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Effects of nitrate and phosphate availability on the tissues and carbonate skeleton of scleractinian corals

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ABSTRACT: To study the effects of nutrient availability on scleractinian corals, fragments of *Montipora digitata* and *Porites cylindrica* were cultured in nutrient-enriched seawater (nitrate: 1.4 to 1.9 µmol l^{-1} and phosphate: 0.1 µmol l^{-1}) for 2 mo under laboratory conditions. For both coral species, the chlorophyll *a* concentration of the endosymbiotic algae increased significantly in both nitrogen only (+N) and nitrogen plus phosphorus (+NP) treatments compared to the control. Endosymbiont carbon (C) content of *M. digitata* increased only under +NP conditions, indicating that phosphorus (P) was limiting the production of endosymbiont cells. Host C and nitrogen (N) contents were not affected by nutrient enrichment for both coral species, suggesting that the moderate nutrient enrichment did not contribute considerably to the production of host tissue. C stable isotope ratios ($\delta^{13}C$) of the endosymbionts and host gradually decreased during the experiment, and even more so in the +N treatment. This suggests that the coral host preferentially catabolized $\delta^{13}C$-enriched organic matter, such as storage lipids, and that this catabolism was enhanced when the N:P ratio of available nutrients was not balanced. Finally, the skeletal $\delta^{13}C$ was also positively correlated with the host and endosymbiont $\delta^{13}C$ values, which implies that the $\delta^{13}C$ of host tissue was at least partially affecting the skeletal $\delta^{13}C$. Overall, moderate nutrient enrichment should not have a negative effect on coral metabolism provided that the N:P ratio of available nutrients is balanced.

KEY WORDS: Corals · Endosymbionts · Nutrient enrichment · N:P ratio · Photosynthesis · Translocation · Calcification · Stable isotope ratios

INTRODUCTION

Coral reefs are found in tropical oligotrophic seawater where the growth rate of primary producers such as phytoplankton is usually limited by the availability of nutrients (Rochelle-Newall et al. 2008). However, modern urbanization and farm land reclamation have increased the amount of nutrient discharged from the land to the coastal ocean through rivers and groundwater throughout the world (Burke et al. 2011). For example, on the Great Barrier Reef, the amount of terrestrial nitrogen (N) and phosphorus (P) runoff has more than doubled since European settlement (Furnas 2003). While particulate material is trapped near the coast, dissolved nutrients such as nitrate (NO_3^-) can disperse for many kilometers into the coastal ocean from river mouths (Devlin & Brodie 2005).

The effects of nutrient enrichment on the physiological status of corals and other coral reef organisms

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have been well studied (Fabricius 2005, Fabricius et al. 2013, D'Angelo & Wiedenmann 2014, Shantz & Burkepile 2014). In most studies, corals were cultured under laboratory conditions where nutrient concentrations were artificially elevated and the effects on coral physiology were evaluated. However, the overall response of corals to nutrient enrichment was not always straightforward and varied by coral taxa, coral morphology, endosymbiont density, and the source of the nutrients (Shantz & Burkepile 2014). Recent studies have payed attention to the N:P stoichiometry of available nutrients in seawater (Wiedenmann et al. 2013, D'Angelo & Wiedenmann 2014, Ezzat et al. 2015, 2016a,b). When only nitrate (NO_3^-) is experimentally added to the seawater, the photosynthetic rate of coral endosymbiotic algae (*Symbiodinium*) and the translocation of photosynthates to the animal host tissue decreases (Ezzat et al. 2015) or additional NO_3^- has no detectable effects (Marubini & Davies 1996, Fabricius et al. 2013). When both NO_3^- and phosphate (PO_4^{3-}) are supplied to corals in a Redfield balanced ratio (N:P = 16), endosymbiont density and photosynthesis, and host calcification are maintained even under thermal stress (Tanaka et al. 2014a, Ezzat et al. 2016a,b). When NO_3^- and PO_4^{3-} are supplied in non-Redfield balanced ratios, the lipid composition of algal cell membranes changes, resulting in photosynthetic malfunctions, the breakdown of the coral–algal symbiosis, and a higher possibility of coral bleaching under thermal stress (Wiedenmann et al. 2013, D'Angelo & Wiedenmann 2014). Overall, these studies show the importance of P and of the N:P ratio in coral physiology. However, the effects of N, P, and the N:P ratio on corals have only been investigated in a limited number of coral species from a limited number of geographic locations in the world, and such studies should be conducted more extensively to understand the general pattern of coral metabolic responses to nutrients.

Another approach for assessing the effects of nutrients on corals is through the use of stable isotopes. Carbon stable isotope ratios ($\delta^{13}C = {}^{13}C{:}^{12}C$ relative to a standard) of the coral animal host ($\delta^{13}C_h$) and its endosymbionts ($\delta^{13}C_e$) are often utilized to understand coral carbon (C) metabolism. Endosymbionts take up dissolved inorganic carbon (DIC) for their photosynthetic production, mainly from the surrounding seawater ($\delta^{13}C_{DIC}$ = 0‰) (Furla et al. 2000, Hughes et al. 2010). Corals also acquire C heterotrophically through feeding on zooplankton (Hughes et al. 2010), which usually has lighter $\delta^{13}C$ values (e.g. $\delta^{13}C_{zoop}$ = −19‰ or more negative; Rau et al. 1989, McMahon et al. 2013). $\delta^{13}C_h$ and $\delta^{13}C_e$ vary as a function of the proportionate contribution of photosynthetic and heterotrophic C to coral tissues (Muscatine et al. 1989, Swart et al. 2005a, Rodrigues & Grottoli 2006, Grottoli & Rodrigues 2011, Levas et al. 2013, Schoepf et al. 2015). However, the effects of inorganic nutrient availability on $\delta^{13}C_h$ and $\delta^{13}C_e$ have only been examined once (Béraud et al. 2013). When endosymbiont photosynthesis was enhanced by nutrient addition, $\delta^{13}C_e$ increased slightly due to increased photosynthetic rate and consequent decreased isotopic fractionation (Béraud et al. 2013). On the other hand, if nutrients are supplied in a non-balanced N:P ratio, the endosymbiotic photosynthetic rate could decrease (D'Angelo & Wiedenmann 2014, Ezzat et al. 2015) and $\delta^{13}C_e$ might decrease. Thus, we hypothesize that $\delta^{13}C_h$ and $\delta^{13}C_e$ will increase with NO_3^- and PO_4^{3-} enrichment but decrease with just NO_3^- addition.

