Marine Pollution Bulletin 118 (2017) 180–187

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Phosphate deficiency promotes coral bleaching and is reflected by the ultrastructure of symbiotic dinoflagellates

Sabrina Rosset [a], Jörg Wiedenmann [a,b], Adam J. Reed [a], Cecilia D'Angelo [a,b,*]

[a] University of Southampton, Ocean and Earth Sciences, Southampton, UK
[b] University of Southampton, Institute for Life Sciences, Southampton, UK

ARTICLE INFO

Article history:
Received 1 August 2016
Received in revised form 12 February 2017
Accepted 15 February 2017
Available online 24 February 2017

Keywords:
Coral bleaching
Dinoflagellates
Eutrophication
Nutrient limitation
Nutrient enrichment
Symbiosis

ABSTRACT

Enrichment of reef environments with dissolved inorganic nutrients is considered a major threat to the survival of corals living in symbiosis with dinoflagellates (*Symbiodinium* sp.). We argue, however, that the direct negative effects on the symbiosis are not necessarily caused by the nutrient enrichment itself but by the phosphorus starvation of the algal symbionts that can be caused by skewed nitrogen (N) to phosphorus (P) ratios. We exposed corals to imbalanced N:P ratios in long-term experiments and found that the undersupply of phosphate severely disturbed the symbiosis, indicated by the loss of coral biomass, malfunctioning of algal photosynthesis and bleaching of the corals. In contrast, the corals tolerated an undersupply with nitrogen at high phosphate concentrations without negative effects on symbiont photosynthesis, suggesting a better adaptation to nitrogen limitation. Transmission electron microscopy analysis revealed that the signatures of ultrastructural biomarkers represent versatile tools for the classification of nutrient stress in symbiotic algae. Notably, high N:P ratios in the water were clearly identified by the accumulation of uric acid crystals.

1. Introduction

The success of coral reefs in oligotrophic environments is owed to the symbiotic association of the habitat-forming scleractinian corals with photosymbionts from the genus *Symbiodinium* (zooxanthellae). These algal symbionts enable the coral host to access the pool of dissolved inorganic nitrogen and phosphorus in the water column in addition to the nutrient uptake by heterotrophic feeding (Crossland and Barnes, 1977; D'Elia and Webb, 1977; Muscatine and D'Elia, 1978; Grover et al., 2003; Titlyanov et al., 2006; Downs et al., 2009; Godinot et al., 2009; Pernice et al., 2012). Moreover, the zooxanthellae recycle ammonium excreted as metabolic waste product by the host, thereby efficiently retaining nitrogen within the holobiont (Muscatine and D'Elia, 1978; Rahav et al., 1989; Wang and Douglas, 1998). The nutrient limitation experienced by the zooxanthellae *in hospite* in oligotrophic conditions results in a skewed chemical balance of the cellular nitrogen and phosphorus content relative to the available carbon. As a result, photosynthetic carbon fixation can be uncoupled from cellular growth, facilitating the translocation of a large proportion of photosynthates to the coral host (Muscatine, 1965; Muscatine et al., 1989; Falkowski et al., 1984; Dubinsky and Jokiel, 1994).

Reefs and the provision of their valuable ecosystem services are globally threatened by climate change and a range of anthropogenic pressures (Goreau and Hayes, 1994; Moberg and Folke, 1999; Sheppard, 2003; Hoegh-Guldberg et al., 2007; Hughes et al., 2007; Baker et al., 2008; van Hooidonk et al., 2013; D'Angelo and Wiedenmann, 2014; Logan et al., 2014). In this context, it has become increasingly clear that the nutrient environment plays a defining role in determining coral reef resilience (D'Angelo and Wiedenmann, 2014; Fabricius, 2005; Szmant, 2002; Brodie et al., 2012; Furnas et al., 2005; Brodie, 1995).

The ratio of dissolved inorganic nitrogen to phosphorus in the marine environment can be interpreted as an indicator of whether photosynthetic primary production is limited by the availability of nitrogen or phosphorus. In coral reef waters, N:P ratios were found in an approximate range from 4.3:1 to 7.2:1 (Smith et al., 1981; Crossland et al., 1984; Furnas et al., 1995) which is lower than the canonical Redfield ratio of 16:1, considered optimal to sustain phytoplankton growth (Redfield, 1958). Consequently, many processes in coral reefs tend to be nitrogen limited (Furnas et al., 2005).

Natural nutrient levels in coral reef ecosystems are impacted by the rising anthropogenic nutrient input into the oceans, especially into coastal waters, via the atmospheric deposition of combustion products, agricultural activities, erosion and sewage discharge (Fabricius, 2005; Brodie et al., 2012; D'Angelo and Wiedenmann, 2014). Since a number of these sources of nutrient enrichment can be influenced at the local scale (Brodie et al., 2010; Kroon et al., 2014; Aswani et al., 2015), the

* Corresponding author at: University of Southampton, Ocean and Earth Sciences, Southampton, UK.
E-mail address: cda1w07@soton.ac.uk (C. D'Angelo).

http://dx.doi.org/10.1016/j.marpolbul.2017.02.044

management of nutrification is a promising tool for coral reef protection which also holds potential to mitigate some of the negative effects of rising sea water temperatures on these ecosystems (D'Angelo and Wiedenmann, 2014).

It has been conceptualised that some direct negative effects of eutrophication on the *Symbiodinium* stress tolerance may be caused, paradoxically, by an associated deprivation of nutrients vital for the physiological functioning of the coral symbionts (Wiedenmann et al., 2013; D'Angelo and Wiedenmann, 2014). The resulting nutrient starvation can occur for example when the availability of one type of essential nutrient (e.g. phosphate) decreases relative to the cellular demand, resulting in imbalanced and unacclimated growth (Parkhill et al., 2001). High nitrate concentrations in combination with low phosphate availability have previously been shown to result in phosphate starvation of the algal symbiont and increased susceptibility of corals to heat- and light-stress-induced bleaching (Wiedenmann et al., 2013). In principle, this condition could not only result from an increased cellular demand due to nutrient (nitrogen) – accelerated cell proliferation rates but also from a selective decrease of one specific nutrient type (Parkhill et al., 2001). Relevant shifts of the nutrient balance in the natural reef environments were reported, for example, for the reefs of Discovery Bay in Jamaica where enrichment with groundwater-borne nitrate resulted in a dissolved inorganic nitrogen to phosphorus ratio of 72:1, coral decline and phase shifts to macroalgal dominance (Lapointe, 1997).

However, the functioning of the coral-*Symbiodinium* association can be severely impaired not only by the imbalanced availability of nutrients, but also by a combined deprivation of both, nitrogen and phosphorus (Rosset et al., 2015). In this light, the expected nutrient impoverishment of oceanic waters that could result from global warming or the rapid uptake of dissolved inorganic nutrients by ephemeral phytoplankton blooms could possibly act in combination with increased heat stress levels to accelerate reef decline (D'Angelo and Wiedenmann, 2014; Riegl et al., 2015).

Due to the fast uptake of dissolved inorganic nutrients by benthic communities it is often difficult to measure the level of nutrient exposure in coral reefs (Furnas et al., 2005). Consequently, biomarkers are required that inform about the nature of the nutrient stress which corals and their symbionts experience under certain conditions (Cooper and Fabricius, 2012; D'Angelo and Wiedenmann, 2014). Recently, we have demonstrated that bleaching and reduced growth of corals resulting from the deprivation of dissolved inorganic nitrogen and phosphorus is reflected by the ultrastructure of zooxanthellae (Rosset et al., 2015). The undersupply with nutrients manifests in a larger symbiont cell size, increased accumulation of lipid bodies, higher numbers of starch granules and a striking fragmentation of their accumulation bodies. We have exploited the potential of these biomarkers to detect nutrient stress imposed on the coral-*Symbiodinium* association and explored the response of the algal ultrastructure to skewed dissolved inorganic nitrogen to phosphorus ratios.

