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Physiological performance of three calcifying green macroalgae *Halimeda* species in response to altered seawater temperatures

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Abstract

The effects of seawater temperature on the physiological performance of three *Halimeda* species were studied for a period of 28 d. Five treatments were established for *Halimeda cylindracea*, *Halimeda opuntia* and *Halimeda lacunalis*, in triplicate aquaria representing a factorial temperature with 24°C, 28°C, 32°C, 34°C and 36°C, respectively. The average F_v/F_m of these species ranged from 0.732 to 0.756 between 24°C and 32°C but declined sharply between 34°C (0.457±0.035) and 36°C (0.122±0.014). Calcification was highest at 28°C, with net calcification rates (G_{net}) of (20.082±2.482) mg/(g·d), (12.825±1.623) mg/(g·d) and (6.411±1.029) mg/(g·d) for *H. cylindracea*, *H. opuntia* and *H. lacunalis*, respectively. Between 24°C and 32°C, the specific growth rate (SGR) of *H. lacunalis* (0.079%–0.110% d^{-1}) was lower than that of *H. cylindracea* (0.652%–1.644% d^{-1}) and *H. opuntia* (0.360%–1.527% d^{-1}). Three *Halimeda* species gradually bleached at 36°C during the study period. Malondialdehyde (MDA) and proline levels in tissues of the three *Halimeda* were higher in 34–36°C than those in 24–32°C. The results indicate that seawater temperature with range of 24–32°C could benefit the growth and calcification of these *Halimeda* species, however, extreme temperatures above 34°C have negative impacts. The measured physiological parameters also revealed that *H. cylindracea* and *H. opuntia* displayed broader temperature tolerance than *H. lacunalis*.

Key words: calcifying macroalgae, climate change, seawater temperature, physiological performance, photosynthesis, calcification

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1 Introduction

Climate change presents various challenges, because of the large scale of its effects and the difficulty of alleviating them by human management (Hughes et al., 2003). Previous studies indicated that seawater surface temperature has continuously and slowly risen by approximately 1°C over the last century in tropical sea areas, and an additional 1–2°C is predicted by the end of year 2100 (Deser et al., 2010; Collins et al., 2013). Marine ecosystems in shallow waters are suffering a variety of environmental stresses, including temperature fluctuations. McKenzie and Campbell (2004) reported that the seasonal seawater temperatures in tropical areas fluctuated from 19.8 to 41.0°C, which has obvious impacts on the temporal and spatial distribution and biomass of marine species. A study of macroalgal herbaria in tropical oceans along the Australian coast showed that in the last 70 years a number of temperate macroalgae showed a poleward shift, and this trend is predicted to continue (Wernberg et al., 2011). Therefore, an improved understanding of patterns of interactions between species and environmental factors is urgently needed (Hofmann et al., 2015; Vogel et al., 2015).

Coral reef ecosystems, one of the most productive and species-rich types of ecosystems, appear to be particularly vulnerable and sensitive to the global increasing seawater temperature (Price et al., 2011). The vast, three-dimensional structures of coral reefs are created by calcifying organisms and provide habitats and shelters for many marine animals (Koch et al., 2013). Within coral reef ecosystems, there are taxonomic differences between marine calcifiers in terms of environmental parameters such as nutrient availability, sunlight irradiance and water currents (Koch et al., 2013). Martin and Gattuso (2009) suggested that a 3.0°C-increase in seawater temperature improved the productivity and net calcification of healthy coralline algae, *Lithophyllum cabiochae*, in autumn and winter when the ambient temperature was 13.3–22.0°C. However, necrosis and mortality occurred when

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the algae were exposed to long period of high temperatures (~25.0°C) in summer. In addition, a significant decrease (~50%) in growth rate was documented in the crustose coralline algae *Porolithon gardineri* under a 2.5–4.5°C temperature increase by Agegian (1985). Thus, it is essential to identify the thermal optima and tolerance ranges of calcareous macroalgae, especially in the tropics, as they have massive impacts on calcium carbonate ($CaCO_3$) formation and primary productivity (Littler et al., 1991; Nelson, 2009).

Calcifying algae within the genus *Halimeda* Lamouroux (order Bryopsidales) are conspicuous components of coral reef ecosystems as they are widely distributed across tropical and subtropical environments which contribute to reef accretion in their habitats (Walters et al., 2002; Campbell et al., 2016). The thalli may be erect, pendant, or sprawling and are anchored by rhizoids produced from inner and outer cortical cells (Wizemann et al., 2015). On coral reefs, *Halimeda* segments accumulated through shedding from living thalli or after holocarpic sexual reproduction to protect lagoons and the reef flat environments (Hillis-Colinvaux, 1980). In certain marine locations, the $CaCO_3$ production rate of this genus can reach 1.4 kg/(m^2·a), which suggest that this genus is a major contributor to carbonate assimilation in reefs (Payri, 1988). Given the importance of *Halimeda* species within coastal habitats, further studies of the responses of *Halimeda* species to elevated temperature is needed. Potin et al. (1990) and Martin et al. (2006, 2007) have reported seasonal changes in algal photosynthesis and calcification in response to warmer seawater during summer months in the Mediterranean. Work by Campbell et al. (2016) has demonstrated that increased temperature (28–31°C) could mitigate the negative effects of ocean acidification by enhancing the metabolic performance of *Halimeda* spp. (photosynthesis and calcification). While these findings have contributed to understanding the effects of elevated temperature on these calcareous algae, the upper lethal and sublethal temperature thresholds for *Halimeda* species still need to be determined.

The South China Sea is located in the northern tropics in China, which is one of the most diverse shallow water marine areas in the world (Morton and Blackmore, 2001). Many offshore islands and archipelagos provide convenient sites for the formation and maintenance of coral communities in South China Sea. However, few studies of high thermal stress on calcareous macroalgae, especially *Halimeda*, have been done. Thus, in this study, the responses of three species of *Halimeda* to elevated temperatures are studied. Using a series of artificial incubations, we examine the effects of thermal stress on photosynthesis, calcification and other measures of physiological performance and analyze the tolerance and ability to adapt to extreme temperature in tropical seawaters of these calcareous macroalgae. The aim of this study was to predict the physiological responses of *Halimeda* to climate changes in the future and provide evidence that conservation of coral reef ecosystems may require more proactive managements.

2 Materials and methods

2.1 *Samples collection*

Thalli of three *Halimeda* species were sampled from reef sites in the South China Sea at depths ranging from 6 m to 8 m in July 2017 (9.53°–9.60°N, 115.34°–115.46°E). The sampling sites were composed of sloping shores which principally consisted of sand, stones, massive dead fragments of branched corals (Fig. 1). All algal specimens were carefully uprooted with the holdfast intact from the substrate. After collection, the samples were placed in a polyethene-free, continuous-flow, seawater system aboard ship for transport to the Tropical Marine Biological Research Station at the Hainan Chinese Academy of Sciences in Sanya, China. The macroalgae were maintained temporarily in three 800 L flow-through mesocosm tanks at the Sanya Station for 2 weeks (salinity 34–35, temperature ~28°C, pH 8.1–8.3). Irradiance for each tank was provided by a series of full spectrum T5 fluorescent bulbs delivering 150 μmol photons m^{-2} s^{-1} to the bottom of the tank on a 12 h/12 h day/night cycle (Hofmann et al., 2014).

