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Diazotroph-Derived Nitrogen Assimilation Strategies Differ by Scleractinian Coral Species

Valentine Meunier^{1*}, Sophie Bonnet², Mar Benavides², Andreas Ravache¹, Olivier Grosso², Christophe Lambert³ and Fanny Houllbrèque¹

¹ Centre IRD Nouméa, UMR ENTROPIE (IRD, Université de La Réunion, CNRS, Université de La Nouvelle-Calédonie, Ifremer), Nouméa, New Caledonia, ² Aix-Marseille Université, Université de Toulon, CNRS, IRD, MIO UM 110, Marseille, France, ³ Univ Brest, CNRS, IRD, Ifremer, LEMAR, Plouzané, France

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*Correspondence:

Valentine Meunier
valentine.meunier@ird.fr

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Reef-building corals generally thrive in nutrient-poor tropical waters, where among other elements, nitrogen (N) availability often limits primary productivity. In addition to their close association with endosymbiotic dinoflagellates of the family Symbiodiniaceae, enabling an effective use and retention of dissolved inorganic nitrogen (DIN), scleractinian corals have developed strategies to acquire new N: (1) They can ingest N-rich sediment particles and preys (from picoplankton to macro-zooplankton) via heterotrophy, including diazotrophs [plankton fixing dinitrogen (N₂) and releasing part of this nitrogen—Diazotroph-Derived N (DDN)—in seawater], a pathway called “heterotrophic nutrition on diazotrophs”; (2) Symbiotic diazotrophs located in the coral holobiont have the molecular machinery to fix N₂, a pathway called “symbiotic N₂ fixation”. Here we used the ¹⁵N₂ isotopic labeling in a series of incubations to investigate the relative contribution of each of these DDN transfer pathways in three worldwide distributed coral species: *Acropora muricata*, *Galaxea fascicularis*, and *Pocillopora damicornis*. We show that N provision via “symbiotic N₂ fixation” is negligible compared to that obtained via “heterotrophic nutrition on diazotrophs,” with DDN assimilation rates about a thousand times lower for *P. damicornis* and *G. fascicularis*, or assimilation rates via “symbiotic N₂ fixation” almost nil for *A. muricata*. Through heterotrophic feeding on planktonic diazotrophs, only *G. fascicularis* and *P. damicornis* can successfully obtain N and fulfill a large part of their N requirements (DDN assimilation rates: 0.111 ± 0.056 and 0.517 ± 0.070 μg N cm⁻² h⁻¹ in their Symbiodiniaceae, respectively). Whereas this contribution is again negligible for *A. muricata*. They also largely consume the picoplankton that likely benefit from this DDN (*Prochlorococcus* and *Synechococcus* cells; respectively, 2.56 ± 1.57 10⁴ and 2.70 ± 1.66 10⁴ cell h⁻¹ cm⁻² for *G. fascicularis*; 3.02 ± 0.19 10⁵ and 1.14 ± 0.79 10⁴ cell h⁻¹ cm⁻² for *P. damicornis*). The present study confirms the different dependencies of the three tested species regarding heterotrophy, with *P. damicornis* and *G. fascicularis* appearing highly efficient at capturing plankton, while *A. muricata*, considered as mainly autotroph, does not rely on these food resources to meet its N and energy needs.

Keywords: dinitrogen fixation, DDN assimilation, scleractinian corals, heterotrophy, diazotrophs, picoplankton, New Caledonia

INTRODUCTION

Reef-building corals generally thrive in nutrient-poor tropical waters, where among others, nitrogen (N) availability often limits primary production (Howarth, 1988). The highly efficient uptake and recycling of nutrients by corals can explain this paradox (Wild et al., 2004; De Goeij et al., 2013). The close association between the coral animal host and its endosymbiotic dinoflagellate of the family Symbiodiniaceae (LaJeunesse et al., 2018) enables an effective use and retention of nutrients and photosynthates (Muscatine and Porter, 1977). Together with their algal symbionts, corals are associated with a variety of other microorganisms, including viruses, protists, Bacteria, Archaea and fungi, an assemblage termed the coral holobiont (Rohwer et al., 2002; Bourne et al., 2016; van Oppen and Blackall, 2019), which also participate in nutrient supply and recycling (e.g., Rosenberg et al., 2007; Rådecker et al., 2015). Among nutrients, N is an essential element of many fundamental molecules of living organisms such as nucleic acids (DNA, RNA) and amino acids (proteins, enzymes) and represents an essential basis for all organic activity. Scleractinian corals developed strategies to acquire new N via different pathways (Goreau et al., 1971; Rådecker et al., 2015). First, algal symbionts efficiently take up dissolved inorganic N (DIN) from surrounding waters and translocate it to coral tissues (Falkowski et al., 1993). Corals can also assimilate dissolved organic N compounds (DON), such as urea and amino acids from the environment (Muscatine, 1990; Mills et al., 2004). Then, the animal host can ingest N-rich sediment particles and prey via heterotrophy (Houlbreque, 2004; Mills and Sebens, 2004). It is well known that corals feed on mesozooplankton (200 μm^{-2} cm; which they catch by using their tentacles (Sebens et al., 1996; Ferrier-Pagès et al., 2003; Houlbreque, 2004; Goldberg, 2018), but are also able to trap pico- and nanophytoplankton (0.2 μm –20 μm ; Houlbreque, 2004; Tremblay et al., 2012; Goldberg, 2018; Sangmanee et al., 2020). Pico- and nanophytoplankton supplies a substantial proportion of the metabolic requirements of most scleractinian corals (Goldberg, 2018; Sangmanee et al., 2020) and represents a continuous available source of food in the reefs (Tremblay et al., 2012). Among plankton, diazotrophs (dinitrogen (N_2)-fixing prokaryotes), are particularly abundant in coral lagoon waters (Turk-Kubo et al., 2015; Messer et al., 2017; Saulia et al., 2020), and heterotrophic nutrition on diazotrophs represent a potential source of N for corals (Benavides et al., 2016; Meunier et al., 2019). This plankton fixes N_2 (Tilstra et al., 2018) transform it into a bioavailable N form (NH_4^+), and releases part of the recently fixed N (Diazotroph-Derived N, DDN) in seawater, providing available N for the development of the planktonic food web (Berthelot et al., 2016; Bonnet et al., 2016). Hence, when corals feed on plankton, it can ingest both planktonic diazotrophs, but also plankton that developed on DDN from planktonic diazotrophs (for simplicity, this pathway will be called hereafter “heterotrophic nutrition on diazotrophs”). Finally, symbiotic diazotrophs located in the coral tissue, coral mucus layer and skeleton, have the molecular machinery to fix N_2 (Lema et al., 2012; Bednarz et al., 2019) and this pathway has been evidenced as a N source in several