2. Materials and methods

2.1. Coral culture

We used *Symbiodinium* clade C1 associated with *Euphyllia paradivisa* as model to establish in long-term experiments the responses of the coral holobiont and zooxanthellae biomarkers to different nutrient environments. We exposed the corals to high nitrogen-low phosphorus (HN/LP) and low nitrogen–high phosphorus (LN/HP) conditions and compared them to corals experiencing nutrient replete (HN/HP) and low nutrient (LN/LP) conditions (Rosset et al., 2015). We note that the attributes "high" and "low" are introduced to facilitate comparison of the nutrient conditions in the context of our experiment and do not necessarily represent all natural reef environments.

Imbalanced nutrient conditions were established in individual aquarium systems within the experimental mesocosm of the Coral Reef Laboratory at the National Oceanography Centre Southampton (D'Angelo and Wiedenmann, 2012): high nitrogen/low phosphorus (HN/LP = ~38 μM NO_3^-/~0.18 μM PO_4^-; N:P ratio = 211:1) and low nitrogen/high phosphorus (LN/HP = ~0.06 μM NO_3^-/~3.6 μM PO_4^-; N:P ratio = 1: 60). The ammonium levels found in our mesocosm are very low (<0.7% of total dissolved inorganic nitrogen) compared to the combined nitrite (~10%) and nitrate concentrations (~90%) (Wiedenmann et al., 2013). Therefore, the measured NO_3^- concentrations (combined NO_2^-/NO_3^-) represent largely the total dissolved inorganic nitrogen pool that could be accessed by the zooxanthellae in the present experiment.

All experimental systems were supplemented with iron and other trace elements by weekly dosage of commercially available solutions (Coral Colours, Red Sea) and partial water changes with freshly made artificial seawater using the Pro-Reef salt mixture (Tropic Marin).

Both the holobiont and the zooxanthellae phenotypes were dominated by the response to the dissolved inorganic nutrient environment and largely unaffected by heterotrophic feeding by the host in our previous study (Rosset et al., 2015). However, to avoid any potential influence of nutrients in particulate form, the corals were not provided with food in the present experiments.

Colonies of *Euphyllia paradivisa* (D'Angelo and Wiedenmann, 2012) were cultured under the two imbalanced N:P ratios for >6 months at a constant temperature of 25 °C and a 10/14 h light/dark cycle. Corals in the HN/LP treatment were first maintained at lower light intensity (~80 μmol m^{-2} s^{-1}) due to the mortality risk caused by prolonged exposure to this nutrient ratio at higher light levels (Wiedenmann et al., 2013). Light intensities were gradually ramped up to ~150 μmol m^{-2} s^{-1} over 7 days and corals were kept under these conditions for 4 months prior to sampling. The corals from the LN/HP treatment experienced a photonflux of ~150 μmol m^{-2} s^{-1} throughout the experiment.

The results of the analyses were contrasted to those described in Rosset et al. (2015) where corals were cultured under comparable light and temperature conditions but at different nutrient levels (high nitrogen/high phosphorus (HN/HP = ~6.5 μM NO_3^-/~0.3 μM PO_4^-) vs low nitrogen/low phosphorus (LN/LP = ~0.7 μM NO_3^-/~0.006 μM PO_4^-).

2.2. Measurements of dissolved inorganic nutrients

Nitrate concentrations were measured by zinc reduction of nitrate to nitrite followed by a modified version of the Griess reaction as described in (Hansen and Koroleff, 1999) using commercially available reagents (Red Sea Aquatics UK Ltd), according to the manufacturer's instructions. The resultant colour change was measured using a custom programmed colorimeter at 560 nm (DR900, HACH LANGE) calibrated with nitrate standard solution in the range 0 to 20 mg l^{-1} NO_3. Phosphate concentrations were measured using the PhosVer 3 (Ascorbic Acid) method (#8048, HACH LANGE) using the same colorimeter (DR900, HACH LANGE) with the program specified by the manufacturer.

2.3. Determination of polyp size

The size of the live polyp (i.e. the part of the corallite covered by tissue) was determined by the end of the treatments. First, the corals were removed from the water to ensure full retraction of the polyp tissue. After a drip-off period of ~2 min, the mean diameters of the individual polyps were measured by averaging the longest and the shortest diameter of oval corallites (Fig. S1). In the case of round corallites, two measurements were taken along two orthogonal lines through the centre. The mean extension of the live tissue cover of the outer parts of the corallites was determined by measuring and averaging its extension at 5 measuring points spaced out evenly around the corallite. The live

polyp volume was calculated using these measurements assuming a cylindrical shape of the polyp.

2.4. Photosynthetic efficiency (Fv/Fm)

A Diving PAM (Walz) was used to determine the Photosystem II (PSII) maximum quantum efficiency (Fv/Fm) as a measure of stress experienced by the zooxanthellae. Measurements were taken under dim light exposure after 12 h of dark acclimation (Warner et al., 2010). A reduction of Fv/Fm below 0.5 was considered to be an indicator of stress as these lower values can indicate PSII damage when measured after dark recovery (Gorbunov et al., 2001).

2.5. Transmission electron microscopy

2.5.1. Sample preparation and imaging

For each experimental treatment, three tentacles of *E. paradivisa* (one per colony) were sampled from fully expanded polyps 1 h after the start of the light period. Tentacles were removed from the centre of each polyp to ensure that they were maximally exposed to light. Specimens were fixed and imaged as described in (Rosset et al., 2015). Briefly, tentacles were fixed (3% glutaraldehyde, 4% formaldehyde, 0.1 M PIPES buffer containing 14% sucrose at pH 7.2) and then cut to obtain only the central section of each tentacle, post-fixed using 1% osmium tetroxide, stained with 2% uranyl acetate and dehydrated with a graded ethanol series before being embedded in Spurr's resin. Semi-thin tentacle sections (~240 nm) were cut and stained with 1% toluidine blue and 1% borax for light microscope observations. For each specimen 3–5 thin sections (<100 nm thick) were obtained that were >20 μm apart from each other to eliminate the possibility of imaging the same algal cell twice. For each experimental treatment, at least nine sections originating from all three tentacles were produced. Sections were stained with lead citrate and imaged on a Hitatchi H7000 transmission electron microscope. For each grid square (Cu200), the 3–4 largest zooxanthellae were imaged in order to analyse only cells that were cut close to their central plane, thus being representative for the maximal cell diameter. For each tentacle, a minimum of 30 zooxanthellae cells were imaged, using 3 or more sections. A total of 100 micrographs of individual zooxanthellae (×6000 magnification) were acquired for each treatment.

2.5.2. Micrograph analysis

All micrographs were analysed using Fiji (Schindelin et al., 2012). The size of individual zooxanthellae cells was deduced from the cell section area ($n = 100$). Furthermore, the area occupied by lipid bodies, starch granules and uric acid crystals was determined for each cell and presented as a percentage of the cell section area ($n = 100$). Accumulation body integrity was measured by the degree of fragmentation by counting the number of fissures in the periphery (Rosset et al., 2015). The accumulation body was only analysed when it was clearly visible in the section. For this parameter, a mean was derived for each processed tentacle per treatment ($n = 3$). The zooxanthellae density was determined by measuring the area of the endoderm and counting the contained zooxanthellae, using semi-thin sections imaged under a light microscope at ×40 magnification ($n = 3$). While the relative differences between samples from the respective treatments are unaltered, the present method produces absolute numbers which are higher compared to published values (Rosset et al., 2015).

