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Partitioning of nitrogen sources to algal endosymbionts of corals with long-term ^{15}N-labelling and a mixing model

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ABSTRACT

Corals are critical in supporting high productivity and biodiversity in oligotrophic seawaters by recycling nutrients. Here, we constructed a model of nitrogen cycling in coral endosymbionts, which provides a mechanism for the efficient nutrient metabolism of corals. First, we conducted a long-term ^{15}N-labelling experiment on corals using flow-through aquaria, where ^{15}N-labelled nitrate was continuously supplied to the corals *Porites cylindrica* and *Montipora digitata* for two months. After the labelling experiment, ^{15}N isotope ratios were measured in the algal endosymbionts and their animal host and the nitrogen (N) dynamics through the endosymbiont cells were modelled. The model calculations showed that the algal endosymbionts in *P. cylindrica* and *M. digitata* derived an average of 80% and 50% of their N from the animal host, respectively. The finding indicates that the large N pool in the coral animal tissue plays an important role in supporting endosymbiotic function, photosynthesis, and consequent coral reef growth. The species-specific difference in available internal N was attributed to N biomass of the animal host per unit coral surface area. Algal N uptake from seawater was enhanced by 20% with the addition of phosphate in seawater. Thus, active endosymbiotic algal photosynthesis in oligotrophic seawater may be related to their dependence on the host-derived phosphorus as well as N.

1. Introduction

Among all marine ecosystems, coral reef areal gross photosynthesis is the highest but net production is close to or slightly above zero due to high respiration rates (Crossland et al., 1991; Gattuso et al., 1999). At the same time, reef nutrient concentrations are relatively low and stable, suggesting that the photosynthetic organisms can actively produce organic carbon (C) with limited nutrients (Atkinson, 2011). To address this paradox, the following reef-specific mechanisms of efficient material cycling have been proposed: (1) the trapping of suspended organic matter with coral mucus (Wild et al., 2004), (2) the absorption of dissolved organic matter by sea sponges (de Goeij et al., 2013), and (3) the recycling and conservation of nutrients within the coral-algal holobiont (i.e., Wang and Douglas, 1998; Tanaka et al., 2006; Reynaud et al., 2009).

The third mechanism has been discussed for half a century. When corals are incubated in prolonged darkness or the photosynthetic activity of their algal endosymbionts is inhibited, they increase the release rate of ammonium (NH_4^+) into the ambient seawater (Wilkerson and Muscatine, 1984; Rahav et al., 1989; Szmant et al., 1990), suggesting that algal endosymbionts recycle NH_4^+ excreted by the animal host (nitrogen recycling hypothesis). However, such an artificial manipulation distorts the normal metabolism of corals: under normal light conditions, endosymbionts translocate most of the C-rich photosynthate to the animal host and the organic matter is used for respiration and short-term metabolic demand of the host (Tanaka et al., 2006; Hughes et al., 2010). When the photosynthetic activity of endosymbionts is artificially inhibited in experiments, the animal host cannot acquire sufficient organic matter, which may prevent NH_4^+ assimilation by the host via glutamine synthetase and/or stimulate it to use amino acids as alternative respiratory substrate (Wang and Douglas,

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1998). To date, these processes have only been observed when the endosymbiotic photosynthesis was severely limited. It is unknown if these metabolic responses occur under natural metabolic conditions, or if instead the animal host does not release NH_4^+ and the endosymbionts do not recycle it (nitrogen conservation hypothesis) (Wang and Douglas, 1998; Piniak and Lipschultz, 2004). To better understand nitrogen (N) recycling and conservation in corals, the N fluxes between the endosymbionts and animal host need to be evaluated under natural conditions.

Algal endosymbionts in corals absorb inorganic N from seawater, synthesize organic N, and translocate it to the coral animal host (Grover et al., 2003; Tanaka et al., 2006; Pernice et al., 2012; Kopp et al., 2013). The acquisition of nutrients from seawater is relatively easy to measure when isotope tracers such as ^{15}N-labelled nutrients are used in coral cultural experiments (Grover et al., 2003; Tanaka et al., 2006; Pernice et al., 2012; Kopp et al., 2013). However, it is suspected that the endosymbionts absorb nutrients not only from seawater but also from animal host N-excretion (Reynaud et al., 2009; Seemann, 2013). Because the endosymbionts reside in the animal host tissue (Wakefield and Kempf, 2001), it has been technically difficult to quantitatively distinguish between the internally-circulating N and externally-acquired N.

To address this issue, we conducted a long-term and continuous ^{15}N-tracer experiment, using ^{15}N-labelled nitrate ($^{15}NO_3^-$) and constructed a simple mixing model of internally-circulating N and externally-acquired N for endosymbionts. This novel approach enabled us to quantitatively partition host- and seawater-derived nutrient fluxes to the endosymbionts. Overall, we show that the reason coral endosymbionts can actively perform photosynthesis in oligotrophic seawater is related to their dependence on the host-derived N.

