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An efficient diazotroph-derived nitrogen transfer pathway in coral reef system

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Abstract

The underlying mechanism for sustaining the high productivity of coral reefs in nutrient deplete water, known as the Darwin Paradox, has long been debated. The input of new nitrogen, for example, derived from N_2 fixation, and efficient nitrogen recycling are hypothesized as two critical factors in resolving the paradox. However, the source of new nitrogen and its connection to nitrogen recycling in the coral reef system remain poorly understood. We measured N_2 fixation rates and investigated the diazotroph community in different components of a tropical coral reef system. Our results show that the reef-building crustose coralline algae demonstrate a significantly higher N_2 fixation rate than the overlying water or the coral holobiont, representing a large source of N_2 fixation to the system. The associated diazotroph community was dominated by the non-cyanobacterial *Rhizobiales*. $^{15}N_2$-based pulse-chase incubation experiments revealed ~ 50% of the diazotroph-derived nitrogen was released from the crustose coralline algae holobiont to the overlying water and coral holobiont within 48 h, which was five fold higher than the fraction of nitrogen released from nitrate-based assimilation by crustose coralline algae. Furthermore, the released diazotroph-derived nitrogen was rapidly transferred to the coral holobiont through grazing and was subsequently released back to the overlying water as dissolved organic nitrogen, revealing an efficient pathway that connects the new nitrogen input with the nitrogen recycling in the coral reef ecosystem. Our results provide new insights into the sources and fates of new nitrogen in the coral reefs, helping to resolve the Darwin Paradox in this unique ecosystem.

Coral reefs, the ecological icon of tropical seas and one of the most productive (5–10 g C m^{-2} d^{-1}) and diverse ecosystems on Earth, provide extensive ecosystem goods and services to more than 500 million people (Moberg and Folke 1999). However, most coral reef ecosystems thrive in oligotrophic waters, like an oasis in the desert, a paradox first noted by Darwin (1842). The underlying mechanism that supports the high productivity in the oceanic "desert" has long been debated. Two hypotheses have been proposed to explain the successful growth, development, and stability of coral reefs in the nutrient-depleted water: (1) the active input of external "new nutrient" to sustain the net growth (Froelich 1983); (2) the efficient nutrient entrapment, translocation, and recycling within the coral reef system (D'Elia and Wiebe 1990). Both processes are likely involved, because both new nutrient supply and efficient nutrient retention are required for the build-up of coral reefs (Rougerie et al. 1992; Benavides et al. 2017; Rädecker et al. 2022).

Nitrogen is a limiting nutrient in many coral reef systems; potential sources of "new nitrogen" in reefs include coastal runoff, upwelling, atmospheric deposition, and nitrogen fixation (O'Neil and Capone 2008). Among these, N_2 fixation has long been proposed as the key new nitrogen source in sustaining the growth of the coral reef ecosystem (Wiebe et al. 1975;

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

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Cardini et al. 2014; Benavides et al. 2017). The earliest measurement of N_2 fixation rates in coral reefs was reported more than four decades ago (Webb et al. 1975), and since then N_2 fixation has been observed in a variety of habitats in the coral reef system, including algal turf, sediments, limestone surfaces, coral skeletons, and coral tissue and mucus (Benavides et al. 2017). In parallel, a growing body of molecular evidence shows a wide distribution of various diazotrophs including autotrophic and heterotrophic bacteria and archaea in diverse habitats, including coral holobiont, benthic algae, sediment, and overlying water in coral reef systems globally (Zehr 2001; Lesser et al. 2004; Morrow et al. 2022). These results suggest that the ubiquitous N_2 fixation process, sustained by diverse N_2 fixers in coral reefs, potentially plays a critical role in supporting the growth of the coral reef system.

However, a key question remains regarding the significance of N_2 fixation to the coral reef. The connection between diazotroph-coral associations and how N_2 fixation benefits corals remain largely uncertain. In particular, whether there are unrecognized diazotrophs and unknown interactions between diazotrophs and other organisms merit further exploration. The discovery of a cnidarian–dinoflagellate–diazotroph association provides a clue for the potential interactions occurring between diazotrophs and other organisms in coral reefs (Lesser et al. 2004). In this example, the diazotroph-derived nitrogen is released and transferred to the symbiotic dinoflagellate within the coral holobiont. Such release and transfer of diazotroph-derived nitrogen was recently observed in a handful of isotope labelling experiments (Rädecker et al. 2022; Moynihan et al. 2022). Likewise, a strong correlation between symbiotic dinoflagellate abundance and the copy number of *nifH* gene indicates a potential contribution of N_2 fixation in supporting the growth of the symbiotic algae (Olson et al. 2009). Furthermore, several studies reveal profound impacts of climate change on diazotrophs and N_2 fixation in coral systems (Cardini et al. 2014; Santos et al. 2014) and a critical role of N_2 fixation in regulating the resistance of corals to climate change. For example, recent reports show an increased contribution of N_2 fixation in supporting primary production and heterotrophic nutrient intake under bleaching conditions, highlighting the importance of N_2 fixation in sustaining the health of the coral holobiont under unfavorable conditions such as rising temperature (Bednarz et al. 2019; Meunier et al. 2019, 2022). Despite the accumulating evidence showing that N_2 fixation and diazotroph-derived nitrogen transfer might constitute a crucial process allowing coral reefs to flourish, these studies are mostly restricted to the coral holobiont, so the source, rate, and pathway of diazotroph-derived nitrogen release and transfer in the complex coral reef ecosystems remain largely unquantified (Benavides et al. 2017).

Coral reefs usually comprise two essential reef-building components: the coral and the crustose coralline algae (Steneck 1986). The reef-building coral is composed of the holobiont, a symbiotic union including the coral host and associated microorganisms including the dinoflagellate algae Symbiodiniaceae (zooxanthellae), bacteria, archaea, fungi, viruses, and protists that play essential roles in coral health, disease, and evolution (Rosenberg et al. 2007; LaJeunesse et al. 2018). The symbiosis between Symbiodiniaceae and reef-building corals forms the trophic and energetic foundation of coral reef ecosystems (Falkowski et al. 1984). Many studies have shown that there is an active bidirectional transport of nutrients between Symbiodiniaceae and the coral host, demonstrating substantial N transfer in the coral holobiont (Falkowski et al. 1993; Tanaka et al. 2018). The crustose coralline algae, a calcifying red alga, represents an important benthic primary producer at the base of the food web in coral reef systems (Haas et al. 2016). Crustose coralline algae are a widely distributed benthic algae in coral reefs in the global ocean (Sneed et al. 2015) with greater areal coverage than coral itself in many coral reef systems (Tâmega and Figueiredo 2019). Crustose coralline algae are also an essential component of limestone within the reef systems, playing a crucial role in coral larval settlement (Sneed et al. 2015) and serving as a hotspot of hosting diverse endolithic and epilithic bacteria and microalgae to form the crustose coralline algae holobiont (Sneed et al. 2015). However, distribution of potential diazotrophs and the rate of N_2 fixation in the crustose coralline algae holobiont, its relative contribution to the gross N_2 fixation rate in the coral reef system, and the rate and pathway of diazotroph-derived nitrogen transfer from crustose coralline algae to coral remain poorly investigated.

To explore the source and fate of N_2 fixation in the coral reef system, we conducted a comprehensive set of isotope labelling incubation and diazotrophic community analysis for samples collected from a tropical coral reef system in the South China Sea (Supporting Information Fig. S1). The primary goals of our study are to (1) quantify the rate of N_2 fixation in different components of the coral reef system including coral holobiont, crustose coralline algae holobiont, and the overlying seawater; (2) identify the associated diazotrophic community; and (3) elucidate transfer and redistribution pathways and rates of diazotroph-derived nitrogen transfer within the coral reef system.

Materials and methods

Study area and sample collection

The Luhuitou fringing reef (18°21′N, 109°47′E), located in Sanya Coral Reef National Marine Nature Reserve, Hainan Island, China, is a typical tropical coral reef ecosystem in the northern South China Sea adjacent to the coral triangle (Supporting Information Fig. S1). The average coverages of hermatypic coral and coralline algae in the research area are 16.9% and 14.6%, respectively (Lei et al. 2018). In our study, coral *Pocillopora damicornis*, the most abundant coral (Li et al. 2012), and *Porolithon onkodes*, the second most

abundant crustose coralline algae in the Sanya Reef system (Lei et al. 2018), were selected as model species to study the N_2 fixation and diazotroph-derived nitrogen transfer processes. *P. damicornis* and *P. onkodes* are also widely distributed in shallow-water habitats throughout the Indo-Pacific region (Gabrielson et al. 2018), thus they are representative species found in the tropical coral reef system.

In September 2018, 32 coral fragments were collected from three *P. damicornis* colonies. For the crustose coralline algae samples, 30 fragments with 2–3 mm of calcium carbonate substrate were collected from five *P. onkodes* thalli by chiseling them out from the rock. Both the coral colonies and crustose coralline algae were randomly picked up in the study area to increase the representativeness and precision of our results. In order to minimize damage to the reef caused by sampling; however, many of coral and crustose coralline algae fragments were sourced from the same sampling unit. This pseudoreplication (Hurlbert 1984) is a compromise between more independent replication and conservation of the subject material. The coral and crustose coralline algae fragments were of similar size, that is, 2–2.5 cm long, 2–2.5 cm wide, area: 4–6 cm^2, which fits best to the size of ceramic chips and our incubation bottles. The similar size also facilitates a direct comparison of biomass and N_2 fixation rates between coral and crustose coralline algae, although the exact size of each fragment was measured for areal rate normalization. After sample collection, the coral fragments were attached to ceramic chips using gel-10 glue and then transferred into a 1000-liter outdoor flow-through incubation tank along with the crustose coralline algae fragments. The seawater was freshly pumped from the Luhuitou coral reef system and supplied to the tank after sand filtering. Shaded screens were used to simulate the in situ light intensity of the sampling depth. The coral and crustose coralline algae were incubated in the tank for 2 weeks to allow for recovery from fragmentation. The number of coral and crustose coralline algae samples used in each of our experiments is listed in Supporting Information Table S1.

