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Nutrient cycling in early coral life stages: *Pocillopora damicornis* larvae provide their algal symbiont (*Symbiodinium*) with nitrogen acquired from bacterial associates

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Abstract

The waters surrounding coral reef ecosystems are generally poor in nutrients, yet their levels of primary production are comparable with those reported from tropical rain forests. One explanation of this paradox is the efficient cycling of nutrients between the coral host, its endosymbiotic alga *Symbiodinium* and a wide array of microorganisms. Despite their importance for the animals' fitness, the cycling of nutrients in early coral life stages and the initial establishment of partnerships with the microbes involved in these processes has received little scrutiny to date. Nitrogen is an essential but limited nutrient in coral reef ecosystems. In order to assess the early nutrient exchange between bacteria and corals, coral larvae of the species *Pocillopora damicornis* were incubated with two coral-associated bacteria (*Alteromonas* sp., or *Vibrio alginolyticus*), prelabeled with the stable nitrogen isotope ^{15}N. The incorporation and translocation of nitrogen from *Vibrio*- and *Alteromonas* bacteria into *P. damicornis* coral larvae and specifically into the coral-symbiotic *Symbiodinium* were detected by nanoscale secondary ion mass spectrometry (NanoSIMS). A significant increase in the amount of enriched ^{15}N (two to threefold compared to natural abundance) was observed in *P. damicornis* larvae within 8 h of incubation for both bacterial treatments (one-way ANOVA, $F_{5,53} = 18.03$, $P = 0.004$ for *Alteromonas* sp. and $F_{5,53} = 18.03$, $P = 0.0001$ for *V. alginolyticus*). These findings reveal that coral larvae acquire nutrients previously taken up from the environment by bacteria. The additional nitrogen may increase the survival rate and fitness of the developing coral and therefore contribute to the successful maintenance of coral reefs.

Introduction

Nitrogen is an essential element for life on Earth due to its requirement for cell maintenance, growth, and functioning. Although nitrogen is the most abundant gas in the Earth's atmosphere, atmospheric nitrogen is not biologically available to most organisms. In oligotrophic environments, such as coral reef systems, mutualistic symbiotic relationships with microorganisms serve as a major source of new nitrogen for invertebrates. Bacteria convert inert atmospheric nitrogen into compounds such as ammonium that can then be readily used by other organisms (Guerinot and Patriquin 1981; Lesser et al. 2004, 2007; Taylor et al. 2007). These coral reef symbiotic associations have coevolved and can influence the ecology of the host through nitrogen cycling processes like nitrogen fixation, nitrification, denitrification, and anaerobic ammonium oxidation (ANAMMOX) (Beman et al. 2007; Kneip et al. 2007; Mohamed

et al. 2010). Some functional genes allowing for these processes have been identified in the coral holobiont through metagenomic analyses (Wegley et al. 2007; Kimes et al. 2010).

Corals can use ammonium as an inorganic nitrogen source (Yellowlees et al. 2008; Sheppard et al. 2009) and Pernice et al. (2012) showed recently that both coral cells and their symbiotic dinoflagellates (*Symbiodinium*) have the capacity to rapidly assimilate this nitrogen compound from seawater. In addition to *Symbiodinium*, corals host a wide range of microscopic organisms including bacteria, fungi, Archaea, and viruses, which together form a collaborative consortium known as the coral holobiont (Rohwer et al. 2002; Rosenberg et al. 2007). Tight nutrient cycling within this complex symbiotic system may allow the coral holobiont to flourish in the nutrient poor waters coral reefs inhabit (Fiore et al. 2010).

