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# Warmer more acidic conditions cause decreased productivity and calcification in subtropical coral reef sediment-dwelling calcifiers

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*Abstract*

The effects of elevated $CO_2$ and temperature on photosynthesis and calcification in the calcifying algae *Halimeda macroloba* and *Halimeda cylindracea* and the symbiont-bearing benthic foraminifera *Marginopora vertebralis* were investigated through exposure to a combination of four temperatures (28°C, 30°C, 32°C, and 34°C) and four $CO_2$ levels (39, 61, 101, and 203 Pa; pH 8.1, 7.9, 7.7, and 7.4, respectively). Elevated $CO_2$ caused a profound decline in photosynthetic efficiency ($F_V : F_M$), calcification, and growth in all species. After five weeks at 34°C under all $CO_2$ levels, all species died. Chlorophyll (Chl) *a* and *b* concentration in *Halimeda* spp. significantly decreased in 203 Pa, 32°C and 34°C treatments, but Chl *a* and Chl $c_2$ concentration in *M. vertebralis* was not affected by temperature alone, with significant declines in the 61, 101, and 203 Pa treatments at 28°C. Significant decreases in $F_V : F_M$ in all species were found after 5 weeks of exposure to elevated $CO_2$ (203 Pa in all temperature treatments) and temperature (32°C and 34°C in all pH treatments). The rate of oxygen production declined at 61, 101, and 203 Pa in all temperature treatments for all species. The elevated $CO_2$ and temperature treatments greatly reduced calcification (growth and crystal size) in *M. vertebralis* and, to a lesser extent, in *Halimeda* spp. These findings indicate that 32°C and 101 Pa $CO_2$, are the upper limits for survival of these species on Heron Island reef, and we conclude that these species will be highly vulnerable to the predicted future climate change scenarios of elevated temperature and ocean acidification.

Since the beginning of the industrial revolution, human activities such as the burning of fossil fuels, industrialization, deforestation, and intensive agricultural activities have raised atmospheric $CO_2$ concentrations (Gattuso and Lavigne 2009). As a consequence, surface seawater temperature has increased by 0.6°C over the last century (Houghton 2009). Moreover, a 35% increase in atmospheric $CO_2$ concentration (from preindustrial levels of 28.4 Pa to approximately 38.9 Pa today) has led to ocean acidification by elevating the dissolved $CO_2$ concentration in the surface ocean, which lowers pH (Solomon et al. 2007). The rate of change is 100–1000 times faster than the most rapid changes in temperature and $CO_2$ in at least the last 420,000 yr (Hoegh-Guldberg et al. 2007). Models parameterized with $CO_2$-emission trends for 1990–1999 (the so-called "Special Report on Emissions Scenarios"; Solomon et al. 2007) predict that $CO_2$ concentrations will rise 150–250% (to $\leq$ 101 Pa) by the year 2100 (Friedlingstein et al. 2006). The surface ocean pH is already 0.1 units lower than preindustrial values (Orr et al. 2005), which is equivalent to a 30% increase in $H^+$ ions (Raven et al. 2005) and is predicted to decrease by a further 0.4 to 0.5 units by 2100 (Raven et al. 2005; Lough 2007).

An increase in sea temperature and atmospheric $CO_2$ will influence the health and survivorship of marine organisms, especially calcifying species, such as molluscs, crustaceans, echinoderms (Doney et al. 2009), corals (Reynaud et al. 2003; Jokiel et al. 2008), calcareous algae (Jokiel et al. 2008), foraminifera (Hallock 2000), and some phytoplankton (Raven et al. 2005; Iglesias-Rodriguez et al. 2008). Temperature influences physiological processes, including photosynthesis, respiration, and calcification (Howe and Marshall 2002; Necchi 2004). In reef-building (scleractinian) corals, warmer temperatures increase the rate of calcification (Lough and Barnes 2000), although increases beyond a thermal threshold as small as 1–2°C above summer averages can lead to mass coral bleaching events (large areas of coral colonies expelling symbiotic algae) and sometimes death (Hoegh-Guldberg 1999).

Ocean acidification has been suggested to have a positive effect on organisms such as seagrass and noncalcifying macroalgae, which utilize $CO_2$ as the substrate for carbon fixation in photosynthesis (Gao et al. 1993; Short and Neckles 1999). However, ocean acidification is likely to have a negative effect on calcified organisms by decreasing the availability of carbonate ions ($CO_3^{2-}$) and hence the organisms' ability to produce their calcium carbonate skeleton (Feely et al. 2004). Acidification has been shown to reduce calcification, recruitment, growth, and productivity in the articulated coralline alga *Corallina pilulifera* Postels and Ruprecht as well as in crustose coralline algae (CCA) when exposed to elevated $pCO_2$ (partial pressure of $CO_2$) seawater (Kuffner et al. 2007; Anthony et al. 2008). Reduced abundance of $CO_3^{2-}$ ions could also lead to an increase in calcium carbonate dissolution in the future (Feely et al. 2004).

Synergistic effects of elevated temperature and $pCO_2$ have had limited examination, but Reynaud et al. (2003) observed 50% lower calcification rates in the scleractinian coral *Stylophora pistillata* Esper compared to either high temperature or low pH conditions in isolation. However, in the corals *Acropora intermedia* Brook and *Porites lobata* Dana, Anthony et al. (2008) found that combined ocean acidification and warming scenarios (rather than ocean

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acidification conditions in isolation) resulted in bleaching, reduced productivity, and calcium carbonate dissolution and erosion in *A. intermedia* and *P. lobata* and in the CCA species *Porolithon onkodes* (Heydrich) Foslie.

Reef-building and sediment-dwelling species, *Halimeda* and symbiont-bearing foraminifera are prominent, co-existing taxa in shallow reef systems and play a vital role in tropical and subtropical ecosystems as producers of sediment in coral reefs (Hallock 1981). However, there is limited evidence of the effects of ocean warming and acidification in these two important carbonate sediment producers. Elevated seawater temperatures of 30°C to 35°C reduced the growth rate in the benthic foraminifera *Rosalina leei* Hedley and Wakefield (Nigam et al. 2008) and induced algal symbiont loss in *Amphistegina gibbosa* d'Orbigny when temperatures reached 32°C (Talge and Hallock 2003). Borowitzka and Larkum (1976) showed an inhibition in calcification in *Halimeda tuna* (Ellis and Solander) Lamouroux when seawater pH was dropped from 8.0 to 7.5. A more recent study found thinner aragonite crystals and higher crystal density in *H. tuna* and *Halimeda opuntia* grown in pH 7.5 as compared to those grown at pH 8.1 (L. L. Robbins unpubl.). Research on symbiotic and nonsymbiotic planktonic foraminifera (*Orbulina universa* d'Orbigny and *Globigerina sacculifers* Brady, respectively) and symbiotic benthic foraminifera (*Marginopora kudakajimensis* Gudmundsson) showed a decrease in shell weight with decreasing availability of the carbonate ion in seawater (Kuroyanagi et al. 2009). These results indicate that a decrease in calcification is likely in these organisms under the acidified conditions that are expected to occur in the future. However, there have been no studies on the combined effect of elevated temperature and $CO_2$ concentration on the photosynthetic marine calcifiers, *Halimeda* spp. and benthic foraminifera.

*Halimeda* spp. precipitate calcium carbonate as aragonite, whereas foraminifera precipitate high-magnesium calcite. The current saturation state of aragonite ($\Omega_a$ = 3 to 4) is greater than that of the high-Mg calcite mineral ($\Omega_c$ = 2 to 3; Kleypas et al. 1999; International Society for Reef Studies 2008), which means that organisms that precipitate high-Mg calcite are expected to have more difficulty in producing their $CaCO_3$ skeleton under elevated $pCO_2$ conditions compared to organisms that precipitate $CaCO_3$ as aragonite (Kleypas et al. 1999). Thus, the hypothesis tested in this study was that the calcifying macroalga *Halimeda* would perform better than benthic foraminifera under high-$CO_2$ conditions and that all organisms would show greater effects under the combined effects of elevated $CO_2$ and temperature.