Temperature (°C) and the photosynthetically active radiation (PAR) of the in situ water were recorded using HOBO® Pendant temperature/light loggers, which were deployed at 2.5 m at the sampling site. Triplicate 50 mL of 0.2-μm filtered seawater samples were collected at 2.5 m depth and stored at −20°C for nutrient and dissolved organic nitrogen (DON) analysis. Triplicate 1-liter seawater samples were collected at the same depth and immediately filtered onto Whatman GF/75 (0.3 μm) filters; the filters were stored at −20°C for particulate nitrogen (PN) measurement.

At the end of the recovery period, three crustose coralline algae (*P. onkodes*) and five coral (*P. damicornis*) fragments were randomly selected and preserved at −80°C for bacterial and diazotrophic community analyses. Triplicate 2-liter samples of in situ seawater from the sampling site were filtered onto 0.2-μm polycarbonate (PC) filters and the filters were preserved at −80°C for bacterial and diazotrophic community analysis.

N_2 fixation rate incubation

To identify the main source of N_2 fixation in our study area to guide the diazotroph-derived nitrogen transfer experiment design (*see* below), a pretest of potential N_2 fixation in crustose coralline algae (*P. onkodes*) and coral (*P. damicornis*) was conducted using the acetylene (C_2H_2) reduction assay (Capone 1993). One crustose coralline algae fragment or one coral fragment with similar size was placed into a 250-mL gas-tight serum bottle (Wheaton) with ~ 150 mL 0.2-μm filtered seawater. Then, 20 mL of acetylene was added to the bottle, which was then incubated for a dark-light cycle (24 h, triplicates). The production of ethylene (C_2H_4) was subsequently measured by gas chromatography (GC). Briefly, after the incubation, 1.5 mL of the headspace gas was extracted using a gas-tight syringe and injected into a GC (Shimadzu GC-8A), the C_2H_2 and C_2H_4 were separated by a Hayesep 80/100 column, and the C_2H_4 was measured by a Flame Ionization Detector. The result showed a significantly higher C_2H_4 production from the crustose coralline algae (4.15 ± 0.12 nmol C_2H_4 cm^{-2} h^{-1}) than from the coral (below the detection limit; Supporting Information Fig. S2), demonstrating the N_2 fixation associated with crustose coralline algae was more active than coral in our study. Therefore, we selected the crustose coralline algae as the source of diazotroph-derived nitrogen in our experiment to study the diazotroph-derived nitrogen transfer between crustose coralline algae and coral.

N_2 fixation rates were measured using the $^{15}N_2$ gas dissolution method (Mohr et al. 2010). The $^{15}N_2$ predissolved in seawater was made with $^{15}N_2$ gas (98.9 atom%, Cambridge Isotope Laboratories) and was prepared following the procedure of Shiozaki et al. (2015). Briefly, seawater was filtered through 0.22-μm filter (Millipore) and degassed using STERAPORE membrane unit for 0.5 h (20M1500A: Mitsubishi Rayon Co., Ltd). Two-liter Tedlar Bags were filled with degassed water without any bubbles, and then 20 mL $^{15}N_2$ gas (98.9% Cambridge Isotope Laboratories) was injected and allowed to reach complete dissolution. N_2 fixation samples were incubated in 1-liter PC bottles (Nalgene, the actual volume is ~ 1225 mL). To increase the ^{15}N enrichment, for the N_2 fixation rate of coral and crustose coralline algae, one coral or one crustose coralline algae fragment was added into ~ 800 mL in situ seawater, and the remaining volume was filled with 425 mL of $^{15}N_2$ predissolved seawater without any headspace, which yielded a high ^{15}N% of tracer N_2 (36% ± 2%, $n = 3$) for the incubation. Such high ^{15}N% enrichment favors the use of $^{15}N_2$ for fixation and is important for the subsequent diazotroph-derived nitrogen transfer experiment. We note, however, as pointed out by White et al. (2020), the dissolution method causes the removal of O_2, CO_2 and other dissolved gases during the degassing process. Although the

change of pH and DO by adding the $^{15}N_2$-enriched water was not measured during our incubation, we tested the impact of our manipulation on the pH and DO concentration by repeating the dissolution method following the same protocol. The results showed that the pH of the in situ water changed from 7.920 ± 0.001 to 8.033 ± 0.002 after the addition of $^{15}N_2$-enriched water, and the DO changed from 8.25 ± 0.10 to 5.92 ± 0.05 mg L^{-1} ($n = 3$). Currently, we are unable to quantify the impact of these variations on our measured rate. However, given the small change of pH (~ 0.1) and the fact that DO concentration remains near saturated, we expect limited impact. More tests to evaluate the effect of the dissolution method on N_2 fixation rate could be pursued in the future. A total of 12 bottles (6 coral and 6 crustose coralline algae fragments) were used for the incubation. The incubation lasted for 0, 24, and 48 h with duplicate samples for each time point (Supporting Information Table S1). N_2 fixation rate of the in situ water was conducted in the same way without coral or crustose coralline algae fragments.

The bottles were deployed in the outdoor flow-through tank with screens to simulate the in situ light. The light intensity was 488 ± 126 μmol quanta m^{-2} s^{-1} at mid-day of our incubation dates (Supporting Information Fig. S3). For the crustose coralline algae and coral samples, at the end of the incubation, seawater was removed gently, crustose coralline algae and coral fragments were reclaimed from each bottle and were preserved at −20°C immediately. For the incubation of N_2 fixation in the in situ seawater (samples without crustose coralline algae and coral), the water was filtered onto a precombusted (450°C, 4 h) GF/75 (Whatman) filter for PN measurement.

Diazotroph-derived nitrogen transfer experiment

A further set of diazotroph-derived nitrogen transfer experiments was conducted to investigate the transfer of diazotroph-derived nitrogen from crustose coralline algae holobiont to the overlying water and coral holobiont. Eight crustose coralline algae fragments were added into two 2-liter PC bottles (four fragments per bottle) with 1.5 liters in situ seawater (Nalgene, the total volume is ~ 2380 mL); the remaining 880 mL was filled with the $^{15}N_2$-saturated seawater and the samples were incubated in the flow-through tank for 48 h. Immediately after the incubation, crustose coralline algae fragments were transferred into eight 250-mL PC bottles (Nalgene), and each bottle was filled with 250 mL of in situ seawater and a coral fragment. Bottles were incubated for 24, 48, and 72 h with duplicates for each time point. The bottles were deployed in the outdoor flow-through tank with screens to simulate the in situ light. At the end of the incubation, crustose coralline algae and the coral were reclaimed from each bottle and were preserved at −80°C immediately. The overlying water was filtered onto a precombusted (450°C, 4 h) GF/75 (Whatman) filter for PN measurement, and triplicate 40 mL filtrate subsamples were collected for NO_x^- ($NO_2^- + NO_3^-$), NH_4^+ and DON analysis, and all samples were preserved at −20°C until analysis.

Transfer of $^{15}NO_3^-$-derived N experiment

N_2 fixation and NO_3^- represent two primary sources of new nitrogen to the coral reef system. To evaluate the fates of these new nitrogen sources, we further compared the rate and pathway of diazotroph-derived nitrogen release and transfer with the nitrate-derived nitrogen from crustose coralline algae to the ambient water and coral holobiont. Similar to the diazotroph-derived nitrogen transfer experiment, four crustose coralline algae fragments were added into a 2-liter PC bottle (Nalgene) with in situ seawater, with 1 mL of $^{15}NO_3^-$ (98% ^{15}N, Sigma Aldrich) amendment to reach a final $^{15}NO_3^-$ concentration of 1 μmol L^{-1} and the bottle was incubated in the flow-through tank for 5 h (7:00–12:00 h). Immediately after the incubation, the crustose coralline algae fragments were rinsed with 0.2-μm filtered seawater to remove any residual $^{15}NO_3^-$ and were transferred into four 250-mL PC bottles, following by the same procedure as the diazotroph-derived nitrogen transfer experiment. The bottles were incubated for 48 h.

Sample preparation for analyses

The crustose coralline algae fragments were freeze-dried and then a small piece of crustose coralline algae tissue (~ 0.001 cm^3) was drilled and ground using an agate mortar. The ground crustose coralline algae were dissolved in 150 μL 4 N HCl (EMSURE®, Merck) for 2 h to remove the $CaCO_3$ and was then transferred onto a precombusted GF/75 filter and dried (50°C, 48 h) for PN concentration and isotope analyses.

For the coral fragment samples, the coral holobiont was separated from the skeleton using Waterpik and airbrush (Tanaka et al. 2018; Bednarz et al. 2019), and the tissue was further separated into coral tissue, Symbiodiniaceae, and microbes via a set of centrifugation and filtration processes (Grover et al. 2002). Briefly, the coral tissue homogenate was centrifuged at $3000 \times g$ for 10 min at 4°C, and the supernatant was centrifuged three times to pellet residual algae. The algal pellet was resuspended and washed three times with filtered seawater to minimize cross-contamination between coral tissue and the Symbiodiniaceae. The coral tissue and the algal pellet were further filtered onto precombusted (450°C, 4 h) GF/D filters (2.7 μm). The filtrate from coral tissue was further filtered onto GF/75 filters (0.3 μm) to collect the microbes, while the filtrate was collected to represent the ruptured coral tissue. Using the above procedures, each incubation bottle was separated into seven different components: crustose coralline algae, coral tissue, Symbiodiniaceae, coral symbiotic microbes, PN in seawater (including planktonic community and nonliving suspended particles), DON (DON and NH_4^+) in seawater and NO_x in seawater. It is worth noting that the size-fractionated filtration method might cause contamination of different sizes onto one filter. Nevertheless, the significant differences in the $\delta^{15}N$ between different components during the

time course incubation suggested any contamination was minimal and did not mask the significant difference in ^{15}N distribution and transfer pattern in our experiment (Supporting Information Table S2).

