carbon dioxide, T = temperature

CO_2 treatment	T (°C)	Salinity	$pH_{(total)}$	TA (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (µmol kg^{-1})	CO_3^{2-} (µmol kg^{-1})	$CO_{2(aq)}$ (µmol kg^{-1})	Ω_{ca}	Ω_{ar}
Expt 1: *Halimeda opuntia*										
Ambient	27.1 ± 0.1	36.3 ± 0.1	8.08 ± 0.01	2049 ± 20	317.8 ± 9.7	1519.1 ± 15.3	209.6 ± 5.5	8.7 ± 0.3	5.0 ± 0.1	3.6 ± 0.3
Level 1	27.1 ± 0.1	36.6 ± 0.1	7.83 ± 0.02	1974 ± 22	611.3 ± 21.8	1647.4 ± 14.9	129.0 ± 4.7	16.8 ± 0.6	3.1 ± 0.1	2.2 ± 0.3
Level 2	27.0 ± 0.1	36.2 ± 0.2	7.68 ± 0.02	2005 ± 52	914.8 ± 49.8	1754.3 ± 45.7	99.5 ± 5.6	25.1 ± 1.3	2.4 ± 0.1	1.8 ± 0.3
Level 3	27.2 ± 0.1	36.6 ± 0.1	7.53 ± 0.04	2093 ± 14	1548.8 ± 61.3	1918.3 ± 13.4	70.1 ± 2.3	41.7 ± 1.5	1.7 ± 0.1	1.2 ± 0.1
Level 4	27.1 ± 0.1	36.6 ± 0.1	7.33 ± 0.02	2140 ± 37	2379.2 ± 98.1	2016.3 ± 34.5	50.1 ± 2.2	65.3 ± 2.4	1.2 ± 0.1	0.9 ± 0.1
Expt 2: *H. simulans*, *H. incrassata*										
Ambient	28.0 ± 0.1	35.6 ± 0.1	7.95 ± 0.01	1925 ± 20	430.2 ± 10.0	1502.4 ± 14.5	165.8 ± 3.8	11.3 ± 0.3	4.0 ± 0.1	2.7 ± 0.1
Level 1	28.1 ± 0.1	35.7 ± 0.1	7.63 ± 0.01	1965 ± 22	1022.5 ± 23.0	1731.3 ± 20.5	92.4 ± 1.7	26.8 ± 0.6	2.2 ± 0.1	1.5 ± 0.1
Level 2	28.1 ± 0.1	35.6 ± 0.1	7.32 ± 0.01	2148 ± 28	2429.6 ± 55.5	2017.5 ± 27.1	52.5 ± 0.8	63.5 ± 1.4	1.3 ± 0.1	0.8 ± 0.1

eters (pH, pCO_2, TA, DIC, and saturation states) were close to their daily mean levels. Weekly water samples were analyzed for TA and pH to provide an independent and complete description of the carbonate chemistry in each tank. TA was determined using open-cell, Gran titration, with measurements checked against certified reference materials (Dickson standard, Scripps Institution of Oceanography), and pH (total scale) was determined using an Orion pH electrode (Thermo Scientific) calibrated against seawater Tris buffer. Seawater carbonate parameters ($CO_{2(aq)}$, HCO_3^-, CO_3^{2-}, Ω_{ca} and Ω_{ar}) were calculated with the program CO2SYS (Lewis & Wallace 1998) using the measured parameters of pH, TA, temperature, and salinity, with the carbonate dissociation constants of Mehrbach et al. (1973), as refit by Dickson & Millero (1987).

Experimental design

Portions of *H. opuntia* were harvested from larger thalli and transplanted into the experimental tanks (5 thalli per tank). Field-collected thalli were cleaned of epibionts and trimmed down to approximately the same size (1.5 g buoyant wt, 4 g wet wt). Each replicate thallus was loosely attached to a 4.5 × 4.5 cm terracotta tile using a cable tie. All thalli were oriented such that the cut base faced the tile surface and the uncut segments faced upward. The tiles were then placed on a plastic egg crate, which elevated the thalli above the experimental tank floor. After the acclimation period, CO_2 enrichment was initiated and continued for 58 d (April 5 to June 1, 2011). At the end of the experiment, thalli were removed from the tanks and processed to quantify calcification and phytochemistry. Upon conclusion, all algae appeared green and healthy with minimal epiphyte loading. Net calcification was calculated as the change in buoyant weight (Davies 1989) of the entire thallus over the enrichment period and is reported as mg g^{-1} d^{-1}. Phytochemical analyses were conducted on new growth, which was determined photographically by comparing images before and after the experimental period. Percent inorganic carbon was measured by ashing aliquot samples of new growth from each algal thallus (12 h in a 500°C muffle furnace) and calculating the ratio of ash dry weight to total dry weight. Contributions of other trace minerals to the ash weight were considered minimal; thus, this ratio was used as a proxy for $CaCO_3$ content (Davis & Fourqurean 2001, Barry et al. 2013).

Terpene analysis

Fresh thalli (368 g wet wt) were extracted in a solvent mixture of ethyl acetate (EtOAc) and methanol (1:1 ratio). *H. opuntia,* like most species of *Halimeda*, produces the diterpene halimedatetraacetate as the major secondary metabolite (Paul & Fenical 1984). This terpene converts to halimedatrial upon thallus damage (Paul & Van Alstyne 1992), and so to prevent this conversion, the algae were extracted fresh in organic solvents. The extract was then fractionated by silica gel column chromatography using non-polar solvent mixtures of EtOAc and hexanes of increasing

polarity. Fractions containing the terpenes were purified by semi-preparative silica HPLC. Two terpenes were isolated, and their identity was confirmed by proton nuclear magnetic resonance (NMR) spectroscopy as a bis-nor diterpenoid and halimedatetraacetate (metabolites No. 5 and No. 4, respectively, from Paul & Fenical 1984). Quantities of the bis-nor diterpenoid were minor; thus, only the major compound halimedatetraacetate was quantified.