2.6. Statistical analysis

For the morphological parameters of zooxanthellae, statistical replication was achieved by analysing 100 distinct algal cells from three tentacles and from different areas within each tentacle ($n = 100$) (Rosset et al., 2015; Table S1). Data from nutrient replete (HN/HP) and low nutrient (LN/LP) treatments (Rosset et al., 2015) were analysed for comparison. A mean value of zooxanthellae density was obtained for each processed tentacle ($n = 3$) (Table S2). Data were tested for normality using the Shapiro-Wilk test and log transformed if found to be non-normally distributed. Statistically significant effects resulting from the difference in dissolved inorganic nutrient availability were determined by one-way analysis of variance (ANOVA) (Table S3), followed by Tukey's post hoc test for pairwise comparison (Table S4). Data that were not normally distributed after transformation were, therefore, determined by the non-parametric Kruskal-Wallis one way ANOVA on ranks. $P < 0.05$ was considered to be significant in all instances.

3. Results

3.1. Effects on the coral holobiont

Corals exposed to the imbalanced, HN/LP conditions, displayed a smaller polyp size and a bleached appearance that closely resembled the phenotype observed in low nutrient water (LN/LP) (Figs. 1, 2a). In contrast, the corals kept under LN/HP imbalanced nutrient levels showed a similar phenotype to the nutrient replete (HN/HP) treatment. The bleached appearance of the polyps from HN/LP conditions was associated with low numbers of zooxanthellae in the tentacle tissue (Figs. 1, 2b, Table 1), similar to the low nutrient LN/LP treatment. In contrast, the symbiont numbers in the tissue of LN/HP exposed corals were comparable to those of corals from nutrient replete (HN/HP) conditions (Figs. 1, 2b).

3.2. Effects on the Symbiodinium ultrastructure

The analysis of TEM micrographs revealed that the size of zooxanthellae from the imbalanced HN/LP treatment and the low nutrient condition were significantly increased compared to those from the nutrient replete and the imbalanced LN/HP treatments (Figs. 1, 3a, Table 1).

The low nutrient (LN/LP) condition and both types of nutrient imbalance increased the content of lipid bodies and starch granules in the symbiont cells (Fig. 3b,c) in comparison to corals from the HN/HP treatment. A biochemical assay using the lipophilic dye Nile Red (see supplementary material for method) confirmed that the increased cellular content of lipid bodies is due to an accumulation of neutral lipids (Fig. S2A). The lipid content remained stable over the day (Fig. S2B).

Only the imbalanced HN/LP condition resulted in a marked increase in the content of uric acid crystals (Fig. 3d, Table 1). Interestingly, none of the imbalanced nutrient treatments caused the fragmentation of the accumulation body characteristic of the low nutrient condition (Fig. 3e).

3.3. Effects on Symbiodinium photosynthetic efficiency (Fv/Fm)

Compared to nutrient replete conditions, zooxanthellae from specimens from the imbalanced HN/LP treatment showed a reduction in the maximum quantum efficiency (Fv/Fm) with values of 0.34 ± 0.05 after dark recovery being indicative of PSII damage or disturbance (Fig. 3f, Table 1). In contrast, Fv/Fm values > 0.5 were recorded for zooxanthellae of corals from the other treatments.

4. Discussion

We used ultrastructural biomarkers of zooxanthellae to gain novel insights into the response of the coral – *Symbiodinium* symbiosis to imbalanced nutrient environments and to analyse the role of nitrogen and phosphorus for the functioning of this association and potential implications for coral reef management. We used the reef coral *Euphyllia paradivisa* harbouring *Symbiodinium* sp. (clade C1) as a model system, exposed the corals to HN/LP and to LN/HP conditions and compared them to specimens from nutrient replete (HN/HP) and low nutrient (LN/LP) treatments (Rosset et al., 2015).

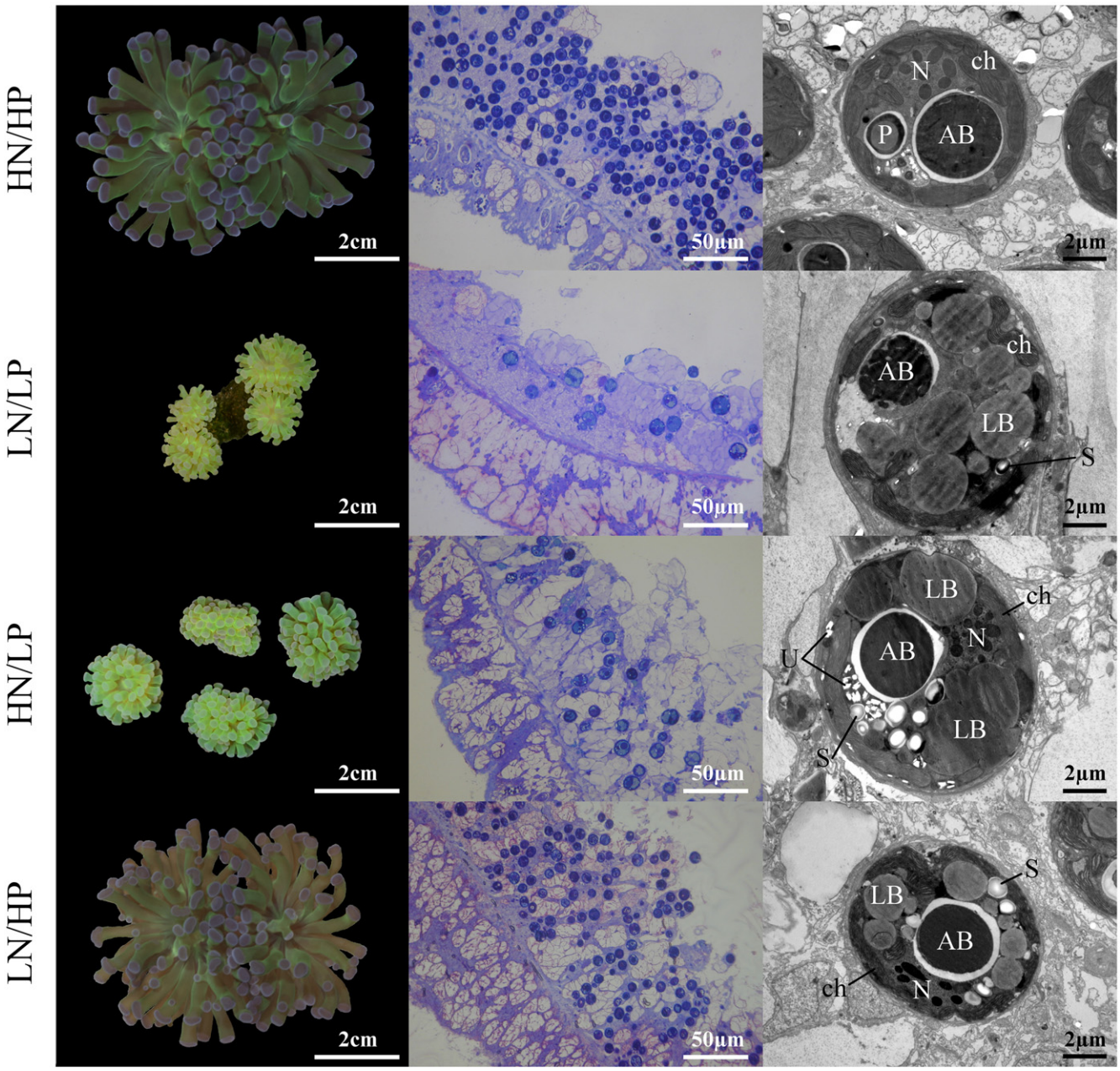

Fig. 1. Effect of dissolved inorganic nutrient availability on polyp size, and on zooxanthellae density and ultrastructure. Panels on the left hand side show representative photographs of *Euphyllia paradivisa* polyps from each experimental treatment. Panels in the central column show light microscope images of tentacle endoderm cross sections (×40 magnification). Panels on the right hand side show micrographs of individual zooxanthellae which represent a mean ultrastructure ($n = 100$) resulting from the respective treatments (×6000 magnification). HN/HP = high nitrogen/high phosphorus, LN/LP = low nitrogen/low phosphorus, HN/LP = high nitrogen/low phosphorus, LN/HP = low nitrogen/high phosphorus. AB = accumulation body, ch = chloroplast, LB = lipid body, N = nucleus with condensed chromosomes, P = pyrenoid, S = starch granule, U = uric acid crystals.