2.2 *Molecular phylogenetic analysis*

Before molecular analysis, all *Halimeda* thalli samples were classified according to their morphological features as described by Dijoux et al. (2012). Each morphotype sample was cleaned 4–5

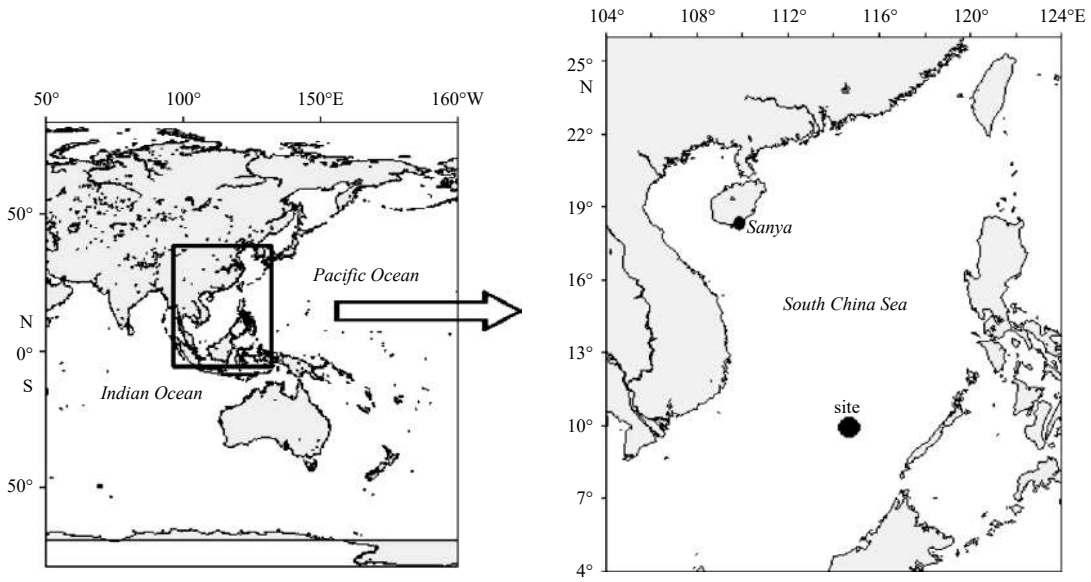

Fig. 1. The sample site located in an outer fore-reef ledge (6–8 m) in the South China Sea and location of the experimental facility in Sanya, China.

times with sterile seawater. Approximately 100 mg of *Halimeda* tissue from each sample was finely ground in liquid nitrogen with a mortar and pestle and total DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Polymerase chain reaction (PCR) was carried out in a total volume of 25 μL containing 2 μL DNA solution, 12.5 μL 2×EasyTaq PCR Supermix, including 1.5 mmol/L of $MgCl_2$ (Transgen Biotech, Beijing, China), and 10 μmol/L of forward and reverse primers with a PCR Eppendorf Mastercler pros (Hamburg, Germany). The following primers were used for PCR of the plastid elongation factor Tu (*tufA*) region (Famà et al., 2002):

tufA–A1 (forward), ATGRCWCGHGMAAAATTTSAACG;
tufA–H2 (forward), ATTTTAGTVGTNTCNGGTGC;
tufA–R (reverse), CCTTCNCGAATMGCRAAWCGC.

The profile of touch down PCR conditions was designed and improved according to Kojima et al. (2015). The nested PCR was performed with conditions described as follows using tufA–A1/ tufA-R and tufA–H2/ tufA–R primers, respectively: an initial denaturation phase at 94°C for 2 min; and 3 cycles of 10 s at 94°C, 30 s at 50°C, 30 s at 68°C; and 3 cycles of 10 s at 98°C, 30 s at 48°C, 30 s at 68°C; and 30 cycles of 10 s at 98°C, 30 s at 42°C, 30 s at 68°C; and a final extension of 10 min at 72°C. The PCR products were tested on ethidium bromide-stained 1.0% agarose gels. After polyethylene glycol (PEG) purification (Lis, 1980), the products were sequenced in both directions by BGI Genomics Co. Ltd., Shenzhen, China. Then, all sequences were aligned using the BioEdit sequence alignment editor (Hall, 1999). Phylogenetic tree analyses were obtained using maximum likelihood (ML) in MEGA6 (Tamure et al., 2007). The reliability of each branch was checked using 1 000 bootstrap replications. Evolutionary distance was computed using the Kimura two-parameter method (Kimura, 1980).

2.3 *Experimental design and treatments*

A factorial experimental design was used, with three species (*H. cylindracea*, *H. opuntia* and *H. lacunalis*) and five temperature treatments (24°C, 28°C, 32°C, 34°C and 36°C). The experiment was conducted in 3 replicates for each treatment. There was 45 L of seawater volume in each aquarium and using a total of 15 aquaria. There was a total of 30 individuals of three species (340–360 g, fresh weight) in one aquarium. Once identified, 10 individuals of each species were designed to each temperature regulated aquarium for 4 weeks (25 August–21 September, 2017). Filtered seawater changes were conducted twice per week to ensure seawater chemistry conditions stable. The seawater temperature was elevated by 1°C every 12 h using water thermostats (TC10, Teco Italy) until the treatment temperature was reached. Samples from each treatment were collected every 4 d during the 28-d experiment.

Seawater parameters, including salinity, temperature, pH (NBS scale) and total alkalinity (TA) was monitored daily. Seawater salinity and temperature were measured with a calibrated handheld YSI meter (YSI, Yellow Springs, OH, USA). Water samples for TA were stored in brown glass bottles and then mixed with a 0.002% volume saturated solution of $HgCl_2$ solution at 4°C in dark conditions before measurement. TA was measured as the Gran titration method using a TitroLine alpha 05 plus titrator (Xylem Analytics, Weilheim, Germany) with an automated sample changer and pH electrode. Salinity, temperature, pH and TA were used to assess dissolved inorganic carbon components (CO_2, CO_3^{2-} and HCO_3^-) with the CO2SYS program (Pierrot et al., 2006). All measurements were completed within 24 h of sampling.

2.4 *Photosynthetic performance and pigment concentrations*

The photosynthetic maximum quantum yield of photosystem (PS) II (F_v/F_m) was measured at the end of the experiment using a DIVING-PAM fluorometer fitted with an 8.0 mm diameter fiber-optic cable (Heinz Walz GmbH, Germany). On the sampling date, haphazard selected mature segments (~3.0 cm) of the three *Halimeda* species in five treatments were removed and dark-adapted for approximately 10 min before measurement. To reduce the possibility of photochemical quenching causing by the measuring light of the diving PAM, the measurement was operated with a constant intensity of measuring light (setting "8") in the "Burst mode" for three *Halimeda* species. Initial measurements of F_v and F_m were recorded to test for bias to elevate the PS II photophysiology activity.

The chlorophyll (Chl) *a* and carotenoid concentrations of each *Halimeda* species were determined using the spectrophotometric methods described by Wellburn (1994) during the experiment. A total of 100.0 mg of mature segments was ground in 10 mL of 90% acetone, and then stored for extraction at 4°C in darkness for 24 h. Then, all samples were centrifuged at 5 000 r/min for 10 min at 4°C (Eppendorf centrifuge 5810 R, Germany). The supernatant was transferred to a quartz cuvette and its absorbance was measured at 664, 647 and 470 nm on a spectrophotometer (UV 530, Beckman counlter, USA).

2.5 *Calcification and tissue mineral content*

Net calcification rates (G_{net}) of each *Halimeda* species were determined by comparing buoyant weights to initial fresh mass at the beginning and end of the experiment. Segments that shed down from the thalli during the experiment were also taken into measurement (Jokiel et al., 1978; Peach et al., 2017). However, the buoyant weights of the *Halimeda* thalli which suffered mortality, with complete segments disarticulating and bleaching, were not measured in this study. A whole *Halimeda* thallus was placed on an acrylic Petri dish hung below an electronic balance (AR224CN, OHAUS, USA; accuracy, ~0.1 mg) using nylon thread suspended in seawater. It involves weighing samples in seawater using the density according to Archimedes' principle to normalize the fresh weight (Jokiel et al., 1978; Langdon et al., 2010).