Our understanding of the onset, establishment and persistence of coral-microbial symbiosis is limited. A recent study revealed the establishment of coral-associated bacterial partnerships in *Pocillopora meandrina* in late developmental stages of larvae (after 76 h) and suggested a functional role of bacteria in processes specific to this life stage, such as coral settlement (Apprill et al. 2009). Another study observed bacterial communities in broadcast spawning corals only after metamorphosis and settlement of the host (Sharp et al. 2010) and the first evidence of vertical transmission of bacteria in brooding corals has been recently reported for the coral *Porites astreoides* (Sharp et al. 2011). Furthermore, it has been suggested that adult corals may release beneficial bacteria with their offspring, a strategy more specific than random horizontal acquisition of bacteria from seawater though less specific than the intergenerational vertical transfer of bacteria via the egg or sperm (Ceh et al. 2013). Apart from this handful of studies, there is a very limited understanding of which bacteria associate with corals in early life stages, and more importantly which, if any, functional roles these communities may have.

In this study, we investigated early interactions between two species of bacteria and larvae of *Pocillopora damicornis*, a brooding coral species that releases competent larvae ready to settle within hours. Nanoscale resolution secondary ion mass spectrometry (NanoSIMS) has previously been employed to image and quantify the metabolic exchange between symbionts, such as nitrogen fixation and transfer by individual bacteria within shipworm host cells (Lechene et al. 2007) and in diatom-cyanobacteria symbiosis (Foster et al. 2011). In this study we used NanoSIMS to follow the incorporation of ^{15}N into bacteria cells and the uptake and passage of labeled nitrogen (^{15}N) from bacteria into coral larvae and *Symbiodinium*.

Materials and Methods

Bacteria isolation & identification

Pocillopora damicornis colonies were collected in March 2009, from Coral Bay, Ningaloo Reef (23°07′S, 113°07′E) and kept in aquaria for 5 days until larvae were released. Planula larvae were collected, washed three times in 0.2 μm filtered seawater (FSW) and crushed before spread plating on Marine Agar (Marine Agar 2210, Difco, Franklin Lakes, NJ). Two morphologically dominant bacterial colonies were isolated to purity and identified by 16S rRNA gene sequencing as *Alteromonas* sp. (accession numbers KF158807-8) and by multilocus sequence typing as *Vibrio alginolyticus* (accession numbers KF158809-10). DNA was extracted from cultures grown overnight in Marine Broth (MB, Difco) at 28°C using a Qiagen (Hilden, Germany) Soil Kit according to the manufacturer's instructions. The 16S rRNA gene of each isolate was amplified using the bacterial-specific primers 63f and 1387r (Marchesi et al. 1998). The three protein coding loci *rpoA* (RNA polymerase a-subunit), *pyrH* (uridylate kinase), and *recA* were used for multilocus sequence identification of the *Vibrio* strain, amplified by PCR, and sequenced according to the methods outlined in Thompson et al. (2007). Cultures were stored in 30% v/v glycerol at −80°C. Obtained sequences were compared to sequences in the GenBank database (Altschul et al. 1997).

Bacteria labeling with stable isotope ^{15}N

Bacterial isolates were grown in ^{15}N (labeled) and unlabeled (control) Celtone Base Powder media (Cambridge Isotope Laboratories, Andover, MA) made up in artificial seawater (ASW). The ^{15}N label was present in the form of ammonium a compound that is naturally available in seawater. Together with the ammonium derived from FSW, ^{15}N-labeled ammonium constituted the only nitrogen source in the growth medium (final concentration, 1 g/L). After 24 h at 28°C bacteria cells were washed three times by resuspending the pellet in 200 mL ASW and centrifuging at 3000 g. After the washing procedure cells were either fixed to confirm the uptake and incorporation of enriched ^{15}N (Fig. S1) or used in coral larval experiments.