4.1. Effect of high nitrate/low phosphate conditions

Recently, we demonstrated that corals exposed to HN/LP conditions were more susceptible to bleaching when exposed to heat stress and/or elevated light levels (Wiedenmann et al., 2013). The detrimental effects were linked to the relative undersupply with phosphorus that can result from the higher demand of the proliferating algal populations rather than to the high nitrogen levels. Phosphate starvation in *Symbiodinium* sp. resulted in a drop of photosynthetic efficiency associated with changes in the ratio of phospho- and sulfo-lipids (Wiedenmann et al., 2013). In other photosynthetic organisms, similar responses to phosphate stress could be attributed to critical changes in the properties of photosynthetic membranes (Frentzen, 2004). Hence, our findings provided a potential mechanistic link between nutrient stress, the malfunctioning of the photosynthetic machinery and the observed bleaching response.

With their low zooxanthellae numbers, bleached appearance and small polyp size, the corals from the HN/LP treatment under elevated light levels resembled the low-nutrient phenotype (LN/LP) previously described (Rosset et al., 2015). These two treatments also had similar effects on the ultrastructure of zooxanthellae, with cell size and the accumulation of carbon-rich storage bodies (lipid bodies and starch granules) being increased in comparison to zooxanthellae from nutrient replete conditions. Similar structural changes were found to be indicative of nutrient limitation in zooxanthellae and free-living microalgae (Hoegh-Guldberg, 1996; Muller-Parker et al., 1996; Hu et al., 2008; Msanne et al., 2012; Weng et al., 2014; Rosset et al., 2015). Those characteristics have been interpreted as indicators of an uncoupling of

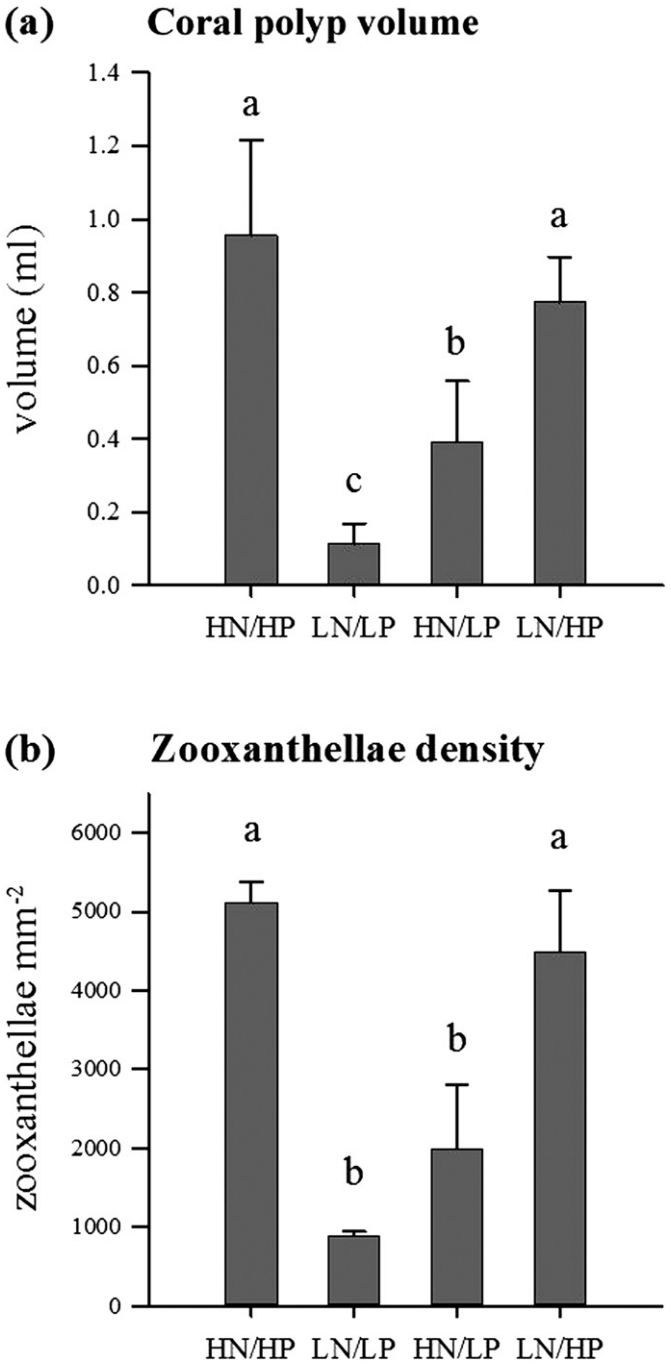

Fig. 2. Effect of dissolved inorganic nutrient availability on polyp size and on zooxanthellae density. (a) Coral polyp volume, (b) zooxanthellae density. HN/HP = high nitrogen/high phosphorus, LN/LP = low nitrogen/low phosphorus, HN/LP = high nitrogen/low phosphorus, LN/HP = low nitrogen/high phosphorus. Mean ± s.d. Statistically significant differences are indicated by the use of different letters (one-way ANOVA, Tukey's test, $P < 0.05$).

carbon fixation from cellular growth. In this state, the nutrient-limited cells sustain a high photosynthetic production while their energy demand is reduced due to slower proliferation rates (Rosset et al., 2015; Hu et al., 2008; Vítová et al., 2015; Vaulot et al., 1987).

Since corals from the HN/LP conditions were supplied with excess nitrogen, the nutrient limitation phenotype of corals and symbionts can be clearly attributed to the undersupply with phosphate. Importantly, under both, nutrient replete and low nutrient conditions, the photosynthetic efficiency measured as Fv/Fm was in the healthy range (> 0.5). In contrast, Fv/Fm was strongly reduced in the imbalanced HN/LP treatment, indicative of failing photosynthesis due to phosphate starvation (Wiedenmann et al., 2013; D'Angelo and Wiedenmann, 2014). At ultrastructural level, the phosphate starvation phenotype resulting from nitrogen enrichment in combination with low phosphate supply can be clearly distinguished from the low-nutrient phenotype by the pronounced accumulation of uric acid crystals. This finding is in line with previous studies that observed comparable deposits in zooxanthellae in response to nitrate enrichment, forming a transitory storage of assimilated nitrogen (Clode et al., 2009; Kopp et al., 2013). Finally, the phosphate-starved zooxanthellae lack the intriguing fragmentation pattern of the accumulation body, characteristic of strongly nutrient-limited zooxanthellae (Rosset et al., 2015).