For quantification of halimedatetraacetate, new growth from each individual thallus (1 to 4 per tank) was extracted 3 times and prefractionated to concentrate the compound of interest prior to analytical HPLC. The thalli were dried (60°C for 24 h), and the dry weight plus crude extract weight equaled the total algal dry weight for each individual thallus. Each crude extract was separated into 2 fractions by silica gel column chromatography (Sep-Pak SPE cartridges) using 2 solvent mixtures: fraction A was eluted in a mixture of 20% EtOAc and 80% hexanes, and fraction B (which contained halimedatetraacetate) was eluted in 100% EtOAc. Halimedatetraacetate was quantified from fraction B using a PerkinElmer HPLC instrument with photodiode array detector and integration of the target peak. Nine concentrations of purified halimedatetraacetate were used to generate a standard curve ranging from 0.05 to 0.9 mg ml^{-1} ($r^2 > 0.97$). The multiple concentrations were derived from 4 independently weighed halimedatetraacetate samples to generate the 9 levels of the standard curve. Samples were solubilized to a concentration of 1 mg ml^{-1}, and 100 µl were injected into the HPLC in a solvent mixture of 17% EtOAc and 83% hexanes with a flow rate of 1 ml min^{-1}. An Econosphere silica column (3 µm, 150 × 4.6 mm) was used for all analyses. An isocratic method with that solvent mixture yielded clean peak separation in fraction B, high levels of absorbance at 254 nm, and repeatable halimedatetraacetate peaks (retention time = 16 min). Individual samples were injected in triplicate and displayed minor variation (±1%), and the results were averaged for statistical analysis. Concentrations of halimedatetraacetate are expressed as mg g^{-1} dry wt.

Expt 2: *H. incrassata* and *H. simulans*

Algal collection

H. incrassata and *H. simulans*, tropical rhizophytic green algae typically occupying soft-bottomed sediments, were collected by hand along the shoreline of Virginia Key, Florida (25.71°N, 80.15° W), in summer 2013. Individuals were located in 3 m of water and were carefully dislodged from the sediments by removing the entire thallus, keeping the holdfast and adhered sediments intact. Individuals were transported in aerated coolers to the Smithsonian Marine Station (SMS), Fort Pierce, Florida, within 24 h and allowed to acclimate in flowing seawater tanks for 48 h prior to experimentation.

CO_2 enrichment: indoor closed system (SMS)

Seawater chemistry was manipulated in 12 independent, temperature-controlled, 37 l tanks at SMS (Table 1). These tanks were housed indoors, allowing for careful control of experimental conditions. Lighting was provided by a series of 220 W Aqua Medic T5 HO light fixtures that replicated natural, broad-spectrum irradiance (PAR = 350 µmol photons m^{-2} s^{-1}). Each tank consisted of a closed seawater system, whereby the water volume was continuously recirculated and thus prevented from mixing with any other tank. Each tank was filled with filtered seawater (<10 µm) collected from an offshore (0.4 km) oceanic location near Fort Pierce. Temperature control was provided by water-jacketed heat exchangers attached to each tank, and temperature was set and maintained at 28° ± 0.1°C by independent dual-stage temperature controllers. Salinity was maintained at 35 by replenishing evaporative losses with deionized water (DI). During the course of the experiment, weekly water changes in each tank (50% volume) were conducted as a means of preventing biologically induced shifts in alkalinity.

CO_2 concentrations were manipulated via a coupled pH stat system (Aqua Medic), which monitored tank pH using separate, independent electrodes. Elevated CO_2 tanks were periodically bubbled with 100% gaseous CO_2 as determined by a series of computer-controlled magnetic solenoids. Thus, as pH levels in the enriched tanks increased toward ambient levels (primarily via CO_2 outgassing), CO_2 was added at a slow rate (25 ml min^{-1}) until the desired pH level was reached. OA treatments consisted of 3 levels (4 replicate tanks randomly assigned to each treatment) set to mimic seawater carbonate forecasts through the year 2300: control pH (8.1), mid pH (7.8), and low pH (7.5) (Caldeira & Wickett 2003). To facilitate comparisons with Expt 1, calculated pCO_2 levels within each tank (means ±1 SE) were 430 ± 9, 1022 ± 23, and 2429 ± 55 µatm, respectively. Measurements of pH (National Bureau of Standards scale) within

each individual tank were logged twice daily near the beginning and end of the light cycle (08:00 and 20:00 h). The mean difference between morning and evening pH values was relatively small, 0.06 unit across all treatments. Additional measurements of pH within each tank were conducted with a separate handheld meter connected to an Orion Ross combination pH electrode (relative accuracy ±0.002 unit). These measurements were conducted 3 to 4 times per week (between 10:00 and 16:00 h) to ensure the proper calibration of the pH stat system. Weekly water samples were collected midday to measure TA via open-cell, potentiometric titration (Mettler Toledo DL15). Certified reference materials (Dickson standard, Scripps Institution of Oceanography) were used to ensure the accuracy of TA measurements. All carbonate parameters within each tank were calculated with CO2SYS, using measured parameters of pH, TA, temperature, and salinity (Table 1). Dissociation constants were the same as those stated in Expt 1.

