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Microbes support enhanced nitrogen requirements of coral holobionts in a high CO_2 environment

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Abstract

Ocean acidification is posing a threat to calcifying organisms due to the increased energy requirements of calcification under high CO_2 conditions. The ability of scleractinian corals to cope with future ocean conditions will thus depend on their ability to fulfil their carbon requirement. However, the primary productivity of coral holobionts is limited by low nitrogen (N) availability in coral reef waters. Here, we employed CO_2 seeps of Tutum Bay (Papua New Guinea) as a natural laboratory to understand how coral holobionts offset their increased energy requirements under high CO_2 conditions. Our results demonstrate for the first time that under high pCO_2 conditions, N assimilation pathways of *Pocillopora damicornis* are jointly modified. We found that diazotroph-derived N assimilation rates in the Symbiodiniaceae were significantly higher in comparison to an ambient CO_2 control site, concomitant with a restructured diazotroph community and the specific prevalence of an alpha-proteobacterium. Further, corals at the high CO_2 site also had increased feeding rates on picoplankton and in particular exhibited selective feeding on *Synechococcus* sp., known to be rich in N. Given the high abundance of picoplankton in oligotrophic waters at large, our results suggest that corals exhibiting flexible diazotrophic communities and capable of exploiting N-rich picoplankton sources to offset their increased N requirements may be able to cope better in a high pCO_2 world.

KEYWORDS
CO_2 seeps, coral holobiont, DDN assimilation, heterotrophy, N_2 fixation, ocean acidification, picoplankton, symbiotic diazotrophs

Valentine Meunier and Laura Geissler are contributed equally to this work.

1 | INTRODUCTION

The global success of tropical corals relies heavily on their symbiotic partnership with photosynthetic intracellular dinoflagellate microalgae (Symbiodiniaceae; LaJeunesse et al., 2018), which supply the animal host with photosynthetically fixed carbon in exchange for nutrients (Muscatine et al., 1981; Muscatine & Porter, 1977). The term coral holobiont encompasses not only the symbiosis between the coral animal host and Symbiodiniaceae, but also denotes a suite of relationships with an array of associated microorganisms (i.e., viruses, protists, Bacteria, Archaea and fungi) (Rohwer et al., 2002; Rosenberg et al., 2007). This highly dynamic system emerges from flexible interactions between its members depending on environmental conditions and its nutritional requirements (Bourne et al., 2016; Knowlton & Rohwer, 2003; van Oppen & Blackall, 2019; Voolstra & Ziegler, 2020; Ziegler et al., 2019). Coral holobionts are highly efficient at acquiring and recycling limiting nutrients such as nitrogen (N), an essential component of nucleic acids and amino acids. The holobiont can fulfil its N requirements through different pathways (Rädecker et al., 2015). First, the symbiosis between corals and their algal symbionts is the most important nutritional interaction, facilitating efficient uptake and translocation of dissolved inorganic N available in the surrounding waters (nitrate NO_3^- and ammonium NH_4^+, called DIN) (Falkowski et al., 1993). The coral holobiont further exploits dissolved organic N (DON) compounds such as urea or dissolved free amino acids from the environment (Muscatine, 1990). Additionally, the coral animal host can ingest N-rich particles and prey via heterotrophy, which appears to be depth-dependent (Ferrier-Pagès et al., 2003; Houlbrèque et al., 2004; Johannes et al., 1970; Meunier et al., 2019; Pogoreutz et al., 2017; Tilstra et al., 2017). Finally, symbiotic diazotrophs, located in the animal tissue, coral mucus layer and skeleton, have the enzymatic machinery to fix atmospheric dinitrogen (N_2) and convert it into NH_4^+ (Bednarz et al., 2019; Lema et al., 2012). This diazotroph-derived N (DDN) has been recognized as an important alternative N source to compensate for dynamic fluctuations in environmental N availability (Cardini et al., 2015; Santos et al., 2014; Tilstra et al., 2019). Labelled $^{15}N_2$ gas allows us to measure net N_2 fixation directly, and to trace the fate of DDN within the different coral compartments (Bednarz et al., 2017; Benavides et al., 2016; Meunier et al., 2019). Among the current threats to reefs, ocean acidification (OA) is a subtle stressor that gradually compromises a suite of biological functions on coral reefs. Currently, much remains to be understood regarding the (long-term) consequences of OA on coral nutrient acquisition and their heterotrophic capacities (Edmunds, 2011; Smith, De'ath, et al., 2016; Towle et al., 2015). While it has been established that the reduced concentration of carbonate ions due to ocean acidification increases the energy demand to maintain calcification and growth rates (e.g. Holcomb, 2009; McCulloch et al., 2012), it remains to be determined whether this increase in energy consumption can be compensated for by altered nutrient cycling in corals and an increase in heterotrophy. Concerning the effects of OA on the different processes of N acquisition by corals, it has been shown that higher pCO_2 (partial pressure of carbon dioxide) has no impact on DIN incorporation rates (Godinot et al., 2011) but virtually nothing is known on the effects on DON uptake rates. For heterotrophy, while some studies have shown that corals reduce their ingestion rates when pCO_2 increases (e.g. Houlbrèque et al., 2015; Smith, Strahl, et al., 2016), others have reported a compensation of the negative effects of OA through increased heterotrophic feeding (e.g. Edmunds, 2011; Towle et al., 2015). Moreover, studies on the effects of OA on coral feeding have only focused on the capture of mesoplankton (200–1000 μm) and not the smallest planktonic size fraction (0.2–2 μm) (Edmunds, 2011; Smith, De'ath, et al., 2016; Towle et al., 2015). In addition, very little attention has been paid to the effects of OA on symbiotic diazotrophs and no pattern emerges from the existing literature (Cardini et al., 2014). Here we investigated the impact of OA on two N acquisition pathways of the coral species *Pocillopora damicornis* collected at volcanic CO_2 seeps in Papua New Guinea (PNG). These volcanic CO_2 seeps provide natural analogues of coral biology under future ocean conditions with respect to more acidic waters. The continuous emissions of near-pure CO_2 in these seeps alter local ocean chemistry and are the most ecologically realistic system for examining responses of corals to OA (Fabricius et al., 2011; Hall-Spencer et al., 2008; Pichler et al., 2019). PNG is also one of the regions with the highest recorded N_2 fixation rates in the world where coastal rates are twice the maximum surface rates measured in the ocean environment (Bonnet et al., 2009; Shiozaki et al., 2010). For coral colonies collected either at an ambient CO_2 site or at a designated high CO_2 site, we (i) evaluated the symbiotic diazotroph community and its activity as well as (ii) measured picoplankton uptake rates, in order to assess the effect on coral holobionts of living in a high CO_2 world.