corals species (Santos et al., 2014; Cardini et al., 2015; Tilstra et al., 2019). While recent works have shown that N acquisition through symbiotic N_2 fixation greatly varies in relation to environmental factors, species, but also corals’ metabolic status (Rådecker et al., 2015; Bednarz et al., 2017; Benavides et al., 2017; Lesser et al., 2018, 2019), heterotrophic nutrition on diazotrophs has only been demonstrated for one coral species, *Stylophora pistillata* in Benavides et al. (2016) and Meunier et al. (2019).

The general aim of the present study was to determine how DDN acquisition through symbiotic N_2 fixation and heterotrophic nutrition on planktonic diazotrophs vary according to coral species. We used the $^{15}\text{N}_2$ isotopic labeling in a series of incubation experiments to investigate the relative contribution of each DDN transfer pathway in three coral species: *Acropora muricata*, *Galaxea fascicularis* and *Pocillopora damicornis*. Widely distributed around the world, these three species have a different heterotrophic capacity. While the genus *Acropora* has always been considered largely autotrophic (Conti-Jerpe et al., 2020), *Galaxea* and *Pocillopora* are conversely considered with higher heterotrophic capacities (Wijgerde et al., 2011; Toh et al., 2013; Conti-Jerpe et al., 2020; Lyndby et al., 2020).

MATERIALS AND METHODS

Study Site and Coral Collection

The study was performed in March 2019 (austral summer). The three coral species tested (*Acropora muricata*, *Galaxea fascicularis*, and *Pocillopora damicornis*) differ in the size of their polyps. A total of 20 apexes (5 cm long) of each the coral species were collected between 3 and 5 m depth at the Crouy reef, New Caledonia (22°15,883'S; 166°19,658'E, sampling license issued by the “Province Sud,” Government of New Caledonia). Coral fragments were transported in individual zip-lock bags to the Aquarium Des Lagons (Nouméa) in a cooler containing freshly collected seawater. Fragments were allowed to recover prior to the start of the incubations, for 24 h in a 20 L aquarium supplied with filtered (porosity 40 μm) seawater, renewed at a rate of 16.5 l h⁻¹ and mixed using a submersible pump (Aquarium system, micro-jet MC 320, Mentor, OH, United States). Seawater was maintained under a constant temperature of 26°C and coral fragments received a constant irradiance of 120 \pm 10 $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$ (photoperiod 12 h: 12 h light: dark) using two Aquablue plus neon bulbs (blue-white, 15.000 K, Giesemann, Germany).

Measurement of Symbiotic N_2 Fixation Rates: $^{15}\text{N}_2$ Incubation Experiments in Filtered Seawater

We used the $^{15}\text{N}_2$ -labeling technique (dissolved $^{15}\text{N}_2$ method, Mohr et al., 2010; Grokopf et al., 2012) as described in Meunier et al. (2019), to quantify symbiotic N_2 fixation and the potential transfer of DDN to the Symbiodiniaceae fraction. Ten coral

fragments from each species were individually incubated for 8 h (from noon to 8 pm, local time) in the dark, into 1 L glass bottles containing $^{15}\text{N}_2$ -enriched 0.2 μm -filtered seawater continuously stirred with magnets. The $^{15}\text{N}_2$ -enriched seawater was prepared as described in Meunier et al. (2019) and its ^{15}N isotopic enrichment was measured by MIMS (Membrane Inlet Mass Spectrometry; Kana et al., 1994) after the 8 h of incubation. All beakers were immersed in a water bath to maintain the temperature constant ($26.1 \pm 0.3^\circ\text{C}$) throughout the experiment.

Measurement of Heterotrophic Nutrition on Diazotrophs Diazotrophy—Feeding Experiments

In parallel experiments, we quantified the assimilation of DDN via heterotrophic nutrition on diazotrophy in the same three coral species. As described in Meunier et al. (2019), we pre-labeled with $^{15}\text{N}_2$, 5 sets of 4.3 L of seawater containing the natural plankton assemblage (collected at the same site as corals). After 24 h of incubation with $^{15}\text{N}_2$, this plankton was collected onto 0.4 μm polycarbonate filters, and vortexed in a 50 mL seawater aliquot to resuspend cells. Each concentrated $^{15}\text{N}_2$ -enriched natural plankton aliquot was provided to 5 coral fragments per species, each of them being incubated for 8 h, in the dark in separated glass beakers (15 beakers in total + controls, see below), continuously stirred with magnets. The natural plankton assemblage was collected at the same site as corals, thus planktonic concentration in the beakers is comparable to natural plankton densities of the sampling site. Three additional control beakers (without corals) were also incubated to quantify pico- and nanoplankton abundance fluctuations in the absence of coral predation (internal grazing, natural cell growth and/or cell death) at the beginning and at the end of the incubations. Incubations were carried out in the dark in order to maximize heterotrophy as, on the reef, coral feeding is exacerbated at night (with polyps more deployed and prey more available) (reviewed by Houlbrèque and Ferrier-Pagès, 2009).