Tissue mineral content was assumed to be equal to $CaCO_3$ content (%) in this study. At the end of the aquaria experiment, the mature (>7 d old) and new apical segments (5–7 d old) were placed in separate, pre-weighed glass vials and rinsed with distilled water 3–4 times to remove dirt and salts, then dried at 60°C in an oven until a constant weight was obtained. Then, all samples were acidified with 5% of 12 mol/L HCl until no effervescence was generated by the addition of acid, after which samples were rinsed with distilled water 3–4 times to remove HCl trances, and then dried in oven to a constant weight. $CaCO_3$ content (%) was determined by the difference between the dry weights before and after removal of calcification.

2.6 *Growth rate and physiological responses*

The fresh weight (FW, g) was measured at the beginning and end of the experiment by blotting whole *Halimeda* thalli dry with paper towels until no change in mass was detectable. The growth rate was calculated using the following formula:

$$\mathrm{SGR} = [(\ln W_t - \ln W_{t0})/d] \times 100,$$

where SGR is the specific growth rate (% d^{-1}), W_t is the fresh

weight obtained at time t, W_{t0} is the fresh weight collected on the day before time t, and d is 28 d between two sampling time points. Production of new segments on mature thalli was also tracked to calculate the new segments proportion of whole thalli.

The concentrations of proline and malondialdehyde (MDA) in *Halimeda* segments were measured to indicate physiological responses to temperature. The proline conferred integrity to maintain the photosynthetic system functioning and to protect the membranes from various damages (Sun et al., 2013). For proline analysis, 500.0 mg of fresh tissue material was ground in 5 mL of 3% sulfosalicylic acid, followed by heating at 100°C for 10 min with continuous shaking. The samples of extract solution were centrifuged at 3 000 r/min for 10 min. Subsequently, 2 mL of supernatant was added with 2 mL each of glacial acetic acid and distilled water and 4 mL of 2.5% acid ninhydrin reagent composed of 2.5 g of ninhydrin, 60 mL of glacial acetic acid and 40 mL of 6 mol/L orthophosphoric acid. Next, the samples were heated at 100°C for 60 min and after cooling to ambient air temperature, 4 mL toluene was mixed to partition the reaction solution. Its absorbance was measured at 520 nm. The values of optical density (OD) were compared with a standard curve to determine the amount of proline (Shan et al., 2007).

The occurrence of MDA was considered as a useful indirect evaluation of general lipid peroxidation under certain environmental stresses (Hodges et al., 1999). Thus, MDA has been used extensively to estimate peroxidation of lipids in membrane and biological systems (Hodges et al., 1999). Approximately 500.0 mg FW of the *Halimeda* segments was ground in 10 mL of 10% trichloroacetic acid (TCA), followed by centrifugation at 4 000 r/min for 10 min. Then, 3 mL of aqueous extract was reacted with 3 mL of 0.6% 2-thiobarbituric acid, heated at 100°C for 15 min, then cooled, and centrifuged at 3 000 r/min for 5 min. Absorbances were measured at 532 and 450 nm. MDA equivalent was calculated as follows:

$$C_{\mathrm{MDA}} = 6.45 \times \mathrm{OD}_{532} - 0.56 \times \mathrm{OD}_{450},$$

where C_{MDA} is the MDA concentration, OD_{532} and OD_{450} represent the absorbance values at 532 nm and 450 nm, respectively.

2.7 *Scanning electron microscopy samples preparation and analyses*

The crystalline microstructure of mature mid-growth segments at 28°C and 34°C was imaged using scanning electron microscopy (SEM). Fresh segments of three *Halimeda* species were soaked in 2% glutaraldehyde and then fixed in 1% OsO_4, subsequently, dehydrated by graded ethanol liquid (20%–100%) and soaked in hexamethyldisilazane. Dehydrated segments of thalli were cut across the segment mid-plane with a razor to expose interior surfaces. Afterwards, the samples were sputter coated with gold and observed using an SEM (S-3400N, Hitachi, Japan) equipped with an energy dispersive spectrometer. All SEM images were measured using image processing software (ImageJ v 1.47).

2.8 *Data analysis*

All statistical analyses were conducted using Microsoft Excel 2010 and Minitab 16.0 software. The figures were constructed using Origin 8.1. All data were tested for normality and equal variances prior to statistical analysis. Data in this study were reported as mean ± standard deviation (mean±SD) and were analyzed by a three-way analysis of variance (ANOVA). Least significant difference (LSD) was used to make post hoc comparisons between different groups. Differences were significant at $P<0.05$ and extremely significant at $P<0.01$.

3 Results

3.1 *Molecular phylogenetic analysis*

Based on morphological characteristics, segments were classified as: *Halimeda cylindracea*, with an obviously cylindrical or trilobed shape; *Halimeda opuntia*, with highly branched thalli and a flat, reniform sometimes ribbed surface; and *Halimeda lacunalis*, with a flat, rounded-oval or cuneate shape. According to molecular identification analysis, the aligned *tufA* sequences of *Halimeda* species were about 800 bp in length. Molecular phylogenetic trees based on ML showed essentially identical tree topologies (Fig. 2). According to the ML tree, the samples of *Halimeda* thalli (Samples H1, H2 and H3) were reproduced with moderate to high statistical support, which showed that the samples fell into distinct clades. Using detailed phylogenetic analysis of ML conducted using *tufA* sequences, samples of H1, H2 and H3 were divided into *H. cylindracea*, *H. opuntia* and *H. lacunalis*.

3.2 *Experimental conditions*

The mean seawater chemistry conditions for each treatment during the study period are shown in Table 1. The pH was very similar across all treatments with mean value of 8.10±0.05 and no significant differences (F=0.284, P>0.05). Seawater salinity did not differ significantly between treatments with a mean value of 34.6±0.1 during the experiment. Calculated values of CO_2, CO_3^{2-} and HCO_3^- values for each treatment ranged between 9.9 and 12.4 μmol/kg, 158.4 and 207.0 μmol/kg, and 1 471.9 and 1 624.5 μmol/kg. The pCO_2 value fluctuated from 375.9 to 528 μatm (1 atm=1.013×10^5 Pa) during the monitoring period.

3.3 *Photosynthetic performance and pigment concentrations*

The photosynthetic performance of the three *Halimeda* species had similar responses to the five seawater temperature treatments (Fig. 3). There was a significant decrease in the F_v/F_m in the 34°C and 36°C treatments (P<0.01), but no obvious change between 24°C and 32°C (P>0.05) (Table 2). The average F_v/F_m values of these species ranged from 0.732 to 0.756 between 24°C to 32°C and had much lower range of 0.122 to 0.457 between 34°C and 36°C. Variations in pigment concentrations also indicate differences in photosynthesis between the *Halimeda* species in each treatment (Fig. 4). At the end of experiment, the average Chl *a* concentrations of the three *Halimeda* species were 2–3 times higher in the 28°C treatment than those in the 34°C and 36°C treatments. Small changes in Chl *a* concentrations were observed between the 24°C and 32°C treatments with mean values of (226.8±10.7) and (241.0±13.7) μg/(g FW); (216.8±9.1) and (176.6±14.3) μg/(g FW); and (184.9±11.2) and (154.0±12.1) μg/(g FW) in *H. cylindracea*, *H. opuntia* and *H. lacunalis*, respectively. Carotenoid concentrations were also affected by temperature differences and were higher for the first 8–12 d, with ranges of 13.7–34.4, 9.1–21.9 and 12.2–28.7 μg/(g FW) for *H. cylindracea, H. opuntia* and *H. lacunalis*, respectively. At the end of the experiment, carotenoid concentrations were lowest in the high temperature treatments (34°C and 36°C) (Fig. 4).

