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Decline in growth of foraminifer *Marginopora rossi* under eutrophication and ocean acidification scenarios

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Abstract

The combination of global and local stressors is leading to a decline in coral reef health globally. In the case of eutrophication, increased concentrations of dissolved inorganic nitrogen (DIN) and phosphorus (DIP) are largely attributed to local land use changes. From the global perspective, increased atmospheric CO_2 levels are not only contributing to global warming but also ocean acidification (OA). Both eutrophication and OA have serious implications for calcium carbonate production and dissolution among calcifying organisms. In particular, benthic foraminifera precipitate the most soluble form of mineral calcium carbonate (high-Mg calcite), potentially making them more sensitive to dissolution. In this study, a manipulative orthogonal two-factor experiment was conducted to test the effects of dissolved inorganic nutrients and OA on the growth, respiration and photophysiology of the large photosymbiont-bearing benthic foraminifer, *Marginopora rossi*. This study found the growth rate of *M. rossi* was inhibited by the interaction of eutrophication and acidification. The relationship between *M. rossi* and its photosymbionts became destabilized due to the photosymbiont's release from nutrient limitation in the nitrate-enriched treatment, as shown by an increase in zooxanthellae cells per host surface area. Foraminifers from the OA treatments had an increased amount of Chl *a* per cell, suggesting a greater potential to harvest light energy, however, there was no net benefit to the foraminifer growth. Overall, this study demonstrates that the impacts of OA and eutrophication are dose dependent and interactive. This research indicates an OA threshold at pH 7.6, alone or in combination with eutrophication, will lead to a decline in *M. rossi* calcification. The decline in foraminifera calcification associated with pollution and OA will have broad ecological implications across their ubiquitous range and suggests that without mitigation it could have serious implications for the future of coral reefs.

Keywords: biomineralization, eutrophication, foraminiferal physiology, high-Mg calcite, ocean acidification

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Introduction

Human activities are causing a rapid increase in atmospheric CO_2, primarily through burning fossil fuel, cement production and land clearing (IPCC, 2007). The ocean acts as a major reservoir in the global carbon cycle by absorbing around 25% of anthropogenically released CO_2 from the atmosphere (Feely *et al*., 2001; Canadell *et al*., 2007). An increased level of CO_2 in the ocean decreases the carbonate ion concentration and lowers the pH, a process known as ocean acidification (OA, Caldeira & Wickett, 2003; Hoegh-Guldberg *et al*., 2007). A meta-analysis normalized to a 140 ppm rise in atmospheric CO_2 compared to preindustrial concentrations, showed a decline in calcification rate in the order of ca. 5–38% (Kleypas & Langdon, 2006). Specifically, marine calcifiers such as planktonic coccolithophorids (Riebesell *et al*., 2000), crustaceans (Gazeau *et al*., 2007), molluscs (Comeau *et al*., 2009), coralline algae (Anthony *et al*., 2008; Kuffner *et al*., 2008), planktonic foraminifera (Moy *et al*., 2009) and corals (Gattuso *et al*., 1998; Langdon & Atkinson, 2005), experience either reduced calcification or enhanced dissolution when exposed to lower pH in ocean waters, yet the effects are variable and life stage dependent (Kroeker *et al*., 2010).

Coral reefs are known to be one of the major producers of carbonate globally, although reef environments only occupy ~0.2% of the ocean's benthos. Benthic foraminifera are among the most abundant protists in the shallow reef marine environment (Murray, 1991), where benthic photosymbiont-bearing foraminifera species produce 80% of the global foraminifera reef carbonate (Langer *et al*., 1997). Such high carbonate production is

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possible due to the symbiosis with algae living within calcifying reef organisms. Zooxanthellae of the genus *Symbiodinium* living within the cytoplasm of many marine hosts make the high calcification rates on reefs possible and it is proposed that the zooxanthellae benefit from the respired CO_2 as well as surfeit nitrate and phosphorus from the host (Be *et al.*, 1977; Jørgensen *et al.*, 1985; Gastrich & Bartha, 1988). Studies in photophysiology propose a close coupling of photosynthesis and respiration in the microhabitat of hermatypic corals (e.g., *Favia spp.* and *Acropora spp.*), planktonic foraminifera (e.g., *Globigerinoides sacculifer* and *Orbulina universa*) and larger benthic foraminifera (*Marginopora vertebralis*, *Amphistegina lobifera* and *A. hemprichii*) (Jørgensen *et al.*, 1985; Rink *et al.*, 1998; Köhler-Rink & Kühl, 2000; Kuroyanagi *et al.*, 2009; Crawley *et al.*, 2010; Sinutok *et al.*, 2011), which suggest that metabolic processes in the host and photosymbiont algae are susceptible to changing pH levels.

Understanding the physiological response of photosymbiont-bearing organisms to eutrophication is critical, as increased host calcification rates are largely attributed to the photosymbiont translocation of photosynthate to the host (Muscatine & Lenhoff, 1983; Muscatine *et al.*, 1984). Unlike perforate species, imperforate foraminifera (such as the photosymbiont-bearing *Marginopora*, *Amphisorus*, *Sorites* and heterotrophic calcareous Miliolida) do not have a large internal carbon pool, therefore cell growth and metabolic activities are likely limited by reduced inorganic carbon diffusion directly from seawater (ter Kuile & Erez, 1987; ter Kuile *et al.*, 1989a,b). *Symbiodinium* cell growth is largely governed by nutrient limitation within the host environment, which redirects the carbohydrates away from the photosymbionts to the host (Dubinsky & Berman-Frank, 2001). Previous studies have shown increased nutrient run-off released photosymbionts from nutrient limitation resulting in reduced growth of the foraminifera test (Uthicke & Altenrath, 2010). Therefore, changes in the concentration of the major biologically limiting nutrients, such as nitrate and phosphorus, from riverine, atmospheric or sedimentary sources will likely have significant impacts on the internal mechanism regulating calcification, a process potentially further hindered by climate changes (Wooldridge & Done, 2009).