Nutrient and isotope analyses

NO_x and NH_4^+ concentrations were measured using the chromogenic method by four-channel Continuous Flow Technicon AA3 Auto-Analyzer with a detection limit of 0.03 and 0.5 μmol L^{-1}, respectively. DON was oxidized to NO_3^- by using the wet digestion method (Knapp et al. 2005). In brief, the DON was oxidized to NO_3^- by using purified persulfate oxidizing reagent (POR) in a 12 mL 450°C precombusted borosilicate glass tube. The persulfate (ACS-grade, Merck) was recrystallized three times and then made up to alkaline POR by dissolving 6 g $K_2S_2O_8$ and 6 g NaOH (ACS-grade, Merck) in deionized water to a final volume of 100 mL. Before the use of POR, the residual NO_3^- concentration in the initial POR (referred to as POR blank) was measured and ensured to be sufficiently low (< 2 μmol L^{-1} in digested solution). The NO_3^- concentration after digestion was measured using the chemiluminescence method with the detection limit of 0.5 μmol L^{-1} (Braman and Hendrix 1989).

The concentration of NH_4^+ after incubation was below the detection limit (<0.5 μmol L^{-1}), hindering the $^{15}N-NH_4^+$ measurement. Alternatively, we adapted the NH_4^+ diffusion method (Holmes et al. 1998) to remove the NH_4^+ to assess the impact of any trace NH_4^+ on the $\delta^{15}N$-DON measurement. Briefly, 10 mL of the filtered seawater sample was transferred into a 120-mL glass bottle (precombusted at 450°C for 4 h), and 0.03 g of MgO (Merk, Reagent grade, precombusted at 450°C for 4 h) was added to reach pH of 9.7. The bottle was kept in an anaerobic jar at 40°C for 2 weeks, the headspace of which was purged with He (>99.999%) every 3 d to remove any diffused NH_3 in the headspace. A parallel set of experiment with mixed NH_4^+ (5 μmol L^{-1}, $\delta^{15}N$ = 187‰) and NO_3^- (5 μmol L^{-1}, $\delta^{15}N$ = −0.5‰) was used to quantify the removal efficiency of NH_4^+. The isotopic content of the mixture was measured using the wet-digestion and bacterial methods (Sigman et al. 2001) before and after the diffusion method. The results showed a NH_4^+ removal efficiency of 94% ± 6% in our experiment (Supporting Information Fig. S4).

Concentrations and isotopic values of the PN samples were analyzed on a Flash EA (Thermo Fisher Flash HT 2000) IRMS (Thermo Fisher Delta V plus) system. During the measurement, an in-house standard (Acetanilide, Sigma-Aldrich) was used to test the linear correlation between the mass and $^{28}N_2$ peak area; and the impact of mass on the measured $\delta^{15}N$-PN. The results showed a strong linear correlation between the mass and $^{28}N_2$ peak area (Supporting Information Fig. S5a) and the $\delta^{15}N$-PN was stable over the range of 13.1–104.5 μg of N, with the measured $\delta^{15}N$-PN of 3.37‰ ± 0.27‰ (Supporting Information Fig. S5b). International reference material (USGS40) with a certified $\delta^{15}N$ value of −4.5‰ was also inserted every eight samples to monitor the drift to ensure the accuracy of the measurements. These tests ensure precise measurements of PN samples. Overall, the reproducibility for $\delta^{15}N$ measurements was better than 0.3‰. Concentration and isotope content of DON were analyzed using the wet digestion method combined with the bacterial method (Sigman et al. 2001; Knapp et al. 2005).

Rate calculations

The N_2 fixation rates of crustose coralline algae, coral, and overlying seawater, and the NO_3^- uptake rate were calculated based on accumulation of ^{15}N in the product pool and were calculated using Eq. (1):

$$R = \frac{1}{\Delta T} \times \frac{A_{PN_f}[PN_f] - A_{PN_0}[PN_0]}{A_{N_2} - A_{PN_0}} \quad (1)$$

where R is N_2 fixation rate, for water column in nmol N L^{-1} d^{-1} and coral or crustose coralline algae in nmol N m^{-2} d^{-1}; ΔT represents incubation time; A_{PN_0} and A_{PN_f} are the ^{15}N-PN enrichment ratio at the start and the end of the incubation, respectively. $[PN_f]$ and $[PN_0]$ represent the nitrogen concentration of different compartments at the end and initial of incubation. We assumed N_2 fixation was homogenous throughout the water column because the sampling site was shallow (i.e., 2.5 m) and the water was well mixed (Supporting Information Fig. S6). The depth integrated N_2 fixation rate was derived by multiplying the measured N_2 fixation rate by the depth.

In the diazotroph- and nitrate-derived nitrogen transfer experiment, the increase of $\delta^{15}N$ ($\triangle^{15}N$, defined as the difference between samples not exposed to ^{15}N-labeled crustose coralline algae (t_0) and samples exposed to ^{15}N-labeled crustose coralline algae after 24, 48, and 72 h) in coral holobiont (coral tissue, Symbiodiniaceae, symbiotic microbes) and overlying seawater (PN, NO_x, and DON) indicated the diazotroph-derived nitrogen from crustose coralline algae transfer to these compartments. The enrichment of ^{15}N in each component during the pulse-chase incubation was calculated using Eq. (2):

$$^{15}N\,\text{enrichment} = A_{PN_f}[PN_f] - A_{PN_0}[PN_0] \quad (2)$$

The diazotroph- and nitrate-derived nitrogen release ratios were calculated using Eq. (3):

$$\text{DDN or NDN release ratio} = \frac{\text{Transfered } ^{15}N}{\text{Total } ^{15}N\,\text{enrichment}} \quad (3)$$

Transferred ^{15}N is the enriched ^{15}N content in the coral and seawater and can be derived from Eq. (4):

$$\text{Transfered}\,^{15}\text{N} = \Sigma\,^{15}\text{N enrichment}_{\text{coral, symbiodinium, symbiotic microbes}}\text{PON, DON, and NO}_x \quad (4)$$

Total ^{15}N enrichment is the total diazotroph-derived nitrogen (enriched ^{15}N content) derived by crustose coralline algae in the incubation system at the end of incubation (5):

$$\text{Total}\,^{15}\text{N enrichment} = \text{Transfered}\,^{15}\text{N} + {}^{15}\text{N enrichment of CCA} \quad (5)$$

DDN, NDN, and crustose coralline algae (CCA) in the above equations denote diazotroph-derived nitrogen, nitrate-derived nitrogen, and crustose coralline algae, respectively. The N_2 fixation rate was reported as the average of four replicate samples for each type of sample (i.e., coral holobiont, crustose coralline algae holobiont, and seawater). For diazotroph- and nitrate-derived nitrogen transfer rates, the rates were calculated as the average of two replicates at each time point. The low number of replicates used in the diazotroph- and nitrate-derived nitrogen transfer incubation introduces uncertainty for the rate estimation, and impedes the using of statistics. Nevertheless, the difference in diazotroph- and nitrate-derived nitrogen transfer rates over the time course between the duplicate samples was lower than the difference in transfer rate between different time points (see diazotroph- and nitrate-derived nitrogen transfer rate in the "Result" section), suggesting the difference in transfer rates was not masked by the uncertainty from low replication. We suggest, however, more biological replicates (≥ 3) should be used in future study to guarantee the use of statistics to increase robustness of the results.

Molecular analysis

DNA extraction

The DNA of coral, crustose coralline algae, and the overlying seawater sample was extracted using the E.Z.N.A.® soil DNA Kit (Omega Biotek) to identify the bacterial and diazotrophic communities associated with crustose coralline algae, coral, and overlying seawater. DNA extract was checked on 1 % agarose gel, and DNA concentration and purity were determined with NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific). The gene-specific (*nifH* and the 16S rRNA) amplicon sequencing was performed at the Research and Testing Laboratory (Majorbio). During each of the DNA extractions, a negative control was performed in parallel to examine any contamination of 16s rRNA and *nifH* genes from the Kit, and the results showed that no laboratory/kit contamination occurred in our polymerase chain reaction (PCR) experiment.

Amplicon tag sequencing

Libraries were PCR-amplified with two primer sets to target both the bacterial 16S rRNA gene and nitrogenase gene (*nifH*). Samples were amplified with (1) the 338F (5′-ACTCCTACGGGAGGCAGCAG-3′; Herlemann et al. 2011) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′; Caporaso et al. 2011) primer pair targeting the variable region V3–V4 of the 16S rRNA gene to assess the overall bacterial community structure; (2) the variable region (360 bp) of the *nifH* gene-specific primers nifH2 (5′-TGYGAYCCNAARGCNGA-3′) and nifH1 (5′-ADNGCCATCA-TYTCNCC-3′; Zehr and McReynolds 1989) to specifically identify the diazotrophic community. The amplicons of 16S rRNA were generated in a 29-cycle PCR as follows: initial denaturation at 95°C for 3 min; amplification of 29 cycles: denaturing at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 45 s; and single extension at 72°C for 10 min, and end at 4°C. The amplicons of *nifH* gene were generated in 37-cycle PCR as follows: initial denaturation at 95°C for 30 s; amplification of 37 cycles: denaturing at 95°C for 30 s, annealing at 53°C for 30 s and extension at 72°C for 45 s; and single extension at 72°C for 10 min, and end at 4°C. All of amplicons purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences).The PCR mixtures contain 5× TransStart FastPfu buffer 4 μL, 2.5 mM Deoxynucleotide Triphosphates 2 μL, forward primer (5 μM) 0.8 μL, reverse primer (5 μM) 0.8 μL, TransStart FastPfu DNA Polymerase 0.4 μL, template DNA 10 ng, and finally ddH_2O up to 20 μL. PCRs were performed in triplicate and a negative control without the template DNA in the PCR was included. The PCR product was extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences) according to manufacturer's instructions and quantified using Quantus™ Fluorometer (Promega).

Purified amplicons were indexed using a Nextera XT Index Kit with eight PCR cycles, following the standard protocols of Majorbio Bio-Pharm Technology Co. Ltd. Following indexing, amplicons were sequenced using paired-end (2 × 300) sequencing on an Illumina MiSeq (Illumina). The raw reads were deposited into the NCBI Genbank database with the accession number of PRJNA799600 for *nifH* gene, and the accession number of PRJNA799477 for 16S rRNA of coral, crustose coralline algae, and seawater microbiome.