In addition, metabolic changes in corals are reflected isotopically in the coral skeletal C ($\delta^{13}C_s$) (Grottoli & Wellington 1999, Grottoli 2002, Rodrigues & Grottoli 2006, Levas et al. 2013, Schoepf et al. 2015), and the skeletal oxygen ($\delta^{18}O_s$) changes in response to temperature and salinity (Suzuki et al. 2005, Omata et al. 2008, Hayashi et al. 2013, Nishida et al. 2014). Because coral skeletons accrete over decades to centuries, skeletal $\delta^{13}C_s$ and $\delta^{18}O_s$ are utilized for paleoenvironmental reconstruction (Suzuki et al. 2003). However, $\delta^{13}C_s$ and $\delta^{18}O_s$ are usually lighter than theoretical values — which are calculated by assuming isotope equilibrium between seawater and calcium carbonate ($CaCO_3$) deposition — and this deviation is known as the 'vital effect' (Urey et al. 1951). Two patterns of the vital effect are known: (1) 'Kinetic isotope effects' can be caused by the chemical reaction of $CaCO_3$ production during calcification. $\delta^{13}C_s$ and $\delta^{18}O_s$ are typically negatively correlated with the skeletal extension rate, a consequence of kinetic isotope effects (McConnaughey 1989). (2) 'Metabolic isotope effects' are caused by the effects of metabolic processes, such as photosynthesis and feeding on zooplankton, on coral skeletal isotopic composition (Grottoli & Wellington 1999, Grottoli 2002, Omata et al. 2008, Schoepf et al. 2014, Tanaka et al. 2014b). About 70 to 75% of the DIC used for coral calcification is derived from CO_2 respired by the animal host and only 25 to 30% is from the external seawater (Furla et al. 2000). However, the isotopic relationship between the host, endosymbiont, and skeletal isotopes has never been evaluated. We hypothesize that $\delta^{13}C_s$ and $\delta^{18}O_s$ correlate with $\delta^{13}C_h$, $\delta^{13}C_e$, and skeletal extension rate.

The objectives of the present study were (1) to evaluate the effects of inorganic N availability, with or without P, on the coral host, endosymbionts, $\delta^{13}C_h$, $\delta^{13}C_e$, and coral calcification rates, and (2) to evaluate the relationship between skeletal isotope ratios ($\delta^{13}C_s$ and $\delta^{18}O_s$), the skeletal extension rate, and metabolism ($\delta^{13}C_h$ and $\delta^{13}C_e$). Two species of scleractinian coral, *Montipora digitata* and *Porites cylindrica*, which are common coral species at the study site, were cultured in either slightly nutrient-enriched seawater or in ambient seawater under laboratory conditions for 2 mo.

MATERIALS AND METHODS

Coral preparation and laboratory culture experiments

Coral preparation and the laboratory experiment has largely been described by Tanaka et al. (2015). Briefly, coral fragments of *Porites cylindrica* and *Montipora digitata* (2 to 3 cm long) were collected from triplicate colonies at Sesoko Island, Okinawa, Japan (26° 37–39′ N, 127° 51–52′ E) in May 2012. They were attached to plastic bolts and allowed to recover from the fragmentation process in an outdoor flow-through tank at Sesoko Research Station (University of the Ryukyus). On 26 June 2012, the coral fragments were transferred to indoor flow-through tanks (6 tanks for each coral species, each tank 5.7 l), where fresh seawater was continuously supplied to each tank at an average rate of 70 ml min^{-1}, and allowed to acclimate for 1 wk. The seawater was filtered with a cartridge-type filter (pore size: 1 µm) before being supplied to the corals in order to minimize any differences in the heterotrophic conditions in the culture tanks. The seawater temperature in the tanks was maintained at 27°C and the water flow in the tanks was generated by submersible pumps producing within-tank flow rates of 3 to 5 cm s^{-1}. The average underwater light intensity was 200 µmol m^{-2} s^{-1}, which was provided with metal halide lamps.

On 3 July 2012, the experiment was started. A mixed solution of potassium nitrate (KNO_3: 1700 µmol l^{-1}) and potassium dihydrogen phosphate (KH_2PO_4: 76 µmol l^{-1}) was continuously supplied to 2 of the 6 flow-through tanks (+NP treatment) and a solution containing only KNO_3 was supplied to 2 other flow-through tanks (+N treatment). The remaining 2 flow-through tanks did not receive any supplementary nutrients (control). NO_3^- (and not ammonium) was used for the N enrichment because it is the major nutrient discharged from land to fringing reefs where the corals in this study were collected. It was not possible to test the effects of PO_4^{3-} alone due to the lack of facilities and manpower. The concentrations of NO_3^-, nitrite (NO_2^-), ammonium (NH_4^+), and PO_4^{3-} in the tanks were measured with a nutrient analyzer (Seal Analytical, QuAAtro) and kept at a constant level throughout the experimental period of 9 wk (Table 1). The experimental tanks were cleaned once or twice a week to prevent algal growth in the tanks. The uptake rate of dissolved inorganic N (DIN) by corals in the +NP and +N treatments was 108 to 132 nmol N cm^{-2} d^{-1} during the experiment (Tanaka et al. 2015). These rates were equivalent to those observed for corals in previous field and laboratory studies (Bythell 1990, Badgley et al. 2006), indicating that the effects of nutrient availability in the present study were realistically assessed.