Experimental setup

Coral colonies were collected from Orpheus Island (Great Barrier Reef, Australia) and transferred to the Australian Institute of Marine Science outdoor aquarium facilities. *P. damicornis* larvae were collected just after their release, washed three times in 0.2 μm FSW (Millipore, Millex-GP,

Billerica, MA), and transferred to 0.2 μm FSW. To allow for coral larvae to acclimatize to experimental conditions, larvae were kept for 12 h in sterile 6-well titer plates (BD Falcon, untreated polystyrene, Franklin Lakes, NJ) at 27°C water temperature and 12:12 light:dark diurnal pattern before bacteria were added to wells at a density of 1×10^6 cells/mL FSW. Twenty larvae per well were coincubated with ^{15}N-labeled *Alteromonas* sp., or *Vibrio alginolyticus* for 8 and 24 h. Incubations with unlabeled *Alteromonas* sp., or *Vibrio alginolyticus* bacteria served as controls. The viability of the bacteria cells in the treatments was assessed through re-isolation, quantification (dilution plating), and gene-based identification (16S rRNA gene similarity) after each treatment.

NanoSIMS sample preparation and analysis

Bacteria and coral larvae for each treatment were sampled and fixed (2.5% glutaraldehyde, 1% paraformaldehyde in ASW), rinsed in phosphate-buffered saline (PBS), and dehydrated in a graded series of ethanol (50%, 70%, 90%, 100%, dry 100%) and dry acetone. After the dehydration samples were infiltrated, embedded in resin (26 g Araldite, 24 g DDSA, 1.4 g BDMA; 2 acetone:1 resin, 1 acetone:2 resin, pure resin), and cured for 36 h at 60°C. Sections of 600 nm thick were cut from resin blocks with glass knives on a Leica EM UC6 Ultramicrotome (Leica Microsystems, Wetzlar, Germany), mounted on silicon wafer disks, and coated with 5 nm gold for NanoSIMS analysis. The distribution of the secondary ion species $^{12}C^{15}N^-$ and $^{12}C^{14}N^-$ in fixed bacteria cells and the sectioned coral larval tissue was mapped and measured using the Cameca NanoSIMS 50 (Gennevilliers, France) ion microprobe at The University of Western Australia. All samples were compared to a yeast standard with known natural $^{15}N/^{14}N$ abundance. The incorporation of ^{15}N tracers was measured by comparing two simultaneously acquired images mapping $^{12}C^{15}N^-$ and $^{12}C^{14}N^-$, and determining the $^{15}N/^{14}N$ compared to the natural isotopic abundance (Lechene et al. 2007).

Images of the samples were acquired at a 256 × 256 pixel resolution, across an area of 20 μm for bacteria, 40 μm for detailed images of *Symbiodinium,* endodermis, and ectodermis regions, and 80 μm for images assembling the mosaic overview (Fig. 1). A beam current of 1pA, corresponding to a beam diameter of approximately 100 nm, was applied. Images were processed and analyzed using ImageJ together with the OpenMIMS data analysis software plugin (http://www.nrims.hms.harvard.edu/software.php). Only images acquired at 20 μm and 40 μm were used for data analysis. Individual images were corrected pixel-by-pixel for 44 ns detector dead time, and data were extracted from the pixels within manually drawn regions-of-interest (ROI). Two images were analyzed to determine the ^{15}N enrichment within the bacteria cultures where ROIs were drawn around individual bacteria. For each coral treatment, three different images were analyzed and each image was separated into three ROIs to include the *Symbiodinium* and the tissue types, endodermis and ectodermis.

Statistical data analysis

All statistical analyses were performed using the free statistical software PAST (Ryan et al. 1995). A one-way ANOVA and Tukey multiple comparison test were conducted to compare differences in $^{15}N/^{14}N$ between (1) bacteria and controls, (2) treatments, and (3) the tissue types ectodermis, endodermis, and *Symbiodinium* within treatments. Data met all the test assumptions.

Results

The uptake and incorporation of the stable ^{15}N isotope from the growth medium into *V. alginolyticus* and *Alteromonas* sp. bacteria were confirmed by NanoSIMS analysis (Fig. S1, Table S1). Both bacterial strains incorporated ^{15}N from the growth medium with a higher level of enrichment for *V. alginolyticus* (^{15}N Atom% 93.3) compared to *Alteromonas* sp. (^{15}N Atom% 88.4).

