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SHORT COMMUNICATION

Diazotrophs: a non-negligible source of nitrogen for the tropical coral *Stylophora pistillata*

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ABSTRACT

Corals are mixotrophs: they are able to fix inorganic carbon through the activity of their symbiotic dinoflagellates and to gain nitrogen from predation on plankton and uptake of dissolved organic and inorganic nutrients. They also live in close association with diverse diazotrophic communities, inhabiting their skeleton, tissue and mucus layer, which are able to fix dinitrogen (N_2). The quantity of fixed N_2 transferred to the corals and its distribution within coral compartments as well as the quantity of nitrogen assimilated through the ingestion of planktonic diazotrophs are still unknown. Here, we quantified nitrogen assimilation via (i) N_2 fixation by symbiont diazotrophs, (ii) ingestion of cultured unicellular diazotrophs and (iii) ingestion of natural planktonic diazotrophs. We estimate that the ingestion of diazotrophs provides 0.76±0.15 µg N cm^{-2} h^{-1}, suggesting that diazotrophs represent a non-negligible source of nitrogen for scleractinian corals.

KEY WORDS: Scleractinian corals, Heterotrophic nutrition, Cyanothece, N_2 fixation, New Caledonia

INTRODUCTION

Tropical scleractinian corals obtain nitrogen via a variety of mechanisms including (i) symbiosis with unicellular dinoflagellates – zooxanthellae – that can take up and retain dissolved inorganic nitrogen (ammonium and nitrate) from the surrounding waters (Falkowski et al., 1993; Marubini and Davies, 1996; Muscatine, 1990), (ii) recycling of animal wastes by the zooxanthellae and subsequent transfer to the coral host as amino acids (Ferrier, 1991), ammonium or urea (Furla et al., 2005), (iii) ingestion of nitrogen-rich sediment particles (Mills and Sebens, 2004; Mills et al., 2004) and plankton (Ferrier-Pagès et al., 2003; Houlbrèque et al., 2004), and (iv) dinitrogen (N_2) fixation by symbiotic diazotrophic communities (Lema et al., 2012; Lesser et al., 2007, 2004; Shashar et al., 1994). Among these potential nitrogen sources, the contribution of free-living planktonic diazotrophs to the nitrogen metabolic requirements of the coral holobiont has not been quantified yet.

Pelagic N_2 fixation is regarded as the most important external source of fixed nitrogen to the ocean (Gruber and Galloway, 2008), fuelling primary production in oligotrophic waters and balancing the global ocean nitrogen reservoir (Mahaffey et al., 2005). In the New Caledonian lagoon, planktonic diazotrophs are very abundant and diverse (Biegala and Raimbault, 2008; Turk-Kubo et al., 2015), accounting for among the highest N_2 fixation rates in the world (Bonnet et al., 2015). Diazotrophic cyanobacteria retain >75% of the N_2 they fix intracellularly (Benavides et al., 2013a; Berthelot et al., 2015), and thus their direct ingestion could provide an important source of nitrogen for corals. Here, we investigated N_2 fixation within the coral holobiont and, for the first time, the ingestion of ^{15}N-labelled cultured and natural diazotrophic assemblages from the New Caledonian lagoon, quantifying their contribution to the nitrogen balance of one of the major scleractinian corals: *Stylophora pistillata*.

MATERIALS AND METHODS

Coral collection and acclimation

Stylophora pistillata samples were collected in the New Caledonian lagoon (22.328°S, 166.410°E; sampling licence issued by the 'Province Sud', Government of New Caledonia). Thirty terminal branch portions (5 cm) were cut from five parent colonies, hung on nylon wires and suspended for 3 weeks in a 20 l aquarium supplied with 100 µm-filtered 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, USA). Temperature (26±0.1°C) and salinity (35.69±0.02) were kept constant using heaters connected to electronic controllers (±0.2°C) and routinely verified using a YSI MPS 556 probe (YSI, Yellow Springs, OH, USA). Corals received a constant irradiance of 120±10 µmol photons m^{-2} s^{-1} (12 h:12 h) using four Aquablue plus neon bulbs (blue–white, 15,000 K, Giesemann, Germany).

Experiment setup and ^{15}N assimilation

After 3 weeks of acclimation, *S. pistillata* branches were incubated in quintuplicate 1 litre sterilized glass containers, filled with filtered (0.2 µm) lagoon seawater and continuously stirred with magnets. The corals were suspended from the top of the containers with the nylon wires. Nitrogen assimilation by the coral holobiont was tested by comparing two mechanisms: (i) the direct uptake of N_2 fixed by endosymbiont diazotrophs (experiment 1) and (ii) the ingestion of planktonic diazotrophs. In the latter case, we tested both a cultured diazotroph strain (experiment 2) and a natural diazotroph assemblage (experiment 3).