At the start of the culture experiment, 12 coral fragments representing 3 coral colonies (4 fragments from each colony) were collected from the control. Six coral fragments representing 3 coral colonies (2 fragments from each colony) were collected from each treatment condition again after 2, 4, and 9 wk (see 'Statistical analyses'). At each sampling interval, the buoyant weight of each coral fragment was measured and then the coral tissues (animal host and endosymbionts) were removed from the carbonate skeleton using the water-pik method and fresh 0.45 µm filtered seawater (FSW). The suspension was centrifuged at 450 × *g* for 5 min to separate the endosymbiont (pellet) and animal host tissue (supernatant) fractions. The endosymbiont pellet was resuspended in FSW and centrifuged again at 450 × *g*

Table 1. Nutrient concentrations in each treatment tank (Tanaka et al. 2015). Mean ± SD is shown (n = 16)

Coral	Nutrient treatments	NO_3^- (µmol l^{-1})	NO_2^- (µmol l^{-1})	NH_4^+ (µmol l^{-1})	PO_4^{3-} (µmol l^{-1})
Montipora digitata	+NP	1.37 ± 0.63	0.11 ± 0.03	0.02 ± 0.02	0.12 ± 0.06
	+N	1.86 ± 0.66	0.14 ± 0.04	0.02 ± 0.02	0.02 ± 0.02
	Control	0.13 ± 0.07	0.08 ± 0.02	0.03 ± 0.03	0.04 ± 0.04
Porites cylindrica	+NP	1.46 ± 0.65	0.12 ± 0.04	0.04 ± 0.04	0.12 ± 0.08
	+N	1.73 ± 0.36	0.14 ± 0.03	0.03 ± 0.03	0.03 ± 0.04
	Control	0.14 ± 0.07	0.09 ± 0.04	0.03 ± 0.03	0.02 ± 0.02

to purify the endosymbiont fraction. The second supernatant was combined with the first one. A sub-sample of the animal host suspension was stored at –20°C for total organic N analysis and the remaining suspension was collected on Whatman GF/F glass fiber filters for isotopic analysis, as described by Tanaka et al. (2015). The endosymbiont fraction was resuspended again in FSW and a sub-sample was collected on GF/F filters for isotopic analysis. The GF/F filters were dried at 50°C and stored frozen for C and N content and $\delta^{13}C$ analyses. The remaining endosymbiont suspension was centrifuged again at 450 × *g* and the pellet was stored with 4 ml of methanol to extract chl *a*. It would have been ideal to analyze the chl *a* and algal cell density of each sample. However, the samples were not large enough to permit the analysis of chl *a*, algal density, and the C and N isotopes. Since the isotopic analyses were a priority, we had to chose between chl *a* and cell density, and we chose chl *a*. Since most previous studies have demonstrated that both chl *a* and algal cell density increase with nutrient enrichment (e.g. Tanaka et al. 2014a), the chl *a* measurements alone are adequate to represent coral photosynthetic potential under the present experimental conditions. The coral skeletons collected at Wk 9 were completely dried at 50°C and then the top 1 mm (or less) of the growing tip was scraped off. The resulting powder was stored at room temperature prior to $\delta^{13}C_s$ and $\delta^{18}O_s$ analysis.

Laboratory analyses and calculations

The amount of organic C and N, and the $\delta^{13}C$ of the organic C on each GF/F filter were analyzed using a Costech Elemental Analyzer (ECS4010) coupled to a Thermo-Finnigan Delta IV Plus stable isotope ratio mass spectrometer (IRMS) via a continuous flow (Conflow III) interface at the Ohio State University. The standard deviation of 62 analyses of USGS40 and USGS41 standards (US Geological Survey) was ±0.09‰ for $\delta^{13}C$. Total organic C and N of the host and endosymbiont samples were calculated using the retention factor of GF/F filters (Tanaka et al. 2015). For $\delta^{13}C_s$ and $\delta^{18}O_s$, at least 1 fragment from each colony from each treatment was analyzed on an automated Carbonate Kiel III device coupled to the same IRMS. Approximately 75 to 95 µg of coral skeletal powder samples were acidified under vacuum with 100% ortho-phosphoric acid, and the resulting CO_2 was cryogenically purified and delivered to the IRMS. The standard deviation of repeated measurements of an internal standard was ±0.03‰ for $\delta^{13}C_s$ and ±0.07‰ for $\delta^{18}O_s$. $\delta^{13}C$ and $\delta^{18}O$ were determined as the per mil deviation of ^{13}C:^{12}C and ^{18}O:^{16}O, both relative to Vienna Peedee Belemnite Limestone standard (VPDB).

The chl *a* concentration in the methanol solution was measured using a spectrophotometer (UV-1800, Shimadzu) and was calculated according to Ritchie (2006). Organic C and N, and chl *a* were normalized to the coral skeletal surface area (cm^2), which was determined by the aluminum-foil method (Marsh 1970). Dry weight of coral fragments was calculated from the buoyant weight using an aragonite density of 2.93 g cm^{-3} (Jokiel et al. 1978) and a seawater density of 1.023 g cm^{-3}. The coral calcification rate was then calculated from the change in dry skeletal weight during a given culture period and normalized to the unit surface area of the skeleton.

Statistical analyses

For the analyses of chl *a* concentration, C and N content, C:N ratio, $\delta^{13}C_h$, $\delta^{13}C_e$, and calcification rate, 6 coral fragments (1 from each of the duplicate tanks for each colony) were collected from each nutrient treatment (+NP, +N, and control) at each culture period (Wk 2, 4, and 9). The coral fragments taken at each time point were independent fragments that were produced at the beginning of the study. Previous studies have shown that colony is usually not a significant effect, and thus colony effects were not included in the final statistical analyses in order to increase statistical power (Rodrigues & Grottoli 2006, Grottoli et al. 2014). In addition, tank effects were not significant for any of the parameters in the present study, and were removed to increase the power of the analyses (Underwood 1997). The average of duplicate fragments for each colony was calculated to eliminate pseudoreplication resulting in an effective sample size of 3 for each nutrient treatment at each culture period. Each variable was then analyzed by 2-way analysis of variance (ANOVA), with nutrient treatments and culture periods as fixed effects. When a significant effect was found, Tukey's HSD test was performed to determine significant differences among the groups. All data were checked for normal distribution of the residual values for each variable using a Shapiro-Wilk's test and for homogeneity of variance using plots of expected versus residual values. Data failing to meet assumption of normality were log-transformed. Correlation analyses of $\delta^{13}C_s$ and $\delta^{18}O_s$ with the skeletal extension rate, $\delta^{13}C_h$, and $\delta^{13}C_e$ were conducted using Pearson's cor-

relation coefficient tests. For the correlation analyses, data were pooled across all treatments within a species, and up to 1 outlier was removed for each coral species according to the Mahalanobis distance. All statistical analyses were performed with JMP software (11.0.0) and significant differences were accepted at $p < 0.05$.