The mosaic of individual NanoSIMS images provided an overview of the location and distribution of ^{15}N nitrogen enrichment through the cross section of a *P. damicornis* larva incubated with *Alteromonas* bacteria (Fig. 1). ^{15}N hotspots were clearly evident in the coral symbiont *Symbiodinium* and in the outer ectodermis (Fig. 2). Hotspots including the *Symbiodinium*, endodermis, and ectodermis tissues imaged in the *P. damicornis* larvae were further investigated with higher magnification and with longer counting times and the $^{15}N/^{14}N$ ratios were determined for the different tissue types after 8 and 24 h of incubation with each bacterial treatment (Fig. 3, Table S1). ^{15}N levels increased significantly in larvae treated with *Alteromonas* sp. (^{15}N Atom% 0.675, one-way ANOVA, $F_{5,53}$=18.03, $P = 0.004$) and *V. alginolyticus*, (^{15}N Atom% 0.861, $F_{5,53} = 18.03$, $P = 0.0001$) compared to controls within 8 h (^{15}N Atom% 0.378). All analyzed tissue types show ^{15}N enrichment, with the highest values in *Symbiodinium* followed by the endodermis and the ectodermis. Larvae treated with *V. alginolyticus* demonstrated significantly higher ^{15}N levels within *Symbiodinium* (^{15}N Atom% 1.132) than in the endodermis (^{15}N Atom% 0.771, $F_{2,8} = 13.51$, $P = 0.018$) and the ectodermis (^{15}N Atom% 0.681, $F_{2,8} = 13.51$, $P = 0.007$).

After 24 h of incubation, ^{15}N levels in *Symbiodinium* decreased in *Alteromonas* sp.–treated larvae (^{15}N Atom% 0.448) but not in larvae treated with *V. alginolyticus*