## Methods

*Experimental design*—Whole specimens of *Halimeda macroloba* Decaisne (thallus size, 13–18 cm long), *Halimeda cylindracea* Decaisne (15–20 cm long), and *Marginopora vertebralis* Quoy and Gaimard (0.3–0.6 cm diameter) were collected by hand from the Heron Island reef flat at low tide at 0.3-m depth in the Southern Great Barrier Reef of Australia (151°55′E, 23°26′S). Symbiont-bearing foraminifera *M. vertebralis* hosts symbiotic alga *Symbiodinium* sp. in interior shell chambers (Pawlowski et al. 2001). The specimens of these species were maintained in a 500-liter aquarium with artificial seawater (pH 8.1, 26°C, salinity 33) under 250 $\mu$mol photons $m^{-2}$ $s^{-1}$ (at water surface) on a 12 : 12 light : dark (LD) cycle. Mature segments of *H. macroloba* (0.8–1.1 cm long) and *H. cylindracea* (1.5–2.0 cm long) from the middle part of the thallus and *M. vertebralis* (320 each species) were randomly allocated to one of four temperature treatments (28°C, 30°C, 32°C, and 34°C) in combination with one of four pH treatments (8.1, 7.9, 7.7, and 7.4; the current and the predicted pH values for the years 2065, 2100, and 2200, respectively, and equivalent to $pCO_2$ 38.5, 60.8, 101, and 203 Pa in this experiment; Houghton 2009). Within each tank, samples of each of the three species were placed in separate, open petri dishes, so that there was no direct physical interaction between specimens. Samples were ramped from 26°C and pH 8.1 to their treatment conditions over 1 week and maintained in the 16 treatments for a further 4 weeks ($n$ = 4). The tanks set at an ambient pH of 8.1 and 28°C acted as controls. The water salinity in the 100-liter experimental tanks was maintained at 33, and the light intensity at the sample height was 300 $\mu$mol photons $m^{-2}$ $s^{-1}$ on a 12 : 12 LD cycle (on at 06:00 h and off at 18:00 h). The treatment tanks were 0.2 m deep, consistent with sampling depth. The carbonate hardness (concentration of $CO_3^{2-}$ + $HCO_3^-$), calcium, nitrate, and phosphate concentration were maintained at 2.3, 10, < 0.0016, and < 0.0005 mmol $L^{-1}$, respectively, and monitored weekly using test kits (Aquasonic Pty). The concentration of $CO_2$ in both treatments and controls was maintained by $CO_2$ gas bubbling through the water held in the sump before it was recirculated to the aquaria containing the samples. $CO_2$ dosing was automated using a pH-controller (Tunze) connected to a solenoid valve on a $CO_2$ gas line. $CO_2$ gas was bubbled through the seawater once pH increased beyond the target pH and was maintained at a precision of ± 0.01 pH units. pH electrodes (National Bureau of Standards scale; Tunze), each connected to a pH-controller, were calibrated every week, during which time no detectable drift in pH was found. Water temperature in each treatment was controlled by water heaters and chillers (Hailea) to ± 0.1°C. Water changes (20%) to each treatment were performed every week using seawater media set to the required pH using $CO_2$ bubbling prior to addition. Salinity was measured daily with a salinity meter (Salt 6, Eutech Instruments), while total alkalinity (TA) was measured weekly by titrating 30 g of seawater with 0.1 mol $L^{-1}$ hydrochloric acid using an autotitrator (Mettler Toledo; Gattuso et al. 1993). From each treatment tank, TA was determined as the average from three independent samples of water. Dissolved inorganic carbon (DIC) concentrations were calculated using the CO2SYS program (version 01.05; Brookhaven National Laboratory; Lewis and Wallace 1998). A summary of the TA, total inorganic carbon (DIC, $CO_2$, $CO_3^{-2}$, $HCO_3^-$), $pCO_2$, and saturation state of seawater with respect to calcite ($\Omega_c$) and aragonite ($\Omega_a$) from each pH (8.1, 7.9, 7.7, 7.4) and temperature (28°C, 30°C, 32°C, 34°C) treatment are shown in Table 1. There

Table 1. Parameters of the carbonate system; total alkalinity (TA), $CO_2$ partial pressure ($pCO_2$), total inorganic carbon ($CO_2$, $CO_3^{-2}$, $HCO_3^-$, DIC), and saturation state of seawater with respect to calcite ($\Omega_c$) and aragonite ($\Omega_a$) from each pH (8.1, 7.9, 7.7, 7.4) and temperature (28°C, 30°C, 32°C, 34°C).

| Treatment | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| pH | Temp (°C) | TA (mmol kg$^{-1}$) | $pCO_2$ (Pa) | $CO_2$ (mmol kg$^{-1}$) | $CO_3^{2-}$ (mmol kg$^{-1}$) | $HCO_3^-$ (mmol kg$^{-1}$) | DIC (mmol kg$^{-1}$) | $\Omega_c$ | $\Omega_a$ |
| 8.1 | 28 | 2.314±0.187 | 32.2±2.6 | 0.008±0.001 | 0.280±0.035 | 1.635±0.135 | 1.923±0.160 | 6.03±0.95 | 4.04±0.61 |
| 8.1 | 30 | 2.647±0.190 | 36.8±2.7 | 0.008±0.001 | 0.340±0.030 | 1.842±0.145 | 2.190±0.166 | 7.40±0.55 | 4.99±0.63 |
| 8.1 | 32 | 2.695±0.189 | 36.9±2.6 | 0.008±0.001 | 0.365±0.042 | 1.835±0.138 | 2.209±0.162 | 8.02±0.73 | 5.44±0.60 |
| 8.1 | 34 | 2.392±0.190 | 32.2±2.6 | 0.007±0.001 | 0.338±0.035 | 1.579±0.133 | 1.924±0.160 | 7.50±0.74 | 5.13±0.60 |
| 7.9 | 28 | 2.685±0.169 | 66.9±4.6 | 0.016±0.002 | 0.231±0.004 | 2.140±0.170 | 2.387±0.170 | 3.34±0.10 | 3.34±0.09 |
| 7.9 | 30 | 2.624±0.168 | 65.1±4.5 | 0.016±0.002 | 0.240±0.003 | 2.058±0.173 | 2.314±0.173 | 3.52±0.15 | 3.52±0.10 |
| 7.9 | 32 | 2.607±0.170 | 64.6±4.5 | 0.015±0.002 | 0.238±0.003 | 2.044±0.180 | 2.297±0.182 | 3.49±0.12 | 3.49±0.10 |
| 7.9 | 34 | 2.309±0.170 | 56.3±4.6 | 0.012±0.002 | 0.235±0.004 | 1.743±0.175 | 1.990±0.176 | 3.57±0.14 | 3.57±0.12 |
| 7.7 | 28 | 2.532±0.110 | 108.0±4.7 | 0.027±0.001 | 0.148±0.018 | 2.172±0.080 | 2.347±0.101 | 2.14±0.44 | 2.14±0.33 |
| 7.7 | 30 | 2.565±0.108 | 110.0±4.7 | 0.025±0.001 | 0.160±0.019 | 2.185±0.082 | 2.370±0.099 | 2.36±0.52 | 2.36±0.32 |
| 7.7 | 32 | 2.427±0.110 | 104.0±4.8 | 0.024±0.001 | 0.162±0.018 | 2.042±0.083 | 2.228±0.110 | 2.41±0.49 | 2.41±0.34 |
| 7.7 | 34 | 2.691±0.109 | 115.0±4.8 | 0.025±0.001 | 0.191±0.020 | 2.244±0.080 | 2.460±0.095 | 2.90±0.49 | 2.90±0.33 |
| 7.4 | 28 | 2.696±0.136 | 247.0±12.5 | 0.062±0.004 | 0.085±0.008 | 2.495±0.145 | 2.642±0.133 | 1.84±0.16 | 1.23±0.15 |
| 7.4 | 30 | 2.568±0.129 | 236.0±12.2 | 0.056±0.005 | 0.087±0.007 | 2.362±0.135 | 2.505±0.132 | 1.89±0.15 | 1.28±0.15 |
| 7.4 | 32 | 2.827±0.134 | 262.0±12.7 | 0.059±0.005 | 0.102±0.008 | 2.588±0.130 | 2.749±0.139 | 2.26±0.18 | 1.53±0.17 |
| 7.4 | 34 | 2.526±0.135 | 235.0±12.5 | 0.051±0.005 | 0.098±0.005 | 2.295±0.140 | 2.444±0.135 | 2.18±0.15 | 1.49±0.16 |

was no significant difference in TA among the 16 pH and temperature treatments, and $pCO_2$, $CO_2$,$CO_3^{-2}$, $HCO_3^-$, DIC, $\Omega_c$, and $\Omega_a$ remained consistent within each pH treatment.