Calcification among Miliolid benthic foraminifera is promoted by elevating intracellular pH to increase carbonate conversion and to overcome Mg^{2+} inhibition (de Nooijer *et al.*, 2009); as observed in corals, this is an energetically expensive process controlled by a series of transport pumps, channels, diffusion gradients and hydrolysis (Cohen & Holcomb, 2009). The kinetic channels enabling calcification (either in the form of aragonite or calcite) may become partially inhibited when there is increased hydration energy of the calcium ion (Lippmann, 1973) or low concentration of carbonate ions (Garrels & Thompson, 1962; Lippmann, 1973). Buffering against large fluctuations in the concentration of internal CO_2 in the calcifying fluids, caused by cycles of photosynthesis and respiration, is possible via the assistance of organic molecules (Teng *et al.*, 1998) or due to the specific skeletal architecture of the species (Holcomb *et al.*, 2009). Even so, ongoing OA may inhibit high-Mg calcite production in benthic foraminifera by hindering the energetic conversion of bicarbonate into carbonate ions or restricting the extraction of Mg^{2+}.

The overall aim of this study was to gain a better understanding of the physiological response of photosymbiosis in foraminifers to future eutrophication and climate scenarios. To our knowledge, this is the first study to test for the interactive effects of increased nutrients and ocean acidification on benthic foraminifera. We studied the combined effect of increased OA (pH 8.1, 7.8, 7.6) and nutrients (+2 μM nitrate and +0.2 μM phosphorus) on the growth and physiological response of *Marginopora rossi*. Specifically, chlorophyll *a*, *Symbiodinium* abundance (cells), growth (calcification), photosynthesis and respiration rates of *M. rossi* were monitored under the experimental conditions.

Materials and Methods

Experimental approach

The imperforate photosymbiont-bearing benthic foraminifer *M. rossi* (Lee *et al.*, in press) was collected from the Heron Island channel (S23°26′99″ E151°51′95″) between 25 and 30 m. *Marginopora spp.* are known to calcify high-Mg calcite tests (>200 mmol mol^{-1}; Raja *et al.*, 2005). The geographical distribution of *Marginopora spp.* populations within the central Great Barrier Reef (GBR) are documented to have high genetic similarity, for up to 100 km between reefs (Benzie & Pandolfi, 1991), suggesting long distance dispersal with some latitudinal differentiation between the northern and southern regions within the Coral Sea (Benzie, 1991). In addition, populations of *Marginopora spp.* are widely distributed among seagrass beds and in the epiphytic zones of the central Pacific (Smith, 1968; Severin, 1987; Langer & Hottinger, 2000), with all species housing *Symbiodinium* dinoflagellate photosymbionts within their endoplasmic tissue (Langer & Lipps, 1995; Lee *et al.*, 1995). Unlike perforate foraminiferal species, imperforate species rely on the uptake of carbon directly from the seawater for calcification (ter Kuile & Erez, 1987).

A total of 180 living specimens of *M. rossi* were used to test the effects of OA and increased dissolved inorganic nutrient concentrations in a fully orthogonal, two-factor closed system experiment, with four replicated 'tanks' as a nested factor and five *M. rossi* per tank (from previous trials, this was found to be the optimal replication for survival, statistical power and maintenance). For 35 days (during July–August 2009),

36 × 500 ml cylindrical experimental tanks were set up in the Coastal Plants Laboratory at The University of Queensland, Australia. Light conditions from the site of collection (midday values of 45–50 μmol quanta m^{-2} s^{-1}) were replicated in the laboratory with 150 W halogen lamps and 18 W 'cool white' fluorescent lamps on a 12:12 hour diel cycle. Water temperature was maintained at 25 °C using a recirculating heater–chiller (TECO RA240, TECO Electric and Machinery Co. Ltd., Taipei, Taiwan). Filtered natural seawater from CSIRO marine laboratories, Cleveland, Australia, was used in the laboratory experiment. The total nitrogen and phosphorus levels measured in the CSIRO seawater (total nitrogen 7.5–12.5 μmol L^{-1} and total phosphorus 0.18–0.38 μmol L^{-1}) were similar to those measured on the Great Barrier Reef (total nitrogen 4.8 – 12 μmol L^{-1} with an average of 7.2 μmol L^{-1} and total phosphorus 0.11–0.84 μmol L^{-1} with an average of 0.34 μmol L^{-1}; De'ath & Fabricius, 2008). No additional food sources were added to the seawater. The pH treatments were based on a control (pH 8.1), an A1B scenario (IPCC, 2007; pH 7.8) and an A1FI scenario (IPCC, 2007; pH 7.6) with pH units in the National Bureau of Standards (NBS) scale. Hereafter, A1B and A1FI refer only to pH predictions and not added temperature changes. The carbonate system was manually adjusted by bubbling CO_2 until it stabilized at the required pH level for the treatment that was measured with a portable pH meter (SevenGo portable pH meter, Mettler Toledo, Switzerland). New treatment seawater was replaced in the closed system tanks every 2 days as needed, with the tank pH monitored daily to an accuracy of 0.01. New treatment water was adjusted to 0.2 μM potassium dihydrogen phosphate (KH_2PO_4), or 2 μM addition of potassium nitrate (KNO_3), or remained unchanged as a control to represent ambient nutrient concentrations. The added nutrients represent typical nutrient peaks experienced during flood plumes in the coastal reef zones of the Great Barrier Reef (Furnas *et al.*, 1995; Schaffelke *et al.*, 2003; Cooper *et al.*, 2007; Uthicke *et al.*, 2010).