2 | MATERIALS AND METHODS

2.1 | Study site

The study was performed during the boreal spring, from May 26 to June 7, 2018, on board the R/V *Alis*. Based on previous studies at the main shallow-water hydrothermal vent area at Tutum Bay (Ambitle Island, New Ireland Province, PNG) (Biscéré et al., 2019; Pichler et al., 1999, 2019; Pichler & Dix, 1996), we selected one reef area (GPS: 4°3′44.82″S, 153°34′56.09″E; within Stations [Sts] 1, 2, 3 and 4) (referred to as high CO_2 site) and a reference site (GPS: 4°4′0.60″S, 153°34′41.43″E; referred to as ambient CO_2 site), located 1.8 km south of the main vent site at the same depth (Figure S1).

2.2 | Measuring environmental conditions

A fine-scale measurement of the seawater carbonate chemistry at Tutum Bay and the adjacent reference reef site was conducted based on samples collected in September 2016 and May 2017 and is fully reported in Pichler et al. (2019) (Supplementary Data,

https://ars.els-cdn.com/content/image/1-s2.0-S0025326X1830780X-mmc1.pdf). During the fieldwork in 2018, we further measured seawater pH_T (total scale) at Sts 1, 2, 3 and 4, and the adjacent ambient CO_2 site (Table S1) during 24-h cycles, using SeaFET pH loggers settled at 5-min intervals.

2.3 | Coral collection

A total of 40 apexes (5 cm long) from 40 parent colonies of *Pocillopora damicornis* were collected using a plier between 3 and 5 m depth, 20 parent colonies at the ambient site, and 20 at the high CO_2 site, spaced at minimum of 5 m (CITES collection permits no. 016232 and 017027). Coral fragments were transported on board the R/V *Alis* within 15 min of collection in individual zip-lock bags to the laboratory, in a cooler containing freshly collected seawater. Fragments were allowed to recover prior to the start of the incubations for 1 h in two aquaria of 20 L supplied with seawater from either ambient or high CO_2 sites and mixed using a submersible pump (Aquarium system, micro-jet MC 320). In the 20 apexes collected at each site: between seven and 10 apexes were used for the incubation experiments with $^{15}N_2$ (sequencing was performed on these same colonies), 10 apexes were used for the feeding experiment, three apexes were stored to analyse later the Symbiodiniaceae density and total chlorophyll content, and three others were used to measure the natural ^{15}N enrichment of the target pool (tissue or Symbiodiniaceae) at the start of the incubation.

2.4 | $^{15}N_2$ incubation experiments

We used the $^{15}N_2$-labelling technique (dissolved $^{15}N_2$ method; Großkopf et al., 2012; Mohr et al., 2010) as described in Meunier et al. (2019) to quantify the DDN assimilation rates into the coral animal tissue and the Symbiodiniaceae algae. The $^{15}N_2$-labelled seawater taken from their respective collection sites was prepared as follows: 4.5 L of prefiltered (0.2 μm) seawater was degassed in a completely filled polycarbonate bottle equipped with a septum cap. Subsequently, 45 ml of $^{15}N_2$ (98.3 atom% ^{15}N, Cambridge Isotope Laboratories) was injected and the bubble was vigorously shaken for 20 min until its complete dissolution and stored at 4°C for 24 h. Coral fragments of *P. damicornis* from each site ($N = 10$ and $N = 7$ from ambient and high CO_2 sites, respectively) were individually incubated in the dark for 6 h (2 p.m. to 8 p.m. local time) into 1-L glass beakers containing 30% volume of this $^{15}N_2$-labelled seawater, and 70% unenriched filtered seawater, continuously stirred with magnets. Coral branches were placed on a support and the glass beakers were sealed air-free using a glass slide. Beakers were immersed in a water bath to maintain constant temperature (28.5 ± 0.3°C) throughout the incubation. Subsequently, at the end of the incubation, corals were rinsed six times with filtered seawater to remove organisms and microbes potentially loosely attached to the coral surface (Houlbrèque et al., 2003; Mills et al., 2004) and subsequently transferred to Ziploc bags and stored at −80°C until further analysis. After incubation, 12 ml was subsampled from each beaker in Exetainer vials, and analysed on a Membrane Inlet Mass Spectrometer (MIMS) to measure the ^{15}N-enrichment of the labelled filtered seawater (Kana et al., 1994). The coral tissue was removed from the skeleton using an air-pick and 20 ml of 0.45-μm filtered seawater. The slurry was homogenized with a Potter tissue grinder. A 10-ml subsample was taken from each homogenate and processed for Symbiodiniaceae density and total chlorophyll concentrations (see below). The rest of each homogenate was centrifuged at 8,000 *g* for 10 min at 4°C to separate the Symbiodiniaceae from the coral tissue. The pellets containing Symbiodiniaceae were dried and ground. Coral tissue solutions were filtered (15 ml) on precombusted GF/F filters under low pressure. The filters and the Symbiodiniaceae crushed were encapsulated in tin cups before analyses using an elemental analyser coupled to an IRMS (EA-IRMS, Integra CN, SerCon Ltd). N assimilation rates into coral tissue and Symbiodiniaceae were calculated as follows (Montoya et al., 1996):