Triplicate aliquots of the ^{15}N -labeled plankton given to corals were also kept for further IRMS analyses to measure the $^{15}\text{N}_2$ enrichment of plankton. Triplicate seawater samples were also collected from each beaker at the beginning and the end of the incubation period for further flow cytometry analyses. Samples were fixed with paraformaldehyde (final concentration of 0.1%) for 30 min at room temperature in the dark, then stored at -80°C . *Prochlorococcus* sp. (0.6 μm), *Synechococcus* sp. (1–2 μm), picoeukaryotes and heterotrophic bacteria abundance were analysed with a FACSVerse Flow Cytometer (Becton Dickinson, CA, United States) as described in Meunier et al. (2019), to quantify the respective ingestion rates by each coral species. Heterotrophic bacteria were detected by diluting the medium 10-fold with filtered seawater (0.2 μm) and staining with SYBR® Green I (DNA). For *Prochlorococcus* sp., *Synechococcus* sp., and picoeukaryotes abundances, quantification was done according to the level of red fluorescence corresponding to Chlorophyll *a* content, and orange fluorescence representative of pigment contents such as phycoerythrin (especially to discriminate

between *Synechococcus* sp. and other pico- and nanoplankton) and also by size-sorting using 1 μm green calibration beads (Marie et al., 1997, 1999).

Sample Preparation

At the end of the incubation, corals were rinsed six times with filtered seawater to remove cells potentially adhered to the coral surface (Houlbrèque et al., 2003), transferred to zip-lock bags and stored at -20°C until analysis. The coral tissue was removed from the skeleton using an air-pick and 20 mL of 0.45 μm filtered seawater and homogenized with a Potter tissue grinder (Rodolfo-Metalpa et al., 2010). Algal symbionts and tissue fractions were separated by centrifugation (Houlbrèque et al., 2003). The pellets containing algal symbionts were dried and ground. Coral tissue solutions were filtered (15 mL) on pre-combusted GF/F filters under low pressure. The filters and the algal symbionts crushed were encapsulated in tin cups for ^{15}N analyses using an elemental analyzer coupled to an isotopic ratio mass spectrometer (EA-IRMS, Integra CN, SerConLtd, Cheshire, United Kingdom). The coral tissue fraction was filtered onto GF/F filters that were also analyzed by EA-IRMS. 10 mL subsample was taken from each homogenate and processed for algal symbionts density and total chlorophyll concentrations (see below). N assimilation rates into coral tissue and algal symbionts were calculated as described in Benavides et al. (2016).

Ingestion Rates of Pico- and Nanoplankton

Ingestion rates were assessed by means of the clearance rate, according to previous studies on corals (Houlbrèque et al., 2004; Tremblay et al., 2012), and calculated using the equations of Ribes et al. (1998), which take into account the natural growth and death of the prey during incubations. Ingestion rates were expressed as the number of prey organisms ingested and normalized to the skeletal surface area (cm^2). Carbon (C) and N content of ingested preys were estimated using conversion factors from the available literature (summarized in Table 1).

Coral Skeletal Surface Area Normalization

Picoplankton ingestion rates were standardized per skeletal surface area (cm^2), estimated using the paraffin wax-dipping method (Stimson and Kinzie, 1991; Naumann et al., 2009).

Statistical Analyses

All tests were performed using R version 3.6.1 within RStudio (Version 1.1.456, 2018). Given the small sample size, non-parametric Kruskal-Wallis tests were first used to test for significant differences in algal symbionts densities, total chlorophyll content and picoplankton ingestion rates between species. When significant differences were found, Wilcoxon-Mann-Whitney tests were performed to test for pairwise differences between each species (Table 2). To look for potential differences in DDN assimilation rates by coral species, DDN transfer pathways and compartments, three-way ANOVAs

TABLE 1 | Carbon and nitrogen cell content (ng cell^{-1}) and related N assimilation rates ($\mu\text{g cm}^{-2} \text{h}^{-1}$) calculated for *Prochlorococcus*, *Synechococcus* using literature conversion factors and the results of the present study (mean \pm SE).