Carbonate chemistry

Total Alkalinity (TA) was monitored twice a week throughout the duration of the experiment by filtering seawater samples with a 0.45 μM syringe into a dark glass vial followed by immediate refrigeration at 0 °C. For each sample, triplicate (~25.0 g) aliquots of seawater were weighed and titrated with 0.1 M HCl to determine the average total alkalinity (±2 uEq kg^{-1}) with a Mettler Toledo T50 automated titrator, with a 1 mL burette, small volume electrode and stirrer. The seawater samples were analysed using the Gran titration method in a two-stage, potentiometric open-cell titration following the method of Dickson *et al.* (2003). Acid concentrations and the alkalinity measurements were calibrated at the beginning of each run on a rotation of every 10 samples using Dickson certified reference seawater standards (Andrew Dickson, SIO, Oceanic Carbon Dioxide Quality Control).

Seawater samples for dissolved inorganic carbon (DIC) analysis were collected twice weekly from each treatment and filtered with a 0.45 μM syringe into glass vials and fixed with 15 μL saturated mercuric chloride. The samples were then opened and delivered with a Kloehn syringe pump (Kloehn Inc., Las Vegas, NV, USA) to a sparging chamber, where the seawater was acidified with 5% phosphoric acid solution. DIC was measured colorimetrically in duplicate with a LI-7000 CO_2/H_2O infrared analyser (LI-COR Biosciences, Lincoln, NE, USA) coupled to a custom-built sample delivery system (Rob Dunbar Laboratory, Stanford University). The accuracy was ±2 μmol kg^{-1} seawater based on Dickson seawater reference samples that were run every seven samples. The measured DIC and Alkalinity were used to calculate the bicarbonate (HCO_3^-), carbonic acid (H_2CO_3) and carbonate ion concentrations (CO_3^-), calcite saturation state ($\Omega_{calcite}$) and pCO_2 levels using the CO2SYS program (Lewis & Wallace, 1998). The Mehrbach *et al.* (1973) dissociation constant was used and adjusted according to temperature (25 °C) and salinity (35) measurements (Dickson & Millero, 1987).

Productivity, O_2 evolution and consumption

At the end of the experiment, a subset of individuals from each treatment (n = 4) was used to conduct the respirometry measurements. All individuals were dark-adapted in their treatment for 30 min prior to commencement of the respirometry assays. Each individual foraminifer was placed in a clear acrylic chamber filled with filtered seawater and fitted with an oxygen optode connected to an optical analyser (Oxy4 v2, PreSens, Regensburg, Germany). A micro stirring bar was placed in each chamber and the chambers were each placed over a magnetic stirrer to ensure dissolved oxygen homogeneity around the foraminifer and optode. The chambers were incubated in a water bath to maintain constant temperature at 25 °C. Dark respiration (R_{dark}) was measured during the first 10 min at 0 μmol quanta m^{-2} s^{-1}. Net photosynthesis (P_{max}) was determined by the greatest rate of oxygen evolution during the exposure of 50 μmol quanta m^{-2} s^{-1} after 20 min. Light-enhanced dark respiration (LEDR) was determined using the oxygen consumption that resumed <1 min post illumination for 10 min. Oxygen measurements were recorded every 10 s. Dark respiration, P_{max} and LEDR were normalized to Chl *a* (moles O_2 mmol Chl a^{-1} h^{-1}). P : R ratios were calculated as (P_{max} + LEDR): (LEDR + R_{dark}) as per Burris (1977). Photosynthetic capacity (P_{gross} = P_{max} + LEDR) was calculated from the oxygen consumption slope and normalized to the foraminifer biomass (g) calculated from the buoyant weight (Davies, 1989) of the foraminifera to give P_{gross} (μmol O_2 g^{-1} h^{-1}). After completing the respirometry assays, the foraminifer were snap frozen in liquid nitrogen and stored at −80 °C until pigment quantification and cell counts were conducted.

Surface area measurements

Each foraminifer was photographed at the beginning and at the end of the experiment (Figure S1). Mortality was low (<1.2%) and was not confined to a particular treatment; foraminifer that died were excluded from analyses. Foraminifer were assumed dead if there was a loss in symbiont colour and when no visible sign of a reticulopodial network was observed, which is the mortality determination method recommended for

experimental work as other methods used to determine mortality are terminal (Bernhard, 2000). The surface area of the foraminifer was measured from the digital images using Image-Pro 5 (Media Cybernetics Inc., Rockville, MD, USA). Following methods of ter Kuile & Erez (1984), the relative growth rate per day was calculated by the following equation:

$$\lambda = \ln\left(\frac{S_f}{S_i}\right) \times \frac{1}{t}$$

$$R = 100 \times \lambda$$

where λ is the growth constant, S_f is the final surface area, S_i is the initial surface area, t is the experimental duration and R is the growth rate in % per day. For the growth calculation, it was assumed that the increment of growth was constant each day.

Chlorophyll concentration and cell count

The foraminifera used in the respirometry measurements from each treatment were decalcified in a 0.5 M ethylenediaminetetraacetic acid (EDTA) solution for 1 week. After the skeleton had dissolved, the samples were placed in a vial with 2–3 mL of fresh seawater and homogenized using a Tissue Tearor™ (Biospec Products, Inc., Bartlesville, OK, USA). Two aliquots were made, one for chlorophyll content and the other for cell density. Aliquots for the chlorophyll analysis were initially spun down to remove the seawater and filled with 1 mL of 100% acetone, half of which was diluted in 5 mL of acetone to be analysed using a UV-visible Cintra 10 spectrometer (GBC Scientific Equipment Pty Ltd., Braeside, Victoria, Australia) with the associated software to obtain the absorbance at 663 and 630 nm. These readings were used in the spectrophotometric equation outlined in Jeffrey & Humphrey (1975), for the determination of chlorophyll *a* (Chl *a*). Algal cells were counted in triplicate using a 0.100 mm Tiefe Depth Profondeur hemocytometer (0.0025 mm^2). Each sample was homogenized before one drop was added to the surface of the hemocytometer. The Chl *a* content and algal cell counts were normalized to the foraminifer surface area previously measured from photographs.