*Mortality assessment*—Mortality in *H. macroloba* and *H. cylindracea* was determined by presence of bleached and disintegrated segments, whereas mortality in *M. vertebralis* was determined by bleached and broken tests. In addition, the lack of variable fluorescence from measures of pulse amplitude modulated (PAM) fluorometry (indicative of photosynthetic activity by algal symbionts) was an indication of mortality.

*Photosynthesis*—Photosynthetic condition was determined through measures of chlorophyll (Chl) *a* fluorescence, oxygen production, photosynthetic pigment concentration, and, for foraminifera, algal symbiont density. To avoid diel and non–steady state variability, steady state light curves (SSLCs), with one irradiance step (372 $\mu$mol photons m$^{-2}$ s$^{-1}$ applied for 300 s) were performed with a 6-mm-diameter fiber-optic on a Diving-PAM fluorometer (Walz) every week at 10:00 h over the duration of the experiment, following 10 min of dark adaptation (Diving-PAM settings: measuring intensity $<$ 0.15 $\mu$mol photons m$^{-2}$ s$^{-1}$, saturating intensity $>$ 4500 $\mu$mol photons m$^{-2}$ s$^{-1}$, saturating width = 0.8 s, gain = 2, damping = 2). Photosystem II (PSII) photosynthetic efficiency was determined through weekly measures of maximum quantum yield, $F_V : F_M$, and effective quantum yield, Y(II). In addition, the capacity for photoprotection (nonphotochemical quenching yield, Y[NPQ]) and level of photoinhibition (nonregulated heat dissipation yield, Y[NO]) were determined through SSLCs (Kramer et al. 2004).

Oxygen production was measured using a needle-type fiber-optic oxygen microsensor PSt1 connected to a Micro TX3 transmitter (Presens). After 10 min of dark adaptation, the samples were placed in 10-mL glass bottles filled to the top with treatment water; then the bottles were sealed and placed in a water bath (Julabo) set to the relevant treatment temperature. The sensor was inserted through a resealable hole in the bottle lid to determine the oxygen production during 5 min under 300 $\mu$mol photons m$^{-2}$ s$^{-1}$ of irradiance, and rates were calculated according to Ulstrup et al. (2005).

Photosynthetic pigment concentration (Chl *a* and Chl *b* for *H. macroloba* and *H. cylindracea* and Chl *a* and Chl $c_2$ for *M. vertebralis*) was determined using the standard spectrophotometric method of Ritchie (2008) at the beginning and end of the 5-week experiment. Chl *a*, Chl *b*, and Chl $c_2$ were extracted by soaking samples in 3 mL of 90% acetone at 4°C in darkness for 24 h. Samples were centrifuged at 1500 $\times$ *g* for 10 min; the supernatant was placed into a quartz cuvette in a spectrophotometer (Varian); and absorbance was measured at 630, 647, and 664 nm. Chlorophyll concentrations were determined using the equations of Ritchie (2008), and the results were expressed in $\mu$g mm$^{-2}$.

Algal symbiont density in the foraminifera was investigated using a confocal microscope (Nikon A1). For each

individual foraminiferan, four randomly selected interrogation areas were chosen from the edge, middle, and center of the test (shell). Algal symbionts in the chambers within each area were counted and expressed in terms of surface area.

*Calcification*—Calcification was determined using the buoyant weight technique (Jokiel et al. 2008), with comparisons made between measurements at the start and end of the experimental period. The buoyant weight technique is a reliable measure of calcification, inferred from changes in skeletal weight (Langdon et al. 2010). It was determined by weighing each sample in seawater of known density and applying Archimedes' principle to compute the dry weight of the sample in the air (Jokiel et al. 1978; Langdon et al. 2010). Weight was measured using an electronic balance with accuracy to 0.1 mg. The samples were placed on a glass petri dish hung below the balance using nylon thread suspended in seawater. The density of water at salinity 33 and 25°C was 1026.42 kg $m^{-3}$ (Jokiel et al. 1978), and the densities of *H. macroloba*, *H. cylindracea*, and *M. vertebralis* at salinity 33 and 25°C were 2052.37, 5384.9, and 2733.98 kg $m^{-3}$, respectively (Jokiel et al. 1978).

Images of aragonite and magnesium calcite crystals were examined for size and abundance analysis using a field emission gun scanning electron microscope (Zeiss Supra 55VP). The instrument was operated at 20 kV with 30-$\mu$m aperture, ~ 4-mm working distance in Hi-Vac mode and imaged using the secondary In-lens detector. Samples were mounted on aluminum stubs using carbon adhesive tape and then placed in a carbon coating unit (Balzers) operated at 40-mm working distance. An area of 9 $\mu m^2$ was selected, and the crystal abundance was determined ($n = 4$ per sample with 10 measurements per replicate) along with crystal width, which was calculated using spatial analysis software (University of Texas Health Science Center, San Antonio, Image Tool version 3; University of Texas).

*Statistical analysis*—To determine any significant differences among treatments in growth rate, Chl *a*, Chl *b*, and Chl $c_2$ concentration, chlorophyll fluorescence parameters ($F_V : F_M$, Y[II], Y[NPQ], and Y[NO]), oxygen production, and crystal density and width over time, repeated-measures ANOVA (rmANOVA) tests were performed. One-way ANOVA tests were used to compare treatments at the initial or final time point (Statistical Package for the Social Sciences version 17). All tests were performed with a significance level of 95%, and Tukey's Honestly Significant Difference post hoc tests were used to identify the statistically distinct groups. If data did not meet the assumptions of normality (Kolmogorov-Smirnov test) and equal variance (Levene's test), the data were transformed using $\log_{10}$ or square root. Differences in symbiont density among treatments were tested using the Friedman test at a 95% significance level.

## Results

*Calcification and mortality*—The calcification rates of *H. macroloba*, *H. cylindracea*, and *M. vertebralis* were slightly positive in the control treatment and were highly negative in the other treatments, indicating dissolution of calcium carbonate. Calcification rate was significantly reduced by elevated temperature (34°C) at all pH levels ($p < 0.05$). Calcification rate of *H. macroloba* at pH 7.4 and 34°C ($-1.24 \pm 0.70$ mg $CaCO_3$ $d^{-1}$) was significantly lower than the control ($0.02 \pm 0.01$ mg $CaCO_3$ $d^{-1}$; $p < 0.001$; Fig. 1A–D). In *H. cylindracea*, calcification rate significantly declined at pH values 7.7 and 7.4 in 34°C treatments ($p < 0.001$; Fig. 1E–H), whereas in *M. vertebralis* calcification rate significantly decreased at pH values 7.9, 7.7, and 7.4 at 30°C, 32°C, and 34°C ($p < 0.05$; Fig. 1I–L). Foraminiferan mortality was first observed at day 21 of the experiment at pH 7.4, 30°C and 34°C; whereas, in *H. macroloba* and *H. cylindracea*, mortality was found at day 28 at pH values 8.1, 7.9, and 7.4 at 34°C and pH 8.1 at 34°C, respectively. *H. macroloba* and *H. cylindracea* had 100% mortality at the end of the experiment at pH 7.4 and 34°C, whereas 100% mortality of *M. vertebralis* was observed at pH values 7.7 and 7.4 in the 32°C and 34°C treatments. At lower pH treatments (all except control) and higher temperature (32°C and 34°C) treatments, the symbiont density of foraminifera significantly decreased ($p < 0.001$; Fig. 2A–E), and foraminifera bleaching and death was observed.