3.4 *Calcification rate and tissue mineral content*

Net calcification rates (G_{net}) varied among the three *Halimeda* species and across the five treatments (P<0.01) (Table 3). Calcification was highest at 28°C, with net calcification rates (G_{net}) of (20.082±2.482) mg/(g·d), (12.825±1.623) mg/(g·d) and

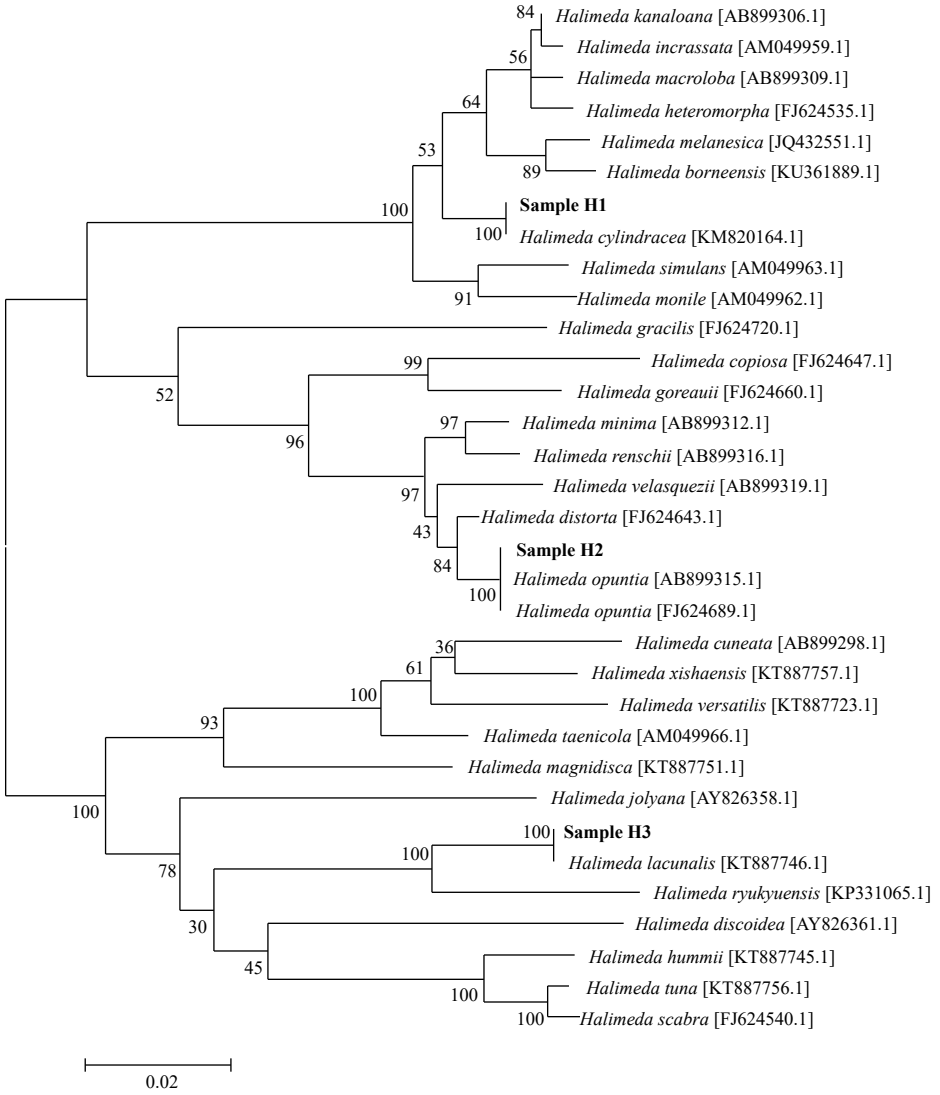

Fig. 2. Maximum likelihood (ML) molecular phylogenetic tree of the genus *Halimeda* based on *tufA*. The scale bar is 0.02 substitutions per site. Numbers on branches indicate bootstrap values from ML analysis. Sample H1, H2 and H3 represented *Halimeda cylindracea*, *Halimeda opuntia* and *Halimeda lacunalis*.

Table 1. Summary of measured and calculated parameters of treatments determined daily during the 28 d aquaria experiment (25 August–21 September, 2017)

Treatment	Measured			Calculated			
	pH_{NBS}	TA/μmol·L^{-1}	SSS	CO_2/μmol·kg^{-1}	CO_3^{2-}/μmol·kg^{-1}	HCO_3^-/μmol·kg^{-1}	pCO_2/μatm
24°C	8.11±0.04	2 023.2±56.3	34.5±0.1	12.4±0.6	158.4±16.7	1 624.5±48.6	425.7±23.5
28°C	8.13±0.03	1 916.5±59.21	34.7±0.1	9.9±0.8	174.6±19.6	1 471.9±52.3	375.9±17.9
32°C	8.14±0.06	2 132.4±64.82	34.3±0.2	10.7±0.6	207.0±20.5	1 619.2±47.1	443.6±19.4
34°C	8.09±0.09	2 078.6±48.93	34.6±0.2	11.1±0.9	198.8±14.8	1 582.0±53.6	483.8±17.2
36°C	8.07±0.04	2 124.4±54.82	34.6±0.1	11.6±0.7	204.4±23.2	1 614.5±55.2	528.0±20.6

(6.411±1.029) mg/(g·d) for *H. cylindracea*, *H. opuntia* and *H. lacunalis*, respectively. The G_{net} of *H. cylindracea* in the 24°C and 28°C treatments were higher than those of the other species, ranging from 14.775 to 20.082 mg/(g·d). The G_{net} of *H. cylindracea* in 32°C treatment showed a significant reduction compared with the 24°C and 28°C treatments. This result should not be over interpreted because excessive segment-shedding of certain *H. cylindracea* thalli restricted our ability to obtain accurate calcification rates resulting in an underestimate of the true rate of G_{net}. No calcification rates were measured for the 36°C treatment, or for *H. lacunalis* in the 34°C treatment, due to the high segment shedding rate at high seawater temperatures. The $CaCO_3$ content was not significantly affected by temperature or species. However, $CaCO_3$ content varied between new and mature segments. The average $CaCO_3$% of new segments was 10.83% lower than the mature segments. Water content of three *Halimeda* species thalli ranged from 51.72% to 63.91% and did not appear to be affected by temperature treatment.

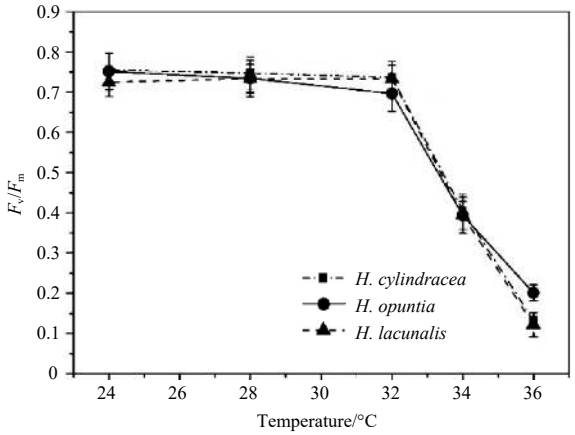

Fig. 3. Photosynthetic maximum quantum yield (F_v/F_m) of three *Halimeda* species incubated in five temperature treatments at the end of the experiment.