2. Materials and methods

2.1. Coral preparation

Fragments of *Porites cylindrica* and *Montipora digitata* (2–3 cm long) were collected from triplicate colonies at a 1.5-m depth around Sesoko Island, Okinawa, Japan (26°37–39′N, 127°51–52′E) in May 2012, transferred to Sesoko Research Station (University of the Ryukyus), attached to plastic bolts (n = 16 from each colony, total n = 48 for each coral species), placed in an outdoor flow-through tank at the research station, and allowed to recover from the fragmentation process (Fig. S1). The tank was shaded with screens to simulate insolation levels at a 1.5-m depth, and the seawater was freshly pumped without filtration from the natural coral reef in front of the research station. On 26 June 2012, the coral fragments were transferred to indoor flow-through tanks (5.7 l), where fresh seawater was continuously supplied at the approximate rate of 70 ml min^{-1} per tank after filtration (pore size: 1 μm) to exclude zooplankton and allowed to acclimate for one week. Four tanks were set up in the laboratory for each coral species and the coral fragments were equally placed among the tanks (Fig. S1). The seawater temperature in the tanks was maintained at 27 °C with flowing seawater. An average underwater light intensity was 200 μmol m^{-2} s^{-1}, which was provided with metal halide lamps.

2.2. ^{15}N-labelling experiment

For each species, a mixed solution of potassium nitrate (KNO_3: 1700 μM) and potassium dihydrogen phosphate (KH_2PO_4: 76 μM) were supplied to two of the four tanks (+NP treatment) at the rate of 0.11 ml min^{-1} approximately and a solution containing only KNO_3 was supplied to the other two tanks (+N treatment) at the same rate. KNO_3 in the supplied solution was prepared by mixing normal KNO_3 and ^{15}N-labelled KNO_3, and the final δ^{15}N of NO_3^- in the solution was 260‰, which was directly measured by drying up the solution and subsequent mass spectrometer analysis. The concentrations of NO_3^-, nitrite (NO_2^-), NH_4^+, and PO_4^{3-} in the tanks were measured with a nutrient analyzer (Seal Analytical, QuAAtro) throughout the experimental period (Table 1).

At the start and after 2, 4, and 9 weeks of the ^{15}N-labelling experiment, coral fragments were collected from each treatment condition (Fig. S1) and the animal host coral tissue including endosymbionts was taken off the carbonate skeleton using the water-pik method. The tissue suspension was centrifuged at 450 × g for 5 min to separate endosymbionts (pellet) from animal host (supernatant). The endosymbiont pellet was suspended with fresh 0.45-μm-filtered seawater (FSW) and the suspension was centrifuged again to purify the endosymbiont fraction. The second supernatant was combined with the first one and the mixed solution was filtered onto a pre-combusted GF/F glass fibre filter (pore size: 0.7 μm) to collect coral animal host. Because all of the animal host tissue was not retained on GF/F filters, a part of the animal host suspension was stored without filtration at −20 °C for total N biomass analysis. The endosymbiont pellet was also filtered onto a GF/F filter after suspended with FSW. All of the glass fibre filters were dried at 50 °C and then frozen.

2.3. Laboratory analyses

The concentration and δ^{15}N of organic N collected on GF/F filters were analyzed on a Costech Elemental Analyzer (ECS4010) coupled to a Finnigan Delta IV Plus stable isotope ratio mass spectrometer via a continuous flow (Conflow III) interface at The Ohio State University. δ^{15}N was determined as the per mil deviation of ^{15}N/^{14}N relative to air. The standard deviation of 62 analyses of USGS40 and USGS41 standards (U.S. Geological Survey) was ±0.09‰. Total organic N in the unfiltered host tissue suspension (TN_h) was oxidized into NO_3^- with borate-buffered persulfate solution by autoclaving (121 °C, 60 min) (Hansen and Koroleff, 1999) and the concentration of tissue-derived NO_3^- was measured from ultraviolet absorbance at 220 nm with a spectrophotometer (Shimadzu, UV-1800) (Collos et al., 1999). From the comparison of an animal host tissue suspension between TN_h and the N amount retained on the filter, the filter retention factor was determined to be 0.36 ± 0.01 for *P. cylindrica* (n = 8) and 0.23 ± 0.00 for *M. digitata* (n = 9) (mean ± s.e.). Total amount of animal host N was then calculated for all the samples using these average retention factors. Endosymbiont and animal host N concentrations were normalized

Table 1
Nutrient concentrations and calculated δ^{15}N of DIN in the culture tanks. Nutrient concentrations (μM) were measured for ^{15}N-labelling tanks and original seawater and the δ^{15}N (‰) of DIN was estimated by calculations (Eq. (3)). Mean ± 1s.d. are shown (n = 16).