DDN transfer pathway: 'symbiotic N ₂ fixation'			
	C (ng C cell^{-1})	References	C assimilation ($\text{ng C cm}^{-2} \text{h}^{-1}$)
Galaxea fascicularis			
<i>Prochlorococcus</i> sp.	$3.6 \cdot 10^{-5(a)}$	Buitenhuis et al., 2012	$9.204 \pm 5.650 \cdot 10^{-1}$
<i>Synechococcus</i> sp.	$2.5 \cdot 10^{-4(a)}$	Buitenhuis et al., 2012	6.749 ± 4.153
Pocillopora damicornis			
<i>Prochlorococcus</i> sp.	$3.6 \cdot 10^{-5(a)}$	Buitenhuis et al., 2012	10.859 ± 0.689
<i>Synechococcus</i> sp.	$2.5 \cdot 10^{-4(a)}$	Buitenhuis et al., 2012	2.842 ± 1.974
	N (ng N cell^{-1})	References	N assimilation ($\text{ng C cm}^{-2} \text{h}^{-1}$)
Galaxea fascicularis			
<i>Prochlorococcus</i> sp.	$9.6 \pm 0.9 \cdot 10^{-6(b)}$	Bertilsson et al., 2003	0.245 ± 0.151
<i>Synechococcus</i> sp.	$3.79 \cdot 10^{-5(c)}$	Redfield, 1958	0.540 ± 0.332
Pocillopora damicornis			
<i>Prochlorococcus</i> sp.	$9.6 \pm 0.9 \cdot 10^{-6(b)}$	Bertilsson et al., 2003	2.896 ± 0.184
<i>Synechococcus</i> sp.	$3.79 \cdot 10^{-5(c)}$	Redfield, 1958	0.227 ± 0.158
DDN transfer pathway: "heterotrophic nutrition on diazotrophs"			DDN assimilation ($\text{ng N cm}^{-2} \text{h}^{-1}$)
Galaxea fascicularis			
Tissue: Symbiodiniaceae			1.727 ± 0.124
Pocillopora damicornis			
Tissue: Symbiodiniaceae			0.316 ± 0.021
Total N ($\text{ng N cm}^{-2} \text{h}^{-1}$)			
<i>Galaxea fascicularis</i>			2.512
<i>Pocillopora damicornis</i>			3.439

^(a)Average, direct, from cultures.

^(b)In axenic cultures (*Prochlorococcus* MED4) in P-limited conditions (mean \pm SD).

^(c)According to the Redfield ratio.

including interactions were conducted. When the ANOVA determined a significant difference, a Tukey's honest significant difference test (HSD) was used to test for pairwise differences while taking into account the interactions between the different variables (Table 3). Given the very large discrepancy in measured values of assimilation rates between the two DDN transfer pathways, a separated two-way ANOVA was used to test the effect of coral species, compartments and DDN assimilation rates on the "symbiotic N₂ fixation" pathway values alone (Table 4). The *ggplot2* package (Wickham, 2008) was used to create the box plot figures. Throughout the manuscript, values given are expressed as mean \pm SE. Statistical significance was accepted at $P < 0.05$.

RESULTS

Nitrogen Assimilation Through the Activity of Symbiotic Diazotrophs

In all three coral species incubated with ¹⁵N₂-enriched seawater, the ¹⁵N enrichment in both compartments (coral tissue and algal symbionts) at the end of the incubation period was higher relative to those at T₀. *A. muricata* presented the lowest ¹⁵N₂ assimilation rates in both coral tissue and symbionts, with no significant differences between both ($2.463 \pm 0.406 \cdot 10^{-5}$ and $1.758 \pm 0.897 \cdot 10^{-5} \mu\text{g N cm}^{-2} \text{h}^{-1}$, respectively, two-way ANOVA, $\text{diff} \leq 0.001$, adj. p -value = 1, Table 4). The highest

assimilation rates were detected in *P. damicornis* symbionts ($3.158 \pm 0.207 \cdot 10^{-4} \mu\text{g N cm}^{-2} \text{h}^{-1}$) compared to those in tissue, for which assimilation rates were below the detection levels. In *G. fascicularis*, assimilation rates were similar between tissue and symbionts ($1.256 \pm 0.575 \cdot 10^{-4}$ and $7.222 \pm 2.634 \cdot 10^{-5} \mu\text{g N cm}^{-2} \text{h}^{-1}$, respectively; two-way ANOVA, $\text{diff} \leq 0.001$, adj. p -value = 1, Table 4). DDN assimilation rates were significantly higher in algal symbionts than in the tissue for *P. damicornis* (two-way ANOVA, $\text{diff} = +0.0003$, adj. p -value < 0.001, Table 4), and assimilation was greater in *P. damicornis* algal symbionts compared to *G. fascicularis* and *A. muricata* algal symbionts

TABLE 2 | Summary of statistical analyses performed with the non-parametric test Mann-Whitney-Wilcoxon test for picoplankton ingestion rates between coral species.

Coral species	Mann-Whitney-Wilcoxon test	
Parameter	W	p-value
Ingestion rates— <i>G. fascicularis</i>		
<i>Prochlorococcus</i> : <i>Synechococcus</i>	11.5	0.91
<i>Prochlorococcus</i> : Picoeukayotes	14.5	0.72
<i>Synechococcus</i> : Picoeukayotes	14.5	0.72
Ingestion rates— <i>P. damicornis</i>		
<i>Prochlorococcus</i> : <i>Synechococcus</i>	25	0.01
<i>Prochlorococcus</i> : Picoeukayotes	25	0.00
<i>Synechococcus</i> : Picoeukayotes	19	0.16

TABLE 3 | Results of a three-way factorial ANOVA with coral species, compartments and DDN transfer pathway as explanatory variables on the dependent variable: DDN assimilation rates.

Source	Degrees of freedom	F-value	p-value
Coral species	2	26.41	<0.001
DDN transfer pathway	1	112.09	<0.001
Compartments	1	49.97	<0.001
Coral species: DDN transfer pathway	2	52.82	<0.001
Coral species: compartments	2	31.09	<0.001
DDN transfer pathway: compartments	1	93.72	<0.001
Coral species: DDN transfer pathway: compartments	2	62.62	<0.001

(two-way ANOVA, diff = + 0.0002, adj. *p*-value < 0.001 and diff = + 0.0003, adj. *p*-value < 0.001, respectively, **Figure 1A**).