Statistical analysis

A nested two-factor ANOVA was conducted with an orthogonal type III sum of squares using the $\log_{10}$ transformed mean growth rates to test the effects of ocean acidification and enhanced dissolved inorganic nutrients. Respirometry assays, algal cell and pigment counts from the subsamples were analysed in a two-way factorial ANOVA with an orthogonal type III sum of squares using the independent values. The assumptions of homogeneity were investigated using Levene's test and a probability plot of the residuals. Where the ANOVA determined significant differences, the *post hoc* Student Newman–Keuls (SNK) test was applied to attribute differences between treatments. All statistical tests were performed using a general linear model procedure in STATISTICA 9 (StatSoft Inc., Tulsa, OK, USA).

Results

Carbonate chemistry

DIC incrementally increased in the CO_2-enriched treatments (Table S1). Saturation state with respect to calcite in the pH 7.6 seawater was calculated to be in the range of 1.98–2.42, which is ~50% lower than the calcite saturation in the pH 8.1 seawater, which was within the range of 3.74–5.20. The HCO_3^- concentration increased from ~1483 to 1967 μmol kg^{-1} as pH fell from 8.1 to 7.6 along with CO_2 concentrations which increased from ~7 to 33 μmol kg^{-1}. On the other hand, the average CO_3^- concentration (expressed as μmol kg^{-1}) range was 156–236 in pH 8.1, 96–195 in pH 7.8 and 73–115 in pH 7.6 (Table S1). Total alkalinity remained within the range of 2036–2127 μmol kg^{-1} among all treatments. The resolution of total alkalinity is greater than the variation predicted by the addition of 0.2 μM phosphate, so no corrections were made for the increase in phosphate ions (Dickson, 1981).

Photosynthetic and respiratory rates

Among the treatments, no significant differences were observed in the ratio of photosynthesis to respiration, the average P : R per treatment ranged between 0.87 and 1.33. Similarly, there was no significant difference in the percentage of respiration when calculated as a percentage of gross photosynthesis, the average of each treatment ranged between 43% and 51% (Fig. 1; *post hoc* supporting information Table S2). There is a clear reduction of P_{gross} as a response of lowering pH, however, there was no effect of nutrients (Fig. 2a; Table S3; $F_{4,27} = 15.05$, $P < 0.001$; *post hoc* supporting information Table S4). There was a significant interaction between the pH and nutrient treatments for net P_{max} per algal cell (Table S3; $F_{4,27} = 3.41$, $P = 0.02$), where net P_{max} per algal cell was significantly greater in the A1B non–nutrient-enriched treatment compared to the A1B nitrate-enriched treatment (*post hoc* supporting information Table S5). However, net P_{max} per Chl *a* declined with lower pH (Fig. 2b; Table S3; $F_{2,27} = 9.37$, $P = 0.001$). Net P_{max} per Chl *a* was not significantly different between the A1B and A1FI pH treatments, but both were significantly lower than the control pH treatment (*post hoc* supporting information Table S6). LEDR per Chl *a* significantly differs among changing pH and nutrients treatments (Fig. 2c; Table S3; $F_{3,27} = 12.95$, $P < 0.001$; *post hoc* supporting information Table S7). There was a significant crossed difference in R_{dark} per Chl *a* (Fig. 2d; Table S3; $F_{4,27} = 3.63$, $P = 0.02$; *post hoc* supporting information Table S8).

Growth rates under the influence of pH and nutrients

A key result of our study is a significant interaction in foraminifer growth due to the cross effect of enriched nutrient and pH treatments (Fig. 3a; Tables 1 and 2). Foraminifer grown in the nitrate-enriched treatments had lower growth rates than those in the nitrate depleted seawater, except under the A1FI scenario. Similar trends were observed among the various pH scenarios under the ambient-, nitrate- and phosphate-treated seawater. The A1FI growth rate per day was significantly less across all nutrient treatments. Growth rate was greatest at ambient pH with ambient- or phosphate-treated seawater, $0.74 \pm 0.01\%$ day^{-1} and $0.75 \pm 0.04\%$ day^{-1} respectively. This was significantly higher (23%) than in the nitrate-enriched treatment as shown by the *post hoc* pairwise comparisons (*post hoc*, supporting information Table S7; $P < 0.05$). At 2 μM nitrate, lower pH reduced growth by 14% (A1B scenario) and 38% (A1FI scenario) compared to the ambient pH–nutrient treatment (Table 1; *post hoc* supporting information Table S9).

Algal cell counts and photosynthetic pigment

Algal cell counts per surface area were significantly different between nutrient treatments (Table S3; $F_{2,27} = 4.05$, $P = 0.03$), with a significantly greater number of cells per surface area in the nitrate-enriched seawater compared to the ambient and phosphate-enriched seawater, except in the A1FI pH treatment (Fig. 3b). Among the nitrate-enriched treatments, there was a drop in the number of cells in the low pH A1FI treatment. No difference was observed between ambient and phosphate-enriched seawater across the different levels of pH. The average algal cell count per surface area among the nutrient treatments was 0.83 ± 0.09 at the ambient nutrient levels, 0.80 ± 0.04 with 0.2 μM phosphate, and 1.03 ± 0.06 with 2 μM nitrate (expressed as algal cells x 10^4 mm^2 ± SE). As a general trend, Chl *a* per algal cell increased with decreasing pH with the exception of nitrate crossed with the A1B pH treatment. Between the non–nutrient-enriched and phosphate-enriched treatments, algal cells per surface area remained constant regardless of the pH, yet both these nutrient treatments increased Chl *a* per algal cell as pH decreased. Whereas, a different trend was observed in the nitrate treatment, in this case cell per surface area decreased with lowering pH, while Chl *a* per algal cell initially dropped and then increased with lowering pH (Fig. 3c; Table S3; $F_{4,27} = 3.85$, $P = 0.01$; *post hoc* supporting information Table S10).