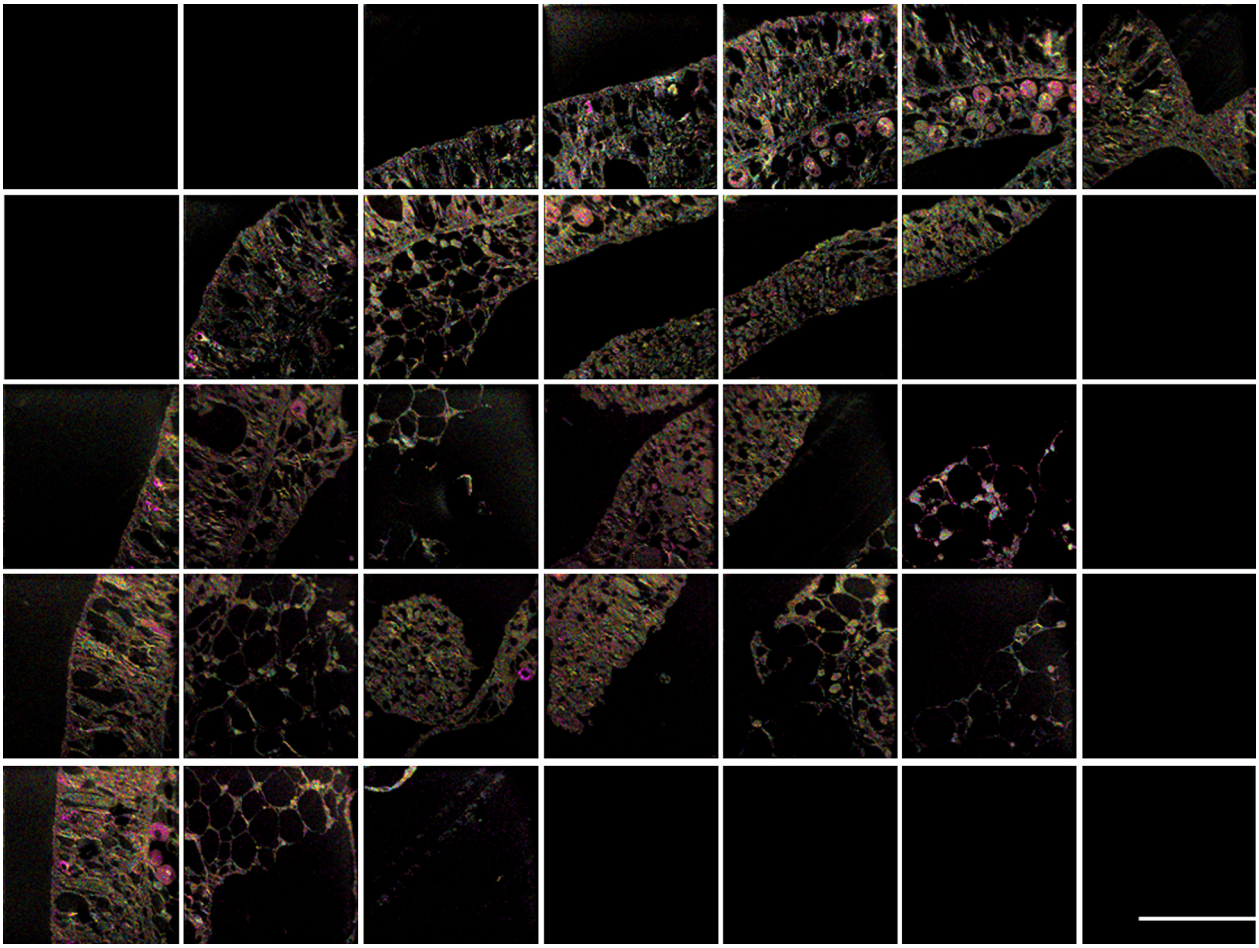

Figure 1. Mosaic of 7 × 5 NanoSIMS images of $^{15}N/^{14}N$ ratio in *Pocillopora damicornis* larva after 8-h incubation with ^{15}N labeled *Alteromonas* sp. The color represents the nitrogen isotope ratio of $^{15}N/^{14}N$ with enrichment of ^{15}N represented by the shift in color toward magenta. Each tile is 80 × 80 μm; 256 × 256 pixels; acquisition time was 30 min, scale bar 50 μm.

(^{15}N Atom% 0.874). Statistical analysis confirmed a significant difference between *V. alginolyticus*-treated larvae and controls ($F_{5,53} = 18.03$, $P = 0.0002$), however, no significant difference was detected between ^{15}N levels within *Alteromonas* sp.–treated larvae and controls. Low standard deviations in *V. alginolyticus*-treated larvae indicate homogenous enrichment levels. Significant differences in ^{15}N levels between *Symbiodinium* (^{15}N Atom% 1.179), the endodermis (^{15}N Atom% 0.756, $F_{2,6} = 8.54$, $P = 0.039$), and the ectodermis (^{15}N Atom% 0.688, $F_{2,6} = 8.54$, $P = 0.021$) tissue types were maintained after 24-h incubation time (Fig. 3).

No signs of mortality or reduced larval fitness were observed in any treatments. The bacterial viability of inoculated cells in the experiments was confirmed by quantification through dilution plating (plate counts 1×10^{-6} cells/mL) and bacteria were identified via 16S rRNA gene sequencing, showing 100% sequence identity with the original inoculated strains (data not shown).

Discussion

The availability of nitrogen influences the biology and ecology of many coral reef organisms and its efficient acquisition ultimately determines the ecological distribution and abundance of these reef inhabitants (Fiore et al. 2010). Corals and their symbiotic dinoflagellate partners, *Symbiodinium*, can acquire nitrogen from a number of sources (Pernice et al. 2012) and we demonstrate that early interactions with bacterial partners provide an additional source of nitrogen for coral larvae, whereby coral larvae acquire nutrients previously taken up from the environment by bacteria. These results identify bacteria as possible key players in early life stages of corals that may benefit the overall fitness of the animal.