Nitrogen Assimilation Through the Heterotrophic Nutrition on Diazotrophs

Very low DDN assimilation rates through heterotrophic nutrition on diazotrophs were measured within *A. muricata* tissue ($0.028 \pm 0.008 \mu\text{g N cm}^{-2} \text{ h}^{-1}$) and no assimilation was detected in their algal symbionts. There was no significant difference between both compartments in *A. muricata* (two-way ANOVA, diff = -0.003, adj. *p*-value = 0.99, **Table 4**). *G. fascicularis* and *P. damicornis* DDN assimilation rates were significantly higher in the symbionts compared to the tissue (two-way ANOVA, diff = + 0.1, adj. *p*-value = 0.014 and diff = + 0.5, adj. *p*-value < 0.001, respectively, **Table 4**). The highest DDN assimilation rates were detected in the algal symbionts of *P. damicornis* ($0.517 \pm 0.070 \mu\text{g N cm}^{-2} \text{ h}^{-1}$), for which no assimilation was measured in the tissue. Among the three species tested, assimilation rates in *P. damicornis* algal symbionts were the highest, 4.6-fold and 5-fold greater than in the same compartments for *G. fascicularis* ($0.111 \pm 0.056 \mu\text{g N cm}^{-2} \text{ h}^{-1}$; two-way ANOVA, diff: + 0.4, adj. *p*-value < 0.001), and *A. muricata*, respectively (two-way ANOVA, diff = + 0.2, adj. *p*-value ≤ 0.001 ; **Figure 1B**).

Ingestion Rates of Picoplankton

In the control beakers without coral colonies, cell concentrations across all plankton groups remained stable or decreased except for bacteria that increased slightly. *Synechococcus* and *Prochlorococcus* were the most abundant organisms in the incubation media at the beginning of the experiment ($5.10 \pm 0.39 \cdot 10^4$ and $8.37 \pm 0.90 \cdot 10^4 \text{ cell mL}^{-1}$, respectively) compared to other pico- and nanoplankton taxa ($3.10 \pm 0.48 \cdot 10^3 \text{ cell mL}^{-1}$). In the beakers containing coral colonies of *P. damicornis* and *G. fascicularis*, the populations of *Prochlorococcus* and *Synechococcus* decreased but not those of picoplankton and bacteria (**Supplementary Figure 1**). Prey increase in beakers containing *A. muricata* colonies, thus the calculated ingestion rates were negligible (**Supplementary Figure 1**). Hence, **Figure 1C** represents only the ingestion rates of *Prochlorococcus* and *Synechococcus* by *G. fascicularis* and *P. damicornis*. *Prochlorococcus* decreased by 72% in beakers containing *P. damicornis* and was the most significantly ingested prey ($3.02 \pm 0.19 \cdot 10^5 \text{ cell h}^{-1} \text{ cm}^{-2}$; Mann-Whitney-Wilcoxon

test; *p* = 0.01, **Table 2**). *G. fascicularis* actively and equally fed on *Prochlorococcus* and *Synechococcus*, with no significant differences between the ingestion rates ($2.56 \pm 1.57 \cdot 10^4$ and $2.70 \pm 1.66 \cdot 10^4 \text{ cell h}^{-1} \text{ cm}^{-2}$; Mann-Whitney-Wilcoxon test; *p* = 0.9, **Table 2**). *Synechococcus* decreased by 24 and 43% in beakers containing the coral colonies of *P. damicornis* and *G. fascicularis*, respectively. The corresponding calculated ingestion rates were $1.14 \pm 0.79 \cdot 10^4$ and $2.70 \pm 1.66 \cdot 10^4 \text{ cell h}^{-1} \text{ cm}^{-2}$, respectively.

DISCUSSION

By simultaneously quantifying the DDN assimilation rates in three coral species through heterotrophic nutrition on planktonic diazotrophs and symbiotic N₂ fixation, this study allows a quantification of the relative contribution of each of these pathways, as well as the distribution of the DDN between two coral compartments (tissue and symbionts). In both *P. damicornis* and *G. fascicularis*, N assimilation rates through heterotrophy on planktonic diazotrophs were found to be a thousand times higher than those obtained via symbiotic diazotrophs. The high assimilation rates obtained here for two species, as well as those previously obtained for *S. pistillata* using the same methodology (Benavides et al.,

TABLE 4 | Results of a two-way factorial ANOVA testing the effect of coral species, compartments and DDN assimilation rates on the DDN transfer pathway.

DDN transfer pathway: "symbiotic N ₂ fixation"		
Source	Diff	adj. <i>p</i> -value
<i>Acropora muricata</i>		
Tissue: Symbiodiniaceae	<0.001	1
<i>Galaxea fascicularis</i>		
Tissue: Symbiodiniaceae	<0.001	1
<i>Pocillopora damicornis</i>		
Tissue: Symbiodiniaceae	+0.0003	<0.001
DDN transfer pathway: "heterotrophic nutrition on diazotrophs"		
<i>Acropora muricata</i>		
Tissue: Symbiodiniaceae	-0.003	0.99
<i>Galaxea fascicularis</i>		
Tissue: Symbiodiniaceae	+0.1	0.014
<i>Pocillopora damicornis</i>		
Tissue: Symbiodiniaceae	+0.5	<0.001

2016; Meunier et al., 2019), demonstrate for the first time that N assimilation originating from natural plankton fixing N_2 or plankton that developed on diazotrophy, seems to be widespread among scleractinian corals. The present study shows that corals with higher heterotrophic capacities compared to *Acropora*, are capable of directly consuming plankton that developed on N_2 fixation. Although we did not measure the grazing of diazotrophs *per se*, we can hypothesize that these planktonic organisms are both diazotrophs, that are abundant in the New Caledonian lagoon (Turk-Kubo et al., 2015; Bonnet et al., 2016), but also picoplankton that is well known to benefit from this DDN in the New Caledonian lagoon (*Prochlorococcus* and *Synechococcus* cells; Berthelot et al., 2016; Bonnet et al., 2016).