Coral	Nutrient conditions	NO_3^- (μM)	NO_2^- (μM)	NH_4^+ (μM)	PO_4^{3-} (μM)	δ^{15}N of DIN (‰)
Porites cylindrica	Original	0.14 ± 0.07	0.09 ± 0.04	0.03 ± 0.03	0.02 ± 0.02	–
	+NP	1.46 ± 0.65	0.12 ± 0.04	0.04 ± 0.04	0.12 ± 0.08	211 ± 23
	+N	1.73 ± 0.36	0.14 ± 0.03	0.03 ± 0.03	0.03 ± 0.04	221 ± 18
Montipora digitata	Original	0.13 ± 0.07	0.08 ± 0.02	0.03 ± 0.03	0.04 ± 0.04	–
	+NP	1.37 ± 0.63	0.11 ± 0.03	0.02 ± 0.02	0.12 ± 0.06	218 ± 16
	+N	1.86 ± 0.66	0.14 ± 0.04	0.02 ± 0.02	0.02 ± 0.02	226 ± 15

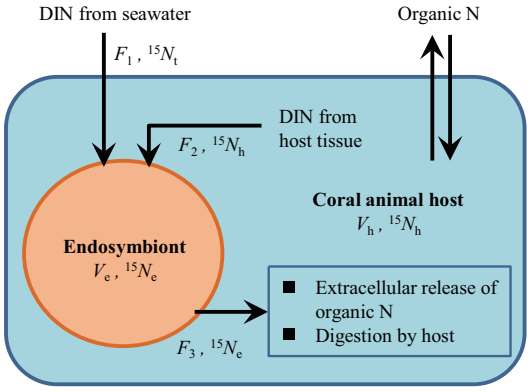

Fig. 1. Nitrogen fluxes in a coral-algal symbiosis. Endosymbionts in coral absorb dissolved inorganic nitrogen (DIN) from seawater and from the host's internal DIN pool and synthesize organic N. The organic N is stored in the endosymbionts or transferred to the animal host. F_1, F_2, and F_3 show N fluxes normalized to coral surface area (μmol cm^{-2} d^{-1}) from seawater to the endosymbionts, from the animal host DIN pool to endosymbionts, and from the endosymbionts to the animal host fraction, respectively. V and ^{15}N indicate average N biomass per unit coral surface area (μmol cm^{-2}) and average δ^{15}N value (‰) of each fraction, respectively, and the changes of these values were expressed as a function of time from regression lines (Table 3). The e, h, and t notations refer to the endosymbionts, animal host, and treatment seawater in a culture tank, respectively (Table 2).

Table 2
List of symbols used in the model equations.

Symbol	Description	Unit
F_1	DIN uptake from seawater	μmol cm^{-2} d^{-1}
F_2	DIN uptake from animal host excretion	μmol cm^{-2} d^{-1}
F_3	Translocation of organic N to animal host	μmol cm^{-2} d^{-1}
V_e	N biomass of algal endosymbionts	μmol cm^{-2}
V_h	N biomass of coral animal host	μmol cm^{-2}
$^{15}N_e$	δ^{15}N of algal endosymbionts	‰
$^{15}N_h$	δ^{15}N of coral animal host	‰
$^{15}N_p$	δ^{15}N of nitrate supplied to tanks	‰
$^{15}N_q$	δ^{15}N of DIN in natural seawater	‰
$^{15}N_t$	δ^{15}N of DIN in treatment seawater	‰
DIN_q	Total DIN concentration in natural seawater	μmol l^{-1}
DIN_t	Total DIN concentration in treatment seawater	μmol l^{-1}

to the coral skeletal surface area (cm^2), which was determined by the aluminium-foil method (Marsh, 1970).

2.4. Model design and calculations

The model describes the endosymbiont N metabolism of inorganic N uptake and organic N release (Fig. 1). The endosymbionts take up dissolved inorganic N (DIN: $NO_3^- + NO_2^- + NH_4^+$) from seawater (F_1: μmol cm^{-2} d^{-1}) and from the internal host-derived DIN pool (F_2: μmol cm^{-2} d^{-1}), and use it to synthesize organic N. The organic N is then either stored in the endosymbiont, or lost from the cell as a transfer product to the host via extracellular release of organic N (F_3: μmol cm^{-2} d^{-1}). N and ^{15}N mass balances of the endosymbionts were expressed as follows:

$$\frac{dV_e}{dt} = F_1 + F_2 - F_3 \quad (1)$$

$$\frac{d^{15}N_e}{dt} \times V_e = \left(^{15}N_t \times F_1\right) + \left(^{15}N_h \times F_2\right) - \left(^{15}N_e \times F_3\right) \quad (2)$$

Symbols in the equations are explained in Table 2. All the parameters, except $^{15}N_t$ (δ^{15}N of total DIN in the treatment tank, see below), change with time. The time-course changes of δ^{15}N ($^{15}N_e$ and $^{15}N_h$), N biomass (V_e and V_h), and their differential values were calculated from exponential or linear fitting to the observed data (Figs. 2 and 3, Table 3). We considered that the independent variable (time) could be used to significantly predict the dependent variable (δ^{15}N and N biomass) when $p < 0.05$. All the parameters were significantly explained with the resulting regression lines, except one N biomass parameter of endosymbionts because the slope was almost zero. In this case, the average value throughout the experiment (2.8 μmol cm^{-2}) was used as a constant for endosymbiont N biomass. δ^{15}N (^{15}N) and biomass (V) values calculated from exponential and linear fitting are supplementarily shown in Tables S1–S4. A parallel model for the animal host was not possible because N fluxes between the animal host and the