4.2. Effect of low nitrate/high phosphate conditions

Despite the relative undersupply of nitrogen in the low nitrate/high phosphate treatment, the polyp size and zooxanthellae density of these corals were comparable to those from the replete nutrient treatment. However, the ultrastructural biomarkers revealed signs of nutrient limitation such as elevated levels of lipid bodies and starch granules in symbiont cells from corals under LN/HP. In the light of previous findings, the effects of the low supply with nitrogen could be interpreted to cause an uncoupling of carbon-fixation and cellular growth that manifests in the increased accumulation of carbon-rich storage products. However, as indicated by the smaller cell size and the high number of symbiont cells within the coral tissue, comparable to those from corals experiencing high nutrient levels (Rosset et al., 2015; Hu et al., 2008; Vítová et al., 2015; Vaulot et al., 1987), cell proliferation rates are still high enough to sustain these zooxanthellae densities. These results, together with the high Fv/Fm values of zooxanthellae from LN/HP corals suggest that the N-limitation sustains a slower but chemically balanced growth while maintaining a functional photosynthesis.

4.3. Differential effects of N and P undersupply and critical thresholds

Our results suggest that symbiotic corals can tolerate an undersupply with nitrogen much better than an undersupply with phosphorus. These findings likely reflect an adaptation of the algal symbionts to the nutrient environment of coral reefs where processes are mostly nitrogen limited (Crossland et al., 1984; Furnas et al., 1995; Smith et al., 1981; Furnas et al., 2005). In agreement with this assumption, previous studies found a trend that nitrogen enrichment stimulates zooxanthellae growth and results in higher zooxanthellae densities, often without obvious negative effects on the corals (Fabricius, 2005).

It cannot be ruled out, however, that nitrogen-fixation by coral-associated microbes in the presence of high phosphate concentration might potentially relieve some of the nitrogen-undersupply of the corals (Rädecker et al., 2015).

The present study clearly shows that phosphate deficiency, alone or in combination with a low supply of nitrate, results in a severe disturbance of the symbiotic partnership as indicated by the loss of coral tissue and zooxanthellae. Phosphate starvation of zooxanthellae induced by nitrogen enrichment and resulting high N:P ratios has previously been shown to disturb the photosynthetic capacity of zooxanthellae and increase the vulnerability of corals to light- and heat stress-mediated bleaching (Wiedenmann et al., 2013). The fact that normal photosynthetic efficiency is retained by zooxanthellae in corals from the LN/LP treatment suggests that an undersupply with phosphate has less severe consequences when the algae become limited by nitrogen. This can be explained by the reduced P-demand of the non-/slow-growing algal population (D'Angelo and Wiedenmann, 2014).

The concentrations of dissolved inorganic nutrients in our LN/LP treatment (~0.7 μM/~0.006 μM) suggest that at measured nitrate concentrations <0.7 μM the impact of skewed N:P ratio becomes less pronounced. In our experiments, a phosphate concentration of ~0.3 μM at a N:P ratio of 22:1 yielded an overall healthy phenotype. Accordingly,

Table 1
Symbiodinium biomarker patterns characteristic for different nutrient environments.

		Nutrient condition			
		Nutrient replete HN/HP	Low nutrients LN/LP	Imbalanced HN/LP	Imbalanced LN/HP
Zooxanthellae ultrastructural biomarkers	Zooxanthellae nutrient status	Nutrient replete growth	N/P co-limitation	P-starved	N-limited
	Zooxanthellae density	Normal	Low	Low	Normal
	Polyp size	Normal	Small	Small	Normal
	Coral health	Normal	Bleached	Bleached	Normal
	Zooxanthellae health (Fv/Fm)	Normal > 0.5	Normal > 0.5	Stressed < 0.5	Normal > 0.5
	Cell size	Small	Increased	Increased	Small
	Lipid body content	Low	Increased	Increased	Increased
	Starch granule content	Low	Increased	Increased	Increased
	Uric acid crystal content	n.d.	n.d.	Increased	n.d.
	Accumulation body fragmentation	n.d.	Increased	n.d.	n.d.

it is likely that the absolute N:P ratio becomes also less critical for the proper functioning of the symbionts when phosphate concentrations exceed a vital supply threshold (>0.3 μM), even when the symbionts are rapidly proliferating.

In contrast, a phosphate concentration of ~0.18 μM at a ~10-fold higher N:P ratio (211:1) yielded a bleached phenotype with the remaining symbionts showing signs of stress (Fv/Fm < 0.4). Therefore, the P-threshold at which corals can become stressed in the presence of high N concentrations can be as high as 0.18 μM. Effects of P deficiency can be expected to become worse if supply from other sources such as particulate food or internal reserves, is low.

4.4. Implications for environmental monitoring and coral reef management

Our study suggests that phosphate can become critically limiting even at concentrations ≤0.18 μM if the N:P ratios well exceed 22:1. This appears surprising since phosphate concentrations in this range are commonly considered ambient or high in natural reef environments. However, Lapointe (1997) reports phosphate concentrations of 0.1–0.18 μM at N:P ratios in the range of 33–72 to be associated with phosphate limitation of macroalgae in the declining reefs of Discovery Bay (Jamaica). These data suggest that the critical threshold values determined by our laboratory study can indeed be found in reef