DDN Assimilation From Planktonic Diazotrophs

Colonies of *A. muricata*, considered to be mainly autotrophic, assimilated very little N through planktonic diazotrophs, whereas the two species *G. fascicularis* and *P. damicornis* greatly benefited from this N source by assimilating, respectively, two or four times more DDN through this pathway, compared to *A. muricata*. After 8 h of incubation in the dark, in *G. fascicularis* and *P. damicornis*, the totality of the assimilated DDN through planktonic diazotroph predation occurred within the algal symbionts compartment. DDN assimilation rates from planktonic diazotrophy measured in *G. fascicularis* ($0.11 \pm 0.05 \mu\text{g N cm}^{-2} \text{h}^{-1}$) and *P. damicornis* ($0.517 \pm 0.07 \mu\text{g N cm}^{-2} \text{h}^{-1}$) symbionts are in the same range as those previously measured using the same methodology within *S. pistillata* after 4 h of incubation also in the dark ($0.76 \pm 0.15 \mu\text{g N cm}^{-2} \text{h}^{-1}$) (Benavides et al., 2016). DDN assimilation rates in *P. damicornis* (belonging to the same family, i.e., Pocilloporidae, as *S. pistillata*) after 8 h of incubation are, however, slightly lower than those of *S. pistillata*. This suggests that either: (i) *S. pistillata* colonies exploit more planktonic diazotrophs as a source of N; (ii) a longer incubation (8 h instead of 4 h for *S. pistillata*) means that more N was transferred from the symbionts to the other coral compartments; or (iii) the planktonic community used at the time of the experiment (more or less abundant, with varying degree of N_2 fixation activity according to the incubation conditions) influence the DDN transfer to the corals. Regardless, the capacity of DDN assimilation through planktonic diazotrophs in corals from Pocilloporidae family (*P. damicornis* and *S. pistillata*) are twice the one of *G. fascicularis*.

DDN Contribution to Coral Nitrogen Daily Needs

N budgets within the coral holobiont remained so far poorly resolved (*sensu* Fiore et al., 2010; Pernice et al., 2012; Rådecker et al., 2015). Our results indicate for the first time that *G. fascicularis* and *P. damicornis* fulfilled a large part of their N requirements through heterotrophic feeding on planktonic diazotrophs or plankton which has benefited from diazotrophy. For example, in colonies of *S. pistillata*, the dissolved inorganic N uptake rates (NH_4^+ and NO_3^- at *in situ* concentrations) are at least $2 \text{ ng N cm}^{-2} \text{h}^{-1}$ (Grover et al., 2002, 2003). For

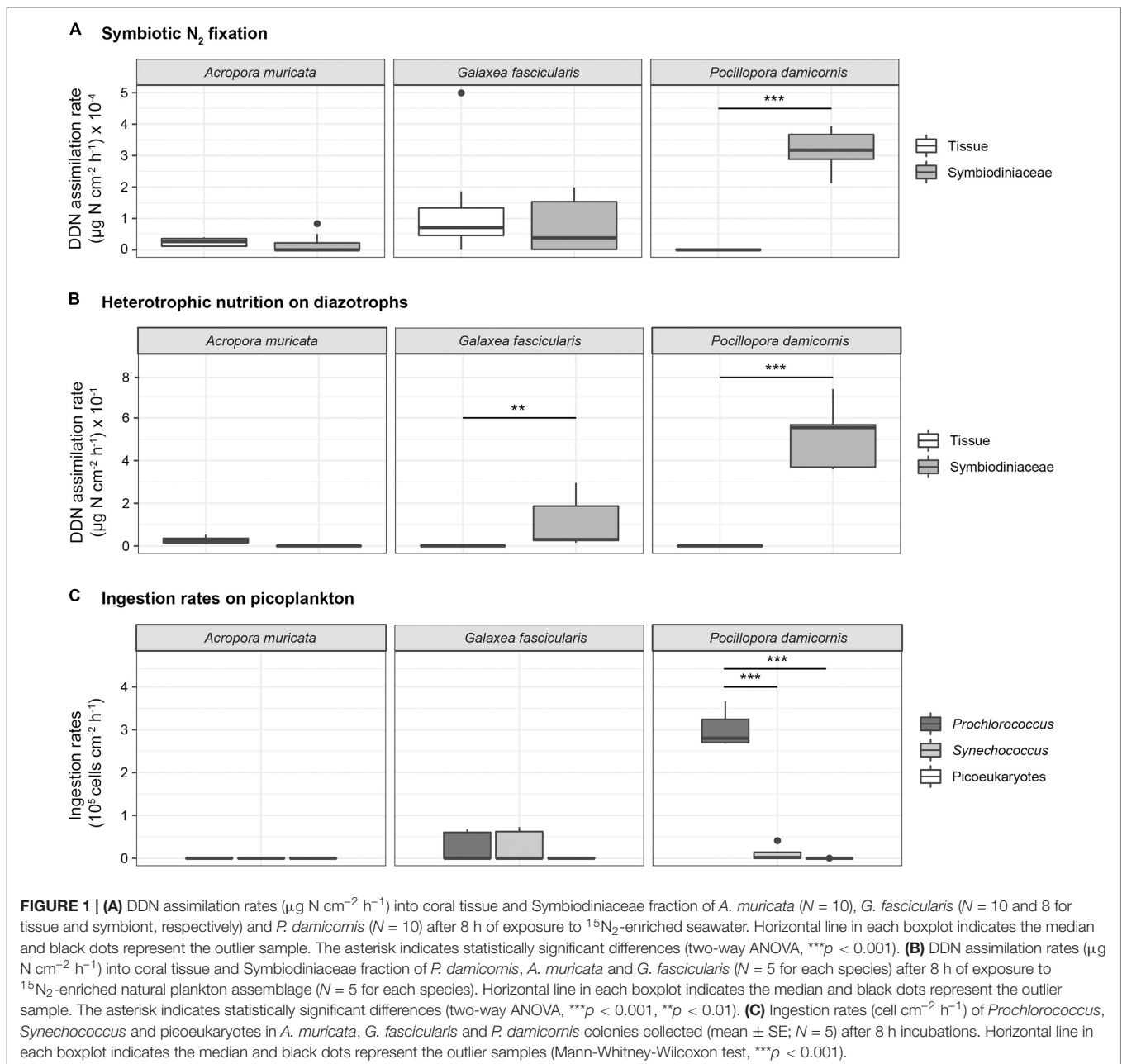
the plankton density tested, we estimate that the amount of N coming from planktonic diazotrophy for *G. fascicularis* and *P. damicornis* (2.51 and $3.44 \text{ ng N cm}^{-2} \text{h}^{-1}$, respectively) would therefore be almost equivalent to the N contribution through inorganic N uptake.