RESULTS

Chl *a* concentrations in *Montipora digitata* and *Porites cylindrica* were significantly elevated by +NP and +N treatments (Fig. 1a,b, see Tables S1 & S2 in the Supplement at www.int-res.com/articles/suppl/m570p101_supp.pdf). Endosymbiont C content in *M. digitata* only increased significantly relative to controls in the +NP treatment (Fig. 1c, Table S1). Animal host C content in *M. digitata* was not affected by nutrients (Fig. 1e, Table S1). Nutrients also had no significant effect on both the endosymbiont and animal C content in *P. cylindrica* (Fig. 1d,f, Table S2). Endosymbiont N content in *M. digitata* increased significantly in the +NP and +N treatments relative to controls, while that in *P. cylindrica* only increased significantly in the +NP treatment (Tables S1 & S2). Animal host N content was not affected by nutrients in both species (Tables S1 & S2). Both C and N content of the animal and endosymbiont fractions declined significantly over time irrespective of treatment (Tables S1 & S2). Endosymbiont C:N ratios of both species were significantly lower in the +NP and +N treatments relative to controls (Fig. 1g,h), while the animal host C:N ratio was lower in the +NP and +N treatments relative to controls only for *M. digitata* (Fig. 1i,j, Tables S1 & S2). $\delta^{13}C_e$ and $\delta^{13}C_h$ of both species decreased over time and were significantly lighter in the +N treatment than in the control at Wk 9 (Fig. 2, Tables S1 & S2).

Calcification rates of *M. digitata* were significantly lower in the +NP and +N treatments during the first 4 wk, but no longer differed after 9 wk (Fig. 3a, Table S1). Calcification rates of *P. cylindrica* were not affected by nutrients at any time during the study (Fig. 3b, Table S2). The $\delta^{13}C_s$ and $\delta^{18}O_s$ of *M. digitata* and the $\delta^{13}C_s$ of *P. cylindrica* were negatively correlated with the skeletal extension rate (Pearson, $p < 0.001$) (Fig. 4a,d, Table S3). A significant positive correlation was found between the $\delta^{13}C_s$ and $\delta^{18}O_s$ of *M. digitata* but not those of *P. cylindrica* (Table S3). The $\delta^{13}C_s$ of both species was positively correlated with the corresponding $\delta^{13}C_e$ and $\delta^{13}C_h$ (Fig. 4b,c,e,f, Table S3).

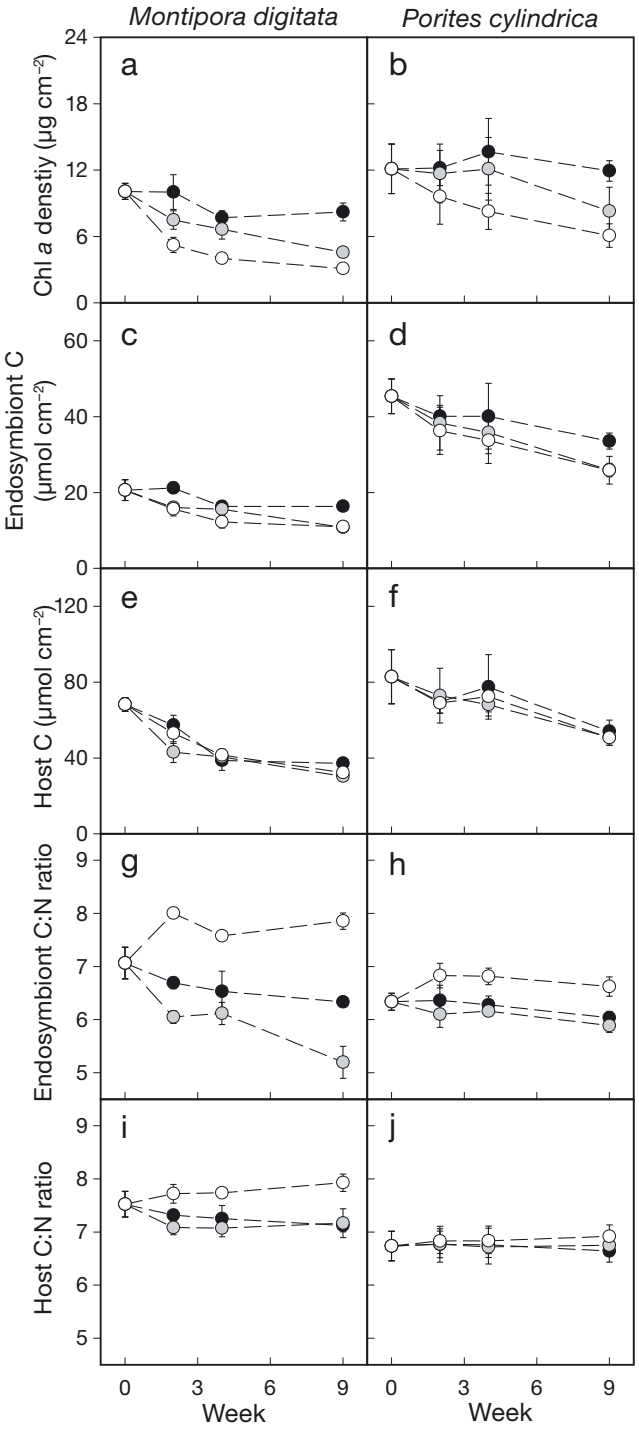

Fig. 1. The time-course changes in (a,b) chl *a* concentration, C content of (c,d) endosymbionts and (e,f) animal host, and C:N ratio of (g,h) endosymbionts and (i,j) animal host of the corals *Montipora digitata* and *Porites cylindrica* in the nutrient treatments of nitrate and phosphate (+NP, ●), nitrate only (+N, ◍), and control (○). Mean ± SD of 3 coral colonies is shown for each sampling time. The time-course changes in N biomass in the +NP and +N treatments have been published by Tanaka et al. (2015)