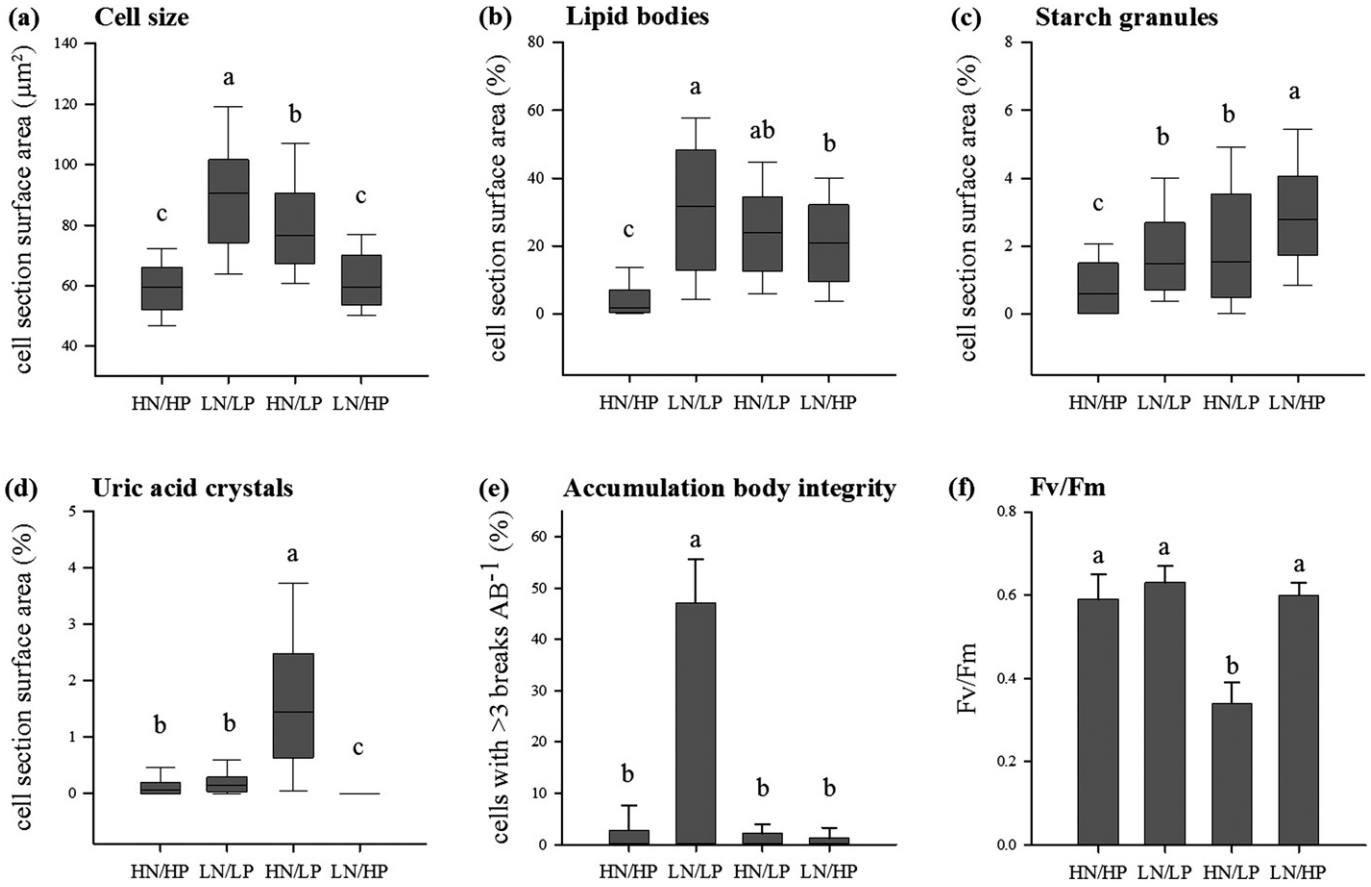

Fig. 3. Effect of dissolved inorganic nutrient availability on zooxanthellae ultrastructure and Fv/Fm. (a) Cell size ($n = 100$), (b) lipid body accumulation ($n = 100$), (c) starch granule accumulation ($n = 100$), (d) uric acid crystal accumulation ($n = 100$), (e) accumulation body fragmentation ($n = 3$), (f) Fv/Fm ($n = 5$). HN/HP = high nitrogen/high phosphorus, LN/LP = low nitrogen/low phosphorus, HN/LP = high nitrogen/low phosphorus, LN/HP = low nitrogen/high phosphorus. Box plots: the vertical line within each box represents the median. The box extends from the first to the third quartile and whiskers extend to the smallest and largest non-outliers. Outliers are not shown. Bar chart: mean ± s.d. Statistically significant differences are indicated by the use of different letters (one-way ANOVA, Tukey's test, $P < 0.05$).

environments impacted by eutrophication. However, it is important to note that nutrient values measured in the water column of natural or experimental mesocosm settings represent a steady-state equilibrium that depends on their production and uptake by organisms. Since these fluxes vary spatially and temporarily among reef regions, the measured nutrient concentrations have to be considered in the context of the respective environment. Consequently, there is an urgent need to refine these thresholds and quantify the absolute amounts of nutrients and the associated fluxes that are responsible for the observed biological effects. These values are required to provide reliable and effective target values for management purposes.

Of particular interest in the context of the present work is also the role of phytoplankton blooms. Stimulated by nutrient enrichment in the first place, coastal blooms can limit primary production by depleting essential nutrients or shifting their ratio over time and space (D'Angelo and Wiedenmann, 2014). Critically, the depletion of dissolved inorganic phosphorus has been reported in the aftermath of phytoplankton blooms that were initially set off by elevated nitrogen levels (Del Amo et al., 1997; Fujiki et al., 2004; Haese et al., 2007). Such a lack of phosphate may render benthic corals more susceptible to stress, bleaching and associated mortality (Wiedenmann et al., 2013). Indeed, previous studies have observed a correlation between elevated nitrogen concentrations, increased phytoplankton densities and coral bleaching (Wagner et al., 2010; Wooldridge, 2009; D'Angelo and Wiedenmann, 2014). Preventing the enrichment of coral reef waters with excess nitrogen should consequently be a management priority. However, it is important to note that also other forms of nutrient enrichment can have a plethora of direct and indirect negative effect on corals and their symbionts (reviewed by D'Angelo and Wiedenmann, 2014). Therefore, the reduction of nutrient enrichment has to be generally high on the agenda of coral reef management (Riegl et al., 2015).

The extended set of cumulative, ultrastructural biomarkers provided here (Table 1) can be used to identify different forms of nutrient stress in *Euphyllia* sp. associated with *Symbiodinium* (C1). These biomarkers hold promise to indicate nutrient stress also in other symbiotic coral species and in various reef settings. Importantly, they have potential to become part of the toolkit that is required for an in-depth understanding of the nutrient environment in coral reefs by bridging knowledge gaps left by traditional measurements of nutrient levels in the water column. Our findings highlight the key role of phosphorus in sustaining zooxanthellae numbers and coral biomass and for the proper functioning of symbiont photosynthesis, thereby contributing to the critical understanding of the importance of phosphorus for the functioning of symbiotic corals (Ferrier-Pagès et al., 2016).

Author contributions

JW and CD provided the research question and the experimental set-up. SR, JW and CD designed the experiments. SR conducted experiments and analysed data. SR, JW and CD interpreted data. AR contributed to the maintenance of the experimental set-up

Acknowledgements

We acknowledge the Biomedical Imaging Unit University of Southampton (A. Page) for the access to the TEM and T. Stead (School of Biological Sciences, Royal Holloway) for discussing zooxanthellae micrographs. We thank Laura Muras (Kopernikus Gymnasium Wasseralfingen) for helping with the polyp size measurements and Luke Morris (University of Southampton) for the conducting the Nile Red assays. The study was funded by NERC (NE/K00641X/1 to JW), European Research Council under the European Union's Seventh Framework Programme (FP/2007–2013)/ERC Grant Agreement no. 311179 to JW and a Vice Chancellor Award studentship to JW. We thank Tropical Marine Centre (London) and Tropic Marin (Wartenberg) for sponsoring the *Coral Reef Laboratory* at the University of Southampton.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.marpolbul.2017.02.044.

References

Aswani, S., Mumby, P.J., Baker, A.C., Christie, P., McCook, L.J., Steneck, R.S., Richmond, R.H., 2015. Scientific frontiers in the management of coral reefs. Front. Mar. Sci. 2:1–13. http://dx.doi.org/10.3389/fmars.2015.00050.

Baker, A.C., Glynn, P.W., Riegl, B., 2008. Climate change and coral reef bleaching: an ecological assessment of long-term impacts, recovery trends and future outlook. Estuar. Coast. Shelf Sci. 80:435–471. http://dx.doi.org/10.1016/j.ecss.2008.09.003.

Brodie, J., 1995. The problems of nutrients and eutrophication in the Australian marine environment. In: Zann, L.P., Sutton, D.C. (Eds.), State of the Marine Environment Report for Australia, Technical Annex 2: Pollution. Great Barrier Reef Marine Park Authority, pp. 1–24.

Brodie, J.E., Devlin, M., Haynes, D., Waterhouse, J., 2010. Assessment of the eutrophication status of the Great Barrier Reef lagoon (Australia). Biogeochemistry 106:281–302. http://dx.doi.org/10.1007/s10533-010-9542-2.

Brodie, J.E., Kroon, F.J., Schaffelke, B., Wolanski, E.C., Lewis, S.E., Devlin, M.J., Bohnet, I.C., Bainbridge, Z.T., Waterhouse, J., Davis, A.M., 2012. Terrestrial pollutant runoff to the Great Barrier Reef: an update of issues, priorities and management responses. Mar. Pollut. Bull. 65:81–100. http://dx.doi.org/10.1016/j.marpolbul.2011.12.012.

Clode, P.L., Saunders, M., Maker, G., Ludwig, M., Atkins, C.A., 2009. Uric acid deposits in symbiotic marine algae. Plant Cell Environ. 32:170–177. http://dx.doi.org/10.1111/j.1365-3040.2008.01909.x.