Experimental design

After the acclimation period, thalli of both species were carefully washed free of epiphytic growth and attached sediment, weighed on an analytical balance (±0.01 g) using the buoyant weight technique (Davies 1989), and transplanted into smaller individual containers filled with sediment from the collection site. These containers were randomly distributed into the 12 tanks, with each tank receiving 5 individuals of each species. Algae were cultured for a total of 49 d (July 16 to September 3, 2013) and then removed from the tanks for growth and phytochemical analysis. All samples appeared green and healthy at the end of the experimental period. Measurements of maximum quantum yield (F_v/F_m) on the distal segments support these findings. Net calcification was measured using the change in buoyant weight before and after CO_2 enrichment and is reported as mg g^{-1} d^{-1}. Percent $CaCO_3$ was assessed with a gravimetric acidification technique (van Tussenbroek & van Dijk 2007). One randomly selected individual of each species from each tank was rinsed with DI and placed in a 60°C drying oven until a constant dry weight was achieved. The aboveground portion of the thallus was then placed in preweighed glass vials and acidified with 10% HCl for 1 h. Afterward, thalli were carefully placed in DI for another hour to remove traces of HCl and dried to a constant weight in the oven. $CaCO_3$ content was determined from the dry weight difference between calcified and decalcified tissues. Terpene analysis was conducted on a separate, randomly selected individual from each tank. Analysis of halimedatetraacetate was similar to that of Expt 1, with a standard curve constructed from purified halimedatetraacetate. Whole thalli without their rhizomes were twice extracted in a solvent mixture of EtOAc and methanol (1:1 ratio), and the dried crude extract was fractionated with silica gel chromatography using solvent mixtures of EtOAc and hexanes (fraction A: 80% hexanes and 20% EtOAc; fraction B: 100% EtOAc). Fraction B was identified as containing the metabolite of interest and was dried, weighed, and solubilized to a concentration of 1 mg ml^{-1} for quantification. Each sample was then filtered through a 0.45 µm filter and analyzed with a Waters HPLC using an Econosphere silica column (3 µm, 150 × 4.6 mm) and a solvent mixture of 20% EtOAc and 80% hexanes. At a flow rate of 1 ml min^{-1}, halimedatetraacetate eluted at 11 min. All samples were analyzed in duplicate and displayed minor variation between repeated injections (±3%).

Expt 3: herbivore responses to phytochemistry

Artificial diet preparation and feeding assays

Sea urchins were collected and held for feeding assays in 2 locations. *Lytechinus variegatus* was collected from West Palm Beach, Florida (26.77° N, 80.04° W), and held in a flow-through seawater system (salinity 31, temperature 27 to 30°C) at SMS. *Diadema antillarum* was collected near Carrie Bow Cay, Belize (16.80° N, 88.08° W) and held in an array of perforated containers anchored on the sea floor near the dock at Carrie Bow Cay Field Station (salinity 34, temperature 28 to 31°C).

To determine the concentration-response of these generalist herbivores to the phytochemical compounds measured in Expts 1 and 2, a series of pairwise-choice feeding assays was performed with artificial foods of varying $CaCO_3$ and secondary metabolite concentrations (n = 11 to 20). Treatment food strips were prepared by mixing different amounts of $CaCO_3$ (Sigma Aldrich C6763-500G) and secondary metabolites (non-polar fraction containing halimedatetraacetate from *H. opuntia*) into an agar-based molten gel, which was poured into a mesh mold. Control food strips consisted of agar-based gels without the phytochemical constituents. To obtain the non-polar fraction of *Halimeda* secondary metabolites, *H. opuntia* was collected from Tavernier, Florida (24.95° N, 80.58° W), and transported

in aerated coolers to SMS. The *H. opuntia* was extracted 3 times overnight in twice its volume of EtOAc and methanol (1:1 ratio). The resultant extract was dried via rotary evaporation, loaded onto a hand-packed silica gel column (normal phase), and rinsed exhaustively with EtOAc. This EtOAc fraction was used to test for the effects of non-polar secondary metabolites on sea urchin feeding preference. The yield of the *H. opuntia* EtOAc fraction was 6.59 mg g^{-1} dry alga. The presence of halimedatetraacetate was confirmed via proton NMR, and quantification of this fraction by HPLC indicated that the concentration of halimedatetraacetate was 0.316 mg g^{-1} dry wt. To generate the treatment foods (Tx), this fraction containing halimedatetraacetate (from the equivalent of 2 g dry wt of alga) was solubilized in 2 ml EtOAc and mixed with 2 g dry wt of powdered *Gracilaria tikvahiae* (a palatable red alga). The carrier solvent, EtOAc, was removed via rotary evaporation, leaving fraction-coated foods. Rotary evaporation was not possible at Carrie Bow Cay Field Station; thus, 1 ml of 95% ethanol was used and mixed into the final diet for the assays conducted there. The control foods (Ctrl) were treated with carrier solvent but did not contain the non-polar fraction or $CaCO_3$. Treatment foods with $CaCO_3$ followed the same recipe and added $CaCO_3$ to the percentage being tested of the total dry recipe (i.e. algal powder, agar, and $CaCO_3$). Control foods tested with foods containing $CaCO_3$, but not the non-polar fraction, were not coated with the carrier solvent. Experimental seaweed powder (2 g) was hydrated with 7 ml of DI, and a molten mixture of 1 g agar and 18 ml DI cooled to 64°C (the lowest workable temperature) was then added and vigorously mixed to create each food. The seaweed-based food was poured into a 1.5 mm thick mold that was laid on a window screen with 7 × 6 squares cm^{-2}. The process was repeated for control foods. Foods were poured side by side (separated by 2 cm) into 2 lanes and cut into strips such that treatment and control foods were each 12 × 12 squares in area and 1.5 mm thick.