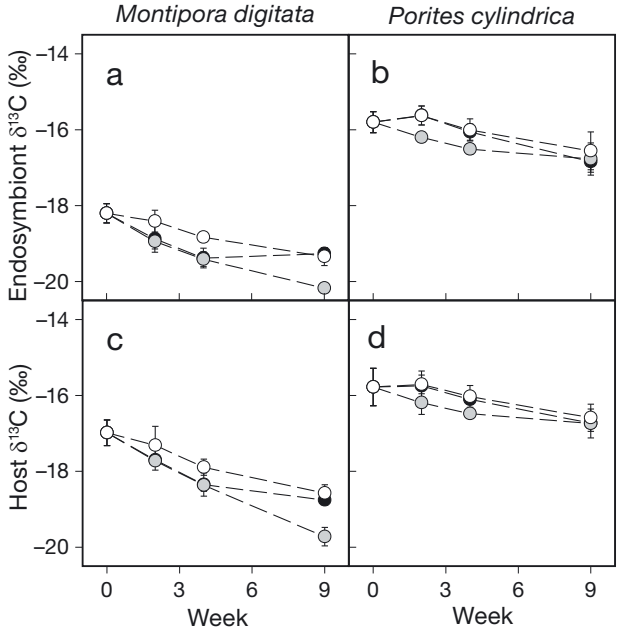

Fig. 2. Time-course changes in the $\delta^{13}C$ of (a,b) endosymbionts and (c,d) animal host of the corals *Montipora digitata* and *Porites cylindrica* in the nutrient treatments of nitrate and phosphate (+NP, ●), nitrate only (+N, ◍), and control (○). Mean ± SD of 3 coral colonies is shown for each sampling time

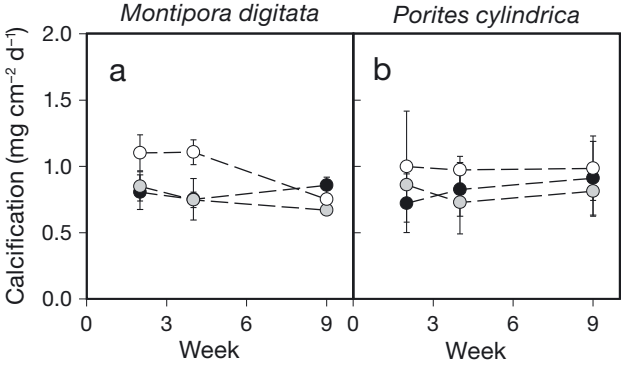

Fig. 3. Time-course changes in the calcification rate of the corals (a) *Montipora digitata* and (b) *Porites cylindrica* in the nutrient treatments of nitrate and phosphate (+NP, ●), nitrate only (+N, ◍), and control (○). Mean ± SD of 3 coral colonies is shown for each sampling time

DISCUSSION

Chlorophyll *a* and organic C and N content

Nutrient enrichment in seawater has been reported to increase chl *a* concentration in coral endosymbionts in many previous studies (reviewed by Fabricius 2005, Shantz & Burkepile 2014). The present study also revealed higher chl *a* concentrations in the +NP and +N treatments than in controls (Fig. 1a,b), indicating that the moderate elevation of nutrient concentrations in the seawater stimulated chl *a* production. The higher chl *a* concentrations in the +NP and +N treatments could be caused by higher densities of endosymbiont cells in the animal host tissue or higher chl *a* per endosymbiont cell (Shantz & Burkepile 2014). The ratio of endosymbiont C to chl *a* was highest in the control and lowest in the +NP treatment for both species (data not shown), suggesting that chl *a* per endosymbiont cell was elevated by nutrients.

P is essential to the synthesis of the nucleus and membranes of endosymbiotic algae and their very high N:P ratio (ca. 50:1) compared to the Redfield ratio (16) indicates that P is limited (Godinot et al. 2011). This is supported by our findings that the C content of endosymbionts only increased significantly in the +NP treatment for *Montipora digitata* (Fig. 1c), indicating that the acquisition of NO_3^- alone did not enhance the production of organic C in their cells, cell volume, or cell number, but that P was also required (Wiedenmann et al. 2013, Ezzat et al. 2015, 2016b). Unlike C, the N content of endosymbionts in *M. digitata* increased in both +NP and +N treatments (see Tables S1 & S2 in the Supplement), suggesting that N can be absorbed without P and is stored as organic N in the form of amino acids or proteins (Fitzgerald & Szmant 1997, Tanaka et al. 2009). Endosymbiont C and N content in *Porites cylindrica* also showed similar trends (Fig. 1d, Tables S1 & S2). Thus, the incorporation of NO_3^- throughout the study is the most likely cause of the decrease in the C:N ratio of endosymbionts for both coral species when exposed to nutrient enrichment (Fig. 1g,h).

Conversely, the C and N content of the animal host was not affected by +NP and +N treatments for both coral species (Fig. 1e,f, Tables S1 & S2), indicating that NO_3^- absorbed by endosymbionts did not lead to any detectable additional production of host tissue. However, several previous studies have shown that NO_3^-- or NH_4^+-derived N can be detected in the animal host tissue (Grover et al. 2003, Tanaka et al. 2006, Pernice et al. 2012, Kopp et al. 2013). Though the incorporation of N was not statistically significant, it was sufficient to significantly lower the C:N ratio of the host tissue of *M. digitata* in both nutrient-enrichment treatments (Fig. 1i, Table S1). Neither the C:N ratio nor N content of the host were significantly affected in *P. cylindrica*. This is probably because (1) the animal host of *P. cylindrica* originally had sufficient N in the tissues and