16S rRNA and nifH *sequence analyses*

The raw fastq files of both 16S rRNA and *nifH* genes were quality-filtered using Trimmomatic (Version 0.36; Bolger et al. 2014) and merged with FLASH (Version 1.2.11; Magoč and Salzberg 2011). For the 16S rRNA gene analysis, the clean sequence data were imported to the QIIME2 artifact (Version 2020.8.0; https://qiime2.org), then demultiplexed and quality filtered using Deblur (Bokulich et al. 2013). The taxonomy analysis was performed using QIIME 2 feature-classifier (classify-sklearn; Bolyen et al. 2019) command against Silva 16S rRNA database (Silva 138 99% operational taxonomic units [OTUs]; https://docs.qiime2.org/2020.11/data-resources/).

For the *nifH* gene, in order to ensure that the primer amplified the proper sequences of the *nifH* gene, the sequences were

filtered with the FrameBot (http://fungene.cme.msu.edu; Fish et al. 2013) with a parameter of 50% of similarity with the protein sequence. The filtered sequences were assigned to TaxaDiva *nifH* database (Gaby and Buckley 2014; Gaby et al. 2018) with 90% similarity using the following steps: firstly, the TaxADivA *nifH* database (Gaby et al. 2018) was imported and trained to fit the naive Bayes classifier using QIIME2 "feature-classifier fit-classifier-naive-bayes" function. Chimeras were removed using the Vsearch plugin in QIIME2 (Bokulich et al. 2013; Rognes et al. 2016; Bolyen et al. 2019), then the homolog sequences were clustered according to the *nifH* representative sequences from TaxADivA *nifH* database (Gaby et al. 2018) based on 90% identity using Vsearch (Rognes et al. 2016) plugin in QIIME2, employing "qiime Vsearch cluster-features-open-reference" function. This is where sequences clustered with existing reference sequences and sequences that do not fit the reference were clustered de novo (Rideout et al. 2014). Furthermore, for the diversity analysis, the clustered sequences were classified and annotated against the TaxADivA *nifH* gene database using "qiime feature-classifier classify-sklearn" (Bolyen et al. 2019).

nifH *diversity analysis*

For the purpose of calculation of alpha and beta diversity analysis, the representative sequences of *nifH* gene that were produced from open-reference OTU picking were aligned using MAFFT (Version 7; Katoh et al. 2002) to construct a rooted phylogenetic tree using the QIIME2 "align-to-tree-mafft-fasttree" command. Based on this phylogenetic tree the alpha and beta diversity analyses were computed using QIIME2 with the qiime diversity alpha-rarefaction and qiime diversity core-metrics-phylogenetic functions were used, respectively, where the subsamples for *nifH* gene were 10,000 and 16S rRNA gene were 36,000 sequences in depth. For the permutational multivariate ANOVA (PERMANOVA) qiime diversity beta-group-significance command was used with same subsample depth for the alpha and beta diversity analysis with 999 permutations.

Statistical analysis

Beta diversity was used to compare bacterial and diazotrophic communities associated with crustose coralline algae, coral, and overlying seawater. The beta diversity is the ratio between regional and local species diversity. It allows us to quantify the degree of community differentiation, in relation to a complex-gradient of environment, or a pattern of environments. Differences in beta diversity of the overall and diazotrophic bacterial community associated with the coral tissue, crustose coralline algae, and overlying seawater were analyzed with PERMANOVA using QIIME 2 at a significance level of 0.05. Beta diversity analyses were based on the weighted and unweighted UniFrac distance metric.

Results

Bacterial and diazotrophic communities associated with crustose coralline algae (*P. onkodes*), coral (*P. damicornis*), and seawater

The beta diversity of OTUs of the overall bacterial communities in coral and crustose coralline algae holobiont were significantly different (PERMANOVA with Bray–Curies distance: $F = 5.170$, $p = 0.027$; Fig. 1a; Supporting Information Fig. S7; Table S3). *Gammaproteobacteria* (30.1% ± 9.1%; dominated by *Burkholderiales* [17.7% ± 9.7%] at the order level) was the dominant phylum in the coral tissue-associated prokaryotic community. The other phyla with relatively high abundance were *Firmicutes* (22.0% ± 19.8%), *Actinobacteriota* (14.0% ± 8.6%), and *Bacteroidota* (13.6% ± 6.9%). The *Alphaproteobacteria* (9.0% ± 2.1%) represented a lower fraction in coral tissue than in crustose coralline algae and seawater. By contrast, the relative abundance of *Gammaproteobacteria* was low in the crustose coralline algae and seawater samples. The bacterial community associated with crustose coralline algae was dominated by *Alphaproteobacteria* (28.3% ± 8.1%; belonging to the *Rhodobacterales* [10.2% ± 6.5%] and *Rhizobiales* [6.3% ± 2.0%]) and *Chloroflexi* (38.5% ± 16.6%; belonging to *Chloroflexia*). The seawater was dominated by *Alphaproteobacteria* (47.4% ± 3.8%; belonging to the potentially diazotrophic orders of *Rhodobacterales* [23.6% ± 4.5%] and *Parvibaculales* [9.2% ± 0.7%]) and *Bacteroidia* (27.2% ± 4.4%; belonging to *Flavobacteriales*).

nifH gene amplicons were obtained from all the crustose coralline algae and seawater samples. However, the gene was detected in only three of five coral samples. Both cyanobacterial and non-cyanobacterial diazotrophs were detected in coral, crustose coralline algae, and seawater. The non-cyanobacterial diazotroph *Rhizobiales* (*Alphaproteobacteria*) was the dominant in crustose coralline algae (32.5% ± 11.8%) and seawater (39.9% ± 2.5%). In contrast, the cyanobacterial diazotroph *Mastigocladus* (33.5% ± 38.6%) was the dominate *nifH* OTU in coral samples (Fig. 1b).

N_2 fixation rates and NO_3^- uptake rate

During our experiment, the NO_x ($NO_3^- + NO_2^-$) concentration was 1.3 μmol L^{-1}, while the concentration of DON and PN was 8.6 ± 0.6 and 3.8 ± 0.1 μmol L^{-1}, respectively. NH_4^+ concentration was below the detection limit (0.5 μmol L^{-1}).

The crustose coralline algae *P. onkodes* demonstrated higher N_2 fixation rate (2.8 ± 1.6 mg N m^{-2} d^{-1}) than the rate in coral *P. damicornis* (0.0 ± 0.1 mg N m^{-2} d^{-1}) during the $^{15}N_2$-labelling incubation, consistent with the C_2H_2-based preliminary result (Supporting Information Fig. S2). The N_2 fixation rate of the overlying water was 21.1 ± 7.4 nmol N L^{-1} d^{-1}, taken the average depth of 2.5 m in our study site, the depth-integrated N_2 fixation rate in the water column was 0.7 ± 0.3 mg N m^{-2} d^{-1}. On the other hand, the NO_3^- uptake rate of crustose coralline algae *P. onkodes* was 22.5 ± 4.1 mg N m^{-2} d^{-1} in our experiment (Fig. 2a).

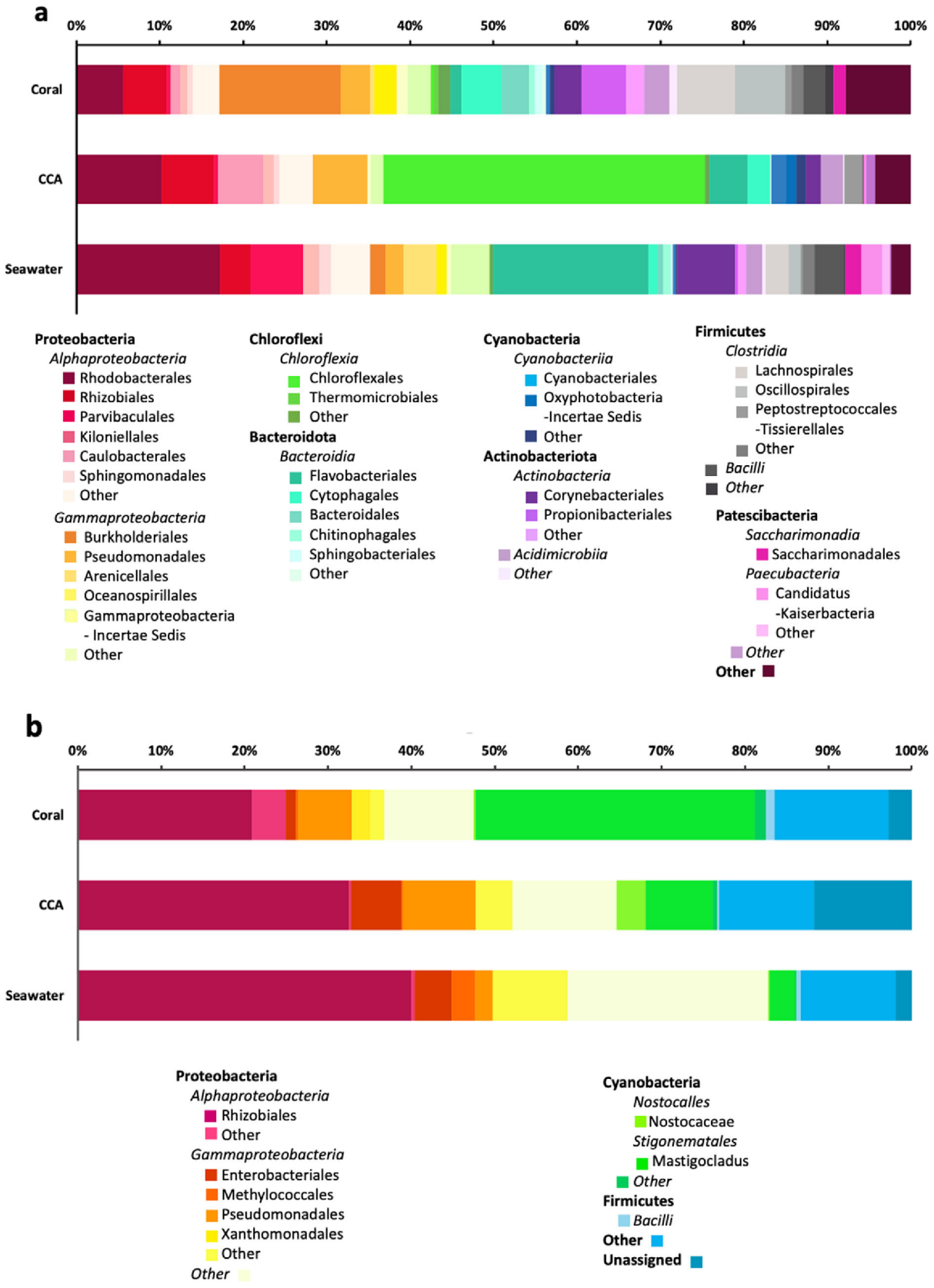

Fig. 1. Overview of the composition of the overall bacterial community (**a**) and the diazotrophic community (**b**) associated with coral *Pocillopora damicornis*, crustose coralline algae (CCA) *Pocillopora onkodes*, and surrounding seawater. The relative abundance of each OTU within the community is indicated in percentages (%).

