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# Translocation and conservation of organic nitrogen within the coral-zooxanthella symbiotic system of *Acropora pulchra*, as demonstrated by dual isotope-labeling techniques

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**Abstract**

Carbon (C) and nitrogen (N) metabolism of the hermatypic coral *Acropora pulchra* and its symbiotic algae (zooxanthellae) was investigated using $^{13}C$ and $^{15}N$ isotope tracers. *A. pulchra* was incubated in seawater containing $^{13}C$-labeled bicarbonate and $^{15}N$-labeled nitrate ($NO_3^-$) for 24 h (pulse period), and subsequently $^{13}C$ and $^{15}N$ isotopic ratios of the host coral and the zooxanthellae were followed in $^{13}C$- and $^{15}N$-free seawater for 2 weeks (chase period). Under our experimental condition of $NO_3^-$ (12 μM), C and N were absorbed by the coral–algal symbiotic system with the C:N ratio of 23 during the pulse period. Taking account of concentration dependence of $NO_3^-$ uptake rates determined by a separate experiment, C:N uptake ratios under supposed in situ $NO_3^-$ conditions (<1.0 μM) would be >3.0 times higher, if the photosynthetic rate did not change. During the pulse period, more than half of the absorbed $^{13}C$ and $^{15}N$ appeared in the host fraction in organic forms. $^{13}C$:$^{15}N$ ratio at the end of the pulse period was similar between the host and the algal fraction, suggesting that algal photosynthetic products were translocated to the host. It is also implied that C:N ratios of the translocated products change depending on N availability for the zooxanthellae. During the chase period, atom % excess (APE) $^{15}N$ of the zooxanthellae constantly declined, while that of the host slightly increased. Consequently, APE $^{15}N$ of the both fractions appeared to approach a common steady state value, suggesting that $^{15}N$ was recycled within the coral–algal symbiotic system. As for C, >86% of C photosynthetically fixed by the zooxanthellae accumulated in the host at the end of the pulse period, and had a turnover time of ca. 20 days for the host C pool during the following chase period. C:N ratios of organic matter newly synthesized with $NO_3^-$ exponentially declined and converged into 5.7 and 4.5 for the host and the zooxanthellae, respectively. This suggests that organic compounds of high C:N ratios such as lipids and carbohydrates were selectively consumed more rapidly than those of low C:N ratios such as proteins and nucleic acids.


*Keywords:* Carbon and nitrogen metabolism; Corals; Nitrate; Turnover; Zooxanthellae; $^{13}C$–$^{15}N$ dual label

## 1. Introduction

Corals and their symbiotic algae (zooxanthellae) have successfully adapted to low-nutrient tropical waters. Zooxanthellae reside in membrane-bound

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vacuoles in the coral gastrodermal cells and can obtain inorganic nutrients from not only ambient seawater but host waste products such as ammonium ($NH_4^+$). Host corals meet their energy sources with organic compounds translocated from autotrophic zooxanthellae as well as feeding of zooplankton by coral polyps. This versatility acquiring nutrients enables coral–algal symbioses to thrive in low nutrient water. However, metabolic mechanisms behind this symbiotic association still have not been well understood, especially for nitrogen (N), which sometimes comes to an issue of excess coastal enrichment of inorganic nutrients in recent years.

$NH_4^+$ and nitrate ($NO_3^-$) are two major inorganic nutrients in reef water, the level of which sometimes elevates due to river input, groundwater discharge (D'Elia et al., 1981; Umezawa et al., 2002). $NH_4^+$ uptake and its effect on symbiotic corals have been relatively well documented (e.g. Achituv et al., 1994; Muller-Parker et al., 1994; Koop et al., 2001). It is mostly accepted that both host corals and zooxanthellae possess anabolic enzymes, glutamine synthetase (GS) and NADPH-dependent glutamate dehydrogenase (NADPH-GDH) (Catmull et al., 1987; Yellowlees et al., 1994), although it is still not clear whether both fractions independently assimilate $NH_4^+$ or not (Roberts et al., 1999a; Lipschultz and Cook, 2002).

On the other hands, $NO_3^-$ assimilation by coral–algal symbiotic systems has been less investigated than $NH_4^+$, though it is sometimes a major form of exogenous N input to coral reefs (Umezawa et al., 2002). The essential enzymes for $NO_3^-$ assimilation, $NO_3^-$ and nitrite ($NO_2^-$) reductases, were detected once in zooxanthellae (Crossland and Barnes, 1977), and recently the appearance of $NO_3^-$ derived $^{15}N$ in zooxanthellae (Grover et al., 2003) indicated that $NO_3^-$ is actually incorporated into algal cells. However, the available data for mechanisms to assimilate $NO_3^-$ and its physiological effects are still very scarce and inconsistent (Miller and Yellowlees, 1989; Marubini and Davies, 1996; Ferrier-Pagès et al., 2001; Normader et al., 2003; Badgley et al., 2006). With the increase in environmental $NO_3^-$, algal density was increased (Marubini and Davies, 1996) or unchanged (Ferrier-Pagès et al., 2001; Normader et al., 2003), and host growth rate was unchanged (Marubini and Davies, 1996; Normader et al., 2003) or decreased (Ferrier-Pagès et al., 2001).