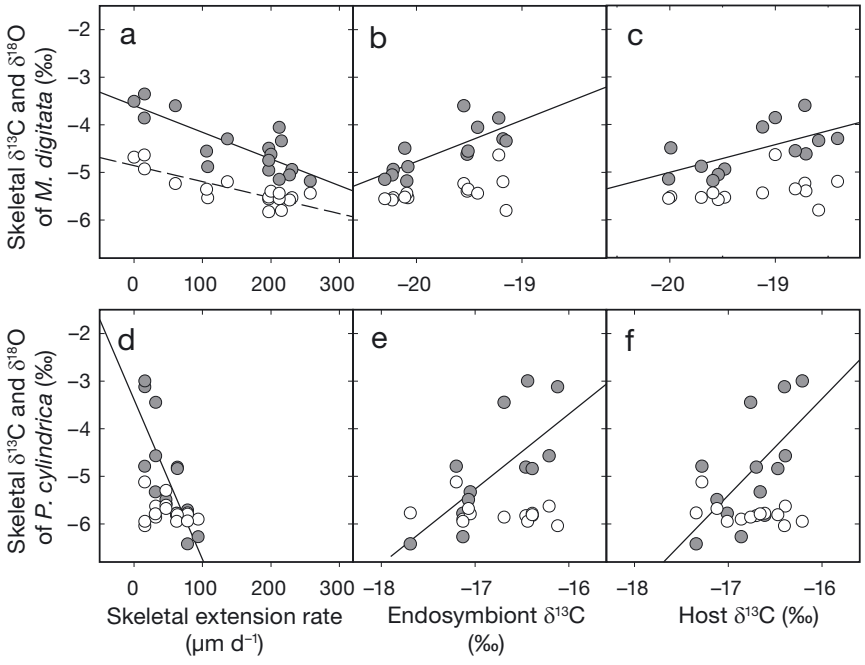

Fig. 4. The relationship between skeletal $\delta^{13}C$ (●) and $\delta^{18}O$ (○) and skeletal extension rate (μm d^{-1}), and endosymbiont and host $\delta^{13}C$ of the corals (a,b,c) *Montipora digitata* and (d,e,f) *Porites cylindrica*. Randomly selected coral fragments were analyzed (see 'Materials and methods'). Significant correlations for skeletal $\delta^{13}C$ (solid line) and skeletal $\delta^{18}O$ (dashed line) are shown

the C:N ratio was already at the lowest level for maintaining the host metabolism and/or (2) the animal host could not utilize the extra N without additional heterotrophically acquired C (Wang & Douglas 1998). This second explanation for the stable N and C:N content in *P. cylindrica* suggests that corals like *P. cylindrica* require sufficient heterotrophically acquired C to assimilate N effectively in the animal host (Piniak et al. 2003, Hughes et al. 2010), while *M. digitata* does not.

The partial removal of heterotrophic C in the present study could have caused the reduction in the C and N content of the host, endosymbionts, and total biomass during the experiment (Fig. 1c–f, Tables S1 & S2). However, because the heterotrophic conditions were the same in all treatment and controls, the assessment of the nutrient effects between treatments and controls is still valid, and the results are still informative of coral metabolism. The chl *a* concentration of the endosymbionts also decreased in most of the treatments over time (Fig. 1a,b). These results are consistent with previous studies that emphasized the importance of heterotrophy for corals (Houlbrèque et al. 2003, Grottoli et al. 2006, Borell et al. 2008, Palardy et al. 2008, Houlbrèque & Ferrier-Pagès 2009). When coral heterotrophy is reduced, endosymbionts do not acquire sufficient nutrients from the host and the chl *a* content per algal cell and/or the endosymbiont density decreases (Houlbrèque et al. 2003, Borell et al. 2008). Thus, the decrease in chl *a* was at least in part due to the absence of plankton in the experimental tanks. Without the algal cell density measurements, it is not possible to determine if the chl *a* reduction was per cell or due to a loss of cells. Although the specific balance between autotrophy and heterotrophy changes with various experimental factors (e.g. light intensity, nutrient concentrations, zooplankton availability), the slightly enriched supply of NO_3^- and PO_4^{3-} in the present study did not appear to compensate for reduced heterotrophy.

Host and endosymbiont $\delta^{13}C$

$\delta^{13}C_h$ and $\delta^{13}C_e$ continuously decreased during the culture experiment, irrespective of the nutrient treatments (Fig. 2). There are 2 possible reasons for this long-term decline in $\delta^{13}C$. First, lower light conditions in the experimental tanks compared to natural conditions could have caused a decrease in the endosymbiont photosynthetic rate, which would have increased the C isotope fractionation during photosynthesis and allowed for lighter C to be preferentially absorbed by the endosymbionts (Muscatine et al. 1989, Alamaru et al. 2009, Lesser et al. 2010). The photosynthetically acquired C could then be translocated to the animal host (Tanaka et al. 2006, Hughes et al. 2010, Tremblay et al. 2012). Thus, lower light levels would result in declines in both $\delta^{13}C_h$ and $\delta^{13}C_e$. The second possibility is that isotopically heavier organic matter was preferentially catabolized leaving the remaining organic C isotopically depleted. For example, lipid $\delta^{13}C$ becomes more depleted in bleached corals, where photosynthesis is dramatically reduced, as heavier-isotope lipids are catabolized (Grottoli & Rodrigues 2011). In the present study, the corals gradually lost their biomass during the culture experiment, suggesting that isotopi-

cally heavier lipids might have been similarly catabolized. Some combination of lower light effects and tissue catabolism is also possible. Considering that autotrophically acquired organic C is largely used for coral holobiont respiration (Hughes et al. 2010), our results suggest that it is this latter mechanism that drove depletions in coral $\delta^{13}C_h$ and $\delta^{13}C_e$ over the course of the study in both species.

The depletion of $\delta^{13}C_h$ and $\delta^{13}C_e$ over time was enhanced in the +N treatment for both coral species, suggesting that the corals in seawater with imbalanced and high N:P ratio of nutrients consumed more storage lipids than the other corals. A recent study showed that a supply of NH_3^+ and PO_4^{3-} with imbalanced high N:P ratios caused a reduction in endosymbiont photosynthetic rates and the translocation of photosynthates to the host tissue of the coral *Stylophora pistillata* (Ezzat et al. 2015). This finding suggests that, compared to the +NP treatment and control corals, the corals in the +N treatment received less photosynthates from their endosymbionts forcing them to consume more isotopically enriched storage lipids (Grottoli & Rodrigues 2011) and consequently drove their $\delta^{13}C_h$ and $\delta^{13}C_e$ values down (Fig. 2, Tables S1 & S2). In the absence of P, corals do not adequately build up adenosine triphosphate (ATP) reserves (Ezzat et al. 2016a), biosynthesize algal phospholipid membranes, or have sufficient available P for vital cellular components such as nucleic acids (Tchernov et al. 2004, Wiedenmann et al. 2013, D'Angelo & Wiedenmann 2014). The increase in the uptake rate of PO_4^{3-} under thermal stress also indicates that P is an essential nutrient for the maintenance of the metabolism of the coral–algal symbioses (Ezzat et al. 2016b).

