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

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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

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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

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