*Pigment content*—After 5 weeks, the Chl *a* and Chl *b* concentration in *H. macroloba* significantly declined at pH values 8.1, 7.9, and 7.7 with 34°C and at pH 7.4 with 32°C and 34°C treatments ($p < 0.001$; Fig. 3A–E). However, no significant change was detected in Chl *a* and Chl *b* concentration at pH values 8.1, 7.9, and 7.7 with 28°C, 30°C, and 32°C treatments over the 5-week experiment ($p > 0.05$; Fig. 3A–E). The Chl *a* and Chl *b* concentration in *H. cylindracea* after 5 weeks significantly declined at pH values 8.1 and 7.9 with 34°C and at pH 7.4 with 28°C and 34°C treatments ($p < 0.001$; Fig. 3F–J). In *M. vertebralis*, Chl *a* and Chl $c_2$ concentration significantly decreased at pH values 7.9, 7.7, and 7.4 at all temperature treatments and at pH 8.1 with 34°C treatment ($p < 0.001$; Fig. 3K–O).

*Chl* a *fluorescence*—Maximum quantum yield ($F_V : F_M$) and effective quantum yield (Y[II]; data not shown) in the control treatment was constant in *H. macroloba*, *H. cylindracea*, and in the symbionts of *M. vertebralis* ($p > 0.05$; Fig. 4A,E,I). A significant decrease in $F_V : F_M$ and Y(II) was found in all species when exposed to elevated $CO_2$ (pH 7.4 in all temperature treatments) and temperature (32°C and 34°C in all pH treatments) over the length of the experiment ($p < 0.001$; Fig. 4A–L). In *H. macroloba*, $F_V : F_M$ declined to zero after being treated at 34°C at all pH levels for 28 d ($p < 0.001$; Fig. 4A–D). $F_V : F_M$ of *H. cylindracea* decreased to zero after 28 d at 34°C, in pH 8.1, 7.9, and 7.7 treatments ($p < 0.001$; Fig. 4E–H). In symbionts of *M. vertebralis*, $F_V : F_M$ significantly decreased at 34°C and in all pH treatments after 14 d of experimentation and also reached zero within 14 d at pH 8.1 and 34°C treatment ($p < 0.001$; Fig. 4I–L). At lower temperatures (28°C and 30°C) with lower pH treatments (pH values 7.7 and 7.4), effective quantum yield (Y[II]; $p <$

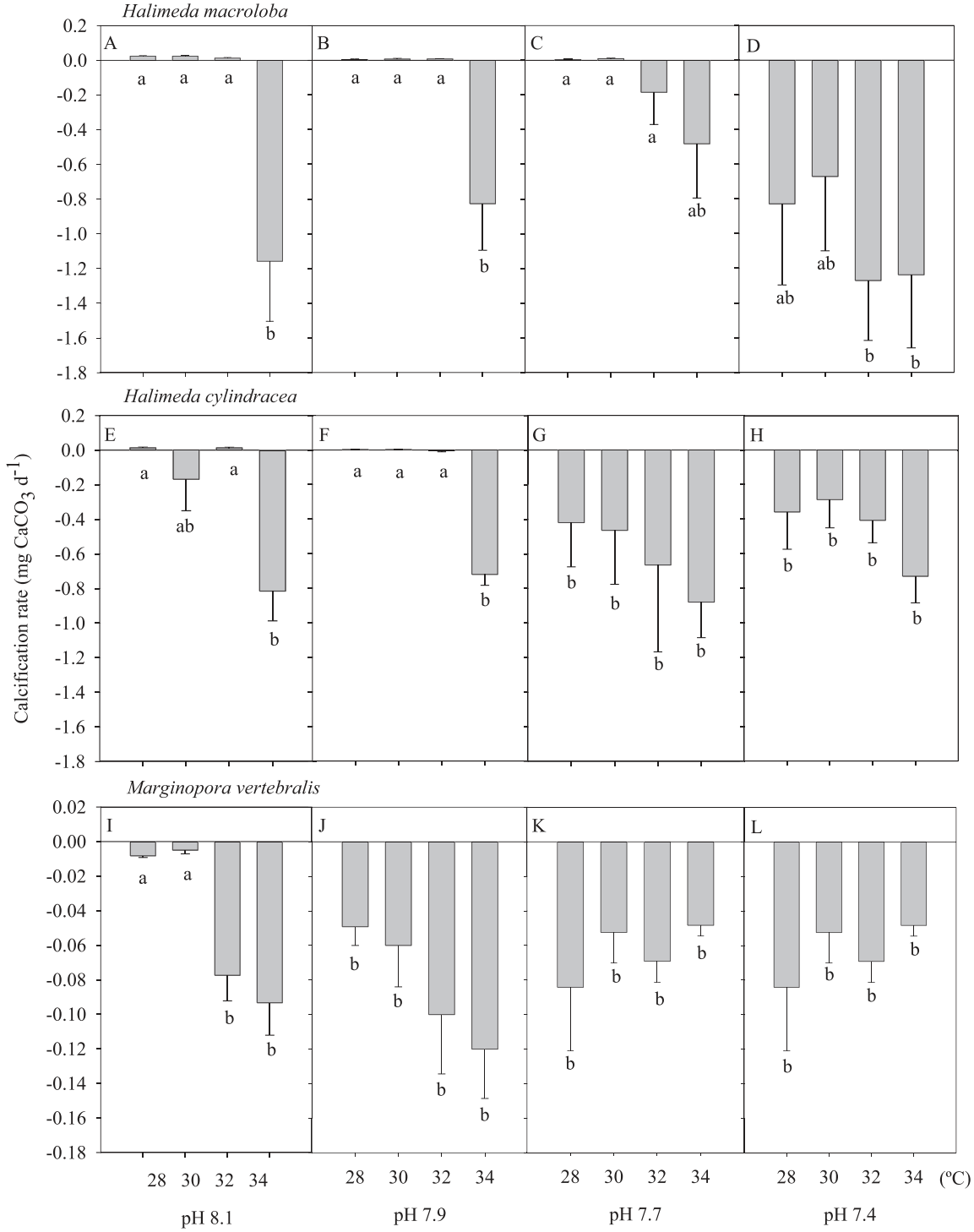


Fig. 1. Calcification rate (mg $CaCO_3$ $d^{-1}$) over the 5-week period in (A–D) *H. macroloba*, (E–H) *H. cylindracea*, and (I–L) *M. vertebralis* in each pH and temperature treatment. Data represent means ($n = 4$, SEM).

0.001; data not shown) and maximum quantum yield ($F_V : F_M$; $p < 0.001$) of *H. macroloba* (Fig. 4A–D), *H. cylindracea* (Fig. 4E–H) and *M. vertebralis* (Fig. 4I–L) significantly decreased after 5 weeks of experimentation. Moreover, there was a greater decrease in $F_V : F_M$ and Y(II) in all species when exposed to the combined treatment of elevated temperature and $CO_2$. The capacity for photoprotection, Y(NPQ), and the level of photoinhibition, Y(NO), were similar over time in all pH and temperature treatments prior to mortality ($p > 0.05$; data not shown).

*Oxygen production*—The rate of oxygen production of *H. macroloba*, *H. cylindracea*, and symbionts of *M. vertebralis* significantly decreased when exposed to elevated