Diazotroph-derived nitrogen release and redistribution from crustose coralline algae to overlying water and coral

During the diazotroph-derived nitrogen transfer experiment, increases of $\delta^{15}N$ in both ambient seawater (PN, NO_x, and DON) and the coral holobiont (coral tissue, Symbiodiniaceae, symbiotic microbes) were observed in the diazotroph-derived nitrogen transfer experiments (Table 1; Supporting Information Table S2). Among all the N species, the $\triangle^{15}N$ in the water column (highest values were 254‰, 242‰, and 51‰ for the PN, DON, and NO_x, respectively) was systematically higher than in the coral (2–7‰). The lower apparent $\delta^{15}N$ increase in the coral samples was largely due to the much higher N content of the coral compartments. Although the use of only two biological replicates ($n = 2$) prevents the use of statistics to compare the significance of ^{15}N accumulation between different timepoints, the precision of our $\delta^{15}N$-PN measurement (< 0.3‰) guarantees the robustness of the $\Delta^{15}N$ observed in our study. The increase of $\delta^{15}N$ during the time course incubation for all the compartments provide direct evidence of ^{15}N-diazotroph-derived nitrogen transfer and redistribution in multiple compartments of the coral reef system. During the time-course incubation, the $\triangle^{15}N$ of tissue and microbes in the coral fragment and the PN and NO_x in the water peaked at 48 h. In contrast, the $\triangle^{15}N$ of *Symbiodimium* and DON showed continuous increase during the 72 h time course incubation. Notably, in order to test the potential contribution of $^{15}N-NH_4^+$ in the DON pool, we compared the $\delta^{15}N$-DON before and after removing the tracer amount of NH_4^+ (*see* "Methods" section), the result showed no significant difference of $\triangle^{15}N$-DON before and after NH_4^+ removal, indicating the ^{15}N measured here was concentrated in the DON pool (Fig. 3).

The increases of $\delta^{15}N$ suggested that ^{15}N from diazotroph-derived nitrogen was transferred from crustose coralline algae into all the N-containing components in our experiment. The distribution of diazotroph-derived nitrogen, however, exhibited large differences in different components and varied over time. At 24 h, a total of 6.2 ± 2.4 nmol ^{15}N was released. The diazotroph-derived nitrogen was mainly distributed in the PN (57.6%, 3.6 nmol N), DON (20.8%, 1.5 nmol N) pools, and the coral tissue (16.6%, 0.9 nmol N; Fig. 4a). At 48 h, the total released ^{15}N increased to 11.9 ± 0.9 nmol N. A significant increase of ^{15}N-diazotroph-derived nitrogen in the coral tissue (30.8%, 3.7 nmol N) was observed accompanied by the decrease of ^{15}N-diazotroph-derived nitrogen in the PN (6.8%, 0.8 nmol N), while the ^{15}N in DON increased to 57.2% (6.8 nmol N) of the released ^{15}N (Fig. 4b). At the end of the incubation (72 h), the total released diazotroph-derived nitrogen (11.3 ± 4.3 nmol N) was close to the value at 48 h. However, the distribution of diazotroph-derived nitrogen was distinct from that of 48 h: over 85.2% (9.6 nmol N) of ^{15}N-diazotroph-derived nitrogen was concentrated in the DON pool followed by coral tissue and Symbiodiniaceae (Fig. 4c). The distribution of ^{15}N-diazotroph-derived nitrogen in the microbes and NO_x^- was low yet detectable during the time-course incubation.

Nitrate-derived nitrogen release and transfer from crustose coralline algae to the coral and overlying water

N_2 fixation and NO_3^- uptake are two key new nitrogen sources to the crustose coralline algae. We further compared the release and transfer rate of diazotroph- and nitrate-derived

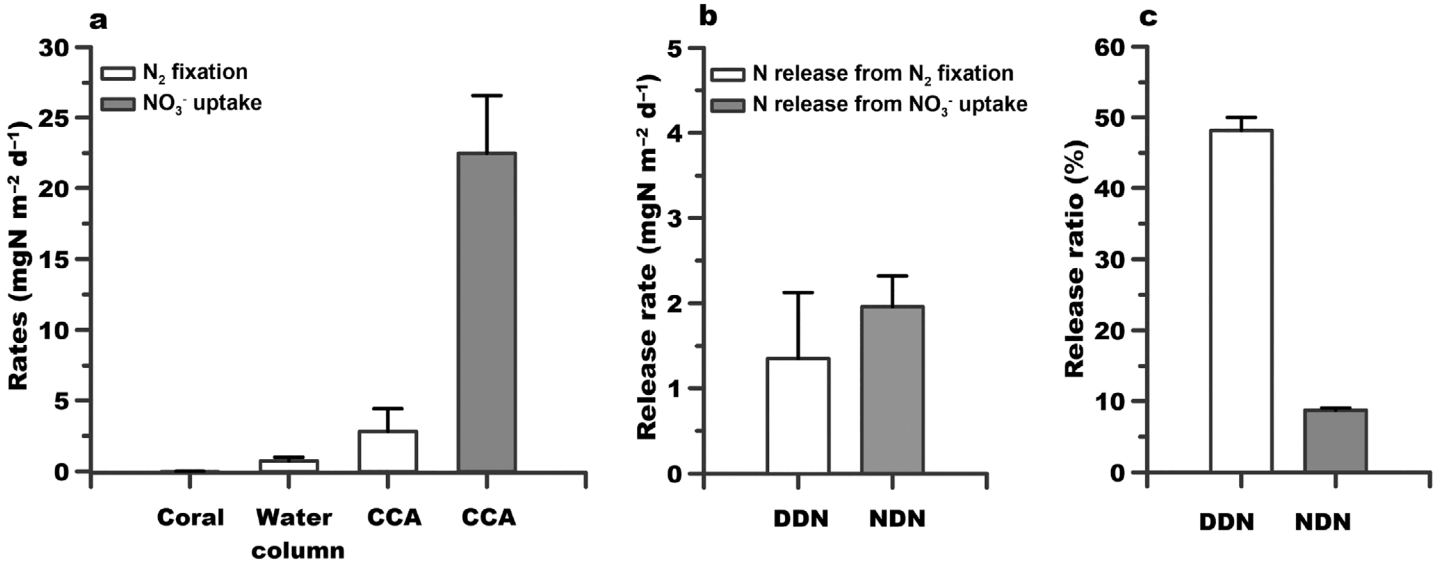

Fig. 2. Comparison between N_2 fixation vs. NO_3^- assimilation. (**a**) N_2 fixation rates in Crustose coralline algae (CCA) *Pocillopora onkodes*, coral *Pocillopora damicornis* and the surrounding seawater (white bars) compared with the NO_3^- uptake rate of CCA P. *onkodes* (grey bar); (**b**) diazotroph-derived nitrogen (DDN) and diazotroph-derived nitrogen (NDN) release rates; (**c**) DDN and NDN release ratio in 48-h incubations. Error bars represent the standard deviation of the rates or ratio (three time points, duplicates in each time point).

Table 1. ^{15}N distribution in the diazotroph-derived nitrogen (DDN) and nitrate-derived nitrogen (NDN) transfer experiment.

Number	Experiment	Total ^{15}N content (nmol ^{15}N)	Released ^{15}N during incubation (nmol^{15}N)	Release ratio (%)
1	DDN transfer, 24 h	21.22	8.57	40.39
2	DDN transfer, 24 h	10.53	3.86	36.66
3	DDN transfer, 48 h	25.65	12.82	49.98
4	DDN transfer, 48 h	23.84	11.04	46.31
5	DDN transfer, 72 h	18.08	6.98	38.61
6	DDN transfer, 72 h	28.84	15.60	54.09
7	NDN transfer, 48 h	67.04	6.05	9.02
8	NDN transfer, 48 h	66.23	5.56	8.39

nitrogen to different compartments of the coral system. The release of the ^{15}N-diazotroph-derived nitrogen from crustose coralline algae was 5.8 ± 0.6 nmol after 48 h incubation, accounting for 8.7% of the assimilated $^{15}N - NO_3^-$ by the crustose coralline algae (66.6 ± 0.4 nmol N; Table 1). Similarly, the ^{15}N-nitrate-derived nitrogen was mainly distributed in the total dissloved nitrogen pool (91.0%), followed by the coral tissue (8.7%). No significant accumulation of ^{15}N-nitrate-derived nitrogen was found in the remaining components (Fig. 4d).