Given lack of $NO_3^-$ and $NO_2^-$ reductases in host corals, $NO_3^-$ can only be utilized by symbiotic algae, and therefore excess $NO_3^-$ input to the reef may lead to overgrowth of the symbiotic algae as previously reported (Marubini and Davies, 1996). However, N recycling hypothesis within coral–algal symbiotic systems (Falkowski et al., 1993) suggests organic N translocation from zooxanthellae to their host corals, which implies that the host may also benefit by increased $NO_3^-$ availability. In addition, it is suggested that the number of symbiotic algae is regulated by the host animal through digestion of algal cells or inhibition of algal overgrowth (Muscatine and Pool, 1979; Titlyanov et al., 1996), which also implies that $NO_3^-$ somehow affects the N metabolism of the host as well as its symbionts. More detailed information on $NO_3^-$ incorporation is, therefore, necessary to understand the nutritional relationship between zooxanthellae and host corals and to evaluate long-term physiological effects.

Consumption of photosynthetically fixed carbon (C) by corals has been often investigated with a $^{14}C$ technique. It is well established that a large part of C fixed by zooxanthellae is translocated to its host coral and consumed for respiration, growth, and organic matter excretion (Muscatine et al., 1984). Many of the previous studies have focused on ratios of the translocated C to total photosynthetically fixed C within relatively short intervals of a few days. However, there are few reports on the fate of stored C over longer periods (2 to 10 days; Cooksey and Cooksey, 1972; Crossland et al., 1980; Szmant-Froelich, 1981). Measuring a long-term metabolic rate of the C pool is necessary to know the rate at which photosynthetically fixed C is turned over through hermatypic corals.

The primary purpose of this study is to investigate the behavior of C and N in coral–algal symbiotic systems. $NO_3^-$ derived N and simultaneously fixed C were followed for 2 weeks in host corals and zooxanthellae using $^{13}C$–$^{15}N$ dual labeling techniques. Simultaneous application of $^{13}C$ and $^{15}N$ tracers has never been performed for coral symbioses but will provide important information on their difference in metabolic rates such as uptake ratios and turnover rates in each fraction. The present study shows how the host coral receive organic compounds synthesized with $NO_3^-$, which have a similar C:N ratio to the organics primarily produced by zooxanthellae.

## 2. Material and methods

The experiment was performed using the zooxanthellate coral, *Acropora pulchra*. They were collected on the reef flat of Shiraho Reef in Ishigaki Island (24° 21′–31′ N, 124° 4′–16′ E), Japan in Aug. 2004. The climate of the island is subtropical with the surface seawater temperature from 21 °C in Feb to 29.1 °C in July (Japan Meteorological Agency). Coral tips of ca.

4 cm in length were obtained from a single community. Collected corals were maintained for 5 days before the experiment in an outdoor aquarium, where pumped seawater surrounding the island was continuously supplied. This seawater includes low nutrients ($NO_2^-$: <0.1 μM, $NH_4^+$:<0.1 μM, $PO_4^{3-}$:<0.1 μM) except for $NO_3^-$, which ranges up to 2 μM. The $NO_3^-$ level in Shiraho Reef is usually between 0.2 and 1.0 μM, therefore this experimental condition did not significantly differ from the natural conditions.

Under natural sunlight, 16 coral tips of *A. pulchra* were incubated from 10:00 for 24 h in a closed aquarium (20 L) without stirring, where $^{15}NO_3^-$ (10 μM) and $H^{13}CO_3^-$ (0.4 mM) were added at the beginning of the incubation (pulse labeling period). Water volume in the aquarium was substantially large for the incubated corals (>100 times, v/v) and the temperature was adjusted to the reef water by flowing seawater outside the aquarium, so there were no evident stresses for the corals such as visible mucus secretion. Seawater subsamples were taken for DIC concentration, its isotopic ratio and nutrient levels at the beginning and the end of the pulse period. Samples for DIC and its isotopic ratio measurements were taken in 15 and 30 ml of a glass vial, respectively, which were sealed with a PTFE (polytetrafluoroethylene)-coated butyl rubber septum and an aluminum seal. They were fixed with saturated $HgCl_2$ to final concentrations of 0.03 and 0.07% (v/v), respectively. Samples for nutrient concentration were taken in 10 ml acrylic tubes and stored at −20 °C until analysis.

After one day labeling period (10:00), tips were transferred into another aquarium (100 L), where seawater was continuously exchanged with pumped seawater from the coast of the island (see above, chase period). Corals were sampled at 0, 6 and 24 h ($t$=0, 0.25 and 1 day, respectively) during the pulse period and at $t$=1.25, 2, 3, 4, 9, 14 days during the chase period. At each sampling time, two coral tips were sampled from the aquarium and processed as follows in order to separate zooxanthellate cells from the host coral tissue: After washing away $^{15}NO_3^-$ and $H^{13}CO_3^-$ from the surface of the coral by flowing fresh seawater, each coral tip was placed in a small glass vial containing 8.0 ml of GF/F filtered seawater, and the vial was sonicated with an ultrasonic cleaner (composite frequency of 24 and 31 kHz, 110 W, inner volume 3.4 L) for 10 min, which resulted in peeling of organic tissue from the skeleton (Piniak and Lipschultz, 2004). It was confirmed by microscopic enumeration that >98% of algal cells remained intact after this 10 min sonication. Tissue suspension was centrifuged at 750 ×$g$ for 5 min to separate zooxanthellae (pellet) from animal tissue (supernatant). The supernatant tissue suspension was stored at −20 °C. The precipitated algal pellet was washed and centrifuged twice with GF/F filtered seawater to minimize contamination by animal tissue, and stored at −20 °C until analysis. The coral skeleton was used for determining the coral surface area by aluminum foil method (Marsh, 1970).