After 8-h incubation time, ^{15}N labels, previously incorporated into *Alteromonas sp* and *V. alginolyticus* bacteria, had accumulated within the coral symbiont *Symbiodinium* and the endodermis surrounding it; lower levels of

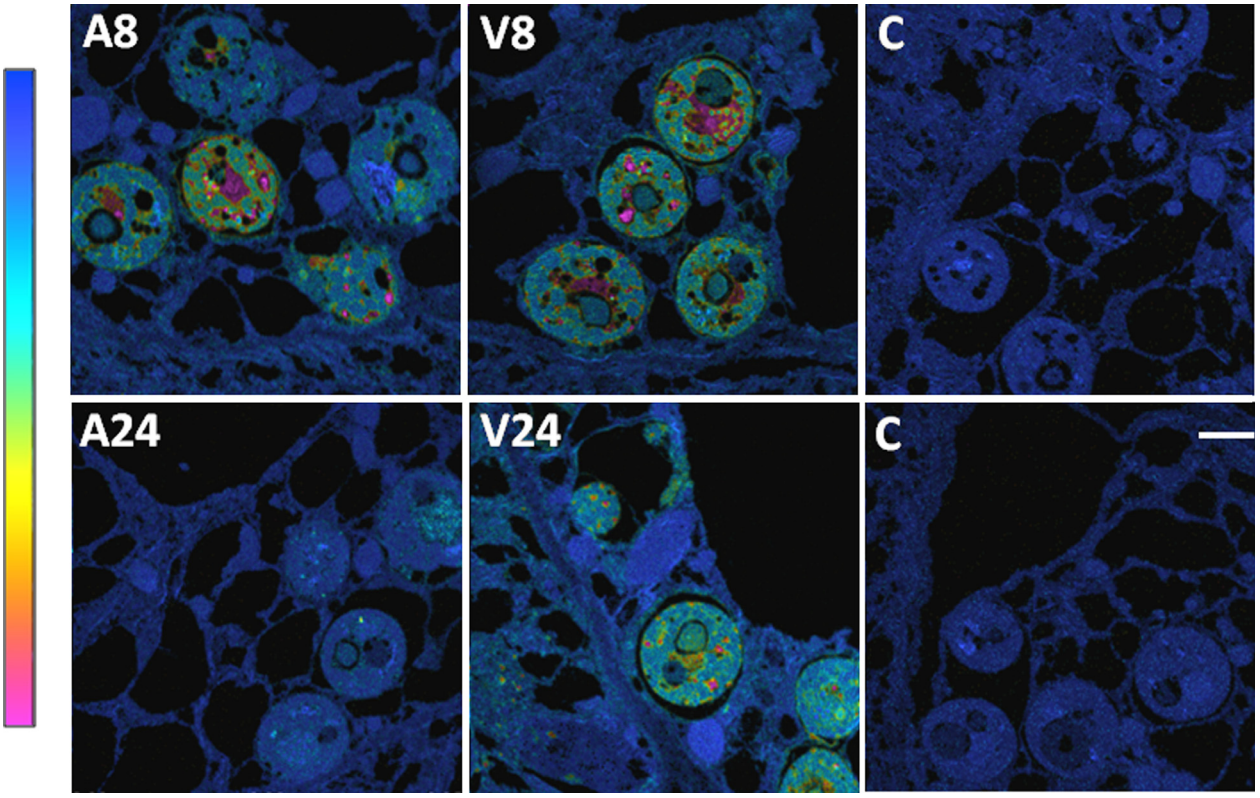

Figure 2. NanoSIMS $^{15}N/^{14}N$ ratio images of *Pocillopora damicornis* larvae after 8 h (8) and 24 h (24) incubation with ^{15}N labeled *Alteromonas* sp. (A), *Vibrio alginolyticus* (V), and unlabeled controls (C); The ratio is expressed on a hue-saturation-intensity scale (HSI), where blue represents the natural isotopic abundance of $^{15}N/^{14}N$ (0.0037) and enrichment of ^{15}N is represented by the shift in color toward magenta (0.05). Each image is 40 × 40 μm, 256 × 256 pixels; acquisition time was 20 ms/pixel, scale bar 5 μm.