Calcification rate

Calcification has been shown to increase, decrease, and not change under moderate nutrient additions (Marubini & Davies 1996, Tanaka et al. 2007, Béraud et al. 2013, Ezzat et al. 2015). Here, the calcification rate of *M. digitata* declined under nutrient-enrichment conditions only during the first 4 wk of the study, and not after 9 wk (Fig. 3a, Table S1). In *P. cylindrica*, nutrient enrichment had no effect on calcification at any time during the study (Fig. 3b, Table S2). These findings are consistent with several other studies on the tropical coral *Turbinaria reniformis* (Béraud et al. 2013, Ezzat et al. 2016a, Hoadley et al. 2016) and the temperate coral *Astrangia poculata* (Holcomb et al. 2010). The lack of moderate nutrient addition effects on calcification may be because endosymbiont growth was not enhanced enough to cause DIC limitation for calcification (Marubini & Davies 1996, Ezzat et al. 2016a). The maintenance of chl *a* in the +NP treatment supports the interpretation that endosymbiont growth was not drastically enhanced by nutrient additions (Fig. 1a,b). However, sufficient supply of plankton for heterotrophy coupled with a balanced inorganic nutrient supply might have increased the coral calcification rate (Ezzat et al. 2016a), though not necessarily (Hoadley et al. 2016).

Whether coral calcification increases, decreases, or does not change in response to nutrient additions may be in part a function of the type of added nutrients and the coral morphology. NH_4^+ is more bioavailable for coral metabolism than NO_3^- because NO_3^- reduction into NH_4^+ is an energy- and electron-consuming process (Dagenais-Bellefeuille & Morse 2013). NH_4^+ enrichment studies might have induced higher endosymbiont and chl *a* density than would be possible with NO_3^-, causing reduced calcification rates due to DIC limitation. Additionally, a meta-analysis showed that the calcification of branching corals is less sensitive to nutrient enrichment than that of mounding corals (Shantz & Burkepile 2014). This is because branching corals have lower endosymbiont densities and faster mass transfer rates between coral tissue and the ambient seawater, which minimizes DIC limitation and any negative impacts on calcification. Because both coral species used in the present study are branching forms, they might not be very sensitive to DIC limitation effects. Overall, our study shows that the calcification rates of *M. digitata* and *P. cylindrica* are not negatively affected by moderate nutrient additions.

Skeletal $\delta^{13}C$ and $\delta^{18}O$

Skeletal $\delta^{13}C_s$ was correlated most strongly with the skeletal extension rate for both coral species (Fig. 4a,d, see Table S3 in the Supplement). Such a negative correlation has also been observed in previous studies, where the slow growing massive corals *Porites* and *Pavona* were surveyed (McConnaughey 1989, Grottoli & Wellington 1999, Suzuki et al. 2005, Omata et al. 2008, Hayashi et al. 2013) as well as faster growing branching corals (Grottoli 2002). This negative correlation is considered to be the result of kinetic isotope effects in calcification: when the skeletal extension rate is slow, the $\delta^{13}C$ of the DIC pool for calcification becomes relatively heavy due to a longer

period of isotopic equilibration with seawater-derived DIC (McConnaughey 1989). But when the skeletal extension rate is fast, the deposition of $CaCO_3$ occurs with minimal isotopic equilibration with seawater DIC and the skeletal $\delta^{13}C_s$ values become more negative due to isotopically light respired CO_2 (McConnaughey 1989). Moreover, the positive correlation between the $\delta^{13}C_s$ and $\delta^{18}O_s$ of *M. digitata* further indicates kinetic isotope effects in this coral species (Omata et al. 2005, 2008, Schoepf et al. 2014).

In addition, this is the first study to evaluate the correlation between skeletal ($\delta^{13}C_s$ and $\delta^{18}O_s$) and organic ($\delta^{13}C_h$ and $\delta^{13}C_e$) isotope ratios of corals. $\delta^{13}C_s$ was positively correlated with $\delta^{13}C_h$ and $\delta^{13}C_e$ for both coral species (Fig. 4, Table S3), implying that these parameters are related to each other (Hughes et al. 2010). Coral calcification is fueled by DIC within the host tissue, most of which is initially derived via photosynthesis (Hughes et al. 2010), then metabolized and made available for calcification through host respiration (Furla et al. 2000, Reynaud-Vaganay et al. 2001, Hughes et al. 2010, Tremblay et al. 2012). This symbiotic C cycling system is the most likely reason for the positive correlation between the skeletal and tissue fraction isotopes. Thus, $\delta^{13}C_s$ is affected by $\delta^{13}C_h$, which fluctuates in response to metabolic conditions (Muscatine et al. 1989, Grottoli & Wellington 1999, Heikoop et al. 2000, Grottoli 2002, Swart et al. 2005b, Krief et al. 2010). For example, previous studies have shown that increased light intensity, which drives endosymbiont photosynthetic rate, results in heavier $\delta^{13}C_s$ (Grottoli & Wellington 1999, Reynaud-Vaganay et al. 2001, Grottoli 2002). While $\delta^{13}C_s$ has been shown to be a reliable proxy recorder of light conditions (i.e. cloud cover) under certain conditions (Grottoli & Wellington 1999, Grottoli 2002), our findings indicate that seawater nutrients could be an additional confounding variable making the interpretation of coral-derived cloud-cover records more difficult to interpret.

Our results show that in both *M. digitata* and *P. cylindrica*, $\delta^{18}O_s$ is not affected by nutrients (Fig. 4, Table S3). This implies that paleotemperature and/or paleosalinity reconstructions based on coral $\delta^{18}O_s$ would be reliable under a range of seawater nutrient conditions. While it is not practical to use branching corals for multi-century paleoceanographic reconstruction because they typically do not have clear banding patterns, they have the potential to provide reliable shorter records spanning a decade or so (Dunbar & Wellington 1981).