To measure bulk C and N content of the host coral and the zooxanthella, other nine coral nubbins without the experimental incubation were prepared and divided in half for each. One part of a nubbin is processed with the ultrasonic cleaner in the same way as samples, and chl. *a* was extracted with methanol from the algal pellet. Another part of the nubbin was also treated with methanol without tissue extraction to determine whole chl. *a* concentration per unit surface area. Bulk C and N content was estimated using the chl. *a* recovery, supposing that extracted tissue with ultrasonic process and remaining tissue on the skeleton had a same ratio of chl. *a* to the organic tissue.

To investigate concentration dependence of $NO_3^-$ uptake rates, 8 glass bottles containing 700 ml of GF/F filtered seawater ($NO_3^-$:<0.1 μM, $NO_2^-$: undetectable, $NH_4^+$:<0.13 μM, $PO_4^{3-}$:<0.01 μM) were prepared. $NO_3^-$ was added in each bottle to get a final concentration of 1, 2, 4, 6, 10, 20, 30, 50 μM. One coral nubbin was incubated in each bottle, where seawater was stirred with a magnetic stirrer from 11:00 to 17:00 under natural sunlight. Subsamples for inorganic nutrient concentration were taken into 10 ml acrylic tubes at the beginning, 14:00 and 17:00, and they were immediately stored at −20 °C until analysis.

## 3. Analysis

Animal tissue suspension and algal pellets were thawed, and then pellets were resuspended in 8.0 ml of distilled water. 100–150 μl of sample solution was infiltrated into a pre-combusted glassfiber filter (Whatman GF/D, 10 mm in diameter). The filters were dried on a hot plate at 80 °C and treated with a vapor of 12 N HCl for 12 h to remove inorganic C. After evaporating extra HCl on the hot plate under vacuum, the filters were dried again in an oven at 50 °C for a few hours. Concentrations and isotope enrichments of C and N were measured by combination of CHN analyzer (Fisons; NA-1500) and IRMS (isotope-ratio mass spectrometer, Finnigan; Delta plusXP) connected via Conflo-III interface (Thermo-electron Co. Ltd.). $NO_3^-$ concentration and its isotopic ratio in animal tissue suspensions were determined with GC-NICI-MS (gas

chromatography/negative-ion chemical ionization/mass spectrometer) after derivatization by pentafluorobenzyl bromide (Tsikas, 2000; with a modification according to Miyajima et al., 2005).

Dissolved inorganic N (DIN: $NO_3^-$, $NO_2^-$, $NH_4^+$) and phosphorus (DIP: $PO_4^{3-}$) were quantified by using a nutrient analyzer AACS-III (BRAN+LUEBBE; Detection limit:<0.01 μM). The isotopic ratio of $NO_3^-$ was determined with GC-NICI-MS (see above).

DIC concentration was measured with a Shimadzu TOC 5000 instrument. The isotopic ratio of DIC was determined with GC-IRMS (Agilent Technolgy GC-6890+Finnigan DELTA plus XP; Miyajima et al., 1995). GC (Agilent Technolgy GC-6890) was equipped with a capillary column J and W GS-GASPRO (30 m length, 320 μm inner diameter) and oven temperature was kept at 80 °C.

## 4. Calculations

### 4.1. Newly synthesized organic C and N

The newly incorporated C during the pulse period ($C_{new}$) was calculated as follows:

$$C_{new} = (\text{bulk C amount} \times \text{APE}^{13}\text{C of a tissue sample}) / (\text{average } APE^{13}\text{C of } DIC), \quad (a)$$

where APE $^{13}$C is atom % excess $^{13}$C (= atom % $^{13}$C−1.108) and average APE $^{13}$C of DIC was determined from the values of the initial (15.4%) and the end (15.0%) of the pulse period. The newly incorporated N during the pulse period ($N_{new}$) was calculated in the same way:

$$N_{new} = (\text{bulk N amount} \times \text{APE}^{15}\text{N of a tissue sample}) / (\text{average APE}^{15}\text{N of } NO_3^-), \quad (b)$$

using 0.366% as the natural atom % $^{15}$N. Average APE $^{15}$N of $NO_3^-$ during the pulse period was 84.7% (initial 84.9% to 84.4% at the end).