Table 2. Results of a three-way ANOVA on F_v/F_m and concentrations of Chl *a* and carotenoid of treatments exposed to the crossed factors of species (*H. cylindracea*, *H. opuntia* and *H. lacunalis*), temperature (Temp: 24°C, 28°C, 32°C, 34°C and 36°C), and time (after 16 and 28 d)

Source of variation	*df*	SS	MS	*F*	*P*
F_v/F_m					
Species	2	0.000	0.000	0.13	0.875
Temp	4	1.467	0.367	154.32	**0.000**
Time	1	0.005	0.005	2.02	0.160
Species×Temp	8	0.017	0.002	0.89	0.530
Species×Time	2	0.036	0.018	7.64	**0.001**
Temp×Time	4	0.022	0.006	2.33	0.066
Species×Temp ×Time	8	0.032	0.004	1.69	0.119
Error	60	0.143	0.002		
Chl *a*					
Species	2	74 760	37 380	141.81	**0.000**
Temp	4	166 946	41 737	158.34	**0.000**
Time	1	3 210	3 210	12.18	**0.001**
Species×Temp	8	31 724	3 966	15.04	**0.000**
Species×Time	2	442	221	0.84	0.438
Temp×Time	4	33 007	8 252	31.31	**0.000**
Species×Temp ×Time	8	6 108	764	2.90	**0.008**
Error	60	15 815	264		
Carotenoid					
Species	2	866.53	433.27	51.14	**0.000**
Temp	4	242.33	60.58	7.15	**0.000**
Time	1	845.43	845.43	99.79	**0.000**
Species×Temp	8	467.31	58.41	6.89	**0.000**
Species×Time	2	19.15	9.57	1.13	0.330
Temp×Time	4	476.32	119.08	14.05	**0.000**
Species×Temp ×Time	8	119.97	15.00	1.77	**0.101**
Error	60	508.35	8.47		

Note: Significant *P* values of factors and interactions ($P<0.05$) are denoted in bold.

3.5 *Growth rate, proline and MDA concentrations*

During the study period, the SGR of *H. cylindracea* and *H. opuntia* were consistently higher than that of *H. lacunalis* in the 24°C, 28°C and 32°C treatments (Table 4). *Halimeda cylindracea* incubated at 28°C had the highest SGR value of (1.644±0.253)% d^{-1}, and the highest SGR of *H. opuntia* occurred in the 32°C treatment at (1.527±0.216)% d^{-1}. Compared with these two species, *H. lacunalis* grew slowly ranging from 0.079% to 0.119% d^{-1} at 24–32°C. However, SGR of *H. cylindracea* and *H. opuntia* decreased to (0.364±0.145)% d^{-1} and (0.118±0.038)% d^{-1} in the 34°C treatment, respectively. In the 36°C treatment, all three *Halimeda* species shed excessively, meaning that no SGR could be obtained at the end of experiment. The proportion of new segment production differed between the three *Halimeda* species and between the five temperature treatments ($P<0.01$). The highest new segment production rates of *H. cylindracea*, *H. opuntia* and *H. lacunalis* occurred at 28°C with 54.55%, 34.78% and 23.08%, respectively. Segment production rates of *H. cylindracea* and *H. opuntia* were significantly decrease at 34°C while *H. lacunalis* was bleaching gradually in the 34°C and 36°C treatments.

The accumulated concentrations of proline and MDA in the tissues of the three *Halimeda* species are shown in Fig. 5. Across all species, proline levels were similar between 24°C and 32°C; however, under high temperature stress tissue proline concentration was 2–4 times higher (Table 5). The concentrations of MDA were assessed in normal and high temperature conditions and were about 2–3-fold higher in the 34°C and 36°C treatments from Day 4 to Day 16. However, after 16 d, the concentrations of proline and MDA greatly decreased as the thalli bleached (Table 5).

3.6 *Aragonite crystal formation*

The aragonite crystals in segments of the three *Halimeda* species under 28°C and 34°C conditions are shown in Figs 6 and 7. There were no obvious differences in crystal shape and size between species in same temperature treatment. Short needles, approximately 5 μm in length, principally occupied the primary inter-utricular space (pIUS) across all three species. Nevertheless, at 34°C, the micro-anhedral carbonate filled less of the pIUS than that at 28°C. The rims of micro-anhedral carbonate in the central medullary utricles (mU) were thinner in the 34°C treatment. Segments of the three *Halimeda* species from the 34°C treatment showed irregular and porous structures of the peripheral utricle surface (Us) compared with those at 28°C, which exhibited a heavily abraded surface. In the 28°C treatment, mU in segments showed thick $CaCO_3$ as the rims of micro-anhedral carbonate gained thickness when segment age increased, and secondary needles and euhedral orthorhombic $CaCO_3$ crystals in mature segments were lager.

4 Discussion

The genus *Halimeda* is widely distributed in the marine tropics, especially in coral reef ecosystem, contributing to the structure and stability of coral reefs and atolls (Beach et al., 2003; Hensley et al., 2013). During the past few decades, the numerous *Halimeda* species have been collected and identified mostly based on morphological and anatomical analyses (Dijoux et al., 2012). The species *H. cylindracea* in this study generally colonized in sandy habitats displayed distinct species-specific morphological features such as cylindrical segments. However, the shapes and size of segments in a *Halimeda* thallus can be strongly influenced by environmental conditions, meaning that identifying biological species by morphological differences is difficult. For instance, the segments on the top half of *H. opuntia*, *Halimeda minima*, and *Halimeda micronesica* thalli all have multiple flat or/and reniform shapes (Dijoux et al., 2012). Since the early 1990s, molecular phylogenetic analysis has been used