$$DDN_{assimilation} = \frac{\left(15N_{target,\,Tf} - 15N_{target,\,T0}\right)}{\left(15N_{enriched\ source} - 15N_{non\text{-}enriched\ source}\right) \times t} \times \left[\frac{PN_{target}}{A}\right]$$

where $^{15}N_{target,\,Tf}$ is the ^{15}N enrichment of the target pool (tissue or Symbiodiniaceae) at the end of the incubation and $^{15}N_{target,\,T0}$ is the corresponding time zero ^{15}N enrichment (natural ^{15}N enrichment of the target pool at the start of the incubation). The $^{15}N_{enriched\ source}$ is the isotopic enrichment of the seawater added to the beakers, the $^{15}N_{non\text{-}enriched\ source}$ is the natural isotopic signature of seawater, t is the incubation time in hours and PN_{target}/A is the mass (μg) of particulate N of the target pool per area (A in cm^2) of the coral skeleton surface (μg N cm^{-2} h^{-1}). To measure NO_x and PO_4^{3-} concentrations, seawater samples were collected in triplicate at the beginning and end of each incubation experiment into acid-washed 20-ml polyethylene tubes, poisoned with 1% $HgCl_2$ and stored at 4°C until analysis with conventional colorimetric methods (continuous flux AA3 Bran +Luebbe autoanalyser) (Raimbault et al., 1990) following JGOFS recommendations (JGOFS, 1994). The detection limits of the instrument were 0.005 and 0.01 μmol L^{-1} for NO_x and PO_4^{3-} respectively.

2.5 | Feeding experiments

In parallel experiments, we quantified the predation on picoplankton of *P. damicornis* colonies. Forty litres of seawater was collected from the ambient and high CO_2 sites at 2 m depth, and prefiltered through a 100-μm mesh to exclude large zooplankton. Five sets of 4.3 L of seawater containing the natural plankton assemblage were collected at the same site as corals. The plankton was then concentrated four times on 0.4-μm polycarbonate filters and vortexed in a 50-ml seawater aliquot to resuspend cells. Each concentrated natural

plankton aliquot was provided to the glass beakers. As for the $^{15}N_2$ incubation experiments, over two consecutive days, we first incubated corals from the high CO_2 site in seawater taken from the same high CO_2 site and then the corals collected at the ambient site in seawater collected at the same ambient site. For each experiment, eight beakers were filled with 1.5 L of preconcentrated seawater, and five coral apexes were incubated for 6 h in the dark continuously stirred with magnets in five beakers while three beakers were used as control beakers (without corals) to quantify plankton fluctuations without any predation (internal grazing, natural cell growth and/or cell death). Triplicate seawater samples were collected from each beaker at the beginning and the end of the period and fixed with paraformaldehyde (final concentration of 0.1%) for 30 min at room temperature in the dark, then stored at −80°C for further flow cytometry analyses on a FACSVerse Flow Cytometer (Becton Dickinson) as described in Meunier et al., (2019). 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. (0.6 μm), *Synechococcus* sp. (1–2 μm) 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 picoplankton) and also by size-sorting using 1-μm green calibration beads (Marie et al., 1997, 1999).

2.6 | Ingestion rates

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. Carbon and N contents of prey were estimated using conversion factors from the available literature (summarized in Table S2).

2.7 | Symbiodiniaceae density and total chlorophyll concentration

For coral samples, Symbiodiniaceae cells were counted three times by light microscopy using a Neubauer's cell. Subsamples of 10 ml from each coral tissue solution were centrifuged at 8,000 *g* for 10 min at 4°C and the supernatant was removed. The pellet containing the Symbiodiniaceae was resuspended in 100% acetone for 24 h (4°C) in the dark to extract chlorophyll *a* and c_2. The extracts were centrifuged at 10,000 *g* for 15 min and absorbances were read at 630, 663 and 750 nm. Chlorophyll *a* and c_2 concentrations were computed according to the spectrometric equations of Jeffrey and Humphrey (1975) and Hoogenboom et al. (2010). Chlorophyll *a* and c_2 are given as total chlorophyll. All measurements were normalized to the skeletal surface area (cm^2), estimated using the paraffin wax-dipping method (Naumann et al., 2009; Stimson & Kinzie, 1991).

2.8 | Coral DNA extraction, *nifH* amplification and sequencing

DNA isolation was performed using the Qiagen DNeasy 96 Blood & Tissue kit (Qiagen) following the manufacturer's instructions with minor adjustments. Briefly, collected coral fragments (seven from the high CO_2 site and nine from the ambient site) were transferred into 50-ml centrifuge tubes, covered in DNA/RNA Shield (Zymo), and stored at −80°C until further analysis. Defrosted coral fragments were transferred into Eppendorf tubes and covered with 400 μl of tissue lysis ATL buffer (Qiagen) and 20 μl of proteinase K, and mixed by vortexing. Samples were then incubated in a ThermoMixer (Eppendorf) at 56°C for 2 h at 600 rpm. Following this, 200 μl of the lysate from each sample was transferred to collection microtubes and incubated at 56°C for 1 h to break down coral mucus. DNA extractions were then continued according to the manufacturer's instructions. DNA concentrations were quantified by Qubit (Qubit dsDNA High Sensitivity Assay Kit, Invitrogen).