Sea urchins were fed mixtures of fresh, palatable seaweeds prior to each assay to minimize indiscriminant feeding due to hunger or nutritional compensation. During the assays, individual urchins were isolated in containers with flowing seawater and allowed to feed on the food strips of control and treatment diets for up to 24 h. Assays were checked every half hour, and individual replicates were terminated when feeding stopped or before 90% of the total amount of food offered (Tx + Ctrl) was consumed. Feeding preference was quantified by counting the number of food squares consumed, and the response variable (% treatment food of total food consumed, %Tx = Tx/(Tx + Ctrl) × 100, was calculated. For example, a %Tx of 50 indicates that urchins consumed equal amounts of treatment and control foods, whereas a %Tx less than 50 indicates that more control food was consumed. Assays in which individuals consumed less than 10% or more than 90% of food offered were excluded from final analyses because in these cases, a true choice cannot be determined (Bolser & Hay 1996).

Statistical analysis

Seawater chemistry was analyzed by comparing the 95% confidence intervals of measured and calculated carbonate parameters during both experiments. Calcification and phytochemical responses measured in Expt 1 were analyzed using linear regression, with pCO_2 as the independent variable. Responses measured in Expt 2 were analyzed using a 2-way ANOVA, with species and CO_2 treatment as the main factors. For both experiments, response metrics measured on multiple individuals within the same tank were averaged to avoid pseudoreplication; thus, individuals were not pooled across tanks, and each tank served as a unit of replication. Post hoc comparisons ($\alpha = 0.05$) for the factor of CO_2 were conducted when significance was detected. Feeding trials were analyzed by 2-way ANOVA, with urchin and phytochemical concentration as the main factors. All data were checked for normality and homogeneity of variance. When these tests were violated, data were either transformed or ranked.

RESULTS

Calcification

OA altered calcification in a species-specific manner. In Expt 1, *Halimeda opuntia* displayed significant reductions (linear regression, $F_{1,9} = 5.69$, $r^2 = 0.42$, $p = 0.04$) in net calcification at elevated pCO_2 (Fig. 1A). Mean rates remained positive for all treatments, including the highest pCO_2 level (2380 µatm). In Expt 2, OA similarly altered calcification (2-way ANOVA, $F_{2,23} = 9.1$, $p = 0.002$) but only for *H. simulans* (CO_2 × species interaction, $F_{2,23} = 7.1$, $p = 0.005$). While remaining positive across all treatments, calcification in *H. simulans* was reduced by elevated pCO_2, with significant differences (Tukey post hoc

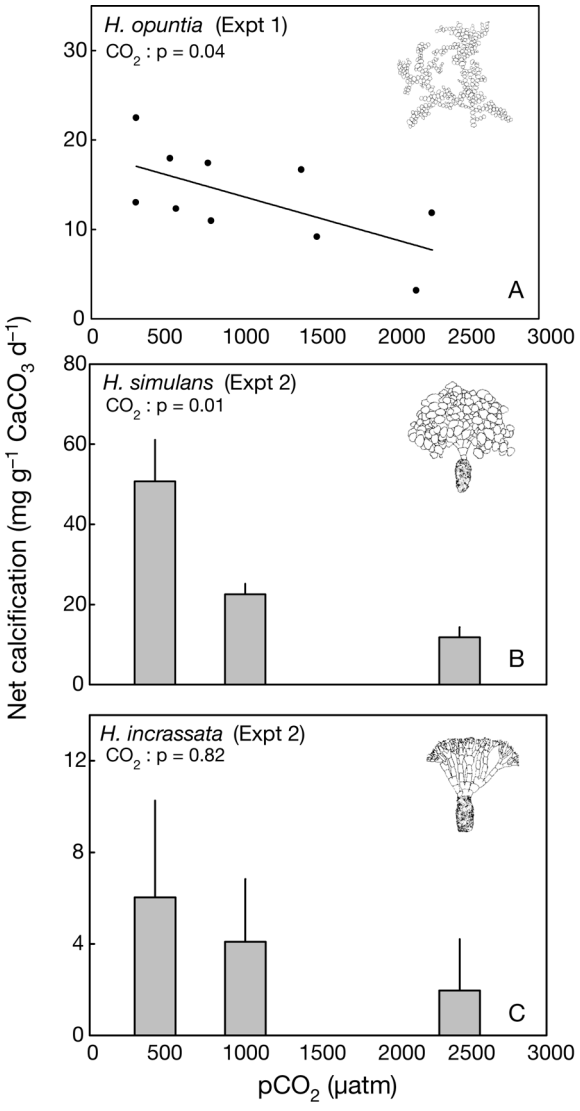

Fig. 1. *Halimeda opuntia*, *H. simulans*, and *H. incrassata*. Net calcification rates in (A) Expt 1 (n = 2 tanks per CO_2 treatment, points represent individual tanks) and (B,C) Expt 2 (n = 4 tanks per CO_2 treatment, bars represent means ± 1 SE of replicate tanks). Note that Expt 1 (*H. opuntia*) contained 2 additional pCO_2 levels (611 and 1548 µatm) as compared to Expt 2 (*H. incrassata* and *H. simulans*). Significant responses were assessed by linear regression in Expt 1 and by ANOVA in Expt 2. Images modified from Verbruggen & Kooistra (2004)