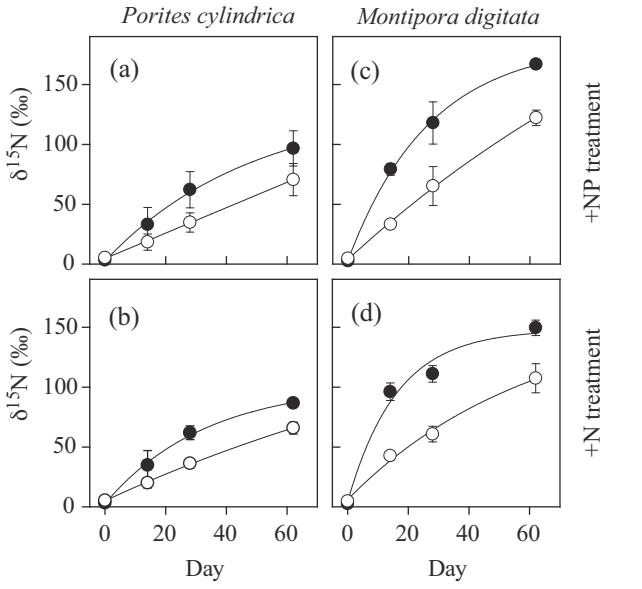

Fig. 2. δ^{15}N in *Porites cylindrica* and *Montipora digitata*. Coral fragments of *P. cylindrica* (a and b) and *M. digitata* (c and d) were continuously supplied with ^{15}N-labelled nitrate and phosphate (a and c) or ^{15}N-labelled nitrate only (b and d), and the δ^{15}N of the endosymbionts (filled) and coral animal host (open) were measured over two months. Mean ± 1s.d. (n = 3) at each sampling time. Parameters of the regression lines are given in Table 3.

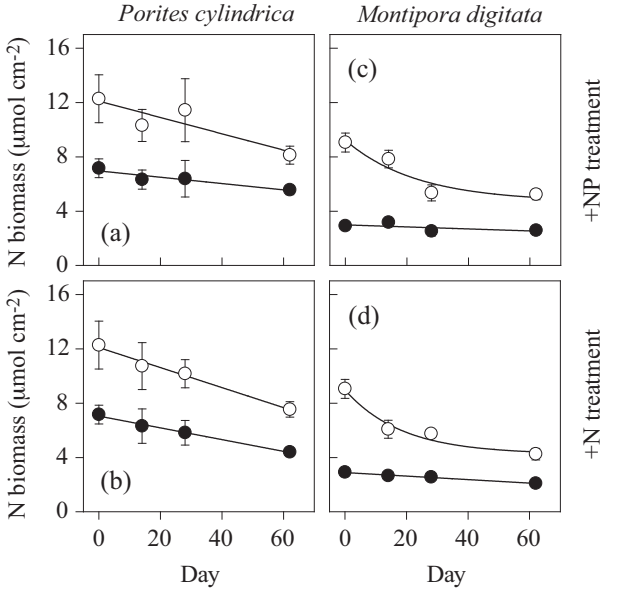

Fig. 3. N biomass in *Porites cylindrica* and *Montipora digitata*. The coral fragments of *P. cylindrica* (a and b) and *M. digitata* (c and d) were continuously supplied with ^{15}N-labelled nitrate and phosphate (a and c) or ^{15}N-labelled nitrate only (b and d), and the N biomass of the endosymbionts (filled) and of the coral animal host (open) were measured over two months. N biomass was normalized to coral surface area. Mean ± 1s.d. (n = 3) at each sampling time. Parameters of the regression lines are given in Table 3.

Table 3
Regression parameters of $\delta^{15}N$ and N biomass. The observed $\delta^{15}N$ and N biomass over time (Figs. 2 and3) were fitted with a linear or curved regression model using one of the following formulae. 1: $y = y_0 + a\,(1 - e^{-bt})$, 2: $y = y_0 + at$, 3: $y = y_0 + ae^{-bt}$, where t is the time (day) and the formula number (f.n.) is shown in the column "Fraction". R^2: the coefficient of determination, P: p-value for the regression line. Only one regression line for endosymbiont N biomass (*Montipora digitata* in +NP) was not statistically significant and thus, the average of the observed values (2.8 μmol cm^{-2}) was used as a constant for the model calculations of Eqs. (1) and (2).