Cooper, T.F., Fabricius, K.E., 2012. Pigmentation of massive corals as a simple bioindicator for marine water quality. Mar. Pollut. Bull. 65:333–341. http://dx.doi.org/10.1016/j.marpolbul.2011.07.019.

Crossland, C., Barnes, D., 1977. Nitrate assimilation enzymes from two hard corals, *Acropora acuminata* and *Goniastrea australensis*. Comp. Biochem. 57:151–157. http://dx.doi.org/10.1016/0305-0491(77)90165-1.

Crossland, C., Hatcher, B., Atkinson, M., Smtih, S., 1984. Dissolved nutrients of a high-latitude coral reef, Houtman Abrolhos Islands, Western Australia. Mar. Ecol. Prog. Ser. 14:159–163. http://dx.doi.org/10.1002/cbdv.200490137.

D'Angelo, C., Wiedenmann, J., 2012. An experimental mesocosm for long-term studies of reef corals. J. Mar. Biol. Assoc. U. K. 92:769–775. http://dx.doi.org/10.1017/S0025315411001883.

D'Angelo, C., Wiedenmann, J., 2014. Impacts of nutrient enrichment on coral reefs: new perspectives and implications for coastal management and reef survival. Curr. Opin. Environ. Sustain. 7:82–93. http://dx.doi.org/10.1016/j.cosust.2013.11.029.

Del Amo, Y., Le Pape, O., Tréguer, P., Quéguiner, B., Ménesguen, A., Aminot, A., 1997. Impacts of high-nitrate freshwater inputs on macrotidal ecosystems. I. Seasonal evolution of nutrient limitation for the diatom-dominated phytoplankton of the Bay of Brest (France). Mar. Ecol. Prog. Ser. 161:213–224. http://dx.doi.org/10.3354/meps161213.

D'Elia, C.F., Webb, K.L., 1977. The dissolved nitrogen flux of reef corals. Proc. 3rd Int. Coral Reef Symp. 1, pp. 325–331.

Downs, C.A., Kramarsky-winter, E., Martinez, J., Kushmaro, A., Woodley, C.M., Loya, Y., 2009. Symbiophagy as a cellular mechanism for coral bleaching. Autophagy 5, 1–6.

Dubinsky, Z., Jokiel, P.L., 1994. Ratio of energy and nutrient fluxes regulates symbiosis between zooxanthellae and corals. Pac. Sci. 48, 313–324.

Fabricius, K.E., 2005. Effects of terrestrial runoff on the ecology of corals and coral reefs: review and synthesis. Mar. Pollut. Bull. 50:125–146. http://dx.doi.org/10.1016/j.marpolbul.2004.11.028.

Falkowski, P., Dubinsky, Z., Muscatine, L., Porter, J.W., 1984. Light and the bioenergetics of a symbiotic coral. Bioscience 34:705–709. http://dx.doi.org/10.2307/1309663.

Ferrier-Pagès, C., Godinot, C., D'Angelo, C., Wiedenmann, J., Grover, R., 2016. Phosphorus metabolism of reef organisms with algal symbionts. Ecol. Monogr. http://dx.doi.org/10.1002/ecm.1217.

Frentzen, M., 2004. Phosphatidylglycerol and sulfoquinovosyldiacylglycerol: anionic membrane lipids and phosphate regulation. Curr. Opin. Plant Biol. 7:270–276. http://dx.doi.org/10.1016/j.pbi.2004.03.001.

Fujiki, T., Toda, T., Kikuchi, T., Aono, H., Taguchi, S., 2004. Phosphorus limitation of primary productivity during the spring-summer blooms in Sagami Bay, Japan. Mar. Ecol. Prog. Ser. 283:29–38. http://dx.doi.org/10.3354/meps283029.

Furnas, M., Mitchell, A., Skuza, M., 1995. Nitrogen and Phosphorus Budgets for the Central Great Barrier Reef Shelf. Great Barrier Reef Marine Park Authority.

Furnas, M., Mitchell, A., Skuza, M., Brodie, J., 2005. In the other 90%: phytoplankton responses to enhanced nutrient availability in the Great Barrier Reef Lagoon. Mar. Pollut. Bull. 51:253–265. http://dx.doi.org/10.1016/j.marpolbul.2004.11.010.

Godinot, C., Ferrier-Pagès, C., Grover, R., 2009. Control of phosphate uptake by zooxanthellae and host cells in the scleractinian coral *Stylophora pistillata*. Limnol. Oceanogr. 54:1627–1633. http://dx.doi.org/10.4319/lo.2009.54.5.1627.

Gorbunov, M.Y., Kolber, Z.S., Lesser, M.P., 2001. Photosynthesis and photoprotection in symbiotic corals. Mol. Ecol. 46, 75–85.

Goreau, T., Hayes, R., 1994. Coral bleaching and ocean "hot spots.". Ambio-J. Hum. Environ. 23, 176–180.

Grover, R., Maguer, J., Allemand, D., Ferrier-Pagès, C., 2003. Nitrate uptake in the scleractinian coral *Stylophora pistillata*. Limnol. Oceanogr. 48:2266–2274. http://dx.doi.org/10.4319/lo.2003.48.6.2266.

Haese, R.R., Murray, E.J., Smith, C.S., Smith, J., Clementson, L., Heggie, D.T., 2007. Diatoms control nutrient cycles in a temperate, wave-dominated estuary (southeast Australia). Limnol. Oceanogr. 52:2686–2700. http://dx.doi.org/10.4319/lo.2007.52.6.2686.