analysis, $p < 0.01$) between the control (430 µatm) and mid pCO_2 (1023 µatm) treatments and the control (430 µatm) and high pCO_2 (2429 µatm) treatments (Fig. 1B). Net calcification in *H. incrassata* was unaltered by pCO_2, with slightly positive values across all treatments (Fig. 1C). Note that calcification rates in *H. incrassata* were lower than those reported for *H. simulans* and *H. opuntia*. As calcification rates were standardized to the initial weight of the thallus, the relatively large size of *H. incrassata* (which included the intact holdfast) likely lowered the calculated rates when compared to other species. Given that *H. incrassata* contains a relatively high number of large basal segments that do not appreciably contribute to segment production (van Tussenbroek & van Dijk 2007), it is likely that the inclusion of these segments reduced calculated rates of net calcification. Initial buoyant weights of *H. incrassata* were 2.5 and 6.1× higher than those of *H. opuntia* and *H. simulans*, respectively.

$CaCO_3$ content

Species-specific responses to OA were further detected in $CaCO_3$ content. Shifts in *H. opuntia* percent $CaCO_3$ were undetected across increasing pCO_2, averaging (±1 SE) 81.4 ± 2.3% ash weight across all treatments (Fig. 2A). In Expt 2, while pCO_2 had an overall effect (2-way ANOVA, $F_{2,23} = 7.78$, $p = 0.004$), significant differences were only detected for *H. incrassata* (species × CO_2 interaction, $F_{2,23} = 12.14$, $p < 0.001$), which displayed lower $CaCO_3$ content at higher pCO_2 levels (Fig. 2C). Post hoc analysis revealed significant differences ($p < 0.01$) between the low (430 µatm) versus high (2429 µatm) and mid (1022 µatm) versus high (2429 µatm) pCO_2 treatments. $CaCO_3$ content was unaffected in *H. simulans*, averaging (±1 SE) 83.9 ± 1.9, 82.9 ± 0.6, and 85.4 ± 0.5% across the low, mid, and high pCO_2 treatments, respectively (Fig. 2B).

Terpene concentrations

Terpene concentrations were unaltered by pCO_2 for all species (Fig. 3). Halimedatetraacetate concentrations in *H. opuntia* did not significantly differ among treatments and ranged from a minimum (means ±1 SE, n = 2) of 1.75 ± 0.09 mg g^{-1} dry wt (1550 µatm pCO_2 treatment) to a maximum of 2.71 ± 0.62 mg g^{-1} dry wt (620 µatm pCO_2 treatment). Responses from *H. incrassata* and *H. simulans* similarly revealed that halimedatetraacetate was unaltered by pCO_2 (2-way ANOVA, $F_{2,20} = 0.50$, $p = 0.617$). Concentrations (means ±1 SE, n = 4) in *H. simulans* were 2.07 ± 0.31, 3.23 ± 0.69, and 4.56 ± 2.16 mg g^{-1} dry wt in the low, mid, and high pCO_2 treatments, respectively. *H. incrassata* displayed values of 4.10 ± 1.18, 3.66 ± 0.39, and 4.48 ± 1.63 mg g^{-1}

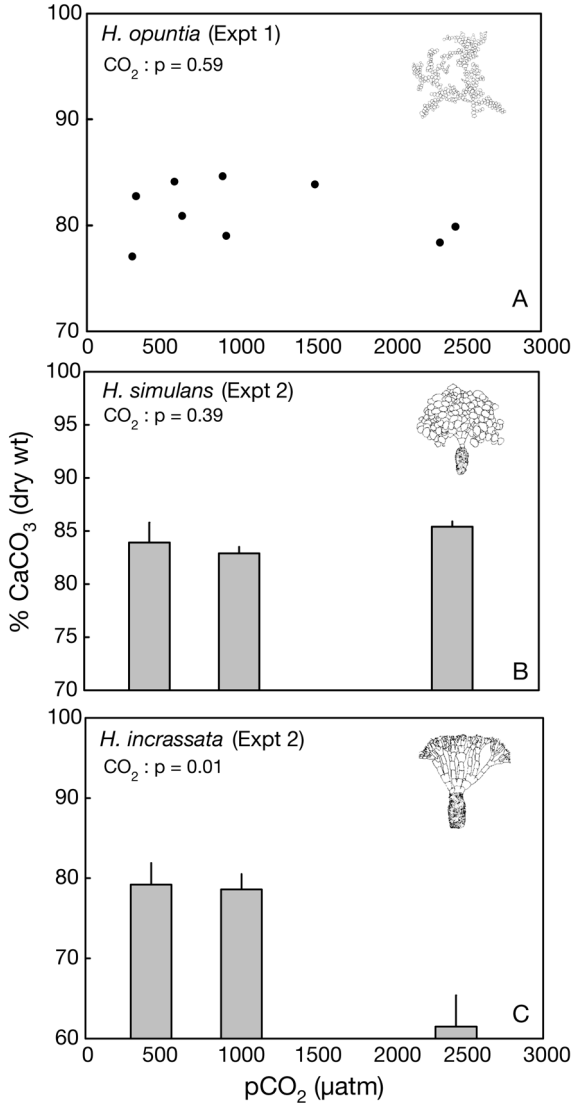

Fig. 2. *Halimeda opuntia*, *H. simulans*, and *H. incrassata*. $CaCO_3$ content (means ± 1 SE) across ocean acidification treatments in (A) Expt 1 (n = 2) and (B,C) Expt 2 (n = 4). Values are expressed as percent $CaCO_3$ of dry weight