Coral	Nutrient conditions	Parameters	Fraction (f.n.)	Regression results				
				y_0	a	b	R^2	P
Porites cylindrica	+NP	$\delta^{15}N$	Symbiont (1)	2.60	131	0.0207	0.92	<0.001
			Host (2)	4.76	1.06	–	0.92	<0.001
		N biomass	Symbiont (2)	6.97	−0.0233	–	0.36	<0.05
			Host (2)	12.1	−0.0601	–	0.46	<0.05
	+N	$\delta^{15}N$	Symbiont (1)	2.69	99.9	0.0302	0.97	<0.001
			Host (1)	5.09	188	0.0063	0.98	<0.001
		N biomass	Symbiont (2)	7.05	−0.0433	–	0.66	<0.01
			Host (2)	12.1	−0.0737	–	0.69	<0.001
Montipora digitata	+NP	$\delta^{15}N$	Symbiont (1)	3.36	181	0.0372	0.98	<0.001
			Host (1)	3.99	342	0.0069	0.97	<0.001
		N biomass	Symbiont (2)	2.99	−0.0074	–	0.27	0.08
			Host (3)	4.70	4.52	0.0430	0.83	<0.001
	+N	$\delta^{15}N$	Symbiont (1)	4.41	144	0.0617	0.97	<0.001
			Host (1)	6.09	157	0.0165	0.97	<0.001
		N biomass	Symbiont (2)	2.89	−0.0129	–	0.69	<0.001
			Host (3)	4.25	4.72	0.0543	0.91	<0.001

ambient sweater were not quantified (see Section 4). However, N fluxes between the host and seawater were taken into account in the observed $\delta^{15}N_h$ ($^{15}N_h$ in Eq. (2)) and thus any uncertainty associated with these pathways did not affect the model calculation results from Eqs. (1) and (2).

Since the seawater residence time in each tank was short (approximately 81 min) and NO_2^- and NH_4^+ concentrations in the tanks were similar to those in the original seawater (Table 1), it was assumed that N of NO_2^- and NH_4^+ were not labelled with ^{15}N. $\delta^{15}N$ of total DIN in the treatment tank ($^{15}N_t$) was calculated from the measured $\delta^{15}N$ of supplied NO_3^- ($^{15}N_p$; 260‰) and an assumed $\delta^{15}N$ of DIN in the original natural seawater ($^{15}N_q$; 3‰, Umezawa et al., 2002) (Table 2). $^{15}N_t$ was calculated as follows:

$$^{15}N_t = \frac{DIN_q}{DIN_t} \times {}^{15}N_q + \frac{DIN_t - DIN_q}{DIN_t} \times {}^{15}N_p \quad (3)$$

where DIN_t and DIN_q are total DIN concentrations (μM) in the treatment tank and original seawater, respectively (Tables 1 and 2).

Finally, F_1 and F_2 can be calculated from day 0 to 62 using the data of $\delta^{15}N$ and N biomass, if F_3 is known. However, it is not reasonable to fix F_3 as a constant in each model calculation because F_3 could change over the experimental period. Since corals were cultured under constant conditions in the laboratory, it was assumed that F_3 was proportionate to V_e throughout the experiment. Thus, a F_3: V_e ratio was fixed and randomly assigned to Eqs. (1) and (2). F_1 and F_2 were repeatedly calculated with allowable F_3: V_e ratios, with a time step of one day (Tables S1–S4). To confine F_1 and F_2 within the range of established coral metabolic rates, the allowable range of F_3: V_e ratios was constrained using previously reported data with an added variability of ±30% to allow for greater uncertainty. As such, F_2/V_e (the ratio of host NH_4^+ excretion to endosymbiont N biomass), F_2/V_h (the ratio of host NH_4^+ excretion to the host N biomass), and $(F_1 + F_2)/V_e$ (N turnover rate in endosymbionts) were regulated to be 0.033–0.17 d^{-1} (Rahav et al., 1989; Szmant et al., 1990), 0.0092–0.13 d^{-1} (Rahav et al., 1989; Szmant et al., 1990), and 0.056–0.10 d^{-1} (Tanaka et al., 2006), respectively. These restrictive conditions confined the N fluxes in the present model within the range of established coral metabolic rates. Consequently, the F_3: V_e ratios were confined to a range of 0.061–0.107 d^{-1} and 0.066–0.110 d^{-1} in the +NP and +N treatment, respectively, for *P. cylindrica* and to a range of 0.079–0.104 d^{-1} and 0.080–0.108 d^{-1} for *M. digitata* (Tables S1–S4). These ranges were similar to those calculated in a recent numerical model on corals (Gustafsson et al., 2013). The percent contribution of host-derived internal N relative to total N influx to endosymbionts (CIN) was calculated as the ratio of F_2 to $F_1 + F_2$. The total amount of N incorporated by endosymbionts via F_1 and F_2 during the experiment was calculated by adding F_1 and F_2 fluxes on each day.

3. Results

In both species of coral and under both nutrient supply conditions, the average $\delta^{15}N$ of the endosymbionts ($\delta^{15}N_e$) and the animal host ($\delta^{15}N_h$) dramatically increased and $\delta^{15}N_e$ consistently exceeded $\delta^{15}N_h$ values (Fig. 2). For *P. cylindrica*, $\delta^{15}N_e$ had values of 97‰ and 87‰ in the +NP and +N treatment, respectively, at the end of the experiment (Fig. 2a and b). $\delta^{15}N_h$ of *P. cylindrica* almost linearly increased and the final values were 71‰ and 66‰ in the +NP and +N treatment, respectively (Fig. 2a and b). For *M. digitata*, $\delta^{15}N_e$ and $\delta^{15}N_h$ were distinctly higher than those of *P. cylindrica*. $\delta^{15}N_e$ of *M. digitata* had the final values of 167‰ and 149‰ in the +NP and +N treatment, respectively, and the corresponding $\delta^{15}N_h$ were 122‰ and 107‰ (Fig. 2c and d). Organic N biomass of animal hosts and endosymbionts per unit coral surface area linearly or exponentially decreased over the experiment, except endosymbionts in the +NP treatment for *M. digitata* (Fig. 3 and Table 3).