Discussion

Crustose coralline algae were the primary source of N_2 fixation in the investigated coral reef system

Crustose coralline algae are generally recognized as an important primary producer to sustain high organic production (0.9–5 g C m^{-2} d^{-1}) in coral reefs (Chisholm 2003) and a habitat for diverse bacteria (Sneed et al. 2015). However, whether active N_2 fixation is directly associated with crustose coralline algae has not been investigated. Our results provide direct evidence showing that the diazotrophs associated with the crustose coralline algae holobiont, probably including both epilithic and endolithic microbial communities, are also a large source of N_2 fixation in the coral reefs. The areal N_2 fixation rate of crustose coralline algae holobiont (*P. onkodes*) was 2.8 ± 1.6 mg N m^{-2} d^{-1}, which was comparable to the highest rates of N_2 fixation in coral reef systems such as algal turfs and microbial mats (Charpy-Roubaud et al. 2001; Rix et al. 2015) and was significantly higher than the rate measured in the dominant coral species (*P. damicornis*; 0.0 ± 0.1 mg N m^{-2} d^{-1}) in the study area. The depth-integrated N_2 fixation in the overlying water was 0.7 ± 0.3 mg N m^{-2} d^{-1}, demonstrating that the crustose coralline algae holobiont was the strongest source of N_2 fixation in the study area. The crustose coralline algae- and coral-associated N_2 fixation incubations were conducted using in situ seawater rather than filtered seawater, which means that any N_2 fixation signal due to planktonic diazotrophs might contribute to the N_2 fixation attributed to crustose coralline algae or coral. Such potential contamination of the N_2 fixation signal by the plankton in the overlying water is unlikely because: (1) the crustose coralline algae or coral fragment was sat at the bottom of the bottles, which were gently mixed in order to distribute the tracer. At end of incubation, the overlying water was gently removed and the crustose coralline algae

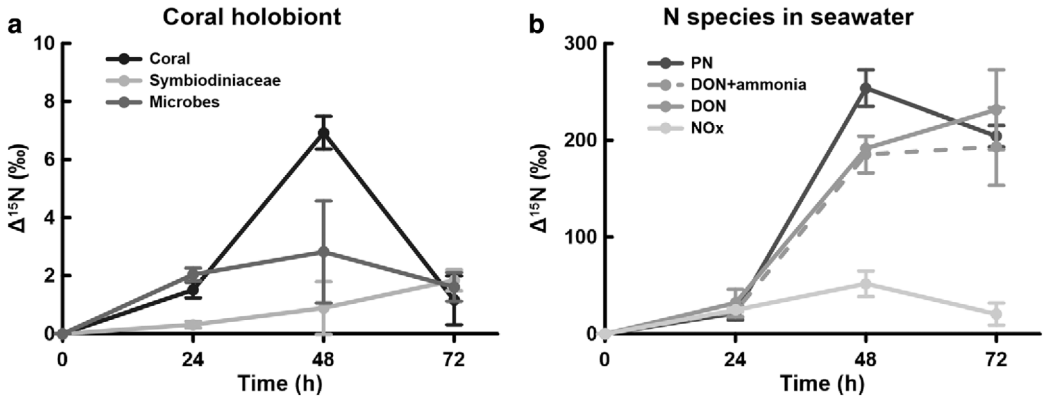

Fig. 3. The increase of $\delta^{15}N$ ($\triangle^{15}N$) of the different components during the diazotroph-derived nitrogen transfer experiment. (**a**) $\triangle^{15}N$ of different components (coral tissue, Symbiodiniaceae, symbiotic microbes) in coral; (**b**) $\triangle^{15}N$ of different components (PN, NO_x, DON (before and after NH_4^+ removal)) in ambient seawater. Symbals and error bars represent the mean and range of the rates or ratio from the duplicate incubation at each time point.

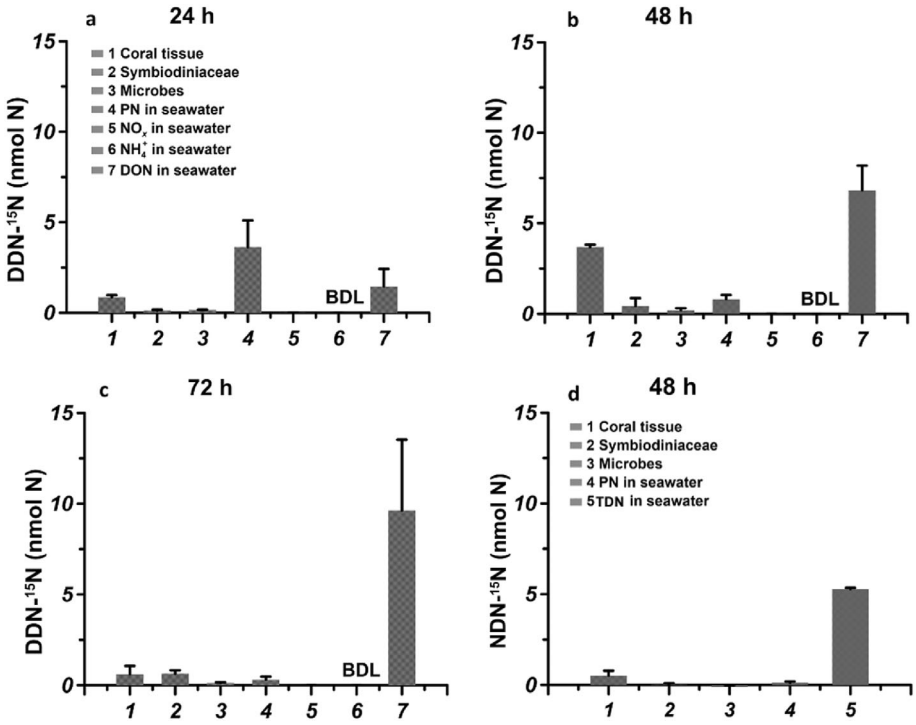

Fig. 4. Distribution of the released ^{15}N of diazotroph-derived nitrogen (DDN) during the time course of the DDN transfer experiment (**a**–**c**) and nitrate-derived nitrogen (DDN) after 48 h (**d**). BDL denotes below the detection limit. Bars and error bars represent the mean and range of the rates from the duplicate incubation at each time point.

and coral fragments were reclaimed using tweezers. Therefore, the plankton biomass and any dissolved components in the overlying water were mostly removed along with the water. (2) The rate of N_2 fixation by the plankton in the seawater was independently measured in the in situ seawater incubations, as described in the methods, that is, in situ seawater without addition of coral or crustose coralline algae fragments. This rate was 21.1 ± 7.4 nmol N L^{-1} d^{-1}, equivalent to ~ 16.7 nmol of N fixed by the 800 mL in situ seawater per day. By comparison, the measured N_2 fixation rate by the crustose coralline algae was 2.8 ± 1.6 mg N m^{-2} d^{-1}. Taking the area of ~ 5 cm^2 in our incubation, the crustose coralline algae fixed ~ 1400 ng N (equivalent to 100 nmol N) per day, which was sixfold higher than the N_2 fixation rate by the plankton in the seawater. (3) No increase in $\delta^{15}N$ was observed in coral samples incubated with in situ seawater (i.e., $\delta^{15}N$ of coral was 8.83‰ ± 0.06‰ and 7.89‰ ± 0.30‰ at 0 and 48 h, respectively), indicating that any transfer of N_2 fixation signal from the overlying plankton community or direct contamination of coral by the plankton did not occur or was below our detection limit. Nevertheless, we suggest that the use of filtered seawater in future studies to measure N_2 fixation associated with the benthic components of coral systems could avoid the potential contamination by the plankton in the overlying water.

N_2 fixation rate in the coral holobiont has been extensively measured, and the reports imply large temporal-spatial variation ranging from 0 to 15 mg N m^{-2} d^{-1} (Shashar et al. 1994; Rädecker et al. 2022; Moynihan et al. 2022). N_2 fixation rate in the coral is controlled by a complex set of environmental conditions, including the coral species, location, depth, irradiance, season, nutrient conditions, and the metabolic status of the coral (Bednarz et al. 2017; Moynihan et al. 2022). For the *P. damicornis* coral, the N_2 fixation rate ranged from 0 to 1.7 mg N m^{-2} d^{-1} in a coral reef system in the Red Sea based on the acetylene reduction method (Shashar et al. 1994). However, in our study, the N_2 fixation rate of *P. damicornis* was at the lower end of the reported rates by both acetylene assay and $^{15}N_2$-labeling incubation, demonstrating that the diazotrophy associated with *P. damicornis* (dominated by cyanobacterial diazotroph) did not actively contribute to N_2 fixation in our study area (Figs. 1b, 2). This result is consistent with a recent survey of N_2 fixation rate associated with three coral species including the *P. damicornis* in New Caledonia, which shows a negligible contribution of symbiotic N_2 fixation to the coral compared to the N_2 fixation in the overlying water, probably due to light limitation on the coral-associated diazotrophs (Meunier et al. 2021). A similar result of undetectable N_2 fixation rate in the coral has been attributed to the high nitrogen loading to the system which inhibits N_2 fixation (O'Neil and Capone 2008). However, whether the absence of N_2 fixation rate in our coral sample is due to nitrogen loading or light limitation should be further investigated.

The depth-integrated N_2 fixation rate of the overlying water (0.7 ± 0.3 mg N m^{-2} d^{-1}) was lower than the N_2 fixation rate measured in the crustose coralline algae holobiont (2.8 ± 1.6 mg N m^{-2} d^{-1}). This result is consistent with previous observations showing that N_2 fixation in the overlying water is lower than in the benthic communities in coral reef systems (O'Neil and Capone 2008; Benavides et al. 2017). Nevertheless, the N_2 fixation rate (21.1 ± 7.4 nmol N L^{-1} d^{-1}) measured in our study is higher than the adjacent Hainan Island coast (0.1–5.6 nmol N L^{-1} d^{-1}; Zhang et al. 2015) and the nearby northern South China Sea (0.1–1.3 nmol N L^{-1} d^{-1}; Chen et al. 2019), indicating more active N_2 fixation in the coral reef than non-coral areas. This is likely due to the stimulation of N_2 fixation in the overlying water by the benthic community in the coral reef system (Wiebe et al. 1975). A direct comparison of N_2 fixation in the water with or without coral finds that N_2 fixation rate is significantly higher in the treatment with coral colonies (Grover et al. 2014). In our study, the diazotroph community of the overlying water and crustose coralline algae holobiont was dominated by the heterotrophic proteobacteria ($64.6\% \pm 28.5\%$ in crustose coralline algae holobiont and $82.8\% \pm 12.0\%$ in seawater), including the members of the class *Alphaproteobactria* within the *Rhizobiales*, and *Gammaproteobactria* within the *Enterobacteriales*, *Methylococcales*, *Psedomonadales*, and *Xanthomonadales*, indicating that the heterotrophic diazotrophs were the main contributors of N_2 fixation in our study area, even though the potential contribution of autotrophic diazotrophs (i.e., cyanobacteria) cannot be ruled out. The higher N_2 fixation rate in the overlying water thus could be attributed to the release of labile organic matter from benthic communities of coral reefs, which stimulated the growth and activity of heterotrophic diazotrophs (Wild et al. 2004; Benavides et al. 2017). This idea is supported by the near twofold higher DOC concentration (141.7 ± 65.8 μmol L^{-1}) in our study area (Luo et al. 2022) as compared to the DOC (73.4–84.6 μmol L^{-1}) in the adjacent northern South China Sea in our sampling season (Meng et al. 2017). Studies on N_2 fixation associated with coral systems show that the composition of the diazotrophic community and N_2 fixation rates are distinct between different coral species (Meunier et al. 2021) and vary across location and depth (Rädecker et al. 2015; Benavides et al. 2017; Tilstra et al. 2019), indicating the need to investigate global coral reef diazotrophy with higher spatial-temporal coverage in the future.