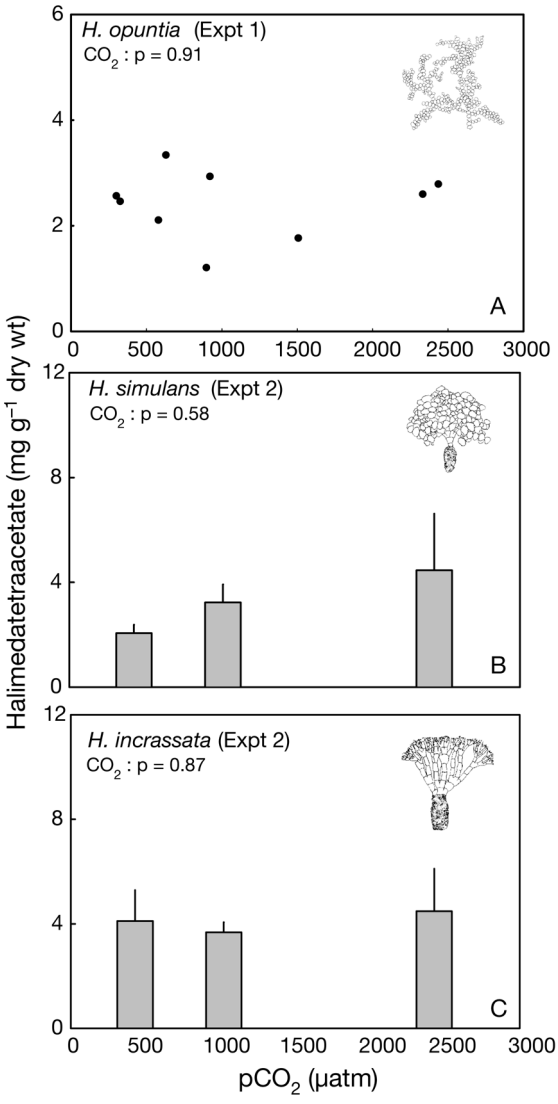

Fig. 3. *Halimeda opuntia*, *H. incrassata*, and *H. simulans*. Terpene concentrations (halimedatetraacetate, means ± 1 SE) across ocean acidification treatments in (A) Expt 1 (n = 2) and (B,C) Expt 2 (n = 4)

dry mass in the low, mid, and high pCO_2 treatments, respectively.

Herbivore responses to phytochemistry

Changes in the $CaCO_3$ content of artificial foods altered the feeding preferences of both *Lytechinus variegatus* and *Diadema antillarum* (Fig. 4A, 2-way ANOVA, p = 0.029). While *L. variegatus* displayed higher consumption of the calcified foods overall than did *D. antillarum* (2-way ANOVA, p = 0.004), both urchins responded similarly to shifts in percent $CaCO_3$ as evidenced by a non-significant interaction term (p = 0.47). As $CaCO_3$ content declined from 80 to 34% dry wt, consumption (%Tx, means ±1 SE) increased from 30.9 ± 6.8 to 50.3 ± 7.8%, repectively, of total consumption in *L. variegatus* and from 21.1 ± 5.1 to 37.9 ± 5.1%, respectively, of total consumption in *D. antillarum*. Increases in secondary metabolites produced similar effects, with a strong decline in preference for foods with higher extract concentrations (Fig. 4B, 2-way ANOVA, p < 0.001). Interactions between urchin species and metabolite concentra-

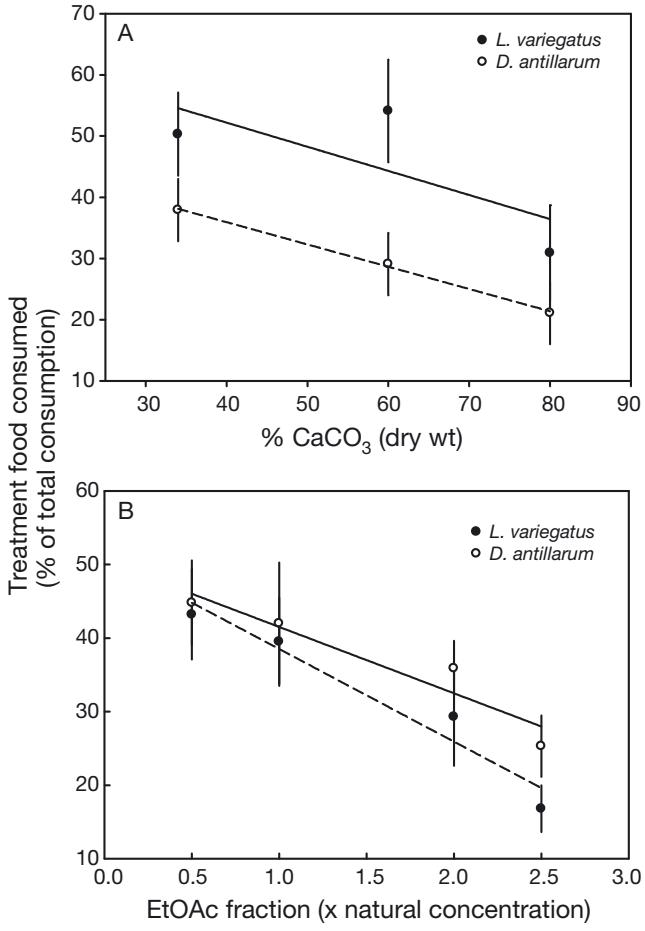

Fig. 4. *Diadema antillarum* and *Lytechinus variegatus*. Concentration response curves (means ± 1 SE, n = 11 to 20) for (A) $CaCO_3$ (by % dry wt of food) and (B) non-polar fractions of *Halimeda opuntia* (by proportion of natural concentration). Values represent the percentage of treatment food consumed relative to total amount of food consumed. Trend lines are displayed for significant shifts in urchin feeding preferences. EtOAc = ethyl acetate

tions were also not significant. Moreover, there were no differences in consumption between species. Over the tested range of EtOAc extract concentrations, declining metabolite concentrations increased consumption of the treatment food (%Tx, means ± 1 SE) from 16.8 ± 3.2 to 43.2 ± 6.1% of total consumption in *L. variegatus* and from 25.3 ± 4.2 to 44.8 ± 5.8% of total consumption in *D. antillarum*.