The model calculations for *P. cylindrica* showed that the contribution of N from the animal host (i.e., F_2) was much higher than that from the ambient seawater (i.e., F_1) (Fig. 4a and b). CIN was 78–88% at the beginning of the study in both +NP and +N treatments (Fig. 4c). During the course of the experiment, the CIN slowly decreased to about 70% due to the reduction of F_2. Overall, the total amount of N incorporated by endosymbionts via the host (i.e., F_2) throughout the experiment was approximately three times higher than that via seawater (i.e., F_1) (Table 4).

For *M. digitata*, the contribution of N from seawater to the endosymbionts (F_1) increased slightly in the +NP treatment, while decreased slightly in the +N only treatment and the final flux of F_1 was higher in the +NP treatment (0.12–0.15 μmol cm^{-2} d^{-1}) than in the +N only treatment (0.069–0.087 μmol cm^{-2} d^{-1}) (Fig. 4d and e) (Table 4). The total amount of N incorporated by endosymbionts via F_1 was also higher in the +NP treatment than in the +N only treatment (Table 4). CIN decreased from 61–70% to 46–48% in the +NP treatment but was almost constant in the +N treatment and at 56–59% at the end of the study (Fig. 4f). In comparing the two coral species, host-derived N to the endosymbionts (F_2) and CIN were

Table 4
N fluxes from seawater and from animal host (F_1 and F_2, respectively) to endosymbionts. The fluxes at the end of the experiment (Day 62), the integrated fluxes over the experimental period, and the proportion of each flux to the total flux ($F_1 + F_2$) was calculated. The highest and lowest range shown in parentheses indicates the results when the maximum and minimum values of F_3: V_e ratio, which was fixed in each model run, respectively, are used (Tables S1–S4). The range of F_3: V_e ratio is indicated in Section 2.

Coral	Nutrient conditions	Flux	Day 62		Integrated from Day 0 to day 62	
			N flux (μmol cm^{-2})	% Of total	N flux (μmol cm^{-2})	% Of total
Porites cylindrica	+NP	F_1	0.13 (0.10–0.15)	29	8.0 (6.9–9.2)	25
		F_2	0.31 (0.21–0.41)	71	24 (15–31)	75
	+N	F_1	0.084 (0.071–0.098)	25	6.7 (5.8–7.5)	23
		F_2	0.26 (0.17–0.34)	75	22 (15–29)	77
Montipora digitata	+NP	F_1	0.14 (0.12–0.15)	53	8.2 (7.4–8.9)	51
		F_2	0.12 (0.10–0.14)	47	8.0 (6.5–9.4)	49
	+N	F_1	0.078 (0.069–0.087)	42	6.8 (6.1–7.4)	49
		F_2	0.11 (0.086–0.13)	58	7.2 (5.6–8.7)	51