To amplify the *nifH* gene, the IGK3 (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCIWTHTAYGGIAARGGIGGIATHGGIA A) and DVV (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ATIGCRAAICCICCRCAIACIACRTC) primers were used (underlined sequences indicate Illumina adapters) (Ando et al., 2005). Triplicate PCRs (polymerase chain reactions) were performed according to Lesser et al. (2018) using the Qiagen Multiplex PCR kit, with a final primer concentration of 1.2 μM in a final reaction volume of 10 μl. Thermal cycler conditions for *nifH* PCR amplification were: initial denaturation at 95°C for 15 min, 40 cycles of 95°C for 45 s, 57°C for 45 s and 72°C for 60 s, followed by a final extension step of 72°C for 10 min. Then, 5 μl of the PCR products was run on a 1% agarose gel to confirm successful amplification. Triplicates for each sample were pooled, and samples were purified using Agencourt AMPure beads (Agencourt Bioscience). Samples were then indexed using the Nextera XT Index Kit version 2 (dual indexes and Illumina sequencing adaptors added). Successful addition of indexes was confirmed by comparing the length of the initial PCR product to the corresponding indexed sample on a 1% agarose gel. Samples were then cleaned and normalized using the SequalPrep Normalization Plate Kit (Invitrogen). The *nifH* libraries were pooled in an Eppendorf tube (6 μl per sample) and concentrated using a CentriVap Benchtop Vacuum Concentrator (Labconco). Following this, the quality of the library was assessed using the Agilent High Sensitivity DNA Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies). Quantification was done using Qubit (Qubit dsDNA High Sensitivity Assay Kit, Invitrogen). Sequencing was performed at 6 p.m. with 20% phiX on the Illumina MiSeq platform at 2×301-bp paired-end V3 chemistry according to the manufacturer's specifications.

2.9 | *nifH* amplicon sequencing data analysis pipeline

Samples analysed here are part of a larger sample set containing multiple species from the same sampling locations (see NCBI bioproject ID https://www.ncbi.nlm.nih.gov/bioproject/PRJNA668126). Initial quality control of MiSeq *nifH* amplicon reads was done using the TaxADivA (TAXonomy Assignment and DIVersity Assessment) perl wrapper script (Gaby et al., 2018) as applied previously in Lesser et al., (2018) (settings: "-y -k -r 26 -l 29 --keepc4 -g 500"). Representative sequences of final MED nodes were translated to the protein level using FRAMEBOT (Wang et al., 2013). In order to obtain putative species-level annotations and to collapse the MED count matrix for downstream analyses, OTU (operational taxonomic unit) clustering at the nucleotide level was conducted via CD-HIT-EST. The clustering cutoff was set to 91.9%, representing the prokaryotic species-level cutoff reported in Gaby et al. (2018). To determine bona fide nitrogenase genes, nitrogenase cluster assignment was accomplished using a three-legged model after Angel et al. (2018). After alignment in MAFFT (Katoh & Standley, 2013), representative sequences for each OTU were first analysed using a Classification and Regression Tree (CART) model (Frank et al., 2016). Secondly, an Evolutionary Placement Algorithm (EPA) Tree (Berger et al., 2011) was constructed using the corresponding RAXML (Stamatakis, 2014) implementation. OTU representatives were aligned with a reference data set from the NifMAP pipeline (Angel et al., 2018) that included representatives of all *nifH*-like gene clusters. Query sequences within this combined reference alignment were then placed on the reference tree from the NifMAP pipeline. Third, in a BLASTP (Camacho et al., 2009) search, OTUnode representative sequences were queried against the Zehr laboratory *nifH* gene database (https://www.jzehrlab.com/nifh) and the results were confirmed against matches in the NCBI nonredundant (nr) protein database. An OTU was considered to be a true *nifH*-representative (i.e., belonging to nitrogenase clusters I–III) if at least two of the three employed characterization methods produced consistent delineations. As a final step, all samples that contained <100 sequences of cluster I–III *nifH* representatives were discarded from subsequent analyses. Relative abundances of retained *nifH* OTUs over samples are available in Table S3. A maximum-likelihood (ML) phylogenetic tree of all final OTUs was constructed using RAXML from 20 starting trees using the PROTGAMMAAUTO method of finding an optimal substitution model (LG + G in this case). Additionally, 1000 bootstrap trees were calculated to support the bipartitions of the best ML tree. Linear discriminant analysis (LDA) was carried out in the LEFSE implementation on the Galaxy web application of the Huttenhower laboratory (Segata et al., 2011). All analyses were carried out at default settings with alpha values for factorial Kruskal–Wallis and subsequent pairwise Wilcoxon tests both at 0.05 and the LDA threshold score at 2.0. OTUs with a score above this threshold were considered significantly different in their relative abundance between sites. Plots of relative abundances were created in R (R Development Core Team, 2018) using the packages "GGPLOT2" and "GGTREE".

TABLE 1 Results of the two-way factorial ANOVA with coral compartments and CO_2 sites as explanatory variables on the dependent variable: DDN assimilation rates. Significant *p*-values (<.05) are in bold

Source	*df*	*F*-value	*p*-value
Coral compartments	1	7.095	**.01**
CO_2 sites	1	4.880	**.04**
Coral compartments: CO_2 sites	1	5.633	**.02**

2.10 | Statistical analyses

All statistical analyses were performed using R version 4.0.1 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 Symbiodiniaceae densities, total chlorophyll content, picoplankton ingestion rates and DDN assimilation rates. When significant differences were found, Wilcoxon–Mann–Whitney tests were performed to test for pairwise differences. To look for potential differences in DDN assimilation rates by CO_2 sites and coral compartments, two-way ANOVAs 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 1; Table S4). The GGPLOT2 package (Wickham, 2008) was used to create box plots. All values provided are expressed as mean ± *SE*. Statistical significance was accepted at $p < .05$.