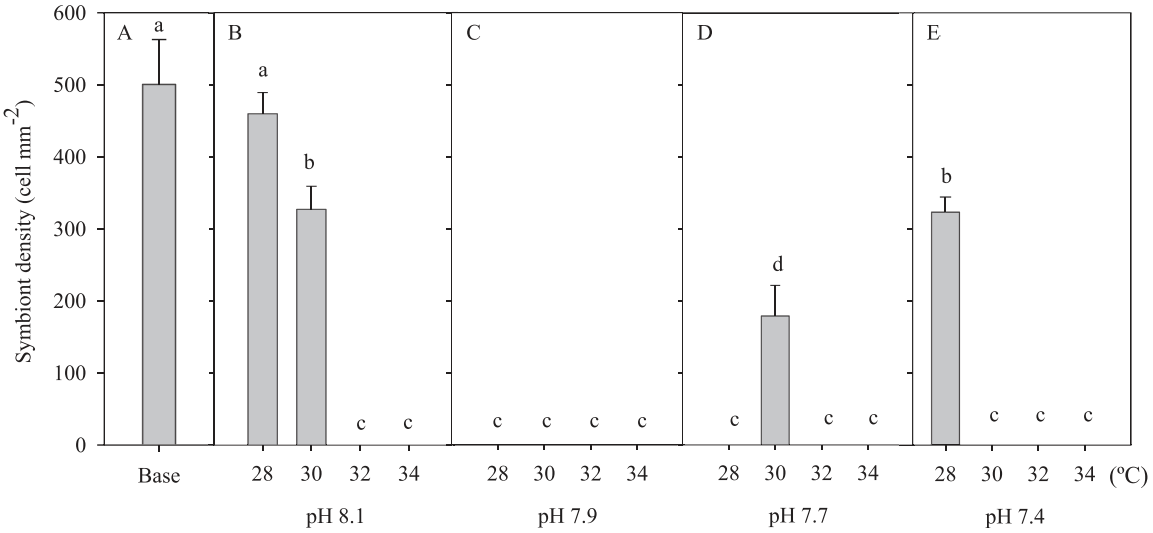


Fig. 2. Symbiont density (cells mm$^{-2}$) of *M. vertebralis* at (A) time zero (base) and each of the four temperature treatments (28, 30, 32, and 34°C) at pH (B) 8.1, (C) 7.9, (D) 7.7, and (E) 7.4. No bar indicates the absence of symbionts. Data represent means ($n = 4$, SEM).

$CO_2$ at pH 7.4 for 18 d ($p < 0.001$, Fig. 5A–L). Higher temperature (34°C) significantly lowered the oxygen production rate in *H. macroloba* only when maintained at a pH of 8.1. Greatest oxygen production of *H. macroloba* was found at pH 8.1, 30°C (14.06 ± 1.47 μmol L$^{-1}$) on day 0, whereas the lowest oxygen production was found at 34°C in all pH treatments on day 35 (0 ± 0 μmol L$^{-1}$; Fig. 5A–D). Similarly, *H. cylindracea* had its highest and lowest oxygen production on day 0, pH 7.9, 34°C and in pH 8.1, 7.9, and 7.4 treatments (14.85 ± 4.82 and 0.0 ± 0.0 μmol L$^{-1}$), respectively; whereas, in symbionts of *M. vertebralis*, the highest and lowest oxygen production was found on day 18, pH 7.9, 34°C and on day 27 and day 35, 32°C and 34°C at all pH treatments (11.71 ± 4.17 and 0.0 ± 0.0 μmol L$^{-1}$; Fig. 5E–L).

*Calcification*—After 5 weeks, the calcium carbonate crystal width of *H. macroloba*, *H. cylindracea*, and *M. vertebralis* significantly decreased when exposed to elevated $CO_2$ at pH values 7.7 and 7.4 ($p < 0.05$; Figs. 6, 7). Elevated temperature had no effect on the crystal width in *H. macroloba* ($p = 0.562$) or *H. cylindracea* ($p = 0.926$) but caused a significant decrease in the crystal width of *M. vertebralis* at 32°C and 34°C in all pH treatments ($p < 0.001$; Fig. 6). In contrast, crystal abundance in the foraminiferans increased significantly at high $CO_2$ at pH values 7.9, 7.7, and 7.4 and high temperature at 32°C and 34°C ($p < 0.001$) from 25.46 ± 0.77 crystals μm$^{-2}$ in the control to 39.93 ± 0.43 crystals μm$^{-2}$ at pH 7.4 and 34°C. However, there was no significant difference in crystal abundance of *H. macroloba* and *H. cylindracea* among pH and temperature treatments ($p > 0.05$).

## Discussion

To our knowledge, this is the first investigation on the combined effects of elevated temperature and ocean acidification on photosynthesis and calcification in the photosynthetic marine calcifying algae *H. macroloba* and *H. cylindracea* and the benthic symbiotic foraminifera *M. vertebralis*. As we hypothesized, the calcifying macroalga *Halimeda* performed better than benthic foraminifera under high $CO_2$ conditions, and the combined factors had a more detrimental (synergistic) effect on growth, photosynthesis, and calcification in all species. Unexpectedly, elevated temperature and lowered pH (34°C and pH 7.4) caused mortality in *H. macroloba* and *H. cylindracea* within 4 weeks and in *M. vertebralis* within 3 weeks. The cause of the mortality of the foraminiferan is likely due to damage to the symbionts, as indicated by changes in photosynthetic pigments, Chl *a* fluorescence ($F_V : F_M$, Y[II]), and oxygen production. There was a decline in Chl *a*, Chl *b*, and Chl $c_2$ concentrations in lower pH treatments (pH values 7.7 and 7.4) after 5 weeks, indicative of chlorophyll degradation, decreased photosynthetic unit size, and/or a decrease in the number of PSII reaction centers. There was also a significant decline in photosynthetic efficiency and primary production after 28 d of exposure to 32°C and 34°C, and pH 7.4 conditions in all three species (Figs. 4, 5). It is clear that elevated $CO_2$ and temperature conditions cause a reduction in the photosynthetic efficiency of PSII. Heat stress is likely to damage PSII, possibly by damaging the D1 protein and disrupting the thylakoid membrane stability (Allakhverdiev et al. 2008), whereas pH stress may disrupt the $CO_2$ accumulation pathway at the site of Rubisco or interfere with electron transport via the thylakoid proton gradients (Anthony et al. 2008).

Lower pH and higher temperature (all treatments except control pH and 28°C, 30°C) significantly triggered the bleaching and death of *M. vertebralis*. Symbiont expulsion is suggested to occur when the symbionts are damaged through photoinhibition (Hallock et al. 2006). Promotion of photooxidative reactions is likely under the stress conditions of pH and temperature applied here, through the degradation of the D1 protein (Talge and Hallock 2003). Alternatively, the symbionts may be digested by the