Ecological implications

The present study had 2 main findings: (1) the response of the organic matter content and $\delta^{13}C$ of corals to nutrient enrichment changes with the N:P ratio of available nutrients and P is an essential factor determining coral physiology, and (2) moderate nutrient enrichment does not affect coral calcification rate (Fig. 5). In the imbalanced N:P treatment (+N treatment), the DIN:DIP ratio in the tanks was 63 to 100 (Table 1), which is comparable to that observed in previous studies on coral physiology that focused on an imbalanced supply of nutrients (i.e. 43, Wiedenmann et al. 2013; 60, Ezzat et al. 2015), and much higher than the Redfield ratio. Such imbalances in N:P ratios are also found in natural settings where groundwater discharges onto coral reefs (D'Elia et al. 1981, Lapointe et al. 1990, Umezawa et al. 2002, Tanaka et al. 2011a,b). For example, at Shi-

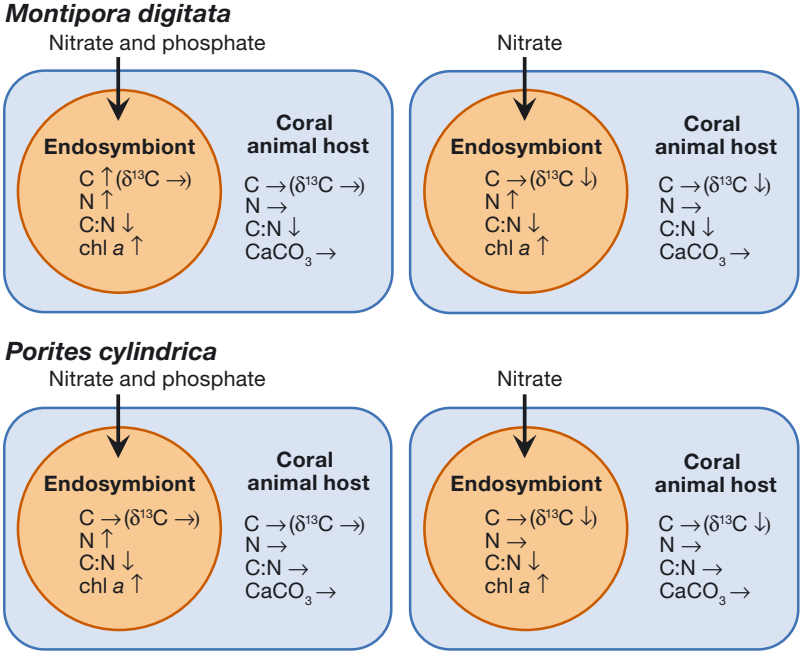

Fig. 5. Summary of the effects of nutrient enrichment on *Montipora digitata* and *Porites cylindrica*. The description of effects (↑: increase, ↓: decrease, →: no effect) is based on the effects found after 9 wk of the culture experiment. C: C content, N: N content, chl *a*: chl *a* concentration, $CaCO_3$: calcification rate. See Tables S1 & S2 in the Supplement for details

raho fringing reef, Japan, the DIN:DIP ratio in seawater is 30 to 62 (average 45) (Tanaka et al. 2011b). According to the present study, this type of imbalanced nutrient enrichment would affect the metabolism of corals like *M. digitata* more than those like *P. cylindrica* (Figs. 1, 2 & 5). The higher impact on *M. digitata* is most likely because it has less N content per area, making the endosymbionts more dependent on both seawater-derived and host-derived inorganic N, while those in *P. cylindrica* are mainly reliant on host-derived inorganic N (Tanaka et al. 2015). When seawater warming occurs simultaneously with eutrophication, corals under imbalanced N:P ratios tend to be more susceptible to the malfunction of coral–algal symbioses and subsequent loss of endosymbionts (Wiedenmann et al. 2013, Ezzat et al. 2016b). The present study indicates that under an imbalanced N:P ratio of nutrients, corals with lower organic matter content like *M. digitata* are more susceptible to bleaching under thermal stress. As a result, imbalanced nutrient supply may cause a shift in coral species composition in the reef (Shantz & Burkepile 2014, Ezzat et al. 2016b).

The present study also shows that imbalanced nutrient supply may change $\delta^{13}C_h$ and $\delta^{13}C_e$. This effect has important implications for stable isotope ecology in coral reefs. When $\delta^{13}C_h$ and $\delta^{13}C_e$ are changed, the $\delta^{13}C$ of organic matter that is released from the coral (e.g. eggs, sperm, and dissolved and particulate organic matter) might also be altered. The $\delta^{13}C$ shift in coral-derived organic matter could be passed on to other organisms through food webs. For example, the $\delta^{13}C$ of pelagic bacteria reflects the $\delta^{13}C$ of dissolved organic C in seawater (Van den Meersche et al. 2009). The $\delta^{13}C$ of muscle tissue and otoliths of coral reef fish also reflects the $\delta^{13}C$ of their food sources (Verweij et al. 2008). Thus, imbalanced nutrient enrichment might also change the $\delta^{13}C$ of coral reef organisms that rely on coral-derived organic matter.

When both inorganic N and P are enriched in seawater in a balanced ratio close to the Redfield ratio, coral endosymbionts can efficiently increase their C and N content, presumably via photosynthesis, and have minimal effect on the host (Fig. 5). These findings suggest that the balanced supply of N and P is not harmful and may even be beneficial to corals as suggested in previous studies (Gil 2013, Shantz & Burkepile 2014, Tanaka et al. 2014a, Ezzat et al. 2016a). However, this does not mean that slight nutrient loading does not alter reef ecosystems. In coral reefs, nutrients are actually taken up by corals as well as other primary producers such as benthic algae. The growth of benthic algae is enhanced with nutrients allowing them to outcompete corals for space, especially when grazing pressure on the benthic algae is low (Szmant 2002). The resulting increase in primary production elevates the concentration of organic matter in reef waters (Tanaka et al. 2011a,b), which might cause detrimental effects on coral survival under thermal stress (Fabricius et al. 2013). Thus, corals on eutrophic reefs would be more critically affected by the indirect effects of nutrients than by the direct effects on their metabolism.

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