3 | RESULTS

3.1 | Seawater carbonate chemistry

Data recorded from the SeaFET and the calculated seawater carbonate chemistry (Table S1) confirmed the data reported by Pichler et al., (1999). Average seawater pH and Ω_{arag} (± *SD*) were respectively 7.76 ± 0.07 and 2.30 ± 0.30 in the high CO_2 area (pooled data), and 8.01 ± 0.03 pH_T and 3.52 ± 0.06 at the ambient station. The highest and lowest pH data measured in the vent site were 7.93 pH_T and 7.56 pH_T, corresponding to a range in Ω_{arag} of 3.0 and 1.5, respectively.

3.2 | Nutrient concentrations

NO_x (NO_2^- + NO_3^-) concentrations were 1.6 times higher at the high CO_2 site (0.921 ± 0.155 μM) compared to those at the ambient CO_2 site (0.562 ± 0.049 μM). PO_4^{3-} concentrations were 1.169 ± 0.143 μM at the ambient CO_2 site and 1.155 ± 0.069 μM at the high CO_2.

3.3 | Symbiodiniaceae densities and total chlorophyll concentration

Coral colonies collected at the ambient and high CO_2 sites had comparable concentrations of Symbiodiniaceae ($1.64 \pm 0.24 \times 10^6$ and $1.72 \pm 0.23 \times 10^6$ cells cm^{-2}, respectively) and total chlorophyll (11.22 ± 0.95 and 11.73 ± 1.13 µg $cm^{-2,}$ respectively).

3.4 | DDN assimilation rates into the coral tissue and the Symbiodiniaceae

DDN assimilation rates in the Symbiodiniaceae of corals from the high CO_2 site were up to 20 times higher than the rates in the coral tissue (two-way ANOVA, diff <0.001, adj. $p = .01$, Table S4), and significantly higher at the high CO_2 site compared to those measured at the ambient CO_2 site (respectively $13.43 \pm 11.75 \times 10^{-5}$ and $2.24 \pm 2.00 \times 10^{-5}$ µg N cm^{-2} h^{-1}; diff <0.001, adj. $p = .02$; Figure 1; Table S4). In contrast, similar DDN assimilation rates were measured in the tissue at both sites, but rates were overall lower than in the Symbiodiniaceae fraction ($1.03 \pm 0.62 \times 10^{-5}$ and $0.63 \pm 0.28 \times 10^{-5}$ µg N cm^{-2} h^{-1}, respectively at both sites; two-way ANOVA, diff = −0.000, adj. $p = 1$; Figure 1; Table S4).

3.5 | Coral diazotroph community composition

A total of 14 bacterial taxa were identified across the 11 coral samples (five from the ambient site and six from the high CO_2 site) that retained more than 100 cluster I–III *nifH* sequences after quality control (of 16 in total) (Figure 2). Diazotroph communities at both sites consisted of 14 different taxa in total with eight of them found in corals from both the ambient and the high pCO_2 sites. Of these eight shared taxa, four belonged to the class Gammaproteobacteria (OTU_05, OTU_19, OTU_22, OTU_23) and four were Cyanobacteria (OTU_31, OTU_83, OTU_85, OTU_86). Among the latter, OTU_31 (NCBI database: Leptolyngbyaceae, 92.9%) was the only OTU with high relative abundances at both sites. Gammaproteobacteria were of higher abundance in samples from the ambient pCO_2 site (LEFSE: LDA effect size = 5.40, $p = .015$), with OTU_03 (Zehr database: *Teredinibacter turnerae*, 96.5%) being a significant discriminant (LEfSe: LDA effect size = 5.13, $p = .036$). Conversely, a single OTU from the family Rhodobacteraceae (Zehr database: *Rhodobacter* sp., 92.9%) was exclusively found in samples from the high pCO_2 site, where it was highly abundant in most samples and accordingly classified as a significant discriminator between sites (LEfSe, LDA effect size = 5.41, $p = .013$) (Table S3).

3.6 | Picoplankton abundances and feeding experiment

The range of picoplankton concentrations in the beakers was: bacteria ($5.97 \pm 0.91 \times 10^5$ and $7.59 \pm 0.13 \times 10^5$ cells ml^{-1}), *Synechococcus* sp. ($5.39 \pm 0.83 \times 10^3$ and $2.54 \pm 0.72 \times 10^3$ cells ml^{-1}), picoeukaryotes ($2.20 \pm 1.27 \times 10^3$ and $2.88 \pm 1.96 \times 10^3$ cells ml^{-1}), and *Prochlorococcus* sp. ($3.92 \pm 1.89 \times 10^2$ and $5.06 \pm 4.38 \times 10^3$ cells ml^{-1}) in the seawater collected at the ambient and high CO_2 sites respectively. *Synechococcus* sp. was at least twice as abundant at the ambient CO_2 site compared to the high CO_2 site. In the control beakers, cell concentrations across all picoplankton groups remained stable in the absence of coral feeding except for bacteria. In contrast, in the experimental beakers with coral fragments, picophytoplankton cell concentrations decreased during the incubation, indicating active coral feeding. *Synechococcus* sp. was the most commonly ingested group,