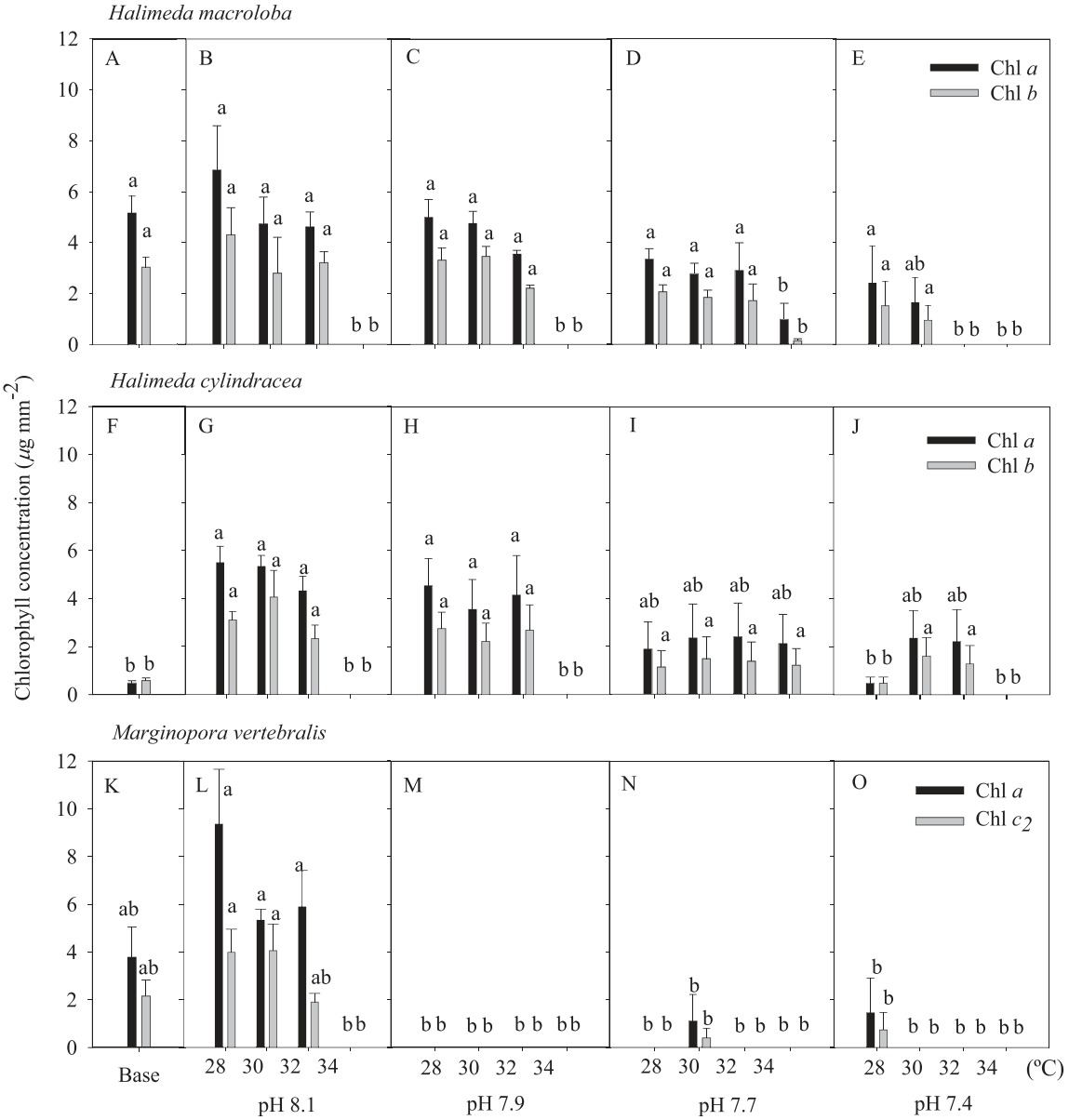


Fig. 3. Chl *a* and Chl *b* concentrations ($\mu$g mm$^{-2}$) in (A–E) *H. macroloba* and (F–J) *H. cylindracea* and Chl *a* and Chl $c_2$ concentrations ($\mu$g mm$^{-2}$) in (K–O) *M. vertebralis* at time zero (base) and each pH and temperature treatment at week 5. No bar indicates the absence of chlorophyll. Data represent means ($n$ = 4, SEM).

host when they are damaged (Hallock et al. 2006). Our results are consistent with the recent study of Talge and Hallock (2003), which demonstrated that bleaching in the foraminifera *Amphistegina gibbosa* is triggered by thermal stress.

Rising $pCO_2$ will inhibit calcification in calcifying organisms by decreasing the availability of $CO_3^{2-}$ ions required for the deposition of calcium carbonate skeletons. However, in photosynthetic organisms (e.g., fleshy algae and seagrass), rising $pCO_2$ is expected to promote photosynthesis and, hence, enhance growth due to the greater abundance of substrate ($CO_2$) required for carbon fixation (Gao et al. 1993; Short and Neckles 1999). Therefore, the relationship between $CO_2$ abundance, photosynthesis, and growth is dependent upon whether or not the organism calcifies.

This study demonstrated that increased $CO_2$ (yielding potentially more substrate available for carbon uptake) did not lead to increased production in any organisms, suggesting that the main effect was one of pH affecting the overall metabolism of the organisms.

There was, however, a dramatic effect on calcification rates. Calcification rate was negative in *H. macroloba*, *H. cylindracea*, and *M. vertebralis* under the highest $pCO_2$ treatment, with high-Mg calcite species experiencing greater decline than aragonite-forming species. The calcification rate of the control did not change over time. The application of heat caused increasingly negative calcifica-

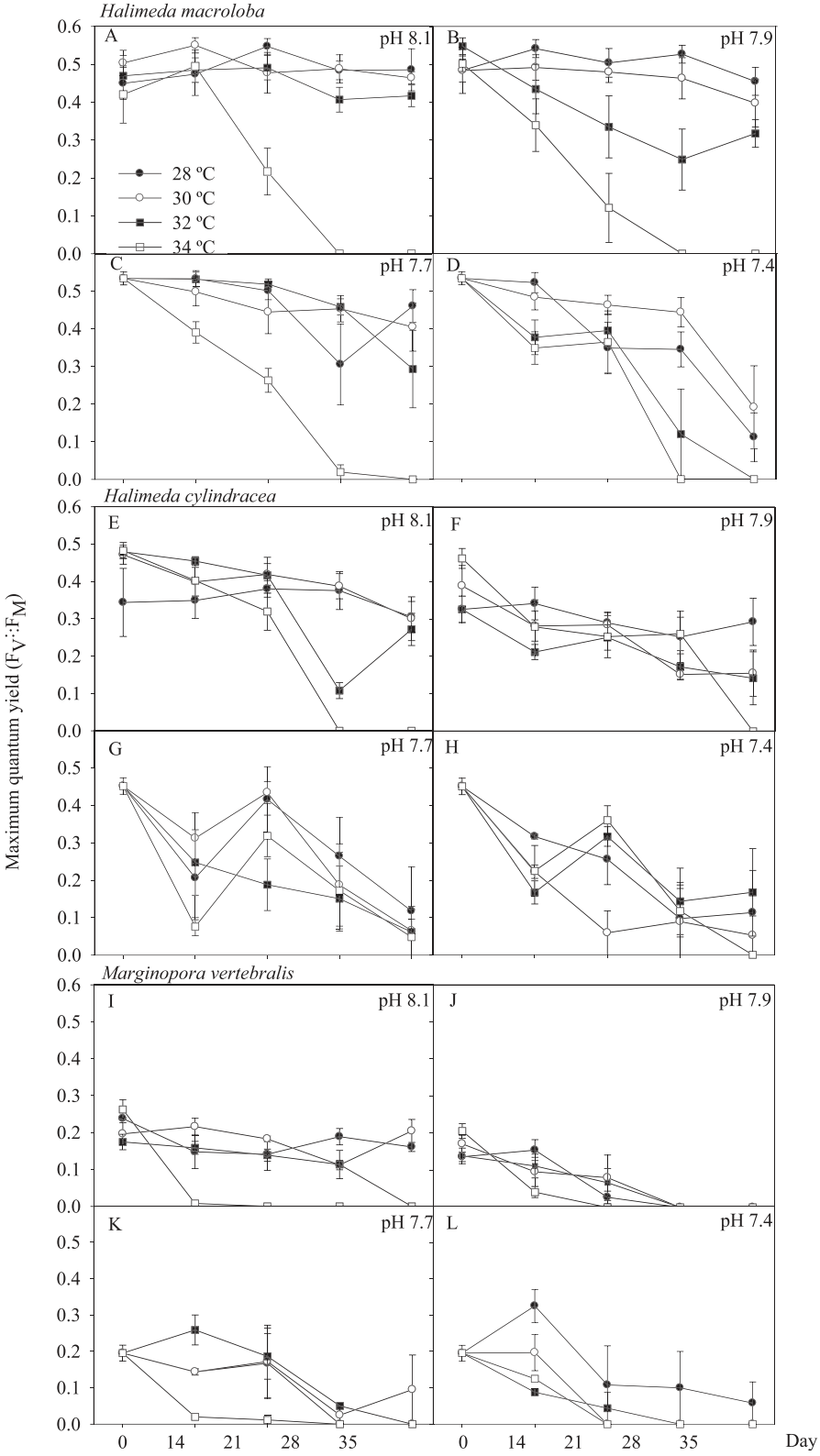


Fig. 4. Maximum quantum yield ($F_V : F_M$) of (A–D) *H. macroloba*, (E–H) *H. cylindracea*, and (I–L) *M. vertebralis* in each pH and temperature treatment over the length of the experimental period. Data represent means ($n$ = 4, SEM).