Picoplankton Ingestion Rates

Consistent with ^{15}N data, the higher heterotrophy degree of *P. damicornis* and *G. fascicularis* is confirmed by the plankton consumption rates. While no ingestion was observed for *A. muricata*, both *G. fascicularis* and *P. damicornis* showed uptake rates ranging from 0.06 to $3.0 \cdot 10^5 \text{ cell h}^{-1} \text{ cm}^{-2}$. Both species did not ingest bacteria or picoeukaryotes but consumed significant numbers of picocyanobacteria: *Synechococcus* sp. and *Prochlorococcus* sp. Our study, focusing only on picoplankton grazing is difficult to compare with previous work interested in pico-nanoplankton as a whole, but ingestion rates of *Prochlorococcus* and *Synechococcus* measured here in *P. damicornis* and *G. fascicularis* are well within the range of cyanobacteria concentrations ingested by *S. pistillata*, *G. fascicularis*, *Montipora digitata*, and *Porites lutea* (Houlbrèque et al., 2004; Tremblay et al., 2012; Sangmanee et al., 2020) in dark conditions also. According to **Table 1**, *Prochlorococcus* and *Synechococcus* ingestion supplied *P. damicornis* with $3.12 \text{ ng N cm}^{-2} \text{h}^{-1}$, which is 4 times higher than for *G. fascicularis* ($0.78 \text{ ng N cm}^{-2} \text{h}^{-1}$). While for *P. damicornis*, *Prochlorococcus* was quantitatively the major prey ingested, as already demonstrated in previous studies for *S. pistillata* (Benavides et al., 2016; Meunier et al., 2019), *G. fascicularis* consumed both *Prochlorococcus* and *Synechococcus*. McNally et al. (2017) had been the first to highlight a selective grazing on specific picoplankton taxa (*Synechococcus*) by *Porites astreoides*. These observed differences in coral species diet highlight that corals may have selective prey acquisition strategies. Sizes of *Synechococcus* and *Prochlorococcus* being comparable, the mechanisms at the origin of this selection remains to be elucidated.

Estimating the DDN Assimilation Through Symbiotic Diazotrophs Activity

N provision via endosymbiotic diazotrophs is negligible compared to that obtained via planktonic diazotrophy, with a factor of $\sim 1,000$ between both pathways for *P. damicornis* and *G. fascicularis*, and almost no symbiotic N_2 fixation for *A. muricata*. These low rates (from $1.76 \pm 0.90 \cdot 10^{-5} \mu\text{g N cm}^{-2} \text{h}^{-1}$ to $3.16 \pm 0.21 \cdot 10^{-4} \mu\text{g N cm}^{-2} \text{h}^{-1}$) are in line with those previously measured in other species (Cardini et al., 2015; Bednarz et al., 2017; Meunier et al., 2019). Using an adapted acetylene (C_2H_4) reduction technique, Cardini et al. (2015) already measured low symbiotic N_2 fixation in *Acropora* sp. (from $3.57 \cdot 10^{-4} \mu\text{g N cm}^{-2} \text{h}^{-1}$ in winter season to $1.17 \cdot 10^{-2} \mu\text{g N cm}^{-2} \text{h}^{-1}$ in summer season). Very low rates have been also obtained for *S. pistillata* using the dissolved $^{15}\text{N}_2$ isotopic labeling method after 12 h ($0.31 \pm 0.77 \cdot 10^{-5} \text{ ng N cm}^{-2} \text{h}^{-1}$ and $0.12 \pm 0.14 \text{ ng N cm}^{-2} \text{h}^{-1}$) (Meunier et al., 2019) and after 72 h ($0.97 \pm 0.14 \text{ ng N cm}^{-2} \text{h}^{-1}$ and $0.39 \pm 0.08 \text{ ng N cm}^{-2} \text{h}^{-1}$) (Bednarz et al., 2017) in coral



tissue and algal symbionts, respectively. Even if we used the same technique as Bednarz et al. (2017), different $^{15}\text{N}_2$ enrichments of enriched seawater (10 atom% vs. 30 atom% in our study) and different incubation times may have affected both the diazotrophic activity and the transfer of DDN into the different coral compartments, justifying the small differences observed between both studies. Although the assimilation rates through symbiotic N_2 fixation measured in our study are low, they are higher in the predominantly heterotrophic corals, *G. fascicularis* (Wijgerde et al., 2011; Conti-Jerpe et al., 2020) and *P. damicornis* (Lewis and Price, 1975; Sebens et al., 1996; Lyndby et al., 2020), compared to the predominantly autotrophic *A. muricata* (Conti-Jerpe et al., 2020).