DISCUSSION

OA reduced *Halimeda* calcification rates and $CaCO_3$ content in a species-specific manner, suggesting that the impacts of OA across this taxonomic group will likely be variable (Table 2). Concentrations of the diterpene halimedatetraacetate, although quite variable among algal thalli, were not altered by OA for any species; thus, shifts in algal composition as related to phytochemistry will primarily result from shifts in mineral content. Feeding assays with generalist herbivores (*Lytechinus variegatus* and *Diadema antillarum*) further demonstrate that the magnitude of OA-induced declines in $CaCO_3$ content across our tested pCO_2 levels may have a significant influence on feeding preference. The implications of this work suggest that certain species of calcareous green algae may face altered palatability and increased grazing pressure because of elevated pCO_2.

Net calcification in *H. opuntia* and *H. simulans* was reduced by OA. Surprisingly, calcification was unaltered in *H. incrassata*, even at the highest pCO_2 level (similar to findings for *H. macroloba* in Comeau et al. 2013, 2014). While increased pCO_2 and reduced Ω_{ar} have been previously shown to reduce calcification in a variety of benthic organisms (Langdon et al. 2000, Orr et al. 2005, Anthony et al. 2008, Hall-Spencer et al. 2008), the variable responses detected in our study support evidence of species-specific responses within *Halimeda* (Price et al. 2011, Sinutok et al. 2011, Comeau et al. 2013). Additional work has displayed non-linear responses of calcification to OA (Ries et al. 2009), with slight increases at intermediate pCO_2 levels (606 ppm) for certain species (i.e. *H. incrassata*). The mechanisms behind the species-specific responses are currently unknown; however, it has been suggested that distinctions in morphological and physiological characteristics may largely drive this variation (Price et al. 2011). Calcification in *Halimeda* occurs within interutricular spaces between medullary filaments that comprise the segment cortex (Hillis-Colinvaux 1980). As these intercellular spaces are semi-enclosed, and partially isolated from the external environment, it has been suggested that the deposition of $CaCO_3$ may be regulated by photosynthetic and respiratory processes (Borowitzka & Larkum 1977, De Beer & Larkum 2001, Lee & Carpenter 2001), which can control seawater pH and $CaCO_3$ saturation states. Newly formed segments do not calcify until chloroplast maturity (Borowitzka & Larkum 1977, Hay et al. 1988), suggesting that there is a link between photosynthesis and calcification and that photophysiological distinctions and segment ultrastructure may contribute to differential OA responses. Similar to Price et al. 2011, we document sensitivity of *H. opuntia* to elevated pCO_2; however, significant effects in our study were only detected at pCO_2 levels beyond what was tested

Table 2. Summary of the responses of *Halimeda* spp. to ocean acidification. Positive effect (+), negative effect (–), and no effect (O) of acidification are indicated for several responses. $CaCO_3$ = calcium carbonate, pCO_2 = partial pressure of carbon dioxide

Species	Calcification	Photo-synthesis	Percent $CaCO_3$	Terpene content	Tested pCO_2 range (µatm)	Experimental duration (d)	Source
H. incrassata	O		–	O	430–2429	49	Current study
	–				409–2856	60	Ries et al. (2009)
H. opuntia	–		O	O	317–2379	58	Current study
	O		–		415–1705	27	Hofmann et al. (2014)
	–		O		416–928	14	Price et al. (2011)
H. simulans	–		O	O	430–2429	49	Current study
H. macroloba	O				449–921	14	Comeau et al. (2014)
	O				391–993	14	Comeau et al. (2014)
	O				430–959	14	Comeau et al. (2014)
	O				262–2062	14	Comeau et al. (2013)
	–	–			381–1208	35	Sinutok et al. (2012)
	–	–			318–2437	35	Sinutok et al. (2011)
H. minima	–				262–2062	14	Comeau et al. (2013)
H. cylindracea	–	–			381–1208	35	Sinutok et al. (2012)
	–	–			318–2437	35	Sinutok et al. (2011)
H. taenicola	–		–		416–928	14	Price et al. (2011)

in Price et al. (2011). Note that at these elevated CO_2 levels (near 2400 µatm), Ω_{ar} values were largely below 1.0; thus, declines in calcification may have resulted from the dissolution of $CaCO_3$, particularly during the night. Furthermore, it is important to note that while light levels were comparable between the 2 experiments of the current study (approximately 350 µmol photons m^{-2} s^{-1}), there are distinctions in light levels between other studies that examine the influence of OA on calcareous green algae. For example, Price et al. (2011) report light levels of 150 µmol photons m^{-2} s^{-1}. Given the potential link between photosynthesis and calcification, the lower light levels of Price et al. (2011) may have altered OA susceptibility and thus potentially account for the different sensitivities of *H. opuntia* to OA, as suggested for some corals (Suggett et al. 2013). Furthermore, apart from OA responses, when compared to other studies, we document relatively large variation in *H. opuntia* calcification under ambient conditions. Price et al. 2011 report *H. opuntia* calcification rates of nearly 7 mg mg^{-1} $CaCO_3$ d^{-1} as compared to our reported rates of 0.018 mg mg^{-1} d^{-1}. Such discrepancies highlight the strong role that geographic region and experimental conditions might play in regulating the growth rates of calcified algae.