Discussion

This study has shown that the cumulative impacts of OA and eutrophication are dose dependent and interactive. Nitrate additions reduced rates of calcification under control levels of acidification, but had a slight mitigating effect on calcification rates under high levels of acidification. These findings actually challenge the notion that reducing eutrophication will improve photosymbiont-bearing foraminifer resilience to OA as an overall decline in growth is observed under the A1FI pH scenario regardless of nutrient treatment. Overall, the growth rate was significantly lower in the A1FI pH scenario compared to the control and the A1B pH scenario, suggesting inhibition of calcite precipitation at the site of calcification.

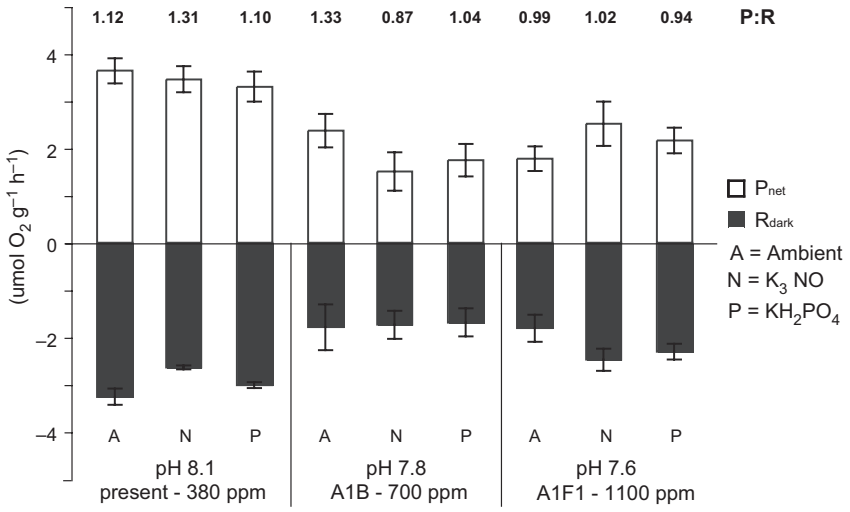

Fig. 1 Net photosynthesis and dark respiration of the mean rates ± standard error for each of the treatments. Average P_{net} : R_{dark} for each treatment is in bold.

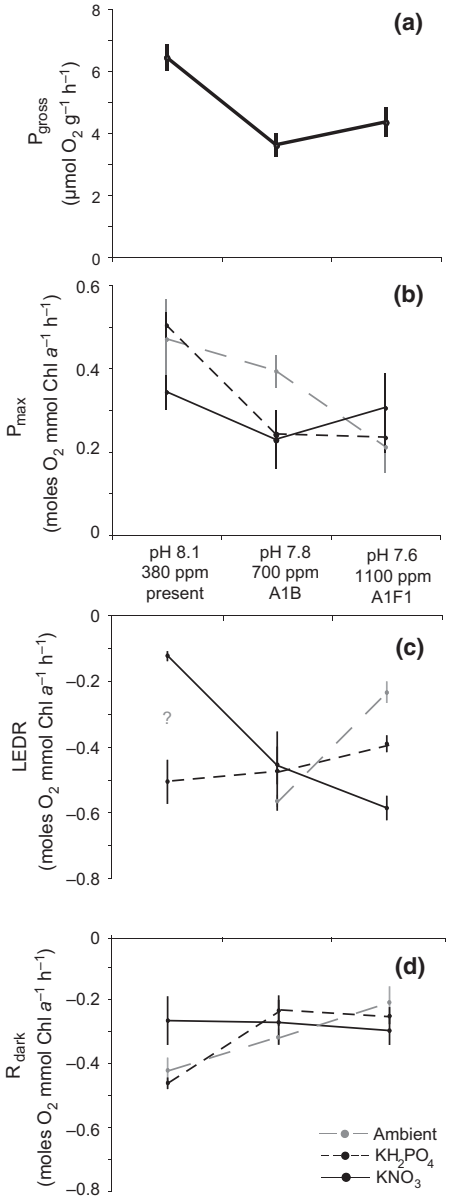

Fig. 2 Summary of the photosynthetic and respiratory response of *Marginopora rossi* to the crossed effects of nutrients (ambient = A; KNO_3 = N; or KH_2PO_4 = P), and pH (present levels; IPCC 2100 scenario A1B; and A1FI) (Solomon, 2007). Panel (a) P_{gross} μmol O_2 g^{-1} h^{-1} (b) P_{max} per Chl *a*, (c) light-enhanced dark respiration (LEDR) per Chl *a*, and (d) dark respiration per Chl *a*.

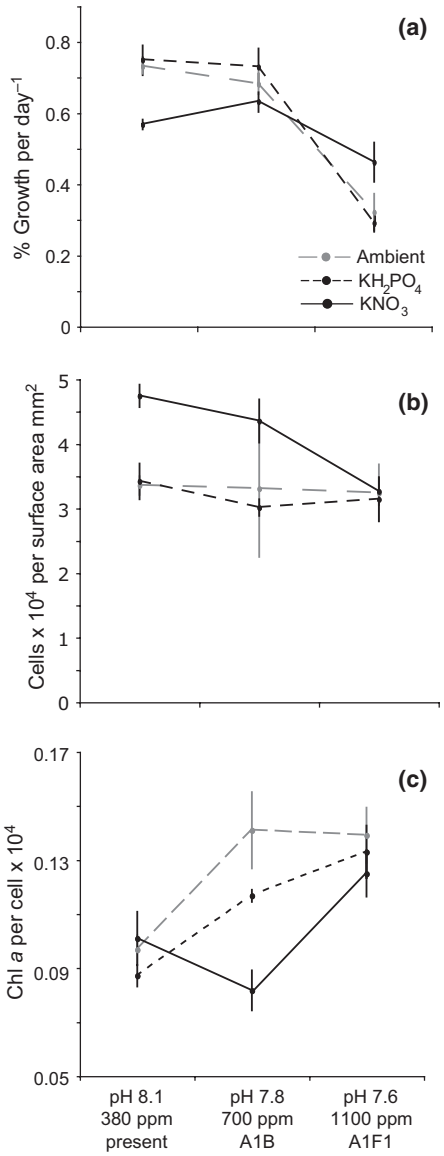

Fig. 3 Calcification, cell counts and Chl *a* interactions plots demonstrating the differences between the experimental treatments. Nutrient concentrations either were unaltered controls, enriched with 0.2 μM KH_2PO_4 or 2 μM KNO_3, while pH was maintained either at present levels (pH 8.1), IPCC 2100 scenario A1B (pH 7.8) or A1FI (pH 7.6). Panel (a) is the percentage growth rate per day of *Marginopora rossi*, (b) the number of cells × 10^4 per surface area mm^2, and (c) the pigment analysis comparing Chl *a* per *symbiodinium* cell × 10^4.