These conclusions are the opposite of those outlined by Pogoreutz et al. (2017b), showing higher symbiotic N_2 fixation rates in autotrophic corals than in the heterotrophic ones. However Pogoreutz et al. (2017b) considered *P. verrucosa* and *S. pistillata* as autotrophic species while corals from Pocilloporidae family are considered as heterotroph in all studies on coral nutrition, showing in particular a group I feeding strategy (feeding by tentacle capture, Lewis and Price, 1975; Ferrier-Pagès et al., 2003; Houlbrèque et al., 2003, 2004, 2015; Houlbrèque, 2004; Lyndby et al., 2020). Results of Pogoreutz et al. (2017b) showed that physiological differences in symbiotic N_2 fixation rates among coral species tested align with relative *nifH* gene copy. So we can hypothesize that more than the functional

group (autotrophic vs. heterotrophic), it is the abundance and the diazotrophic community associated with each species that seems to influence N uptake by symbiotic N₂ fixation. *A. muricata* and *P. damicornis* host contrasted communities in the Great Barrier Reef (GBR) (Lema et al., 2012), which might harbor different diazotrophic activity. Thus in our study, differences in the diazotroph community associated with those two species, may explain the observed differences in DDN assimilation via symbiotic N₂ fixation.

It should be noted that in the present study, N₂ fixation rates by symbiotic diazotrophs were obtained at night, and are therefore maybe slightly lower than rates measured during the day. Bednarz et al. (2017, 2018), as well as Tilstra et al. (2019), found that the ecological dependence of corals on diazotrophy might depend on depth, and so on light availability, with a decrease in relative *nifH* gene copy numbers with increasing depth. This may be linked to reduce photosynthate translocation by Symbiodiniaceae to the coral host, thus decreasing the fueling of the energy-expensive activity of coral-associated diazotrophs (Pogoreutz et al., 2017a). However, no study until now has measured day/night differences in N₂ fixation in corals. Like the rates measured during the day by Cardini et al. (2015), or during day/night cycles by Bednarz et al. (2017), N₂ fixation rates by symbiotic diazotrophs in our three tested species remain low and very negligible compared to what is contributed by diazotrophic plankton. *P. damicornis* colonies preferentially accumulate N in their symbionts than in their tissue, confirming that symbiotic diazotrophs have a close relationship with algal symbionts which rapidly benefit from the fixed DDN (Lesser et al., 2007; Olson et al., 2009; Pogoreutz et al., 2017b). Unlike *P. damicornis*, in *G. fascicularis*, a significant proportion of DDN coming from symbiotic diazotrophs is also found in the tissue compartment.

CONCLUSION

The present study investigated the patterns of DDN acquisition in three different coral species and allowed us, among other things, to identify whether these patterns changed according to their dependence on heterotrophy. *P. damicornis* and *G. fascicularis* appear to be highly efficient at capturing plankton (either diazotrophs and/or picoplankton that developed on diazotrophy), while *A. muricata* does not rely on these food resources to meet its N and energy needs. As climate change destabilizes symbiosis, thus blocking the main source of energy and nutrients, the heterotrophic ability of corals as an alternative nutrient resource plays an essential role in their survival (e.g., Sebens et al., 1996; Grottoli et al., 2006; Anthony et al., 2009; Tremblay et al., 2016; Conti-Jerpe et al., 2020; Fernandes de Barros Marangoni et al., 2020; Rådecker et al., 2021). From our results, we can expect that coral species such as *P. damicornis* and *G. fascicularis*, which are able to meet their N equally by planktonic diazotrophy and dissolved inorganic sources, will be able to maintain their energy reserves to cope with climate change. On the opposite, corals that are mainly autotrophic such as *A. muricata* may not adapt and survive, as heat stress events will become more frequent and intense. A previous

study (Meunier et al., 2019) has demonstrated that bleached colonies of *Stylophora pistillata* were able to increase their feeding rate precisely on these planktonic diazotrophs but also on *Synechococcus* cyanobacteria, whose cells are rich in N (Bertilsson et al., 2003; Jacquet et al., 2006). So our study reveals that coral species that highly depend on heterotrophy could, in the event of bleaching, rely not only on the supply of nutrients and energy from meso-macroplankton but also on a major supply of N by planktonic diazotrophs and picoplankton. Taking advantage of the consumption of planktonic diazotrophs or picoplankton that have developed on diazotrophy could be one of the main strategies for coral recovery facing bleaching, as both the activity and geographical distribution of diazotrophs will likely increase with future rising sea surface temperature (Breitbarth et al., 2007; Levitan et al., 2007; Hutchins et al., 2017).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/ **Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

VM, FH, and SB conceived the ideas and designed the methodology. VM and FH performed the experiments and led the writing of the manuscript. OG and CL carried out some of the laboratory analyses. VM and AR performed the statistical analyses. All authors contributed critically to the drafts and gave the final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2021.692248/full#supplementary-material>

Supplementary Figure 1 | Abundances (cell mL⁻¹) of *Prochlorococcus*, *Synechococcus* and picoeukaryotes in the beakers containing (A) *A. muricata* colonies, (B) *G. fascicularis* colonies and (C) *P. damicornis* colonies before (T₀) and after (T₁) the 8 h incubations.

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