$CaCO_3$ content was influenced by OA but only for *H. incrassata*, which interestingly did not display declines in calcification. Furthermore, the 2 species that did display declines in calcification (*H. opuntia* and *H. simulans*) did not show any shifts in $CaCO_3$ content. Thus, in addition to interspecific variation, we further document distinctions between declines in net calcification and declines in $CaCO_3$ content regarding OA responses. Price et al. (2011) similarly showed OA-induced declines in calcification in *H. opuntia*, with no resultant shift in $CaCO_3$ content. These findings indicate that some species of *Halimeda* may respond to OA via increased production of segments with lower $CaCO_3$ content (i.e. *H. incrassata*) versus reduced production of segments with unaltered $CaCO_3$ content (i.e. *H. opuntia* and *H. simulans*). These distinctions may similarly relate to morphology and photophysiology; however, additional research will be needed to specifically determine the factors driving such variation. As calcification rates in *Halimeda* integrate both segment production and $CaCO_3$ content, future work may benefit by simultaneously measuring both metrics to accurately assess OA responses. While $CaCO_3$ content in *H. incrassata* declined by nearly 20% on a dry weight basis, note that such a response was only detected at the highest pCO_2 level (2429 µatm); thus, associated shifts in algal palatability for this species likely will not occur until beyond the year 2100.

Terpene concentrations were unaltered by OA in all species, suggesting that OA-induced shifts in palatability will be primarily driven by shifts in $CaCO_3$ content. All species displayed comparable concentrations of the extracted diterpene, halimedatetraacetate, ranging from 1.0 to 6.7 mg g^{-1} dry wt. This range was slightly higher than concentrations

reported in field-collected samples of *Halimeda* spp. (1.0 mg g^{-1} dry wt) (Paul & Fenical 1986). The synthesis and storage of these compounds has been suggested to be an energetically costly process (Hay & Fenical 1988). Thus, we anticipated that OA-related declines in photosynthetic performance may have indirectly altered terpene production. As previously demonstrated for *Halimeda* spp., elevated pCO_2 can result in significant declines in photosynthetic performance (Sinutok et al. 2011, 2012); for this study, however, these declines did not translate into altered secondary metabolite content. It is possible that either (1) the duration of this study was not sufficient to detect shifts in secondary compounds, or (2) OA-induced photosynthetic impairment was not of sufficient magnitude to influence metabolite content.

High $CaCO_3$ content and non-polar secondary metabolite concentrations deterred feeding by *L. variegatus* and *D. antillarum*. These findings are in general agreement with prior work demonstrating the anti-herbivore properties of these phytochemicals (Pennings & Paul 1992, Hay et al. 1994, Schupp & Paul 1994). Within the context of OA, our work suggests that declines in $CaCO_3$ content may increase algal palatability and herbivore feeding preferences for certain species. Many conspicuous algae in reef habitats are heavily calcified, thus indicating that mineral defenses serve as a viable strategy for escaping intense herbivory (Paul & Hay 1986, Paul et al. 2001). While the mechanisms underlying the effectiveness of $CaCO_3$ as a feeding deterrent are unclear, it has been suggested that calcified tissues exert a buffering effect on the gut pH of many herbivores, thus interfering with digestion (Hay et al. 1994). Such a mechanism is purportedly increasingly effective in herbivores with acidic guts, such as *D. antillarum* (Lewis 1964). Other herbivores, such as parrotfish, have been shown to be unaffected by $CaCO_3$ content or even display increased preference for foods containing higher concentrations of $CaCO_3$ (Hay et al. 1994, Schupp & Paul 1994, Pennings et al. 1996). Thus, OA-induced shifts in mineral content will only influence algal palatability with respect to specific herbivores. Our feeding assays with non-polar secondary metabolites revealed an increased urchin preference for foods containing lower halimedatetraacetate concentrations. Such trends are likely associated with documented toxic effects of diterpenoid secondary metabolites from *Halimeda* spp. (Paul & Fenical 1986, Hay & Fenical 1988). While OA did not alter algal terpene concentrations, we note that interactions between $CaCO_3$ content and secondary metabolites have been noted for some species of reef fishes (Hay et al. 1994, Schupp & Paul 1994); thus, declines in algal $CaCO_3$ may influence the effectiveness of terpenoid chemical defenses.

Pairing the experimental OA work with herbivore feeding trials allowed us to (1) place shifts in algal phytochemistry within the context of herbivore responses, and (2) evaluate whether the magnitude of detected shifts in chemical/mineral composition will be of importance to select herbivores. While our results support the hypothesis that OA will influence the palatability of calcareous algae, we highlight that the prevalence of this phenomenon will be variable and strongly dependent on algal species and herbivore identity. Furthermore, our documented shifts in mineral content were only significant at the highest of pCO_2 levels, thereby restricting herbivore responses to OA beyond the year 2100. We note that interactions with other stressors, such as elevated temperature, may largely magnify OA responses and elicit greater effects on resident herbivores, as documented in Johnson & Carpenter (2012). Additional research will be needed to comprehensively evaluate how OA alters algal palatability and herbivore feeding preferences. Such work will contribute to our broader understanding of the community and ecosystem effects of OA in the marine environment.

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