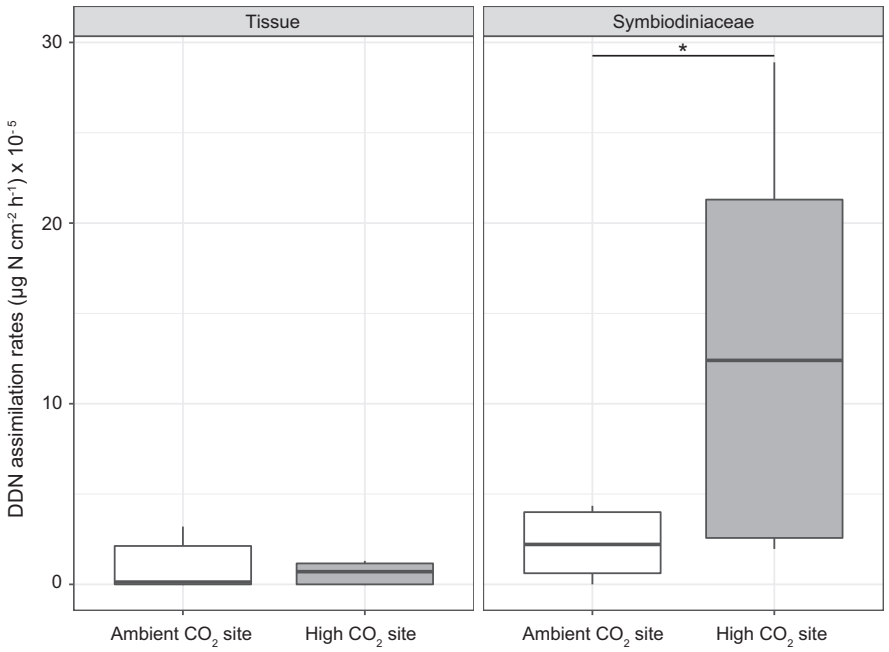

FIGURE 1 DDN assimilation rates (µg N cm^{-2} h^{-1}) into coral tissue and the Symbiodiniaceae fraction of *Pocillopora damicornis* ($N = 10$ and $N = 7$ for ambient and high CO_2 sites, respectively) after 6 h of exposure to $^{15}N_2$-enriched seawater. The horizontal line in each boxplot indicates the median. The asterisk indicates statistically significant differences ($p < .05$)

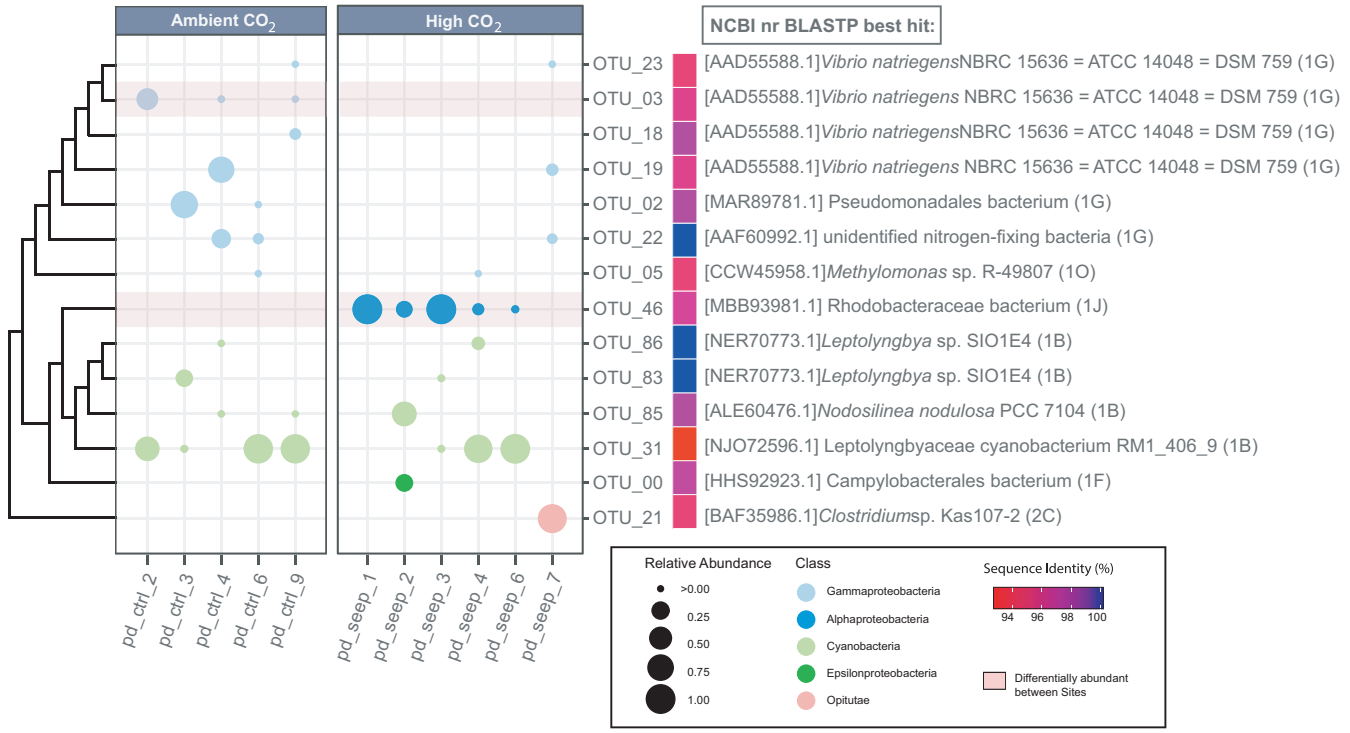

FIGURE 2 Diazotroph community composition of corals collected from the ambient and high CO_2 sites. Depicted is the relative abundance (indicated by dot-size) of *nifH*-based bacterial OTUs for all samples with at least 100 sequences assigned to *nifH* clusters I–III. OTUs are sorted by their phylogenetic relationship as indicated by the dendrogram on the left. Best hit results from a BLASTP search against the NCBI nonredundant database are shown next to the OTU identifiers with coloured boxes indicating protein sequence identity. OTUs with a red-shaded background are significantly different in abundance between the ambient and high CO_2 sites