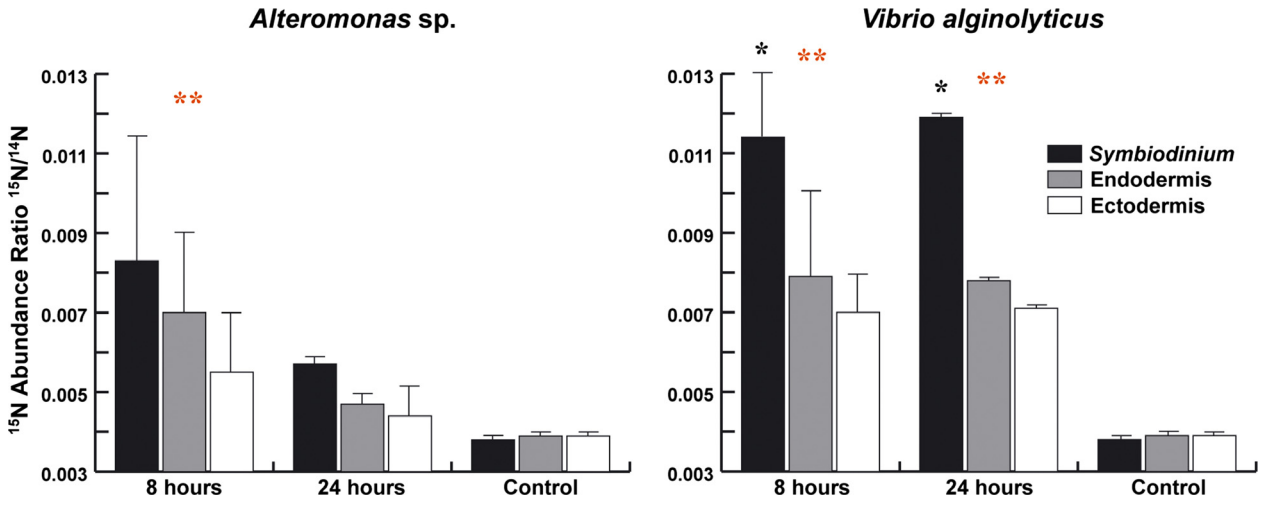

Figure 3. The $^{15}N/^{14}N$ isotope ratio within *Pocillopora damicornis* larvae exposed to two different ^{15}N-labeled bacteria, *Alteromonas* sp. and *Vibrio alginolyticus*. Data are presented as means +SD and asterisks indicate significant differences between treatments and control (**) and between tissue types within individual treatments (*) as assessed by one-way ANOVA and Tukey test ($P < 0.05$).

enrichment were found in the ectodermis. This distribution was clearly defined in all treatments, however, the pattern was most prominent in *V. alginolyticus* treatments where ^{15}N levels were significantly higher in *Symbiodinium* compared to coral tissue. These findings support a recent study that reported the rapid uptake of ^{15}N-labeled

ammonium from seawater into *Symbiodinium* and host cells of the coral species *Acropora aspera* (Pernice et al. 2012). *Symbiodinium* cells possessed significantly more nitrogen than their coral host cells although the role that bacteria may have in this uptake and processing was not investigated by Pernice et al. (2012).