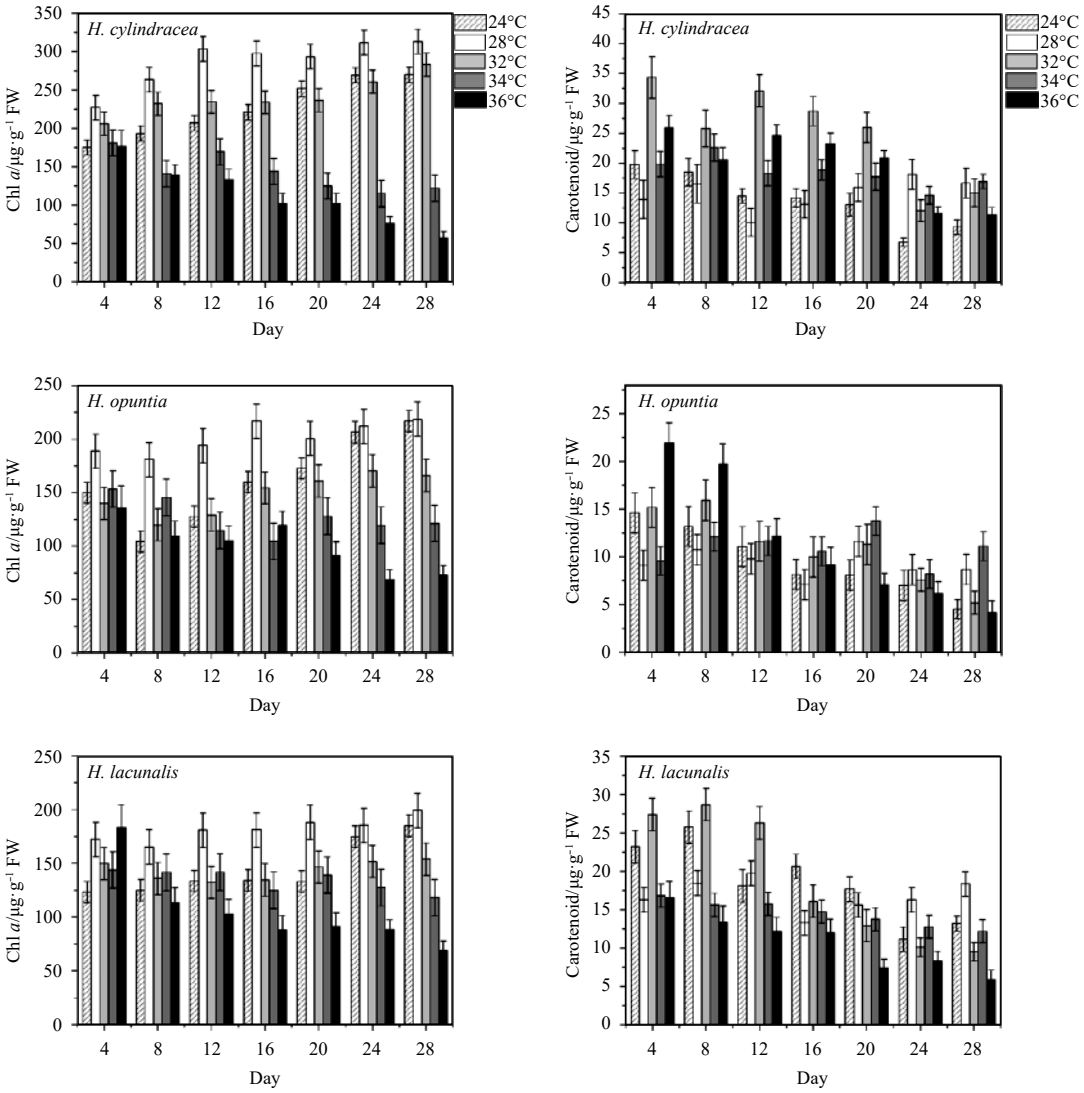

Fig. 4. Variations in concentrations of Chl *a* and carotenoid between the tissues of three *Halimeda* species from five temperature treatments during the 28 d experiment.

Table 3. Calcification responses of three *Halimeda* species to five temperature-related treatments over the 28 d experiment

Group		G_{net}/mg·g^{-1}·d^{-1}	New segment $CaCO_3$/%	Mature segment $CaCO_3$/%	Water content/%
24°C	*H. cylindracea*	14.775±2.003	81.22±3.12	89.38±1.81	58.91±3.26
	H. opuntia	4.579±1.023	80.71±2.73	92.18±2.11	51.72±2.61
	H. lacunalis	1.457±0.392	72.83±1.62	84.72±1.02	63.91±3.10
28°C	*H. cylindracea*	20.082±2.482	81.28±1.22	91.82±2.33	55.73±4.15
	H. opuntia	12.825±1.623	83.21±3.52	84.11±3.26	53.28±3.15
	H. lacunalis	6.411±1.029	73.49±3.21	87.51±5.21	58.36±1.91
32°C	*H. cylindracea*	8.282±1.934	73.91±2.45	89.98±2.81	63.23±4.22
	H. opuntia	8.296±1.236	85.12±2.37	90.29±3.45	54.23±3.84
	H. lacunalis	1.521±0.294	82.33±1.92	86.28±1.09	57.23±4.19
34°C	*H. cylindracea*	3.246±0.923	64.23±4.11	92.17±2.62	54.12±1.23
	H. opuntia	0.979±0.063	75.73±2.42	85.26±3.11	59.23±2.81
	H. lacunalis	–	–	88.41±3.12	55.32±3.77
36°C	*H. cylindracea*	–	–	75.83±5.23	56.38±2.73
	H. opuntia	–	–	84.76±4.17	61.34±1.54
	H. lacunalis	–	–	83.35±2.81	56.61±3.55

Note: Net calcification rate (G_{net}) is shown as total $CaCO_3$ production compared to initial fresh thalli weight. Tissue mineral content of new (5–7 d old) and mature (>7 d old) segments is shown as $CaCO_3$ percentage. Water content represents the reduction in dried weight compared to fresh weight.

Table 4. The growth responses of three *Halimeda* species to five temperature-related treatments over the 28 d experiment

Group		Maximum new Segment/%	SGR/% d^{-1}
24°C	*H. cylindracea*	34.78	0.652±0.102
	H. opuntia	21.05	0.360±0.089
	H. lacunalis	7.69	0.079±0.006
28°C	*H. cylindracea*	54.55	1.644±0.253
	H. opuntia	34.78	0.885±0.192
	H. lacunalis	23.08	0.109±0.021
32°C	*H. cylindracea*	37.50	1.157±0.302
	H. opuntia	27.71	1.527±0.216
	H. lacunalis	11.76	0.110±0.051
34°C	*H. cylindracea*	15.49	0.364±0.145
	H. opuntia	10.45	0.118±0.038
	H. lacunalis	–	–
36°C	*H. cylindracea*	–	–
	H. opuntia	–	–
	H. lacunalis	–	–

Note: Maximum new segment production rate (%) is the maximum proportion of the thalli that produced new segments. Specific growth rate (SGR) is expressed as % d^{-1}.

for species identification. In recent years, the plastid *tufA* sequence was put forward as the DNA barcode of delimitation for the Ulvophyceae (Saunders and Kucera, 2010) and the genus *Halimeda* (Verbruggen et al., 2006, 2007). In this study, use of the *tufA* gene for molecular analysis allowed three species-level clades to be distinguished, corresponding to *H. cylindracea*, *H. opuntia* and *H. lacunalis*.

The results of the present study regarding photosynthetic performance and concentrations of Chl *a* and carotenoid reveal that the physiological performance of *Halimeda* varied between species and between temperature treatments. During the experimental period, the seawater conditions were kept relatively stable in order to allow that tolerance of *Halimeda* to elevated temperatures to be determined accurately (Table 1). The three *Halimeda* species exhibited similar photosynthetic responses to fluctuations in seawater temperature. Average F_v/F_m values of these species were higher at 24–32°C than that at 34–36°C. The concentrations of Chl *a* and carotenoid were also high in the 24–32°C treatments, which could increase the ability of photosystem II to harvest light (Teichberg et al., 2013). Together with lower pigment concentrations, opposing photosynthetic characteristics were obvious in the 34–36°C treatments, especially after 28 d of exposure. These results agree with those of the previous