### 4.2. Nitrate uptake rate

$NO_3^-$ uptake rate in the 2nd experiment to determine concentration dependence was calculated as follows: $NO_3^-$ uptake rate [μmol N cm$^{-2}$ h$^{-1}$]=$(C_i - C_e) \times V/(3 \times S)$, where $C_i$ and $C_e$ are the initial and end of $NO_3^-$ concentration [μM], respectively, during the period of 3 h. $V$ and $S$ represent a water volume [L] and a coral surface area [cm$^2$]. An obtained value of the uptake rate was considered as the one when $NO_3^-$ concentration was an average of $C_i$ and $C_e$.

### 4.3. Statistical analysis

Tissue data are expressed as an average value ± a data range of duplicates. Exponential regressions for temporal changes in APE during the chase period (2–14 days of the incubation) were conducted with Sigma Plot 8.02 (SPSS Inc.).

## 5. Results and discussion

### 5.1. $NO_3^-$ incorporation

A few previous studies suggested that $NO_3^-$ was taken up by zooxanthellae, and therefore only affects algal metabolism. To investigate whether or not $NO_3^-$ derived N is transferred to host corals through algal metabolism, we focused on accumulation rates of $^{15}$N in both fractions of the hosts and zooxanthellae during the pulse period.

During the pulse period, $^{15}$N appeared in both the host coral and the zooxanthellae within 6 h (Fig. 1). APE $^{15}$N was 4.7 times higher in the zooxanthellae (7.2%) than the corals (1.5%) at the end of the pulse period. Considering that the percent of the zooxanthellate N biomass (4.4±0.4 μmol N cm$^{-2}$) to the host N biomass (62.9±5.5 μmol N cm$^{-2}$) was 7.0%, excess $^{15}$N amount which appeared in the host accounted for 75% of totally

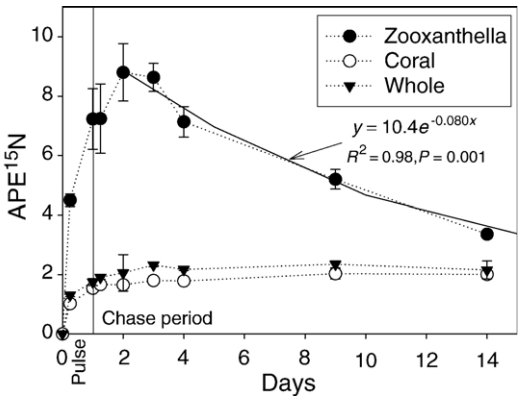


Fig. 1. Temporal changes in APE $^{15}$N of the zooxanthella, the host coral and the whole coral–algal tissue (average±range of duplicates) during the pulse-chase experiment. The exponential curves are regression for the data between 2 and 14 days. During the pulse period, it was nighttime from 0.38 to 0.83 days.

incorporated excess $^{15}N$ (1.3 μmol N $cm^{-2}$) at the end of the pulse period.

The enzymes for incorporating $NO_3^-$, $NO_3^-$ and $NO_2^-$ reductases, were only found in zooxanthellae once by Crossland and Barnes (1977). Grover et al. (2003) observed with a $^{15}N$ technique that APE $^{15}N$ and also $^{15}N$ amount in zooxanthellae increased greater than host corals after 12 h incubation with 0.3 and 3 μM $^{15}NO_3^-$. These suggest that zooxanthellae are the initial site to take up $NO_3^-$ and synthesize it into organic N. In our study, while APE $^{15}N$ of the zooxanthellae during the pulse period increased more rapidly than that of the host (Fig. 1), more than half of the incorporated $^{15}N$ was found in the host fraction. Here, there was a question of whether this $^{15}N$ in the host was in the form of organics or $NO_3^-$. It has been observed that $NO_3^-$ concentration in the haemolymph of the symbiotic invertebrate *Tridacna gigas* increased within a few hours under 20 μM $NO_3^-$ (Shepherd et al., 1999). In our study, however, $NO_3^-$ was not detected in the host fraction throughout the whole labeling period (<0.01 μmol N $cm^{-2}$), indicating that most of the excess $^{15}N$ in the host was organic N. Therefore, organic N, which was synthesized from $NO_3^-$ by the zooxanthellae, is suggested to be translocated to the host during the pulse period.

It has been well recognized that zooxanthellae release a large part of photosynthetic products to their host under N-deficient conditions, and these released products have been believed to have high C:N ratios (Muscatine et al., 1972). In contrast, under sufficient N conditions for zooxanthellae, organic N translocation to the host has long been controversial (Lewis and Smith, 1971; Falkowski et al., 1993; Markell and Trench, 1993; Hawkins and Klumpp, 1995; Wang and Douglas, 1998; Roberts et al., 1999a,b; Lipschultz and Cook, 2002). Because $NO_3^-$ and $NO_2^-$ reductases have not been found in host corals (Crossland and Barnes, 1977; Muscatine et al., 1984), it was considered that $NO_3^-$ was exclusively utilized by zooxanthellae. However, our results showed that the zooxanthellae release organic N, synthesized from $NO_3^-$ through photosynthetic activity, to the host fraction in a short term.