These results contrast with those obtained by Vogel & Uthicke (2012), where it was reported that acidification at 1925 ppm pCO_2 had no effect on the calcification rate of *M. vertebralis*. However, Kuroyanagi *et al.* (2009), Fujita *et al.* (2011) and Hikami *et al.* (2011) found reduced weight and diameter with lower pH among cultured photosymbiont-bearing imperforate Miliolida. In this context, reduced calcium carbonate production is likely a combination of dissolution of previously deposited calcium carbonate (high-Mg calcite showing the greatest solubility sensitivity compared to low-Mg calcite and aragonite e.g., Brown & Elderfields, 1996), OA driven cellular acidosis along with reduced proton export at the site of new calcification on the peripheral margin of the foraminifera (e.g., Glas *et al.*, 2012).

Table 1 Growth rates of *Marginopora rossi* from the two-factor laboratory experiment (ambient = A; KH_2PO_4 = P; KNO_3 = N). Mortality was low (<1.2%) and was not confined to a particular treatment; foraminifer that died were excluded from analyses. The experiment was run for 35 days. Values are an average ± standard error of the beginning surface area, ending surface area and the % growth per day calculated following the methods of ter Kuile & Erez (1984)

Treatment	Beginning surface area (mm)	Final surface area (mm)	% growth day^{-1}
A – pH 8.0–8.1	11.49 ± 0.98	14.88 ± 1.27	0.74 ± 0.01
A – pH 7.8–7.9	11.00 ± 0.89	14.06 ± 1.18	0.69 ± 0.03
A – pH 7.6–7.7	15.19 ± 1.36	16.87 ± 1.40	0.32 ± 0.04
P – pH 8.0–8.1	10.89 ± 1.36	14.00 ± 1.08	0.75 ± 0.04
P – pH 7.8–7.9	10.42 ± 0.78	14.29 ± 0.99	0.73 ± 0.05
P – pH 7.6–7.7	15. 05 ± 1.03	16.58 ± 1.07	0.29 ± 0.03
N – pH 8.0–8.1	13.28 ± 1.36	16.22 ± 1.65	0.57 ± 0.01
N – pH 7.8–7.9	12.54 ± 0.92	15.62 ± 1.11	0.64 ± 0.03
N – pH 7.6–7.7	15.91 ± 0.79	18.96 ± 1.10	0.46 ± 0.06

Table 2 Statistics of *Marginopora rossi* growth rates from the two-factor laboratory experiment. The data were analysed with a nested two-factor (pH and nutrients) ANOVA, with a type III partial sum of squares. All growth rates were $\log_{10}$ transformed before analysis. Significant differences are in bold ($P < 0.05$ level)

Effect	SS	DF	MS	*F*	*P*
Nested mean effect					
pH	0.738	2	0.369	65.130	**<0.001**
Nutrient	0.001	2	0.000	0.048	0.953
Nested (pH × nutrient)	0.138	4	0.034	6.083	**0.001**
Error	0.153	27	0.006		

The calcification mode among high-Mg imperforate benthic foraminifera is achieved by biologically controlled exocytosis of seawater to the site of calcification (e.g., de Nooijer *et al.*, 2009; Fig. 4a). As previously shown by Hikami *et al.* (2011) changes in calcification among imperforate species may be due to $CaCO_3$ saturation state rather than lower pH or increased pCO_2. Precipitation of the high-Mg calcite is likely to be inhibited due to changes in HCO_3^- and H^+ concentration, which alter the intracellular chemistry needed for calcification, metabolism, protein synthesis and ion exchange (Fig. 4b). In addition, ter Kuile (1991) observed that the external sources of HCO_3^- are more important than enzymatic processes in determining foraminiferal calcification rates.

Photosymbiont-bearing benthic foraminifera occur principally in high-light and oligotrophic conditions. Similar to corals, their success is attributed to the translocation of photosynthate from the photosymbiont to the host (Muscatine & Lenhoff, 1983; Muscatine *et al.*, 1984; ter Kuile *et al.*, 1989a,b). Photosynthetically driven CO_2 reduction provides energy-rich carbohydrates used to support metabolic activities and cell growth. Previous studies, from field and comparative laboratory experiments, showed that increased exposure to nutrients derived from terrestrial runoff negatively affected the growth rate of *M. vertebralis* (Reymond *et al.*, 2011). This was explained by a destabilization between the coupling of photosynthesis and growth within the photosymbiont and the restriction of photosynthate translocation to the host (Fig. 4c–d); as originally proposed for corals (Dubinsky & Berman-Frank, 2001). This appears to be the case in this study because of the increase in algal cells per surface area, which has also been previously observed in corals (Falkowski *et al.*, 1993; Steven *et al.*, 1997). Phosphate does not have the same impact as nitrates as there was no difference in photosymbiont cell densities between the ambient and phosphate-enriched treatments, leading to the conclusion that even in peak flood conditions phosphate is not a limiting nutrient.