Together, our results revealed that the diazotrophy associated with the crustose coralline algae demonstrated higher N_2 fixation intensity than the overlying water and the adjacent coral holobiont in our study area (Fig. 2). The high organic matter content appears to stimulate the heterotrophic diazotrophs in crustose coralline algae holobiont and the overlying water. Given that crustose coralline algae are widely distributed in global reef systems (Sneed et al. 2015) and have greater areal coverage than coral itself in many coral reefs (Tâmega and Figueiredo 2019), this result indicates a potentially huge yet historically overlooked source of N_2 fixation in the coral reef system that should be explored with higher spatial and temporal resolution in the global coral reef systems. Future studies are also warranted to distinguish the contribution and fate of diazotroph-derived nitrogen released by the epilithic and endolithic diazotrophic community, and to discern the role of autotrophic and heterotrophic diazotrophs to N_2 fixation in the crustose coralline algae holobiont.

Distinct fate of diazotroph- and nitrate-derived nitrogen in the coral reef system

For the diazotroph-derived nitrogen transfer experiment, the ^{15}N originating from N_2 fixation in crustose coralline algae holobiont was transferred into all components of the ambient water and coral in the incubation within the first 24 h (Fig. 4a). Around $38.6\% \pm 1.8\%$ (6.2 ± 2.4 nmol ^{15}N) of the fixed ^{15}N-N_2 in crustose coralline algae was released and redistributed in different components in the incubation during the first 24 h. At 48 h, the released ^{15}N increased to $48.1\% \pm 1.8\%$ (11.93 ± 0.89 nmol ^{15}N), which was comparable to the released ^{15}N ratio of $46.4\% \pm 7.7\%$ (10.83 ± 4.78 nmol ^{15}N) at 72 h. The result demonstrated rapid release and efficient transfer of diazotroph-derived nitrogen from crustose coralline algae to the ambient seawater and coral holobiont. Release of diazotroph-derived nitrogen has been extensively reported in the water column the open ocean (Mulholland 2007) and inside the coral holobiont (Rädecker et al. 2022; Moynihan et al. 2022). However, direct measurement on diazotroph-derived nitrogen release and transfer in crustose coralline algae remain lacking. To our knowledge, this is the first report on the active N_2 fixation and rapid release of diazotroph-derived nitrogen from the diazotrophy associated with crustose coralline algae to the overlying water and coral holobiont. These experimental results reveal an overlooked source of new nitrogen and its transfer pathway that is likely important in the coral reef system.

To further elucidate the importance of diazotroph-derived nitrogen release from diazotrophy associated with crustose coralline algae to support the growth of coral, we compared diazotroph-derived nitrogen release rate with nitrate-derived nitrogen release rate. In general, N_2 fixation and NO_3^- represent the two most important new nitrogen sources in oligotrophic coral reef systems. Our results showed that although the N_2 fixation rate (2.8 ± 1.6 mg N m^{-2} d^{-1}) was lower than the NO_3^- uptake rate (22.5 ± 4.1 mg N m^{-2} d^{-1}) in the crustose coralline algae (Fig. 2a), the diazotroph-derived nitrogen release rate (1.4 ± 0.8 mg N m^{-2} d^{-1}) was comparable with nitrate-derived nitrogen release rate (2.0 ± 0.4 mg N m^{-2} d^{-1}; Fig. 2b), resulting in sixfold higher diazotroph-derived nitrogen release ratio than nitrate-derived nitrogen release ratio (48.1% for diazotroph-derived nitrogen vs. 8.7% for nitrate-derived nitrogen, defined as the ratio of release rate to N_2 fixation rate or

NO_3^- assimilation rate, see methods; Fig. 2c). The large difference in N-release ratio highlights the distinct fates of the two sources of new nitrogen, N_2 fixation by the diazotrophs and NO_3^- assimilation by the crustose coralline algae. The new nitrogen produced by the diazotrophs was rapidly released into the ambient water and subsequently utilized by phytoplankton and transferred into the coral holobiont. This result demonstrated a highly efficient diazotroph-derived nitrogen release and transfer from the symbiotic diazotrophs to other plankton in the coral reef system, consistent with observations in the open ocean (Mulholland 2007; Caffin et al. 2018 and reference therein). By contrast, the low release ratio of nitrate-derived nitrogen suggested that NO_3^- was mainly assimilated and retained by the coralline algae itself for growth. These results suggested the distinct fates of diazotroph- and nitrate-derived nitrogen in the crustose coralline algae, highlighting a substantial yet unappreciated role of crustose coralline algae in sustaining the net growth of coral holobiont.

Diazotroph-derived nitrogen transfer pathway in the coral reef system

To date, studies on diazotroph-derived nitrogen transfer in the coral reef system have primarily focused on the internal diazotroph-derived nitrogen flows in the coral holobiont (i.e., diazotroph-derived nitrogen translocation between skeleton, mucus, coral tissue, and Symbiodiniaceae) (Rädecker et al. 2022; Moynihan et al. 2022). Only a few studies have investigated the transfer of external N_2 fixation into the coral holobiont through grazing (Benavides et al. 2016; Bednarz et al. 2017). However, the specific pathways and rates of diazotroph-derived nitrogen exchange between external diazotrophs, overlying water and coral holobiont, particularly the fate of N_2 fixation associated with crustose coralline algae and its transfer to coral holobiont remain unclear.

During the diazotroph-derived nitrogen transfer experiment, ^{15}N sourced from the crustose coralline algae was observed in nearly all of the N-containing components during the first 24 h, indicating a rapid release and translocation of diazotroph-derived nitrogen (Fig. 4a). The redistribution of ^{15}N, however, showed large differences among different components and varied over time, implying distinct pathways of diazotroph-derived nitrogen transfer in the complex coral reef system. The significant increase in $\delta^{15}N$ of PN, DON, and NO_x in the overlying water at 24 h (Fig. 3) suggested that the diazotroph-derived nitrogen was firstly transferred into pelagic microorganisms before being further transported into the benthic coral holobiont. NH_4^+ and DON have been proposed as key forms of reactive nitrogen of diazotroph-derived nitrogen in the marine environment (Berman and Bronk 2003; Berthelot et al. 2016) and the coral holobiont (Benavides et al. 2016, 2017). In our study, the rapid transfer of diazotroph-derived nitrogen to PN and NO_x in the first 24 h indicated that NH_4^+ was likely a key component as NH_4^+ is a preferential nutrient for phytoplankton and the substrate for nitrifiers, although the NH_4^+ and DON in the diazotroph-derived nitrogen pool remains unresolved still. Nevertheless, NH_4^+ was below the detection limit during our incubation and there was no significant change of $\delta^{15}N$ in DON before and after NH_4^+ removal. Combined observations suggested that crustose coralline algae-associated NH_4^+ release and reutilization were tightly coupled in the overlying water. On the other hand, we observed a stepwise accumulation of ^{15}N in the DON pool during the time DIN course experiment, while the ^{15}N in the PN pool peaked at 24 h and then decreased afterward, indicating DON released from coral was not a major source for the growth of microorganisms in the overlying seawater.

As for the coral holobiont, the transfer of ^{15}N into the Symbiodiniaceae was lower than the coral tissue during our incubation period, suggesting not only the ^{15}N signal in coral tissue was not sourced from the Symbiodiniaceae but also the diazotroph-derived nitrogen was not actively accessed by the Symbiodiniaceae inside the coral during our incubation period. The lack of ^{15}N accumulation in the Symbiodiniaceae might also reflect the efficient scavenging of diazotroph-derived nitrogen by the phytoplankton in the overlying water, preventing the transfer of dissloved inorganic nitrogen from overlying water into the coral holobiont. The $\delta^{15}N$ of the microbes covaried but was consistently lower than coral tissue during the time course incubation, indicating the assimilation of coral sourced organic nitrogen by the microbes in the coral holobiont.

The stepwise change of ^{15}N distribution in different components of investigated N-containing components during the 72 h time course incubation allowed us to examine the main pathways of diazotroph-derived nitrogen transfer from crustose coralline algae to the overlying water and coral holobiont in the incubation. The diazotroph-derived nitrogen was concentrated in the PN followed by DON in the overlying water during the first 24 h, indicating efficient release and transfer of diazotroph-derived nitrogen to the planktonic microorganisms in the overlying water. It is worth noting that the increase of $^{15}N-NO_3^-$ indicated at least part of the diazotroph-derived nitrogen was released as NH_4^+ and then further converted into NO_3^- via nitrification (Fig. 5a). At 48 h, the diazotroph-derived nitrogen signal was mainly distributed in the DON pool of the overlying seawater, while the ^{15}N in PN significantly decreased, accompanied by the increase of ^{15}N in coral tissue, suggesting a transfer of ^{15}N from PN in the overlying water into the coral tissue through grazing (Fig. 5b). This is consistent with the observation of the high grazing rate of coral on phytoplankton (Tremblay et al. 2011).