Hansen, H.P., Koroleff, F., 1999. Determination of nutrients. In: Grasshoff, K., Kremling, K., Ehrhardt, M. (Eds.), Methods of Seawater Analysis. Wiley-VCH Verlag GmbH, Weinheim, Germany:pp. 159–228 http://dx.doi.org/10.1002/9783527613984.ch10.
Hoegh-Guldberg, O., 1996. Nutrient enrichment and the ultrastructure of zooxanthellae from the giant clam Tridacna maxima. Mar. Biol. 125:359–363. http://dx.doi.org/10.1007/BF00346316.
Hoegh-Guldberg, O., Mumby, P.J., Hooten, A.J., Steneck, R.S., Greenfield, P., Gomez, E., Harvell, C.D., Sale, P.F., Edwards, A.J., Caldeira, K., et al., 2007. Coral reefs under rapid climate change and ocean acidification. Science 318:1737–1742. http://dx.doi.org/10.1126/science.1152509.
van Hooidonk, R., Maynard, J.A., Planes, S., 2013. Temporary refugia for coral reefs in a warming world. Nat. Clim. Chang. 3:508–511. http://dx.doi.org/10.1038/nclimate1829.
Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., Darzins, A., 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J. 54:621–639. http://dx.doi.org/10.1111/j.1365-313X.2008.03492.x.
Hughes, T.P., Rodrigues, M.J., Bellwood, D.R., Ceccarelli, D., Hoegh-Guldberg, O., McCook, L., Moltschaniwskyj, N., Pratchett, M.S., Steneck, R.S., Willis, B., 2007. Phase shifts, herbivory, and the resilience of coral reefs to climate change. Curr. Biol. 17:360–365. http://dx.doi.org/10.1016/j.cub.2006.12.049.
Kopp, C., Pernice, M., Domart-Coulon, I., Djedat, C., Spangenberg, J., Alexander, D., Hignette, M., Meziane, T., 2013. Highly dynamic cellular-level response of symbiotic coral to a sudden increase in environmental nitrogen. MBio 4, e00052-13. http://dx.doi.org/10.1128/mBio.00052-13.
Kroon, F.J., Schaffelke, B., Bartley, R., 2014. Informing policy to protect coastal coral reefs: insight from a global review of reducing agricultural pollution to coastal ecosystems. Mar. Pollut. Bull. 85:33–41. http://dx.doi.org/10.1016/j.marpolbul.2014.06.003.
Lapointe, B., 1997. Nutrient thresholds for bottom-up control of macroalgal blooms on coral reefs in Jamaica and southeast Florida. Limnol. Oceanogr. 42, 1119–1131.
Logan, C.A., Dunne, J.P., Eakin, C.M., Donner, S.D., 2014. Incorporating adaptive responses into future projections of coral bleaching. Glob. Chang. Biol. 20:125–139. http://dx.doi.org/10.1111/gcb.12390.
Moberg, F., Folke, C., 1999. Ecological goods and services of coral reef ecosystems. Ecol. Econ. 29:215–233. http://dx.doi.org/10.1016/S0921-8009(99)00009-9.
Msanne, J., Xu, D., Konda, A.R., Casas-Mollano, J.A., Awada, T., Cahoon, E.B., Cerutti, H., 2012. Metabolic and gene expression changes triggered by nitrogen deprivation in the photoautotrophically grown microalgae *Chlamydomonas reinhardtii* and Coccomyxa sp. C-169. Phytochemistry 75:50–59. http://dx.doi.org/10.1016/j.phytochem.2011.12.007.
Muller-Parker, G., Lee, K.W., Cook, C.B., 1996. Changes in the ultrastructure of symbiotic zooxanthellae in fed and starved sea anemones maintained under high and low light. J. Phycol. 32:987–994. http://dx.doi.org/10.1111/j.0022-3646.1996.00987.x.
Muscatine, L., 1965. Symbiosis of hydra and algae. III. Extracellular products of the algae. Comp. Biochem. Physiol. 16, 77–92.
Muscatine, L., D'Elia, C.F., 1978. The uptake, retention and release of ammonium by reef corals. Limnol. Oceanogr. 23, 725–734.
Muscatine, L., Falkowski, P., Dubinsky, Z., Cook, C.B., McKloskey, L.R., 1989. The effect of external nutrient resources on the population dynamics of zooxanthellae in a reef coral. Proc. R. Soc. Lond. B 236:311–324. http://dx.doi.org/10.1098/rspb.1989.0025.
Parkhill, J., Maillet, G., Cullen, J.J., 2001. Fluorescence based maximal quantum yield for PSII as a diagnostic of nutrient stress. J. Phycol. 37:517–529. http://dx.doi.org/10.1046/j.1529-8817.2001.037004517.x.
Pernice, M., Meibom, A., Van Den Heuvel, A., Kopp, C., Domart-Coulon, I., Hoegh-Guldberg, O., Dove, S., 2012. A single-cell view of ammonium assimilation in coral-dinoflagellate symbiosis. ISME J. 6:1314–1324. http://dx.doi.org/10.1038/ismej.2011.196.
Rädecker, N., Pogoreutz, C., Voolstra, C.R., Wiedenmann, J., Wild, C., 2015. Nitrogen cycling in corals: the key to understanding holobiont functioning? Trends Microbiol. 23:490–497. http://dx.doi.org/10.1016/j.tim.2015.03.008.
Rahav, O., Dubinsky, Z., Achituv, Y., Falkowski, P., 1989. Ammonium metabolism in the zooxanthellate coral, *Stylophora pistillata*. Proc. R. Soc. Lond. B 236:325–337. http://dx.doi.org/10.1098/rspb.1989.0026.
Redfield, A.C., 1958. The biological control of chemical factors in the environment. Am. Sci. 46, 205–221.
Riegl, B., Glynn, P.W., Wieters, E., Purkis, S., d'Angelo, C., Wiedenmann, J., 2015. Water column productivity and temperature predict coral reef regeneration across the Indo-Pacific. Sci. Rep. 5:8273. http://dx.doi.org/10.1038/srep08273.
Rosset, S., D'Angelo, C., Wiedenmann, J., 2015. Ultrastructural biomarkers in symbiotic algae reflect the availability of dissolved inorganic nutrients and particulate food to the reef coral holobiont. Front. Mar. Sci. 2:1–10. http://dx.doi.org/10.3389/fmars.2015.00103.
Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al., 2012. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9:676–682. http://dx.doi.org/10.1038/nmeth.2019.
Sheppard, C., 2003. Predicted recurrences of mass coral mortality in the Indian Ocean. Nature 275:272–275. http://dx.doi.org/10.1038/nature01987.
Smith, S.V., Kimmerer, W.J., Laws, E.A., Brock, R.E., Walsh, T.E.D.W., 1981. Kaneohe Bay sewage diversion experiment: perspectives on ecosystem responses to nutritional perturbation l. Pac. Sci. 35, 279–395.
Szmant, A., 2002. Nutrient enrichment on coral reefs: is it a major cause of coral reef decline? Estuaries 25:743–766. http://dx.doi.org/10.1007/BF02804903.
Titlyanov, E.A., Titlyanova, T.V., Yakovleva, I.M., Kalita, T.L., 2006. Rhythmical changes in the division and degradation of symbiotic algae in hermatypic corals. Russ. J. Mar. Biol. 32:12–19. http://dx.doi.org/10.1134/S1063074006010020.
Vaulot, D., Olson, R.J., Merkel, S., 1987. Cell-cycle response to nutrient starvation in two phytoplankton species, *Thalassiosira weissflogii* and *Hymenomonas carterae**. Mar. Biol. 630:625–630. http://dx.doi.org/10.1007/BF00393106.
Vítová, M., Bišová, K., Kawano, S., Zachleder, V., 2015. Accumulation of energy reserves in algae: from cell cycles to biotechnological applications. Biotechnol. Adv. 33:1204–1218. http://dx.doi.org/10.1016/j.biotechadv.2015.04.012.
Wagner, D., Kramer, P., van Woesik, R., 2010. Species composition, habitat, and water quality influence coral bleaching in southern Florida. Mar. Ecol. Prog. Ser. 408:65–78. http://dx.doi.org/10.3354/meps08584.
Wang, J., Douglas, A., 1998. Nitrogen recycling or nitrogen conservation in an alga-invertebrate symbiosis? J. Exp. Biol. 201, 2445–2453.
Warner, M.E., Lesser, M.P., Ralph, P.J., 2010. Chlorophyll fluorescence in reef building corals. In: Suggett, D.J., Prášil, O., Borowitzka, M.A. (Eds.), Chlorophyll *a* Fluorescence in Aquatic Sciences: Methods and Applications. Springer, pp. 209–222.
Weng, L.-C., Pasaribu, B., Ping Lin, I., Tsai, C.-H., Chen, C.-S., Jiang, P.-L., 2014. Nitrogen deprivation induces lipid droplet accumulation and alters fatty acid metabolism in symbiotic dinoflagellates isolated from *Aiptasia pulchella*. Sci. Rep. 4:1–8. http://dx.doi.org/10.1038/srep05777.
Wiedenmann, J., D'Angelo, C., Smith, E.G., Hunt, A.N., Legiret, F., Postle, A.D., Achterberg, E.P., 2013. Nutrient enrichment can increase the susceptibility of reef corals to bleaching. Nat. Clim. Chang. 2:1–5. http://dx.doi.org/10.1038/nclimate1661.
Wooldridge, S.A., 2009. Water quality and coral bleaching thresholds: formalising the linkage for the inshore reefs of the Great Barrier Reef, Australia. Mar. Pollut. Bull. 58:745–751. http://dx.doi.org/10.1016/j.marpolbul.2008.12.013.