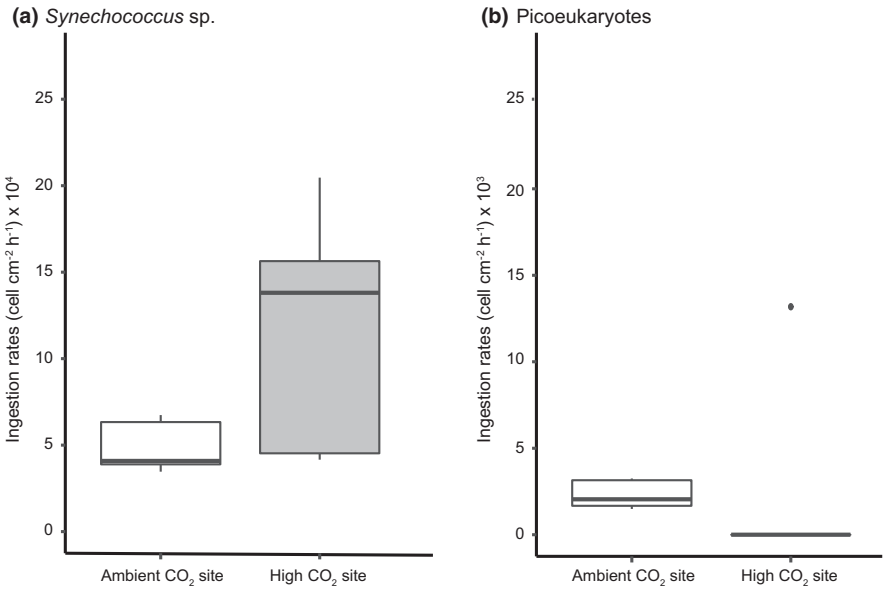

FIGURE 3 Ingestion rates (cells cm^{-2} h^{-1}) of (a) *Synechococcus* sp. and (b) picoeukaryotes in *Pocillopora damicornis* colonies collected at the ambient and high CO_2 sites ($N = 5$) after 6 h of incubation. The horizontal line in each boxplot indicates the median and black dots represent the outlier samples

with ingestion rates being 2.4 times higher in samples collected at the high CO_2 site than in those collected at the ambient one ($0.49 \pm 0.01 \times 10^5$ and $1.17 \pm 0.72 \times 10^5$ cells cm^{-2} h^{-1} respectively) (Figure 3). Picoeukaryotes were the second most ingested group, regardless of the coral collection site. *Prochlorococcus* sp. and heterotrophic bacteria were not ingested by coral colonies.

4 | DISCUSSION

Little is known regarding the effect of OA on N acquisition pathways of corals. Although Rädecker et al. (2014) suggested that coral-associated N_2-fixing organisms could be affected by OA, at present no evidence has emerged from the literature. By combining isotope

tracer studies, flow cytometry and molecular analyses, this study demonstrates for the first time that under high pCO_2 conditions, the N assimilation pathways of *Pocillopora damicornis* colonies are significantly modified: both DDN assimilation rates and picophytoplankton prey ingestion increase, concomitant with a site-specific diazotroph community assembly.

At the high CO_2 site, the DDN assimilation of Symbiodiniaceae associated with *P. damicornis* is highly variable but significantly higher compared to the ambient CO_2 site. Coral holobionts thus appear to have a higher dependency on microbially fixed N_2 to fulfil their N requirements in a high CO_2 environment. A previous study measuring the physiological parameters of this same coral species, on the same sampling sites, has shown that rates of photosynthesis by algal symbionts are higher at the high CO_2 site compared to the ambient site (Biscéré et al., 2019). This increased productivity of algal symbionts probably translates into higher N requirements of the holobiont as produced photosynthates need to be supplemented with N for amino acid synthesis and to sustain photosystem functioning (Béraud et al., 2013; Falkowski et al., 1993; Rädecker et al., 2015). The stimulated DDN assimilation, although only measured over a short period (6 h) and in a single coral species, suggests that higher inorganic N concentrations at the high CO_2 site are insufficient to compensate for the increased N requirements of the holobiont. Under such N-limited conditions, diazotrophs could potentially support the nutrition of other holobiont members as well. Unlike the algal symbionts, the host may be more energy-limited, as more energy is needed to maintain calcification rates under more acidic conditions (McCulloch et al., 2012). Under high CO_2 conditions, the coral tissue compartment, thus, probably does not have more carbon at its disposal and does not have an increased demand for fixed N. This is confirmed by similar DDN assimilation rates measured in the coral tissue at the high and ambient CO_2 sites. In general, N_2 fixation rates measured in our study were in the same range as those measured by Cardini et al., (2015) in *Acropora* sp. (3.57×10^{-4} μg N cm^{-2} h^{-1}) and by Meunier et al. (2021) also on *P. damicornis* Symbiodiniaceae under ambient pCO_2 conditions (3.16×10^{-4} μg N cm^{-2} h^{-1}).