C:N ratios of newly incorporated organic matter ($C_{new}$:$N_{new}$) also supported the above observation (Fig. 2). $C_{new}$:$N_{new}$ in the host indicates C:N ratios of newly translocated organic matter from the zooxanthellae, and it was quite similar to the $C_{new}$:$N_{new}$ ratio found in the zooxanthellate cells at the first sampling time (Fig. 2; 23.2 ± 1.3 and 21.3±1.2, respectively). This suggests that organic compounds the host received had a similar composition to the organics primarily produced by zooxanthellae. In other words, the

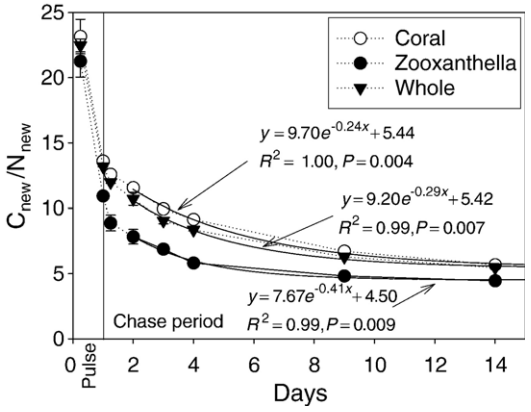


Fig. 2. Temporal changes in C:N ratios of newly produced organic C and N (average±range of duplicates). The exponential curves are regression for the data between 2 and 14 days. During the pulse period, it was nighttime from 0.38 to 0.83 days.

zooxanthellae constantly translocated a large part of photosynthates, even though N was contained in them. Therefore, translocated products could have variable C:N ratios depending on N availability for zooxanthellae. Muscatine et al. (1984) also showed changes in C:N ratios of translocated products to the host coral, though it was caused by different irradiance of their habitat and consequent change in C:N uptake ratios for zooxanthellae.

Though translocation of organic N, derived from $NO_3^-$, from the zooxanthellae to the host has been suggested in our study, the partitioning ratio of $^{15}N$ (algal $^{15}N$/coral $^{15}N$=0.31–0.33 during the pulse period) was significantly higher than the ratio of biomass N (algal N/coral N=0.070; see above). This implies that if the high $NO_3^-$ concentration continued and sufficient light was also provided, it might enhance the algal growth rather than the host as compared with N-depleted conditions, and subsequently might disrupt the inherent balance of the host and the zooxanthellae causing algal population increase (Marubini and Davies, 1996).

### 5.2. N recycling in coral–algal symbiotic systems

After $^{15}N$ acquisition by both the host coral and the zooxanthellae, N turnover was followed under natural $^{15}N$-free seawater. During the chase period, APE $^{15}N$ of the zooxanthellae increased during the first 1 day and then it clearly declined from 8.8% to 3.4% for 12 days (Fig. 1), while that of the host slightly increased from 1.5% to 2.0%. The occurrence of this period of the algal APE $^{15}N$ to reach maximum after the pulse period suggests that a part of $^{15}N$ that had already been

incorporated by the algae was not instantaneously synthesized into steady organic N but remained for a while as unstable compounds such as $NH_4^+$ and volatile amines. These unstable intermediates must have been lost during sample preparation for the analyses (Cooksey and Cooksey, 1972).

The clear decline of APE $^{15}N$ of the zooxanthellae during the chase period suggests: (1) preferential transfer of $^{15}N$-enriched products from the zooxanthellae to the host (Falkowski et al., 1993; Hawkins and Klumpp, 1995; Roberts et al., 1999a) as mentioned in the above section, and/or (2) algal uptake of isotopically lighter $NH_4^+$ excreted from the host coral (Rahav et al., 1989; Szmant et al., 1990; Lipschultz and Cook, 2002; Piniak et al., 2003), and/or (3) DIN uptake from ambient seawater.

APE $^{15}N$ increase in the host coral can occur by three processes: (1) transfer of $^{15}N$-labeled products from zooxanthellae, and/or (2) host excretion of isotopically lighter $NH_4^+$, and/or (3) host digestion of algal cells (Muscatine and Pool, 1979; Titlyanov et al., 1996). Muscatine and Pool (1979) and Titlyanov et al. (1996) investigated into regulation of the symbiotic algal number by their host, where one of the mechanisms was digestion of increased algal cells by the host. Although previously reported doubling times of zooxanthellae are relatively low (e.g. 0.040–0.082 $day^{-1}$ for *Seriatopora hysrix* and 0.028–0.032 $day^{-1}$ for *Stylophora pistillata*; Hoegh-Guldberg and Smith, 1989; 0.077 $day^{-1}$ for *Acropora cervicornis*; Szmant et al., 1990), it would be stimulated by sufficient nutrient supply (e.g. 0.43 $day^{-1}$ for *Montipora verrucosa*; Chang et al., 1983). Since our labeling experiment was conducted under relatively N sufficient condition, the excess algal cells, which proliferated with N uptake during the pulse period, might have been digested by the host during the following chase period.