The efficiency of biological pumps and channels are controlled by the acquisition of essential elements through a series of depletion-diffusion gradients (Pörtner *et al.*, 2004; Thornton, 2009), and is an important process to create the necessary conditions for calcification. Under control acidification levels, the addition of nitrate leads the photosymbiont to hold onto the fixed carbon therefore reducing the energy supply to the host's glycolysis and oxidative phosphorylation, necessary for the production of ATP required to drive pumps associated with proton extrusion and DIC or Ca^{2+} acquisition at the site of calcification. In this way, both photosymbiont cell proliferation and reduced skeletal growth rates in *M. rossi*, can be explained. In particular, *Symbiodinium* play a critical role in producing these conditions by withdrawing CO_2 for photosynthesis and energy for proton and calcium pumping (Goreau *et al.*, 2004). Yet the process is dynamic and naturally fluctuates in response to day–night cycles, for example, organisms with symbiotic dinoflagellates are subject to near

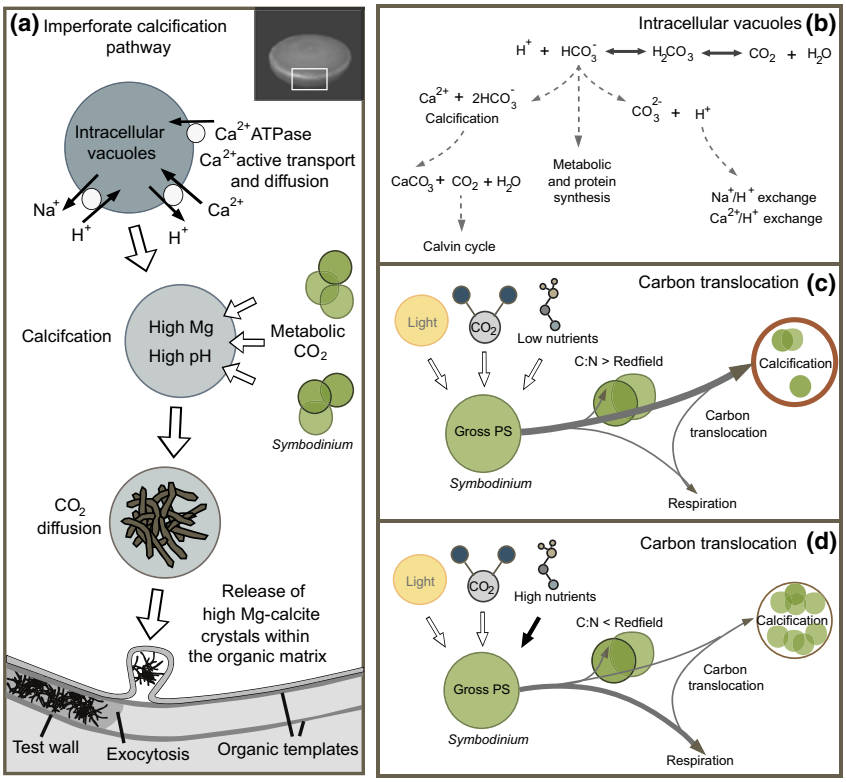

Fig. 4 Summary of calcification mechanisms in a generalized model for a dinoflagellate bearing porcelaneous foraminifera adapted from Erez (2003), Pörtner *et al.* (2004), and de Nooijer *et al.* (2009). (a) Seawater vacuoles are formed by exocytosis and move through the cell altering pH, Ca^{2+} and Mg^{2+} through various pumps and channels. During calcite precipitation pH rises and subsequently is lowered at the site of biomineralization. (b) A breakdown of the pathways utilizing the various carbonate species in the intracellular vacuoles. A schematic diagram of the relationship between the photosymbiont and the host displaying the effects of elevated CO_2 and nutrient levels on calcification and *Symbiodinium* abundance under (c) "normal" oligotrophic conditions, and (d) when exposed to eutrophication (after Berman-Frank & Dubinsky 1999, Dubinsky & Berman-Frank, 2001). The uncoupling of the production of carbohydrates and cell growth in the algae is largely governed by nutrient limitation within the photosymbiont. Coupling occurs between photosynthesis and cell growth within the algal photosymbiont as the proportion of nutrients become closer to the Redfield ratio within the photosymbiont; therefore, the carbohydrates are retained by the photosymbiont instead of being translocated to the host.

anoxic conditions at night (Kühl *et al.*, 1995) and to greater night-time reductions in intracellular pH, than aposymbiotic organisms (Venn *et al.*, 2009). As shown among planktonic foraminifera, photosynthesis by day activity increases pH and O_2 concentrations in the macro environment (Rink *et al.*, 1998), however, the potentially negative effects of symbionts by night may explain the observation that the growing margins are locally devoid of symbionts. Likewise, this may explain the positioning of symbionts in planktonic foraminifera in extruded pseudopodia as opposed to within the main cellular body of benthic foraminifera such as *M. rossi*. Potentially, being able to rearrange photosymbionts within the intracellular space over diel cycles enhance the ability of their hosts to experience rapid calcification, however, the potential for such photosymbiont movement requires further investigation.

When the pH gradient increases within the vacuoles, via photosynthesis, so does the nutrient diffusion rate, which causes a reduction in the pH gradient within the intracellular spaces and therefore lowers the diffusion rate of nitrate and phosphorus from seawater (Miller & Yellowlees, 1989; Jackson & Yellowlees, 1990). Given that CO_2 can only diffuse along a concentration gradient, it has been suggested that *Symbiodinium* possess a carbon-concentrating mechanism to provide the CO_2 concentrations required for carbon fixation (Raven, 1997; Leggat *et al.*, 2002). This mechanism for the acquisition of DIC is present in most aquatic microalgae (Colman & Rotatore, 1995; Korb *et al.*, 1997) and frequently involves the enzyme carbonic anhydrase, which greatly accelerates the conversion rate of CO_2 to HCO_3^-. However, the rise in H^+ in seawater may counter the effect of the extra DIC and hinder the efflux of H^+ between the location where calcification occurs and the calicobalstic

epithelial space (Al-Horani *et al*., 2003; Jokiel, 2011). Crossed effects of internal regulation of pH gradients, light, nutrient and temperature may complicate the response of foraminifer and their photosymbionts to changes in inorganic carbon species and could explain some of the variation in the photosynthesis and respiration rates of the photosymbionts and the reallocation of carbon to the host.