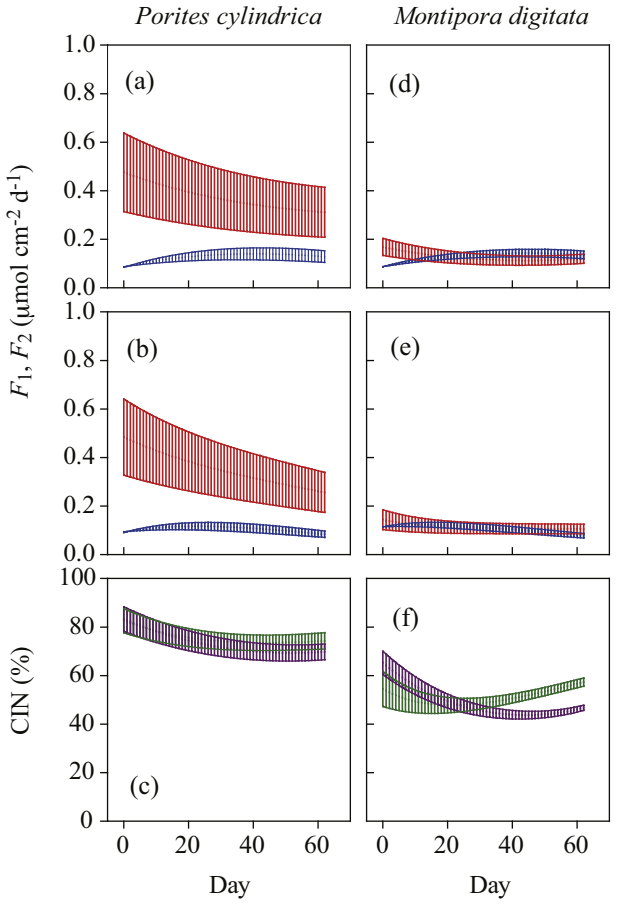

Fig. 4. Nitrogen fluxes calculated from the nitrogen mixing model. N fluxes from seawater to endosymbionts (F_1, blue), from coral animal host to endosymbionts (F_2, red), and the ratio of F_2 to $F_1 + F_2$ (CIN) are shown as the function of time (day) for *P. cylindrica* (a: +NP, b: +N, c: CIN) and *M. digitata* (d: +NP, e: +N, f: CIN). In panels c and f, both the +NP (purple) and +N (green) model results are shown. The highest and lowest values at each time point correspond to the results when the maximum and minimum values of F_3: V_e ratio, which were fixed in each model run, were used (Tables S1–S4). The range of F_3: V_e ratio is indicated in Section 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

distinctly higher in *P. cylindrica* than in *M. digitata*, while F_1 was similar between them (Fig. 4 and Table 4).

4. Discussion

In both species of corals, $\delta^{15}N_e$ consistently exceeded $\delta^{15}N_h$ (Fig. 2), suggesting that the endosymbionts had a faster ^{15}N turnover rate. Previous isotope-labelling studies with $^{15}NO_3^-$ also observed that endosymbionts were labelled much faster than the animal host (Grover et al., 2003; Tanaka et al., 2006; Kopp et al., 2013). Because only the endosymbionts can directly incorporate nitrate (NO_3^-) through their enzymatic activity (Crossland and Barnes, 1977; Leggat et al., 2007), the experimentally supplied $^{15}NO_3^-$ would first have to have been absorbed by the endosymbionts and then translocated to the coral animal host as organic N (Grover et al., 2003; Tanaka et al., 2006; Pernice et al., 2012; Kopp et al., 2013). Kopp et al. (2013) recently reported that N incorporated by endosymbionts of the coral *Pocillopora damicornis* was first stored as uric acids in the algal cells, translocated to the animal host, and utilized in specific cellular compartments of the host. Since endosymbionts in corals generally have a turnover time of 10–50 days (Wilkerson et al., 1983; Hoegh-Guldberg and Smith, 1989; Rahav et al., 1989; Szmant et al., 1990; Tanaka et al., 2006) and the present experimental period was 62 days, a complete turnover of all endosymbiont cells should have taken place during the study, the endosymbiont cells should have been fully labelled, and had an average $\delta^{15}N_e$ value equivalent to that of the seawater DIN (211–226‰). Given that the average $\delta^{15}N_e$ was much lower than expected from the $\delta^{15}N$ of seawater DIN, the endosymbionts were thus incorporating both external labelled seawater DIN and internally-circulating DIN excreted by the animal host most likely in the form of NH_4^+ (Rahav et al., 1989; Szmant et al., 1990).

Our novel approach of long-term stable-isotope labelling and subsequent model calculation has succeeded in partitioning these N sources for coral endosymbionts. Algal endosymbionts in *P. cylindrica* and *M. digitata* derive 78–88% and 47–70% of their N from the animal host N pool, respectively, at the start of the experiment (Fig. 4). This is consistent with a recent numerical model of several hypothetical scenarios of seawater nutrient concentrations and coral feeding rates showing that algal endosymbionts must be deriving >50% of their N from the animal host (Gustafsson et al., 2013). In addition, the CIN range for *P. cylindrica* (78–88%) was comparable with the previously estimated value of 90% for *Stylophora pistillata*, which was calculated from the excretion rate of NH_4^+ from the animal host under the suspension of endosymbiotic photosynthesis (Rahav et al., 1989). Moreover, the finding that $\delta^{15}N$ of coral animal host was not enriched compared to the prey zooplankton also suggested that N was not excreted from the symbiosis but recycled by the endosymbionts (Reynaud et al., 2009). Our approach of ^{15}N-labelling has demonstrated N recycling in corals using empirically derived data and strengthens the argument that the endosymbiotic algae in corals are heavily dependent on host-derived recycled N.

The model results showed that F_2 and CIN were higher in *P. cylindrica* than in *M. digitata* (Fig. 4), which is most likely related to the larger host N biomass of the former (Fig. 3). A larger N biomass could provide higher amounts of NH_4^+ excretion (Szmant et al., 1990), a larger stable internal N pool for the endosymbionts, and enhanced stability for endosymbiont photosynthesis. This might

help to explain why bleached *Porites compressa* corals continue to depend on photoautotrophically acquired energy, but bleached *Montipora capitata* corals shift from endosymbiont autotrophy to animal host heterotrophy (Grottoli et al., 2006). The gradual decline of F_2 over the course of the study was most likely due to the gradual decrease in N biomass over that time (Fig. 3), which could have been driven by the absence of zooplankton in the seawater due to the filtration system (Houlbrèque and Ferrier-Pagès, 2009). With sufficient heterotrophic activity, N biomass and F_2 might have been kept at the initial levels throughout the experimental period. Although the present experimental design did not quantify the effect of heterotrophic feeding, the time-course change of F_1 and F_2 fluxes implies that the absence of heterotrophy might gradually decrease the uptake of host-derived N by the endosymbionts but not dramatically affect CIN (Fig. 4).