N recycling in coral–algal symbiotic systems has been a controversial mechanism (Falkowski et al., 1993; Roberts et al., 1999a), where the most equivocal process could be translocation of organic N from zooxanthellae to their host. If N recycling occurred in our experiment, simultaneous observations of algal APE $^{15}N$ decline, host APE $^{15}N$ increase and their approach to a steady-state value should be expected as time passed. On the other hand, in the light of conservation concept of coral symbiosis (e.g. Wang and Douglas, 1998), host corals and zooxanthellae each prevent acquired N from being lost, and therefore their APE $^{15}N$ would keep constant or decrease for both fractions depending on external N availability. Although the decrease in algal APE $^{15}N$ might be not sure whether $NH_4^+$ uptake from the host coral or DIN uptake from ambient seawater (see below), the increase in the host APE $^{15}N$ suggests that N was not conserved at least within the algal fraction.

While APE $^{15}N$ of the zooxanthella significantly declined (5.4% during 2–14 day), the increase in APE $^{15}N$ of the host during the corresponding period was small (1.65 to 2.00%). However, considering the percent of algal N biomass to the host (7.0%; see above), the observed 5.4% decrease in the algal fraction could make an increase in the host APE $^{15}N$ as little as 0.38% on the supposition that the whole N biomass was constant. The value corresponded well to the actual increase in the host APE $^{15}N$ (0.35%). Additionally, APE $^{15}N$ of the whole symbioses was almost constant during the chase period (2.2% on average during 2–14 days), suggesting that most of the incorporated $^{15}N$ was retained in the coral–algal symbiotic system for at least 2 weeks.

Algal APE $^{15}N$ decrease might be caused by DIN uptake from ambient seawater as mentioned above. Supposing that the decline was completely caused by external $NO_3^-$ uptake, 2.3 μM is constantly necessary in the seawater, using the equation in Fig. 3. This is nearly the upper limit of $NO_3^-$ contained in the pumped seawater which was used in the experiment. Therefore, DIN uptake from ambient seawater could be not the only N source to reduce algal APE $^{15}N$. Both N sources, i.e. $NH_4^+$ from the host coral and $NO_3^-$ from seawater, can be considered for the dilution.

The algal turnover rate estimated from exponential curve was 0.080 $day^{-1}$ (13 days; Fig. 1). In the meanwhile, the host APE $^{15}N$ change was quite slow. Supposing that the host N biomass was constant during the chase period, excess $^{15}N$ increase in the host is calculated to be 0.022 μmol $cm^{-2}$ $day^{-1}$ and therefore

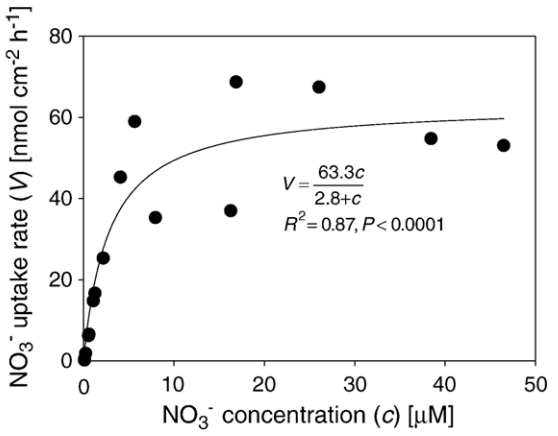


Fig. 3. The relationship between $NO_3^-$ concentration ($c$) and its uptake rate per unit surface area of the coral ($V$). Data are fitted to the Michaelis–Menten kinetic curve.

0.027 μmol $cm^{-2}$ $day^{-1}$ as $N_{new}$. In reality, not only $N_{new}$ but other organic N produced before and after the pulse period in the algal cells must have been translocated to the host. Roughly, given 10 times of $N_{new}$ from the aspect of the chase period, 0.3 μmol N $cm^{-2}$ $day^{-1}$ could be translocated to the host. It gives turnover time of 210 days for the host N pool. This difference in turnover time between the zooxanthellae and the host imply that the zooxanthellae grow faster than the host and seriously digested or that there is a turnover pool in the zooxanthellae of small metabolites that temporarily stores small organic metabolites synthesized from external DIN and $NH_4^+$ excreted by the host and the zooxanthellae ship them back to the host without involvement of macromolecular synthesis.

From the algal APE $^{15}N$ at the end of the pulse period (7.2%), the rate of $NO_3^-$ assimilation ($N_{new}$) in the zooxanthellae was calculated with the Eq. (b) to be 0.38 μmol N $cm^{-2}$ $day^{-1}$ at our $NO_3^-$ condition of 12 μM. Considering the loss of unstable $^{15}N$ during sample preparation (see above), the value would increase up to 0.50 μmol N $cm^{-2}$ $day^{-1}$. Because $NO_3^-$ uptake rate is strongly dependent on its concentration (Fig. 3), the uptake rate under supposed in situ levels (<1.0 μM) would be <1/3 of the pulse period, i.e. <0.17 μmol N $cm^{-2}$ $day^{-1}$ into the zooxanthellae. On the other hand, the host N turnover (210 days) implies its $NH_4^+$ excretion of 0.3 μmol N $cm^{-2}$ $day^{-1}$, which exceeds external $NO_3^-$ uptake by the zooxanthellae. Therefore, $NH_4^+$ excretion from the host could be an important N source for the symbiotic algae.