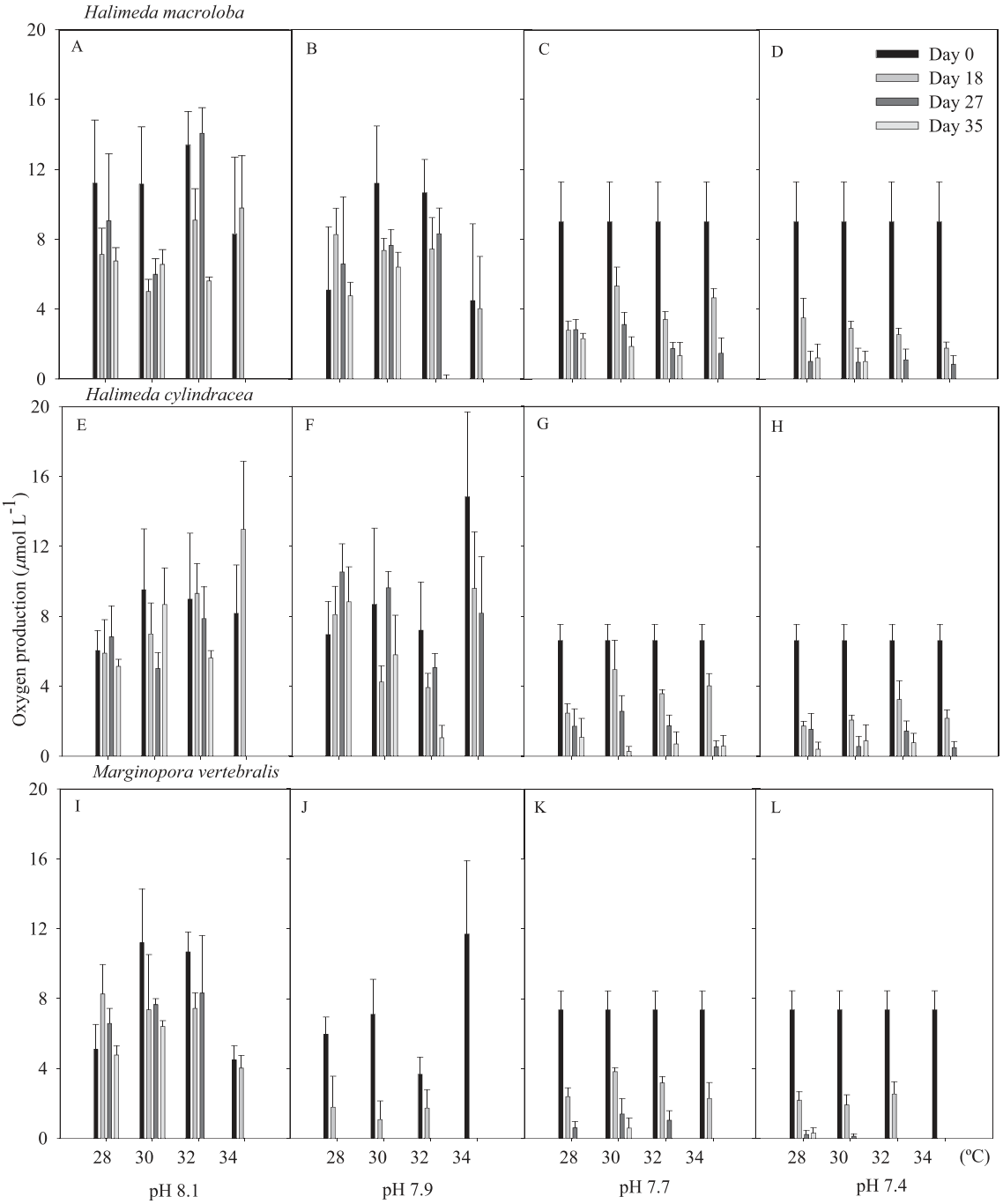


Fig. 5. Oxygen production ($\mu$mol $O_2$ $L^{-1}$) in (A–D) *H. macroloba*, (E–H) *H. cylindracea*, and (I–L) *M. vertebralis* in each pH and temperature treatment over the length of the experimental period. Data represent means ($n$ = 4, SEM).

tion rates in all species, with *M. vertebralis* being most sensitive under all $pCO_2$ treatments at the highest temperature (pH values 8.1, 7.9, 7.7, and 7.4, and 34°C; Fig. 1), whereas calcification rate of the control did not change over time. The extreme $CO_2$ treatment of our experiments created the greatest reduction in $CO_3^{2-}$ saturation state (to 1.23 ± 0.15 for $\Omega_a$ and 1.84 ± 0.16 for $\Omega_c$; Table 1), which virtually prevented calcification in all three organisms and increased the potential for dissolution of the calcium carbonate structure. Increased temperature above the optimum temperature for these species will have a negative effect on calcification by decreasing enzyme activity and photosynthetic $CO_2$ fixation (Borowitzka 1986; Hallock 2000; Gonzalez-Mora et al. 2008). Thinner aragonite and calcite crystals were observed in the two *Halimeda* species when exposed to high $pCO_2$ (pH values 7.7 and 7.4) and in *M. vertebralis* when exposed to high $pCO_2$ and elevated temperature conditions (pH values 7.7 and 7.4, and 32°C and 34°C; Figs. 6, 7). Crystal density in *M. vertebralis* increased with higher $pCO_2$ and

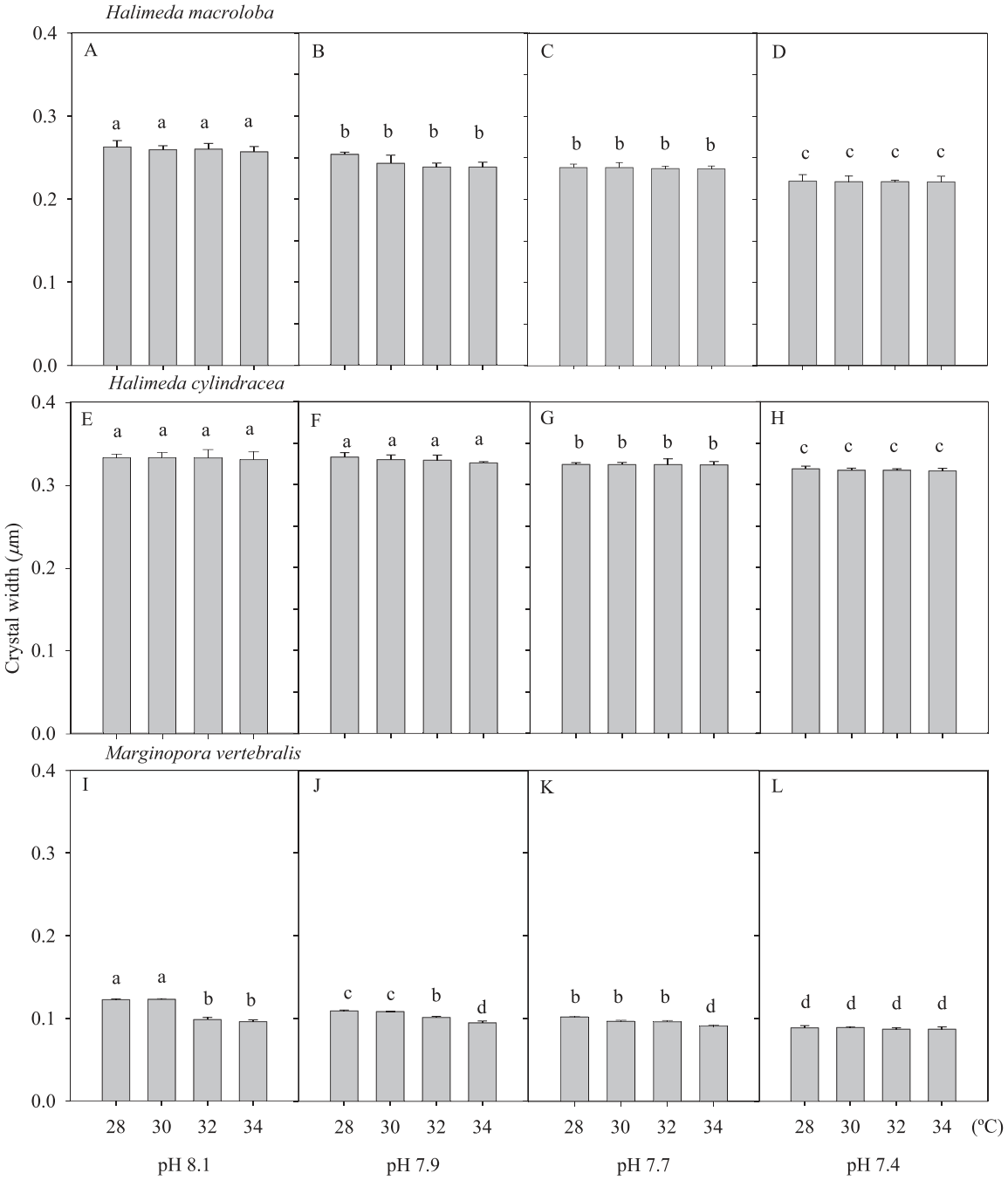


Fig. 6. Crystal width ($\mu$m) in (A–D) *H. macroloba*, (E–H) *H. cylindracea*, and (I–L) *M. vertebralis* under pH and temperature conditions at the end of the 5th week. Data represent means ($n$ = 4, SEM).