Prompted by our finding that N_2 assimilation rates were increased in Symbiodiniaceae from corals from high CO_2 sites, we sought to assess coral diazotroph community composition. Despite the putative importance of diazotrophs in the coral holobiont (e.g. Pogoreutz et al., 2017; Rädecker et al., 2015), the diazotroph community was poorly conserved across colonies and of low diversity. Only a few bacterial taxa comprised the diazotroph community associated with corals at high and ambient CO_2 sites, in line with explorations of coral-associated diazotroph communities from earlier studies (Lema et al., 2012; Lesser et al., 2018). The high abundance of cyanobacteria in the diazotroph community and their stable presence across sites aligns with previous reports that symbiotic cyanobacteria are functionally important holobiont members supporting the nutrition of algal symbionts (Lesser et al., 2007). While the functional importance of cyanobacteria in the coral holobiont may limit the flexibility of this association to counter environmental change (Voolstra & Ziegler, 2020), we nevertheless found clear differences between the high CO_2 and ambient CO_2 diazotroph communities. This shift was predominantly driven by the loss in diversity and abundance of Gammaproteobacteria at large and the prevalence of an Alphaproteobacterium (OTU_46) at the high CO_2 site. Similar shifts from Gammaproteobacteria to Alphaproteobacteria for microbiomes at large have been previously reported from stressed coral holobionts during coral bleaching or eutrophication events (McDevitt-Irwin et al., 2017; Pootakham et al., 2018; Ziegler et al., 2017). These authors argue that the increase in relative abundance of Alphaproteobacteria during the high seawater temperature period (Pootakham et al., 2018) may simply reflect an opportunistic colonization of this group of bacterial communities on coral tissues damaged from the heat stress, which is not the case here for corals subjected to a higher pCO_2. Hence, the structure of diazotroph communities is probably subject to the same or similar dynamics and selective processes as the overall bacterial community associated with coral holobionts. While such bacterial community shifts have been proposed to be opportunistic in nature (Zaneveld et al., 2017), our findings suggest that differences in the diazotroph community composition between high and ambient CO_2 sites directly affect the assimilation of fixed N_2 in the coral holobiont. However, additional experiments are needed to unequivocally determine whether these higher N_2 fixation rates associated with increased relative abundance of Alphaproteobacteria are consistent across other coral species living in a high CO_2 environment. Dynamic fluctuations in diazotroph abundance and community composition may enable coral holobionts to rapidly adapt to altered environmental conditions and maintain stable N availability required for holobiont functioning (Reshef et al., 2006; Voolstra & Ziegler, 2020). Corals that exhibit high levels of microbiome flexibility (sensu Voolstra & Ziegler, 2020) may have advantages in a changing environment.

In addition to higher DDN assimilation rates in Symbiodiniaceae, we show that corals fully acclimatized to elevated CO_2 throughout their entire post-settlement lives exhibit increased feeding rates on picoplankton and in particular on one specific group, *Synechococcus* sp. So far, studies that have investigated the effects of elevated CO_2 on coral heterotrophy have focused primarily on the largest size fraction of plankton, the mesoplankton (Edmunds, 2011; Houlbrèque et al., 2015; Towle et al., 2015). The only study comparable to ours, carried out under in situ conditions (in Upa-Upasina Reef, PNG) shows, contrary to our results, reduced mesozooplankton consumption rates under elevated pCO_2 conditions (Smith, Strahl, et al., 2016) for unknown reasons. It is worthwhile pointing out that *Synechococcus* is significantly not the most abundant at the high CO_2 site or in the open ocean, yet corals preferentially selected these cells, known to be rich in N, for feeding. Moreover, Fu et al., (2007) demonstrated that the N content of *Synechococcus* even increases with rising CO_2. Surprisingly, the same preferential consumption of this type of cells has been revealed for colonies of *Stylophora pistillata* under thermal stress (Meunier et al., 2019) and colonies of *Porites astreoides* under controlled conditions (Pacherres et al., 2013). Such selectivity is known for corals feeding on mesozooplankton (Sebens et al., 1996; Smith, Strahl, et al., 2016), but has

never been observed for picoplankton and is awaiting further experimental verification. Feeding behaviour is usually mediated by chemical cues and could be also related to the nutritional value of the ingested prey, but is unknown for the case of *Synechococcus*.

With doubled rates of photosynthesis, corals in acidified waters have an increased demand for N. Our results, even if they were obtained with relatively short incubations of a single coral species, argue that colonies meet this additional N requirement mainly through heterotrophic feeding on *Synechococcus* (uptake rates are three times higher at the high CO_2 site relative to the ambient CO_2 site) and through increased availability of fixed N_2 by symbiotic diazotrophs (assimilation rates show a 4-fold increase). We can compare the amount of N provided by DDN assimilation and picoplankton ingestion to other sources of N acquisition in the coral holobionts. For example, for *Stylophora pistillata*, a coral species of the same family (Pocilloporidae), the absorption of dissolved inorganic N (nitrate +ammonium) brings 2 ng N cm^{-2} h^{-1} (Grover et al., 2002, 2003). Based on our data, we estimate that the amount of N coming from DDN and picoplankton (5.6 ng N cm^{-2} h^{-1}) for corals at high CO_2 vents would make up about 19% of the inorganic N uptake, underlining the importance of these N sources for the N requirements of corals under acidified conditions. As such, we argue that strategies for corals to maintain high productivity under ocean acidification incorporate selected feeding on N-rich picoplankton cells as well as the acquisition of highly efficient symbiotic diazotrophs. Given the high abundance of picoplankton-sized unicellular cyanobacteria (*Synechococcus* sp.) and prochlorophytes (*Prochlorococcus* sp.) in oligotrophic waters, corals capable of utilizing this nutrient source may be able to cope better with high pCO_2 conditions by offsetting the concomitant increased N demand through selective feeding and an enhanced productivity of their algal symbionts (Fu et al., 2007; Newbold et al., 2012; Sala et al., 2016). It remains to be determined to what degree selective feeding and diazotroph community assembly can be adjusted across coral holobionts as a means of acclimation to living in a high CO_2 world.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

V.M., F.H., R.R.M., S.B. and C.R.V. conceived the ideas and designed the methodology. V.M., F.H., L.G. and C.R.V. performed the experiments, V.M., F.H., L.G., N.R. and C.R.V. wrote the manuscript. N.R., C.R.V., G.P., O.G. and C.L. carried out some of the laboratory analyses. All authors helped draft the manuscript and gave the final approval for publication.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supporting Information and in the Dryad Digital Repository: https://doi.org/10.5061/dryad.rfj6q57bj

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