A recent study proposed that the broadcast spawning coral *Acropora tenuis* and the brooding coral *P. damicornis* release specific and potentially beneficial bacteria with their offspring to secure their maintenance across generations. This passing on of selected coral partners to the next generation might indicate highly evolved associations in which the presence of bacteria is advantageous and fulfills important roles in the fitness and survival of the coral host (Ceh et al. 2013). Interestingly, along with *Roseobacter*, *Alteromonas*, and *Vibrio* affiliated species were the most abundant bacterial taxa released with planulating *P. damicornis* colonies (Ceh et al. 2013). The current study supports the notion that *V. alginolyticus* and *Alteromonas* sp. might be beneficial to *P. damicornis* larvae in serving as first providers of nitrogen or even as established players within nitrogen cycling processes. Apprill et al. (2009) confirmed the establishment of coral-associated bacterial partnerships in *Pocillopora meandrina* in late developmental stages and suggested a functional role which may include supplementation of nitrogen. Furthermore, Olson et al. (2009) identified diazotrophic *Vibrio* spp. as highly abundant in corals and positively correlated with *Symbiodinium*. To unambiguously clarify, however, whether *V. alginolyticus* and *Alteromonas* sp. are indeed involved in the internal nitrogen cycling as part of a highly specific long-term symbiosis within the coral holobiont, studies identifying marker genes coding for nitrogen cycling processes are required.

It cannot be excluded that lysing bacterial cells provide residual labeled nitrogen which then passes directly to the coral partner *Symbiodinium* without the direct and active involvement of bacteria (Pernice et al. 2012). However, the active uptake of bacteria into coral larval tissues has been reported before (Apprill et al. 2009). In this study, the viability of inoculated cells was confirmed after all treatments and bacteria were washed before the experiment to avoid residual ^{15}N labels; it is therefore unlikely that nitrogen from lysed bacteria was introduced in the beginning of the experiment. This leaves us with two possible mechanisms for the movement of nitrogen from bacteria to coral larvae: (1) bacteria live in coral tissues and transport nitrogen to and within the coral host or (2) the coral host kills some bacterial cells to obtain nitrogen from the bacterial cytosol. In either case, the overall bacterial population would remain viable. Shorter experimentation time intervals are needed to show the step-by-step uptake of ^{15}N into coral larvae and the combination of Fluorescence *in situ* Hybridisation and NanoSIMS techniques, where bacteria and ^{15}N enrichment can be colocalized in the same image, could further clarify the presence and active involvement of living bacteria in nitrogen cycling processes in corals.

The exact pathway of ^{15}N from bacteria into the coral tissue and *Symbiodinium* and the mechanisms involved in this transfer remain to be elucidated. *Symbiodinium* photosynthates are directly transferred into coral mucus (Meikle et al. 1988), where they attract motile bacteria (Ducklow and Mitchell 1979). *V. alginolyticus,* chemotactically responds to algal products like acrylate, glycolate, and dimethylsulfide (Sjoblad and Mitchell 1979) and might be attracted by *Symbiodinium* exudates in the coral mucus layer. Therefore, the high ^{15}N levels in ectodermal larval tissues might indicate either an accumulation of *V. alginolyticus* in the coral mucus layer or nitrogen transferred from *V. alginolyticus* to coral larvae.

This study presents a novel approach to investigate the functional role of bacteria associated with corals. Coincubation of bacteria enriched with the stable isotope ^{15}N with coral larvae was used to demonstrate the passage of nitrogen between bacteria, the coral animal, and its symbiotic dinoflagellate partner *Symbiodinium*. Coral–microbial interactions were established rapidly in the brooding coral *P. damicornis* and the uptake of nitrogen appeared to be largely related to the presence of *Symbiodinium*. The ability of coral larvae to acquire nitrogen from bacteria provides additional essential nutrients that may increase the survival rate in early coral life stages and therefore provides a competitive advantage in the nutrient poor environments reef-building corals reside in.

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Conflict of Interest

None

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. NanoSIMS images of $^{15}N/^{14}N$ in fixed *Alteromonas* sp. and *Vibrio alginolyticus* bacteria. The ratio is expressed on a hue-saturation-intensity scale (HSI), where blue represents the natural isotopic abundance ($^{15}N/^{14}N = 0.0037$) and enrichment of ^{15}N is represented by the shift in color toward magenta ($^{15}N/^{14}N = 15$ and $^{15}N/^{14}N = 25$, respectively). The image is 20×20 μm and 256×256 pixels; acquisition time was 20 min.

Table S1. Isotopic composition of bacteria/coral tissue (mean).