temperature levels (pH values 7.9, 7.7, and 7.4, and 32°C and 34°C); however, there was no change in calcium carbonate crystal abundance in *H. macroloba* and *H. cylindracea* at elevated $pCO_2$ and temperature conditions. Previous studies on *H. opuntia* and *H. tuna* that found a reduction in crystal width and an increase in crystal abundance with decreasing pH indicate that the crystallization may be initiated and terminated more frequently with increasing $pCO_2$ (L. L. Robbins unpubl.). The decrease in crystal width and increase in crystal density in *M. vertebralis* in this study shows that calcification in this high-Mg calcite species (*M. vertebralis*) is more sensitive to lower pH and higher temperature than in the aragonite-forming species of *Halimeda* spp. This finding is consistent with the prediction of Kleypas et al. (1999) based on calcium carbonate saturation state, in which the saturation threshold is lowest in high-Mg calcite–depositing species.

Furthermore, photosynthetic marine calcifiers may experience conditions that reduce calcification rate but enhance photosynthetic rate (e.g., low pH, high $CO_2$

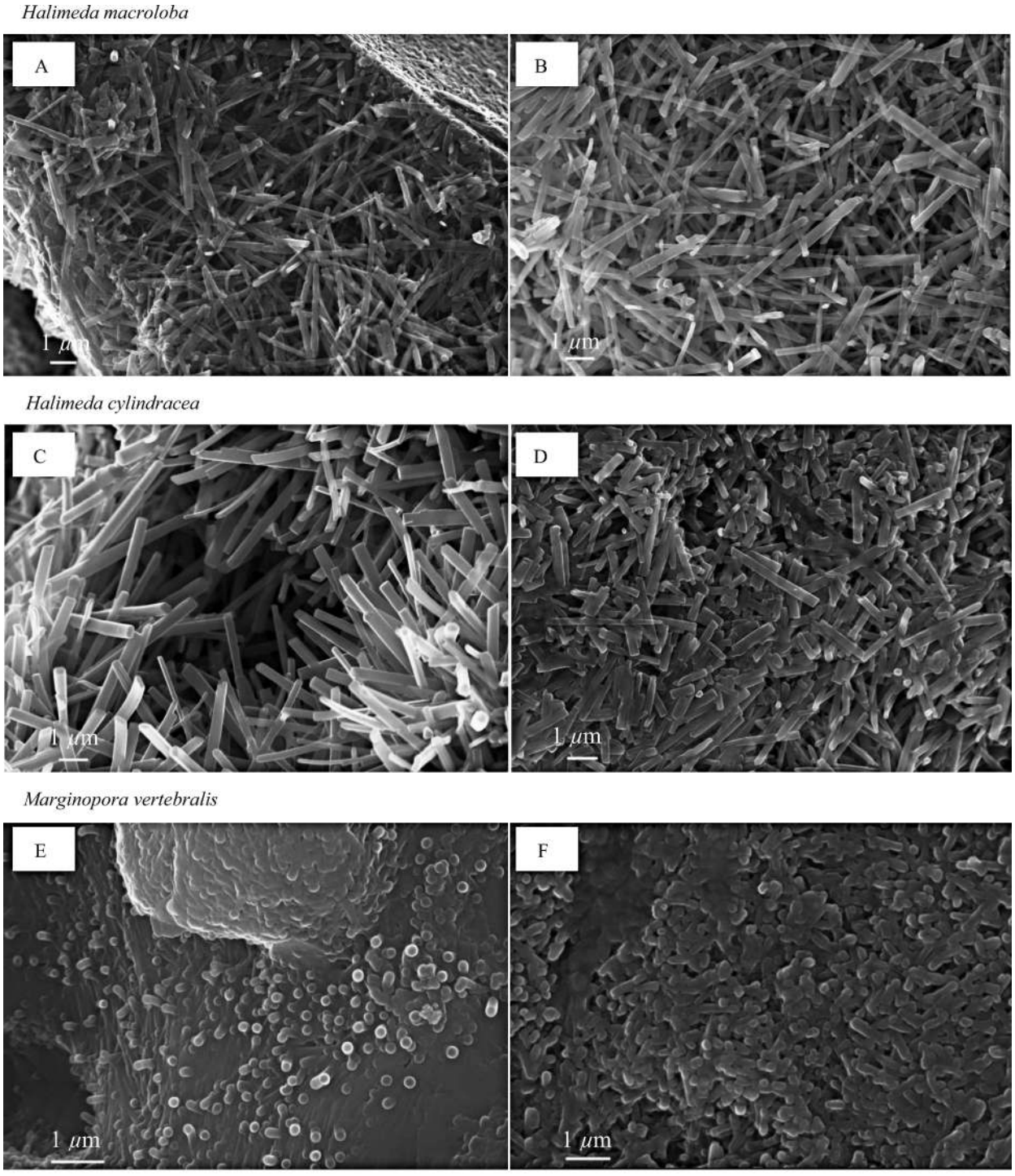


Fig. 7. SEM photographs of crystals in (A–D) *H. macroloba*, (E–H) *H. cylindracea*, and (I–L) *M. vertebralis* in control (pH 8.1, 28°C) and pH 7.4, 34°C treatment at the end of the 5th week.

availability). In this study, although rising $pCO_2$ resulted in an increase in dissolved $CO_2$ and $HCO_3^{2-}$ availability (substrates for photosynthesis), the increases in these carbon species might be too small to promote photosynthesis and growth but large enough to reduce calcification (Reynaud et al. 2003).

Exposure to elevated temperature (32°C and 34°C) alone or reduced pH (7.7 and 7.4) alone reduced photosynthesis

and calcification in *H. macroloba, H. cylindracea,* and *M. vertebralis.* However, there was a strong synergistic effect of elevated temperature and reduced pH, with dramatic reductions in photosynthesis and calcification in all three species. It is suggested that rising temperature and $pCO_2$ exceeds the threshold for survival of these species. Subsequent mortality may be the cause of the reduced calcification and photosynthesis. A simultaneous reduction in pH and higher temperature will result in the greatest effect on these species. It is likely that the elevated temperature of 32°C and the $pCO_2$ concentration of 101 Pa are the upper limit for survival of these species at our site of collection (Heron Island on the Great Barrier Reef, Australia). However, when taking into account the effects of high solar radiation (including ultraviolet light), this upper limit of survival may be an overestimate (i.e., the upper limit of survival may be below 32°C and 101 Pa $pCO_2$); Gao and Zheng (2010) showed that photosynthesis and calcification are dramatically reduced under high irradiance in combination with elevated $CO_2$ concentration.

Under the predicted climate change scenarios of rising ocean temperatures and ocean acidification, the vulnerability of calcifying algae and foraminifera is of great concern. With some predictions estimating that atmospheric $CO_2$ concentrations will reach 101 Pa by 2100 and 203 Pa by 2200 (Friedlingstein et al. 2006; Houghton 2009) and that the ocean temperature will rise by 2–6°C over the next 100–200 yr (Houghton 2009), the survival of these photosynthetic marine calcifiers is under threat. Furthermore, noncalcifying macroalgae, which may benefit from near-future climate change scenarios (Gao et al. 1993; Hobday et al. 2006), are expected to exhibit a competitive advantage over calcifying species (Fabry et al. 2008; Martin and Gattuso 2009). The loss of these calcifying keystone species will affect many other associated species, such as fish communities, in the future (Kleypas and Yates 2009). Consequent changes in community structure and habitat structure for many marine organisms could very well influence trophic interactions and habitat availability for other coral reef organisms. The loss of these sediment-producing species will also reduce the sediment turnover rate and decrease the amount of carbonate sands in the marine environment.

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