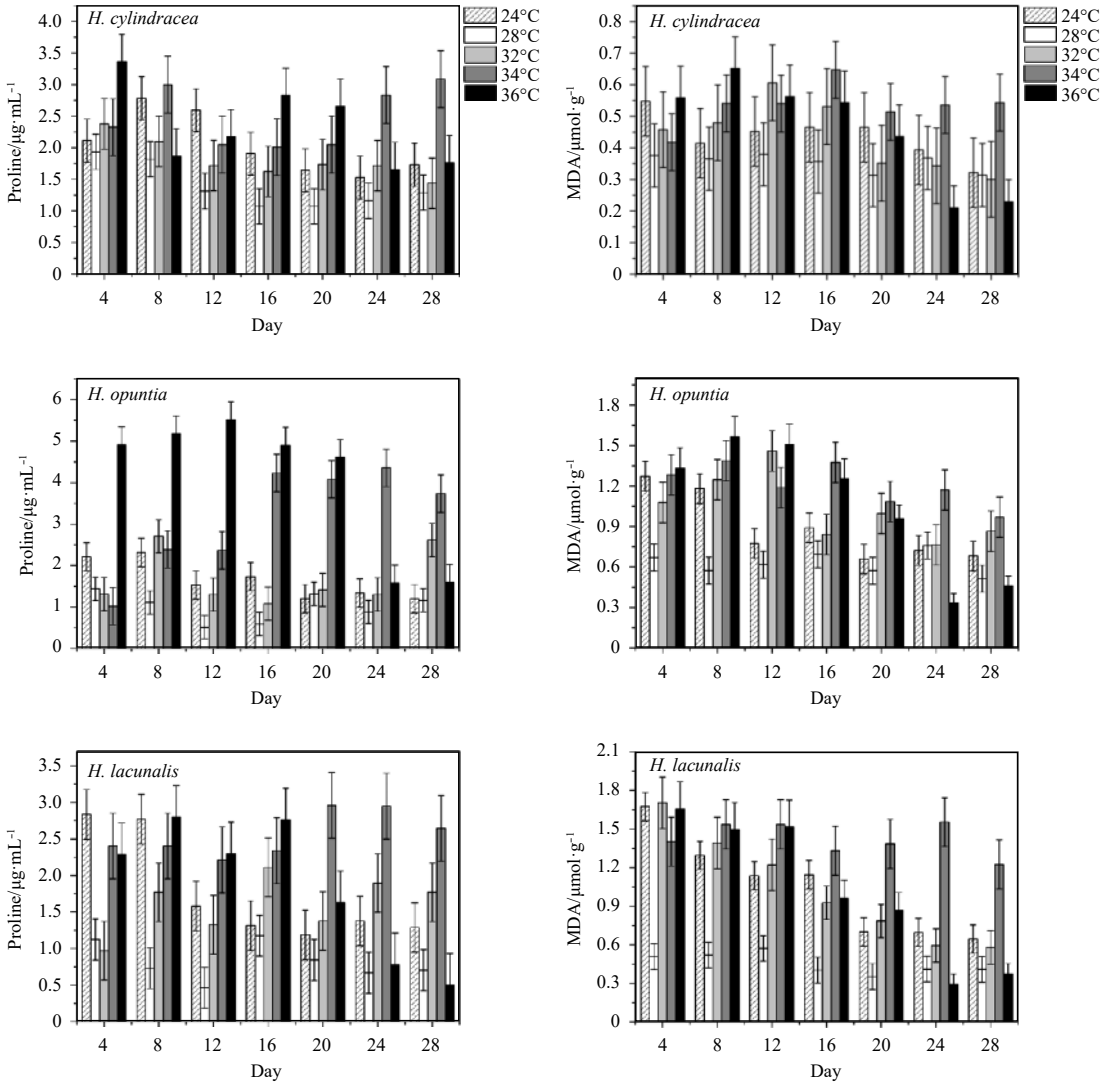

Fig. 5. Proline and MDA concentrations in tissues of the three *Halimeda* species exposed to five temperature treatments for 28 d.

Table 5. Results of a three-way ANOVA on proline and MDA concentrations of *Halimeda* in five treatments exposed to the crossed factors of species (*H. cylindracea*, *H. opuntia* and *H. lacunalis*), temperature (Temp: 24°C, 28°C, 32°C, 34°C and 36°C), and time (after 16 and 28 d). Significant *P* values of factors and interactions ($P<0.05$) are denoted in bold.

Source of variation	*df*	SS	MS	*F*	*P*
Proline					
Species	2	5.248	2.624	9.08	**0.000**
Temp	4	33.078	8.269	28.61	**0.000**
Time	1	2.509	2.509	8.68	**0.005**
Species×Temp	8	13.262	1.658	5.74	**0.000**
Species×Time	2	0.132	0.066	0.23	0.797
Temp×Time	4	15.736	3.394	13.61	**0.000**
Species×Temp×Time	8	7.457	0.932	3.22	**0.004**
Error	60	17.342	0.289		
MDA					
Species	2	5.977	2.988	145.61	**0.000**
Temp	4	3.021	0.755	36.79	**0.000**
Time	1	2.718	2.718	132.41	**0.000**
Species×Temp	8	1.110	0.139	6.76	**0.000**
Species×Time	2	0.731	0.366	17.82	**0.000**
Temp×Time	4	1.142	0.353	17.20	**0.000**
Species×Temp×Time	8	0.353	0.044	2.15	**0.044**
Error	60	1.231	0.021		

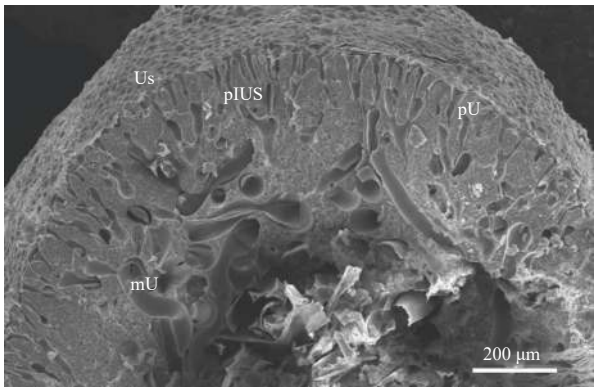

Fig. 6. Representative segment of *H. cylindracea* fractured on the segment vertical mid-plane to expose internal microstructure. Image shows locations of aragonite crystal formation in primary inter-utricular spaces (pIUS), primary utricles (pU), utricle surface (Us) and central medullary utricles (mU).

temperature-related studies. Lee and Hsu (2009) demonstrated that the F_v/F_m values of siphonous green algae *Codium edule* dropped slowly in the first 4 h at 35°C and then decreased rapidly after 8 h, indicating that the 35°C temperature inhibited and impaired photosynthetic activities that the effects were more severe after a long period of exposure. Another calcareous species in this genus, *Halimeda incrassata* showed an obvious decline in photosynthesis at 32°C conditions and succumbed to 75.0% mortality at 34°C (Anderson, 2006).

Previous studies have shown that the chemical reactions of calcification are influenced by the saturation state of $CaCO_3$ (Ω_{Ca}), seawater temperature, and salinity, among other factors (Millero et al., 2006; Millero, 2007). The results of this study point to the existence of upper temperature thresholds for calcification, indicating that additional warming is likely to disrupt intrinsic metabolic processes, with a parabolic relationship forming between seawater temperature and calcification of calcareous organisms (Lough and Barnes, 2000; Muehllehner and Edmunds, 2008; Edmunds et al., 2012). Castillo et al. (2014) found that the calcification rate of the reef-building coral *Siderastrea siderea* exhibited a thermal performance curve, accelerating from 25°C to 28°C and declining from 28°C to 32°C. The G_{net} of *H. cylindracea* in this study was highest at 24–28°C while the maximum G_{net} of *H. opuntia* was obtained between 28°C and 32°C. *Halimeda lacunalis* showed lower calcification rates at 24–32°C during the experiment and no obvious G_{net} could be obtained above 34°C, suggesting that the temperature thresholds for calcification vary between *Halimeda* species. Peach et al. (2017) measured the calcification rates of six *Halimeda* species and found that the G_{net} of *H. opuntia* (~44 mg/(g·d)) were extremely higher than *Halimeda copiosa*, *Halimeda goreauii*, *Halimeda incrassata*, *Halimeda monile* and *Halimeda tuna* rates, ranging from 2 to 5 mg/(g·d), which was also higher than the G_{net} of *H. opuntia* measured in this study. Marine calcareous macroalgae have multiple crystalline forms of $CaCO_3$ and species-specific rates of calcification (Koch et al., 2013). The crystals of genus *Halimeda* were majorly formed by calcification in intercellular spaces (Peach et al., 2017). In this study, SEM images show obvious effects of changes in seawater temperature on the inter-utricular space due to physiological changes in the calcification metabolism and the shapes and sizes of the $CaCO_3$ microstructures in *Halimeda* segments. It is likely that primary needle formation and organic matrix components are actively enhanced by calcification (Wizemann et al., 2014). Optimal seawater temperature leads to elevated external carbonic anhydrase activity in *Halimeda* so that HCO_3^- is used more effectively by calcifying proteins in $CaCO_3$ biomineralization (Bertucci et al. 2013; Hofmann et al., 2014).