Respiration is a complex and essential metabolic process needed for the growth and maintenance of photosymbiotic cells (reviewed in Amthor, 2000). While oxygen consumption is widely known to be a determining factor regulating metabolic rate, body size and ventilatory efficiency among marine organisms (Pörtner, 2002), foraminifera have a lower respiration rate compared to other microbenthic groups (Geslin *et al*., 2011). In addition, temperature, light and pH are believed to explain ~60% of the net oxygen production variance among *M. vertebralis* (Uthicke & Fabricius, 2012). In this study, a typically lower R_{dark} in comparison to LEDR was observed, as has been observed in other photosymbiont-bearing organisms (Crawley *et al*., 2010). The individuals maintained in lower pH conditions increased Chl *a* density per algal cell in comparison to the controls, but there was no change in photosymbiont cell density. The increase in Chl *a* per algal cell under decreased pH suggests that the symbionts were likely carbon limited (Fitt *et al*., 2000). This is further supported by the increased LEDR rate maintained in low pH seawater and the failure to observe an increase in the rate of dark acclimated respiration (R_{dark}). With corals, it has been demonstrated that slowly growing cells will respire less than rapidly growing ones (Falkowski *et al*., 1985), although respiration may increase under stressful conditions leading to utilization of up to 60% of the gross photosynthate in the process (Geider, 1992). Compared to the controls, both OA treatments showed a reduced efficiency of net O_2 evolution as shown by the increased symbiont Chl *a* concentrations and P_{max} per Chl *a* decrease. Therefore, the investment in Chl *a* is not an optimal acclimation method given that the symbionts could modulate light levels by moving back and forth in the host cell (Leutenegger, 1977; personal observations).

The P : R ratio is a well-established physiological measurement used to assess the proportion of consumption and production of marine microalgae (Burris, 1977). This ratio has been previously measured for several species of unicellular marine algae and ranged between ca. 1.5 and 18 (Humphrey, 1975), and among 70 species of photosymbiotic corals, with an average P : R ratio of ~2.4 (Battey, 1992). The lack of changes in the P : R ratio in this study indicates that there is a limiting effect of changing water quality in the downstream activation of carbon dioxide fixation during photosynthesis and oxygenation during photorespiration. A decline in LEDR was observed under the non–nutrient-enriched A1FI treatment (cf to A1B), suggesting a decline in photorespiration (Burris, 1977) and possibly mitochondrial respiration, which is consistent with previous work on *Symbiodinium* in hospite within a reef-building coral (Crawley *et al*., 2010). As the average P : R ratio was close to or >1 among *M. rossi*, we can assume that the energy produced via photosynthesis was consumed within a 24-h period, which shows no preference to store excess energy. Photosymbiont-bearing foraminifer maintained their potential to survive autotrophically without the need to feed heterotrophically regardless of the water quality treatment. The fact that respiration did not exceed the photosynthetic output suggests that there was no additional maintenance or repair respiration required due to the treatments, however, overall P_{gross} (μmol O_2 g^{-1} h^{-1}) was reduced in declining pH conditions.

Overall, the growth rate of the photosymbiont-bearing foraminifer, *M. rossi* is highly sensitive to ocean acidification. Declining pH led to decline in P_{gross} and foraminifer growth rates despite increased Chl *a* per algal cell, which should have lead to an ability to harvest more light. Algal cells are more abundant per surface area in the nitrate-enriched treatments, suggesting release from nitrogen limitation, however, cell density fell with declining pH, which implies that pH has a more potent effect. Overall, there appears to be a threshold as the growth decline in the lower pH treatments became evident only in the A1F1 scenario. This indicates the potential for *M. rossi* to maintain a level of tolerance to some variation in pH and nutrients. These results have boarder implications for foraminiferal community structure and sediment production within coral reef systems, as previously observed among eutrophic gradients (Hallock *et al*., 2003; Schueth & Frank, 2008; Uthicke & Nobes, 2008; Uthicke *et al*., 2010). However, the potential of acclimation to changing conditions needs further investigation to evaluate the energetic cost of compensation mechanisms among imperforate species. We recommend that future experimental studies should consider manipulating a broader gradient of phosphate and nitrate concentrations crossed with pCO_2 treatments. Measuring buoyant weight instead of surface area will also give a greater accuracy of the dissolution and total calcification rate, in addition to cross section SEM or μCT scans.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Before and after images of the same foraminifera from each treatment from the eutrophication and ocean acidification experiment.
Table S1. Carbonate chemistry parameters of the experimental nutrient and pH treatments.
Table S2. Raw data of the P_{max}, R_{dark} and P : R from Fig. 3.
Table S3. ANOVA results for the photophysiological measurements of *Marginopora rossi* from the two factor (pH and nutrients) laboratory-controlled growth experiments.
Table S4. Newman–Keuls *post hoc* test of the pairwise comparisons of P_{gross} μmol^{-1} O_2 g^{-3} h^{-1} (MSE = 1.7133, DF = 27).
Table S5. Newman–Keuls *post hoc* test of the pairwise comparisons of P_{max} per cell (MSE = 1.505, DF = 27).
Table S6. Newman–Keuls *post hoc* test of the pairwise comparisons of P_{max} per Chl *a* (MSE = 0.013, DF = 27).
Table S7. Newman–Keuls *post hoc* test of the pairwise comparisons of LEDR per Chl *a* (MSE = 0.013, DF = 24).
Table S8. Newman–Keuls *post hoc* test of the pairwise comparisons of R_{dark} per Chl *a* (MSE = 0.295, DF = 27).
Table S9. Newman–Keuls *post hoc* test of the pairwise comparisons of the growth rate of *Marginopora* (MSE = 0.006, DF = 27).
Table S10. Newman–Keuls *post hoc* test of the pairwise comparisons of Chl *a* per cell (MSE = 0.00030, DF = 27).

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