### 5.3. C turnover

C partitioning ratios between the zooxanthellae and the host were measured, and subsequent C turnover in each fraction was estimated with a $^{13}C$ technique. At the first sampling time (6 h, 16:00) in the pulse period, APE $^{13}C$ of the zooxanthellae (1.51%) was 2.7 times higher than the host coral (0.56%) (Fig. 4). Considering that the percent of the algal C biomass (38±3 μmol C $cm^{-2}$) to the host (495±40 μmol C $cm^{-2}$) is 7.7% per unit coral surface area, 83% of the totally synthesized organic C ($C_{new}$) accumulated in the host for 6 h. At the end of the pulse period, APE $^{13}C$ of the zooxanthellae (1.10%) was 2.2 times higher than the host (0.51%), which means that 86% of $C_{new}$ accumulated in the host coral.

A part of $C_{new}$ could have been continuously consumed for the host and algal respiration during the pulse period. Assuming that the respiratory ratio of the zooxanthellae to the host was identical to the C biomass ratio (algal C/coral C=7.7%), >86% of photosynthetically fixed C could have been actually translocated from the zooxanthellae to the host. Not only labeled organic C ($C_{new}$) but unlabeled, previously stored organic C might also have been translocated during the pulse period, which leads to underestimation of the proportion of the translocated $C_{new}$ to the photosynthetically fixed C. However, this unlabeled fraction can be considered minor because newly fixed C is observed with a $^{14}C$ technique to be largely consumed for respiration within first 24 h (Cooksey and Cooksey, 1972; Crossland et al., 1980). Considering that respiration during daytime was stimulated compared to night (6 times; Kühl et al., 1995), most of the $C_{new}$ could be consumed immediately after produced in the light. Therefore, the remaining $C_{new}$ at the end of the pulse period was probably materials transferred to a stored C pool in each fraction. The decrease in APE $^{13}C$ during the chase period would mostly reflect a change in isotopic ratios of their C pools assigned to biomass production.

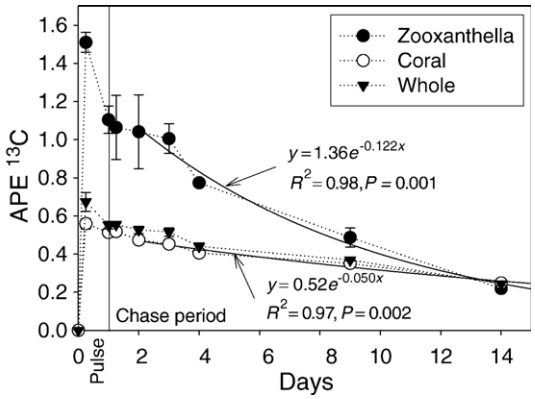


Fig. 4. Temporal changes in APE $^{13}C$ of the zooxanthella, the host coral and the whole coral–algal tissue (average±range of duplicates) during the pulse-chase experiment. The exponential curves are regression for the data between 2 and 14 days. During the pulse period, it was nighttime from 0.38 to 0.83 days.

The turnover rate of this stored C pool of the zooxanthellae was 0.12±0.01 $day^{-1}$ (or turnover time of 8.2±1.0 days; Fig. 4). Using the algal C biomass (38 μmol C $cm^{-2}$), the rate of net algal biomass production is calculated as 4.6 μmol C $cm^{-2}$ $day^{-1}$, supposing that C in the zooxanthellae was completely mixed. Assuming that 86% of $C_{new}$ accumulated in the host per day as discussed above, gross C flux from the zooxanthellae to the host could be 28 μmol C $cm^{-2}$ $day^{-1}$. C turnover rate for the host C pool (495 μmol C $cm^{-2}$) is, therefore, calculated to be 0.057 $day^{-1}$ (18 days). If complete mixing did not occur in the algal cells, that is to say, there was a turnover pool in the zooxanthellae of

small metabolite as mentioned about N (see above), 4.6 μmol C $cm^{-2}$ $day^{-1}$ of the algal biomass production could be overestimated. In this case, C flux from the zooxanthellae to the host could be <28 μmol C $cm^{-2}$ $day^{-1}$ and C turnover rate for the host C pool are >18 days.

On the other hand, $C_{new}$ accumulated in the host at the end of the pulse period was calculated to be 18 μmol C $cm^{-2}$ $day^{-1}$, which seems to be less than the estimated C flux from the zooxanthellae during the chase period (28 μmol C $cm^{-2}$ $day^{-1}$). One possibility is that there existed sufficient $NO_3^-$ during the pulse period, so the zooxanthellae allocated photosynthetic products to themselves larger than the allocation ratio of the chase period. Another is that photosynthetic C fixation rate declined due to preferential use of energy to take up and assimilate $NO_3^-$ in algal cells under sufficient $NO_3^-$ supply (Lean et al., 1982; Turpin, 1991). Both of them could have reduced the $C_{new}$ accumulation in the host during the pulse period.

### 5.4. C and N differences in metabolic rates

The merit to apply dual labeling techniques is to assess C–N differences in various stages. In this section, we bring up C:N uptake ratios and subsequent C–N consumption rates on metabolic processes. To our knowledge, this study is the first application of $^{13}C$–$^{15}N$ dual labeling techniques for hermatypic corals.