Together, these reallocations of ^{15}N during the time course incubations revealed efficient diazotroph-derived nitrogen transfer in the coral reef system. Diazotroph-derived nitrogen originated from crustose coralline algae holobiont was rapidly released into the overlying water as NH_4^+ and DON, of which the NH_4^+ was rapidly scavenged by phytoplankton and nitrifiers in the overlying water. The diazotroph-derived nitrogen was then

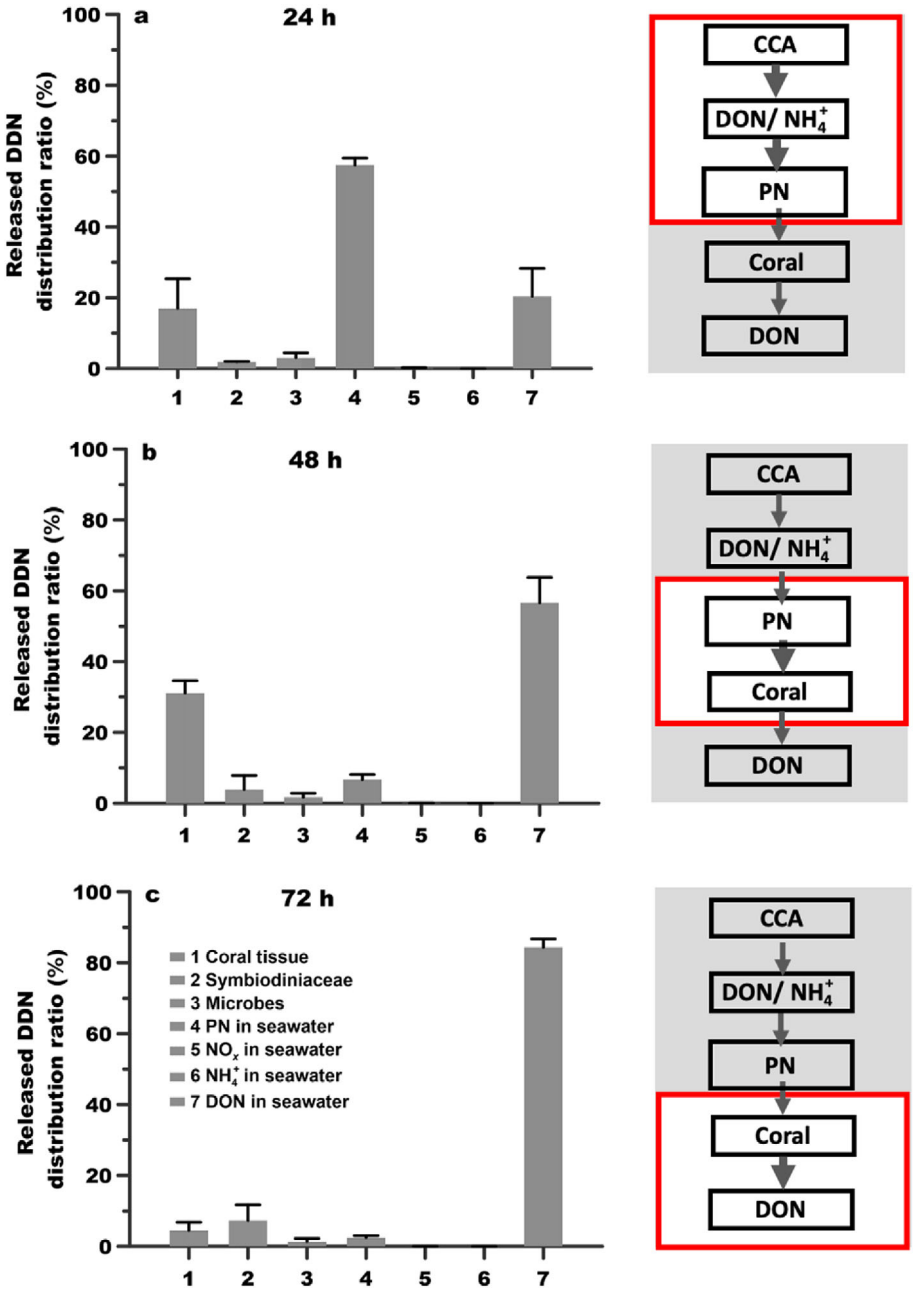

Fig. 5. Percentage distribution of diazotroph-derived nitrogen (DDN) from crustose coralline algae (CCA) to different components of the coral (coral tissue, Symbiodiniaceae, symbiotic microbes) and N species in ambient seawater (PN, NOx, NH_4^+, DON) during the time-course incubation of the DDN transfer experiment. (**a**–**c**) Results at 24, 48, and 72 h, respectively. Bars and error bars represent the mean and range of the rates from the duplicate incubation at each time point. The red box in the right panel illustrates the main DDN transfer pathway at each time point.

transferred from the overlying water into the coral through feeding on the PN from the overlying water. However, a large fraction (83.4 %) of the grazed ^{15}N was released back into the environment. The ^{15}N in the Symbiodiniaceae increased stepwise but with a much slower accumulation rate, indicating a less efficient utilization of coral-released diazotroph-derived nitrogen by the Symbiodiniaceae. By the end of the incubation, nearly all the released ^{15}N was in the DON pool, indicating rapid digestion of PN by coral and transfer of ^{15}N back to the overlying water as DON (Fig. 5c).

Elucidating the efficient diazotroph-derived nitrogen transfer pathway in coral system and the ecological implication

Our comprehensive set of N_2 fixation and transfer incubations revealed a complex yet highly efficient diazotroph-

derived nitrogen release and transfer pathway in the coral reef system. Within this system, the diazotrophy associated with crustose coralline algae represents a large source of N_2 fixation. The newly fixed nitrogen was rapidly released (~ 50% of diazotroph-derived nitrogen was released in 48 h) into the overlying water, and was subsequently scavenged by the planktonic microorganisms such as phytoplankton and nitrifiers. The diazotroph-derived nitrogen was then transferred into the coral holobiont through grazing, and was released back into overlying water by excretion, demonstrating a rapid and efficient diazotroph-derived nitrogen transfer pathway that connects the new nitrogen input with the nitrogen recycling in the coral reef ecosystem (Fig. 6).

Both coral and algae release large amounts of dissolved organic matter (DOM), which constitutes one of the largest sources of organic matter in coral reef systems (Wild et al. 2004; Rix et al. 2017). However, recent studies show that the DOM produced by algae is utilized at a significantly higher rate than the coral-derived DOM, suggesting different composition and bioavailability of the DOM sourced from coral and crustose coralline algae (Rix et al. 2017; Silva et al. 2021). Consistent with these observations, the ^{15}N was mainly distributed in the PN pool at the first 24 h in our study, indicating the crustose coralline algae-derived DON was rapidly assimilated by the phytoplankton in the overlying water; by contrast, the low content of ^{15}N in the PN and accumulation of ^{15}N in the DON pool indicated the coral derived DON was less available to the phytoplankton. These results implied that coral plays a crucial role in regulating the bioavailability of DON in the coral reef system through grazing and excretion. It also suggested that the more labile DON produced by the algae tends to promote the growth of pathogenic microbes (Nelson et al. 2013) and the less labile DON helps to maintain the community structure through reducing macroalga and bacterioplankton blooms in the coral reef system (Nelson et al. 2013; Silva et al. 2021). The conversion of more labile crustose coralline algae-derived DON to less bioavailable coral-derived DON may function to maintain the homeostasis and the health in the coral reef system.

In concert, our finding of the efficient diazotroph-derived nitrogen transfer pathway between crustose coralline algae-associated diazotrophy and coral holobiont provides new evidence of efficient N recycling, revealing a tight connection between new nitrogen input and recycling to sustain the high productivity in the coral reef system, helping to explain the "Darwin Paradox" in this unique ecosystem.

Data availability statement

All data needed to evaluate the conclusions in the paper are deposited in the Zenodo database that can be accessed through https://doi.org/10.5281/zenodo.6095269. The raw reads of Bacterial and diazotrophic communities associated with crustose coralline algae (*P. onkodes*), coral (*P. damicornis*), and seawater were deposited into the NCBI Genebank database with the accession number of PRJNA799600 for *nifH* gene, and the accession number of PRJNA799477 for16S rRNA gene.

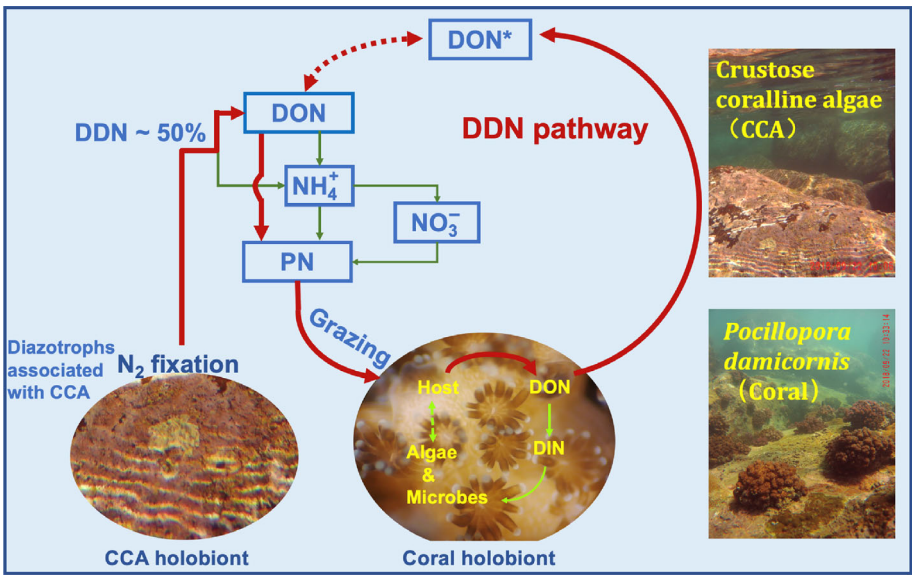

Fig. 6. Schematic diagram of the proposed coral-mediated diazotroph-derived nitrogen (DDN) transfer in the coral reef system. Within this system, the diazotrophy associated with crustose coralline algae (CCA) represents the dominant source of N_2 fixation, and the newly fixed nitrogen is rapidly released into the overlying water, fueling the growth of the phytoplankton. The DDN is then transferred into the coral holobiont through grazing and excretion, which drive the DDN flow from overlying water into the coral and convert the PN into DON, forming a rapid and efficient DDN release and transfer pathway. The major DDN transfer pathway is represented in the red arrow and the green arrow denotes the remaining pathways. The panels on the right are photos of CCA and coral used for incubation during our sampling period.

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Conflict of Interest

None declared.

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