In the context of organic osmolytes, proline could protect cellular structures and combine with other defense-related enzyme systems under environmental stresses (Sun et al., 2013). In this study, high concentrations of proline may have kept the physiological systems functioning and relieved stresses in 34°C treatment. A similar conclusion can be drawn regarding the 36°C treatment over the initial ~20 d. However, at the end phase of the experiment (Day 20–28), the concentrations of proline decreased as a result of *Halimeda* thalli bleaching and breaking down gradually. Better growth of the three *Halimeda* species was correlated with lower lipid peroxidation, which was revealed by low MDA concentrations in the 24–32°C treatments, demonstrating that the *Halimeda* suffered less oxidative damage under thermal stress conditions (Sun et al., 2013). In the first 4–8 d, MDA levels of *Halimeda* at 32°C were high, after which the levels declined, demonstrating that the algae were adjusting in response to temperature stress. All these results in the present study suggest that *Halimeda* species positively altered physiological responses to thermal stresses, and that the upper sublethal and lethal temperatures were 34°C and 36°C, respectively. This corresponds with the optimal temperature range (28–32°C) determined in previous studies (Beach et al., 2003; Biber and Irlandi, 2006; Peach et al., 2017) and the thermal threshold (34°C) for growth (Sinutok et al., 2011). Thorhaug (1976) reported that many macroalgae (e.g., *Halimeda*, *Udotea*, *Penicillus*) in tropical marine ecosystems at Turkey Point exhibited fast growth in the summer when seawater temperatures reached up to 31–32°C, extremely close to the upper thermal thresholds (~32–38°C), and pointed out that thermal acclimation did not alter algal thermal limits.

Most studies on *Halimeda* species, including the present study, indicate that they have great capacity to adapt quickly to

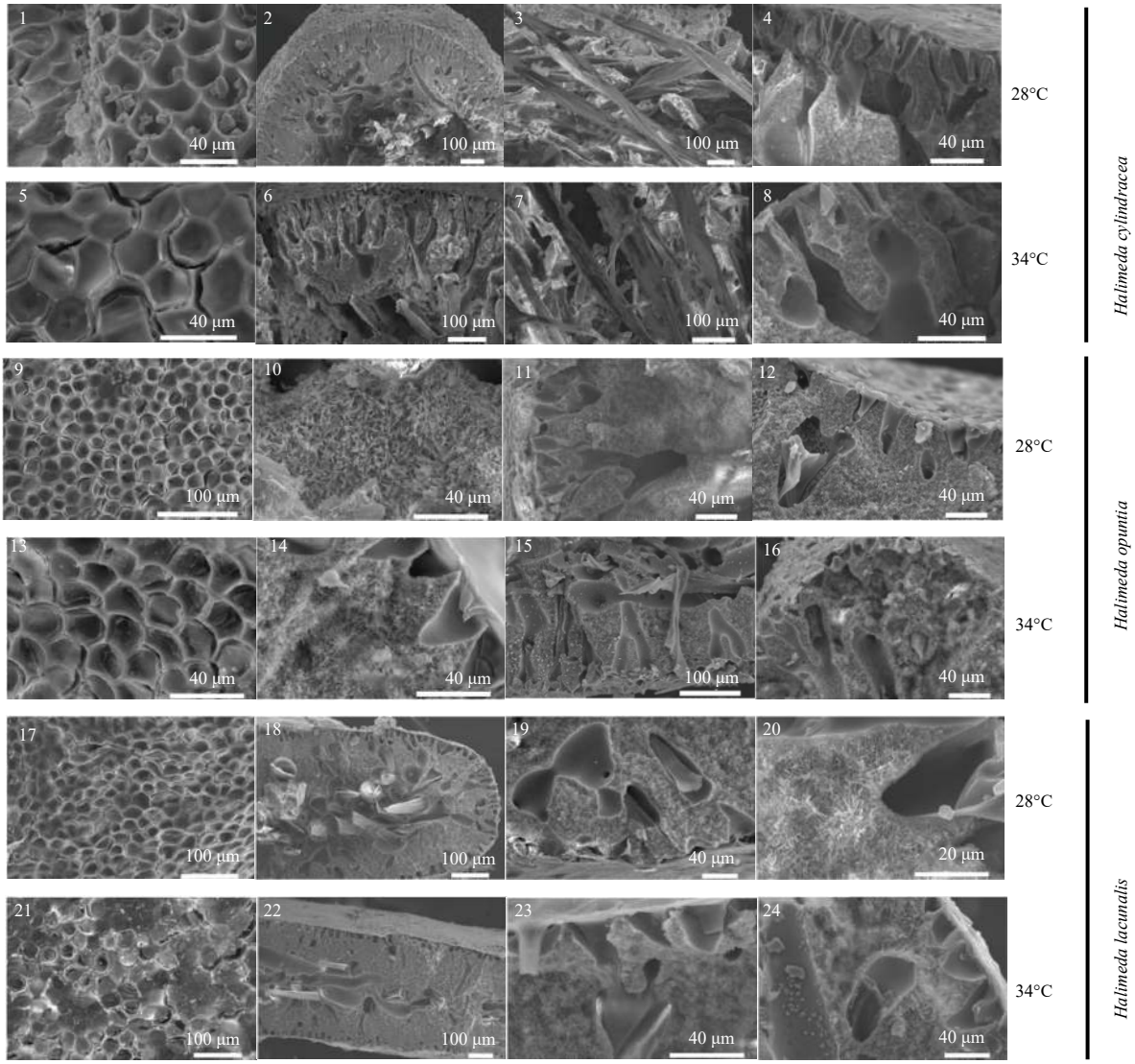

Fig. 7. SEM vertical-section images of mid-growth segments of three *Halimeda* species sampled under 28°C (1–4, 9–12 and 17–20) and 34°C (5–8, 13–16 and 21–24) treatments. 1, 5, 9, 13, 17, and 21 corresponding utricle surface (Us). 2, 6, 18, and 22 cross-sectional overviews of the microstructure of a complete segment. 10 and 14 magnification of segments showing the location of utricles (U) and primary inter-utricular spaces (pIUS). 3–4, 7–8, 11–12, 15–16, 19–20, and 23–24 detailed analyses of central medullary utricles (mU) and the thin calcified segment rim.

temperature variations (Reynaud et al., 2003; Hoegh-Guldberg et al., 2007; Manzello, 2010; Sinutok et al., 2011; McCulloch et al., 2012). The seawater temperature at our sampling sites ranges from 26.5°C to 32.0°C throughput the year (unpublished data). In the 24°C treatment, which was below the average temperature at the sampling sites, the concentrations of proline and MDA in the *Halimeda* tissues declined slowly after 8 d, indicating that acclimation was taking place. Campbell et al. (2016) has reported that, in experiments which couple ocean acidification and waring, moderate increases in temperature could improve metabolic performances (e.g., photosynthesis and calcification) and alleviate the detrimental effects of shifts in seawater chemistry. These findings are strongly relevant to coral reef ecosystems that are facing climate changes caused by human activities, as growth and distribution of common calcareous algae may significantly affect coral survival and growth (Teichberg et al., 2012). The wide distribution of calcifying organisms, including *Halimeda* species, is likely not only due to seawater temperatures, but also light intensities, nutrient availability and so on (Kleypas et al., 2006; Hurd et al., 2009; Hofmann et al., 2014). Furthermore, most previous work has focused largely on Pacific species, which may be acclimated to cooler seawaters than their Caribbean counterparts (Campbell et al., 2016). As a result, conclusions regarding the physiological effects of temperature may strongly depend on organismal acclimation and regional environmental factors (Campbell et al., 2016).

In conclusion, this study demonstrated that from 24°C to 32°C, elevated temperature enhanced growth and while extreme high temperatures (34–36°C) damaged *Halimeda* tissues. Baseline measurements of SGR and calcification rate in 34°C treatments, only *H. cylindracea* and *H. opuntia* could grow even if these physiological performances were slowly, which displayed broader tolerance to a range of temperatures than *H. lacunalis*. However, upper lethal temperature thresholds for the three *Halimeda* species was 36°C with all thalli disarticulating and bleaching. High concentrations of proline in *Halimeda* tissues

were released to keep the physiological systems functioning under environmental stresses. As temperature changes do not occur in isolation, it will be important to conduct further experiments which combine temperature with other factors such as acidification, light and nutrient availability in order to determine the likely interactive and/or cumulative impacts these parameters have on the physiology of calcareous *Halimeda* species.

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