C:N ratios of accumulated organic C and N in the zooxanthellae and the host coral were 21.3±1.2 and 23.2±1.3, respectively, after 6 h of the pulse period (Fig. 2). These are not significantly different, and the average C:N uptake ratio for the whole coral–algal symbiotic system is calculated to be 23. It can be proposed that the used *A. pulchra* removed DIC and $NO_3^-$ from seawater for organic production with this ratio under sufficient $NO_3^-$ supply, though the obtained value could be underestimated some because a part of $C_{new}$ had been already consumed for respiration (see above).

C:N uptake ratios by phytoplankton drastically vary with environmental conditions such as DIN concentration (Lean et al., 1982), nutritional status of algal cells (Turpin, 1991), and irradiance and cell size (Frenette et al., 1998). Likewise, the $NO_3^-$ uptake rate of *A. pulchra* strongly depended on its concentration (Fig. 3). Because in situ $NO_3^-$ level is usually <1.0 μM, C:N uptake ratios, and also C:N ratios of translocated organic matter from the zooxanthellae to the host, would be >3.0 times higher than the observed value (23), assuming that the photosynthetic C fixation rate did not change. This is consistent with the estimated C:N ratio of translocated organic matter from the zooxanthellae to the host during the chase period, i.e. >90, which was calculated from the estimated C and N flux (28 and 0.3 μmol $cm^{-2}$ $day^{-1}$, respectively; see above).

Changes in $C_{new}$:$N_{new}$ during the chase period show the difference in metabolic rates between simultaneously incorporated C and N (Fig. 2). Clear exponential decay indicates that organic C was consumed faster than N for both the host coral and the zooxanthellae. This must be an adaptive character of organisms living in oligotrophic environments such as coral reefs, where nutrients such as N and P are relatively difficult to acquire compared to C. Organic C is easily obtained for symbiotic corals by algal photosynthesis. This character might not be seen for non-symbiotic corals, which cannot obtain organic C as readily as symbiotic ones and consequently consume low C:N compounds for respiration (Szmant et al., 1990).

The $C_{new}$:$N_{new}$ ratios drastically declined during the first night of the pulse period, and then exponentially decreased at a rate constant of 0.24 and 0.41 $day^{-1}$ for the host coral and the zooxanthellae, respectively (Fig. 2). This implies that high C:N compounds such as lipids and carbohydrates were selectively consumed as time passed. In contrast, organics with low C:N ratios (5.4 and 4.5 for the host and the zooxanthellae, respectively) such as proteins seemed to persist for at least 2 weeks. Assuming that APE $^{15}N$ of the whole coral–algal symbiotic system was constant at 2.2% during the chase period (average of 2–14 days; Fig. 1) and that $N_{new}$ was completely retained in the system during that time, 24–41% of the $C_{new}$ at the first sampling point ($t$=0.6) were retained for at least 2 weeks as low C:N compounds, considering the possibility that APE $^{15}N$ of the whole ranged 1.3–2.2% at '$t$=0.6.'

## 6. Future problems

In this study, $NO_3^-$ concentration was set at one level in the pulse period and C was synthesized into organics under sufficient N supply. C partitioning ratios into various organic components such as structural proteins, storage lipids and excretory mucus materials in coral and algal tissue might vary depending on N availability. As C turnover rates are likely to be different among respective components, the average turnover of newly synthesized organic C may also be dependent on ambient N status. Thus, C and N turnover and retention efficiency in each fraction should be elaborated further under different nutrient conditions. It will also provide information on how C and N dynamics through coral

colonies are affected by increasing nutrient enrichment in coastal environments.

## 7. Conclusion

Pulse-chase experiments using $^{13}C$ or $^{15}N$ have been employed to reveal gross fluxes of C or N from and to organisms by monitoring isotope dilution or enrichment in relevant pools with time. In this study, by applying $^{13}C$–$^{15}N$ dual labeling techniques, we succeeded in directly demonstrating how conservatively the coral-zooxanthella symbiotic system could exchange and metabolize N relative to C. This dual labeling technique should be a useful method to analyze the relationship between C and N for their uptake, metabolism and release rates. Moreover, the technique will give important information on a metabolic relationship in symbiotic systems such as hermatypic corals and zooxanthellae, where complicated fluxes in various forms of materials occur. In this study, it is concluded that:

During algal photosynthesis, >70% of organic N synthesized from $NO_3^-$ by zooxanthellae was translocated to their host coral as soon as produced. The organic matter translocated to the host was similar, at least in C:N ratios, to that remaining within the algal cells. However, $NO_3^-$ derived N accumulated more in the zooxanthellae compared to the algal:coral N ratio, suggesting a decrease in the translocated organic matter to the host with increasing $NO_3^-$ availability for the zooxanthellae.

Once incorporated, organic compounds of higher C:N ratios were consumed more rapidly than those of lower C:N ratios in both of the host coral and zooxanthellae. The difference in C–N metabolic rates was most prominent during the first night after incorporation, and then exponentially diminished. $NO_3^-$ derived organic N was retained for >2 weeks with simultaneously incorporated C as organic compounds of low C:N ratios in both fractions. Thus, the coral-zooxanthella symbiotic system could be highly conservative for N.

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