The species-specific difference in host N biomass further affected the proportionate contribution of each nutrient source for endosymbionts. Compared with *P. cylindrica*, CIN of *M. digitata* largely decreased in the +NP treatment (Fig. 4), indicating that a host-derived N in *M. digitata* was not sufficient for the endosymbionts to absorb phosphorus available in the seawater and animal host tissue. Thus, we hypothesize that endosymbionts in lower host N biomass species such as *M. digitata* shift their N source from the internal host N pool to the external seawater DIN, depending on the seawater nutrient condition. In fact, at the end of the experiment, *M. digitata* in the +NP treatment absorbed more nutrients from seawater than those in the +N only treatment (Fig. 4d and e, and Table 4). Overall, endosymbionts of both coral species exposed to NO_3^- and PO_4^{3-} absorbed 20% more NO_3^- from seawater than when only NO_3^- was supplied (Table 4). This suggests that the uptake of NO_3^- by endosymbionts is enhanced by phosphorus, and that N uptake by endosymbionts in the +N treatment was somewhat limited by the absence of phosphorus. Similarly, Godinot et al. (2011) reported that the saturated uptake rate of NH_4^+ increased with the addition of PO_4^{3-} to seawater, demonstrating that the endosymbionts uptake of DIN from seawater was limited by intracellular PO_4^{3-}.

In previous studies, the release of NH_4^+ from the animal host has been observed only when the endosymbiotic photosynthesis was artificially inhibited (Wilkerson and Muscatine, 1984; Rahav et al., 1989; Szmant et al., 1990). The present findings are the first to quantitatively demonstrate that N is recycled in coral-algal symbiosis in a normal diel cycle and that the coral animal host is an important N source for the endosymbionts (recycling hypothesis). However, this conclusion does not necessarily exclude the property of N conservation by the animal host: endosymbiotic photosynthesis supplies the animal host with C-rich organic matter, which can help the host to assimilate NH_4^+ in the host tissue and/or to utilize this photosynthetic product for the host respiration. These effects of endosymbiotic photosynthesis might have more or less reduced NH_4^+ excretion from the animal host as proposed in the N conservation hypothesis (Wang and Douglas, 1998; Piniak and Lipschultz, 2004). Additional study is needed to more definitively determine the extent to which this N conservation functions in animal hosts.

Our data, in combination with published work, indicate that both seawater derived and heterotrophically derived N are critical sources of N for coral endosymbionts. The animal host acquires heterotrophic N by capturing zooplankton and absorbing dissolved organic matter (Piniak et al., 2003; Houlbrèque and Ferrier-Pagès, 2009), stores the acquired N with a long turnover time (Tanaka et al., 2006), and excretes very little of it slowly (Szmant et al., 1990; Seemann, 2013). Our findings further reveal that coral algal endosymbionts incorporate two to four times more internally derived N than seawater DIN (Fig. 4 and Table 4). Therefore, endosymbiont photosynthesis is supported not only by oligotrophic seawater DIN but also by microorganisms such as zooplankton via the coral animal host. Given a larger-scale perspective, the high primary production of coral reefs is maintained by both inorganic and organic nutrients. This helps to explain the nutrient paradox of coral reefs–nutrient recycling within corals helps to maintain reef production in an oligotrophic environment.

Furthermore, the negative effect of ocean acidification on coral calcification (Hoegh-Guldberg et al., 2007; Pandolfi et al., 2011) has been observed to be partially or totally offset by an elevated supply of inorganic nutrients to seawater (Holcomb et al., 2010; Chauvin et al., 2011; Tanaka et al., 2014). Nutrients basically enhance the photosynthesis of endosymbionts, which consequently help the animal host to perform calcification (Holcomb et al., 2010; Chauvin et al., 2011; Tanaka et al., 2014). In theory, endosymbionts in corals with a larger pool of animal host N such as *Porites* should be likely to acquire more nutrients from the host and to more steadily perform photosynthesis and thus calcification at low pH. This concept is supported by previous findings showing that massive *Porites* corals dominate reefs in naturally low pH areas due to volcanic carbon dioxide seeps (Fabricius et al., 2011). In addition, other coral genera that are tolerant of acidified seawater may also be efficient nutrient recyclers within their symbiosis (Schoepf et al., 2013). Thus, the degree of dependence on internally-circulating and externally-acquired N for corals may be an axis of bifurcation in coral survival in the near future. In the present study, a parallel model for the animal host was not possible because dissolved organic N fluxes between the animal host and the ambient sweater (Grover et al., 2008; Tanaka et al., 2009), zooplankton feeding rates (Houlbrèque and Ferrier-Pagès, 2009), and N_2 fixation by bacteria associated with corals (Lesser et al., 2004) were not possible to quantify with the current experimental design. Future research that included these additional measurements would be needed to more completely model the N metabolic pathways. At the end, the present approach of using a blend of stable-isotope labelling and modelling calculations could also be applied to the other organisms and ecosystems to determine other nutrient cycling systems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ecolmodel.2015.04.017

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