For experiment 1 we used the dissolved $^{15}N_2$ method (Mohr et al., 2010) as outlined in Großkopf et al. (2012). The quintuplicate glass containers were filled to 20% volume with ^{15}N-enriched seawater, 80% unenriched filtered seawater, and incubated for 4 h.

In experiment 2, we incubated corals with a culture of unicellular diazotrophs isolated from the lagoon and cultured in autoclaved filtered seawater supplemented with nutrients in the same

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proportion as for the YBCII medium (Chen et al., 1996). The strain used was a 5–6 µm unicellular diazotroph classified as *Cyanothece*, which is regularly found in the New Caledonian lagoon (Turk-Kubo et al., 2015). The day before experiment 2 started, *Cyanothece* cells were pre-labelled with $^{15}N_2$ by incubating triplicate 20 ml culture aliquots (~10^6 cells ml^{-1}) with 20% volume $^{15}N_2$-enriched seawater (as explained above). These aliquots were incubated for 24 h at 27°C, 100 µmol photons m^{-2} s^{-1} on a 12 h:12 h light:dark cycle. After 24 h, triplicate 3 ml subsamples were filtered through pre-combusted (450°C, 5 h) GF/F filters (Whatman, St Louis, MO, USA) to obtain the initial ^{15}N-enrichment of the culture (source pool). The rest of the culture aliquots were concentrated by gentle filtration onto 2 µm polycarbonate filters. The filters were added to 50 ml filtered seawater and vortexed to obtain a concentrated suspension of ^{15}N-labelled cells (~9×10^5 cells ml^{-1}); 20 ml of this concentrate was added to the quintuplicate glass containers before filling them with filtered seawater and hanging the corals from the top. In parallel, triplicate containers with the same cell concentration but without corals were used as a control.

In experiment 3, we tested the ingestion of diazotrophs using a natural planktonic assemblage. We note that as *Cyanothece* is a usual member of the planktonic diazotrophic community in the New Caledonian lagoon (Turk-Kubo et al., 2015), the natural sample used may be partly composed of *Cyanothece* cells. Unfortunately, we did not perform strain-specific quantitative analyses that allow us to report the abundance of *Cyanothece* in our sample. Plankton was sampled from the lagoon the day before the experiment by prefiltering seawater through a 100 µm mesh to exclude larger cells. The sample was split into eight 4.3 l polycarbonate bottles: three were immediately filtered to obtain the initial ^{15}N-enrichment of the source pool and five were incubated with 20% volume $^{15}N_2$-enriched seawater for 24 h as described above. At the end of the incubation, the content of the bottles was filtered onto 0.2 µm polycarbonate filters which were placed in 50 ml filtered seawater and vortexed to resuspend the cells. Aliquots of 5 ml were kept for cell counts (flow cytometry, see below), while the remaining 45 ml was added to the incubation containers with corals.

In all experiments, the temperature was checked for optimal conditions before attaching the coral branch to the top of the containers. Subsequently, the containers were closed hermetically using a glass slide. At the end of each experiment, corals were rinsed six times with filtered seawater to remove cells potentially adhered to the coral surface (Houlbrèque et al., 2003) and subsequently transferred to Ziploc bags and stored at −20°C until analysis.

We considered nitrogen assimilation in zooxanthellae and tissue separately as both 'compartments' use nitrogen for their own needs. Zooxanthellae have the ability to take up and retain nitrogen from surrounding water (Grover and Maguer, 2002; Rädecker et al., 2015) and transfer it to the host (Kopp et al., 2013); corals can also obtain nitrogen via the ingestion of external food sources (Houlbrèque and Ferrier-Pagès, 2009); hence, it was important to calculate the assimilation of nitrogen by the tissue and the zooxanthellae separately. Nitrogen assimilation rates were calculated as described in Montoya et al. (1996) (Eqn 1):

$$N_{\text{assimilation}} = \frac{(^{15}N_{\text{target},T_f} - {}^{15}N_{\text{target},0_f})}{(^{15}N_{\text{enriched source}} - {}^{15}N_{\text{non-enriched source}}) \times t} \times [PN_{\text{target}}/A], \quad (1)$$

where $^{15}N_{\text{target},T_f}$ is the ^{15}N enrichment of the target pool (tissue or zooxanthellae) at the end of the incubation, $^{15}N_{\text{target},0_f}$ is the corresponding 'time zero' ^{15}N enrichment (natural ^{15}N enrichment of the target pool at the start of the incubation). The source pool varies between the three experiments and both the $^{15}N_2$-labelled ($^{15}N_{\text{enriched source}}$) and non-labelled ($^{15}N_{\text{non-enriched source}}$) atom % values are considered in Eqn 1 as follows: (i) in experiment 1 the source pool is seawater and the isotopic enrichment of the filtered seawater added to the containers, (ii) in experiment 2, it is the *Cyanothece* culture and (iii) in experiment 3 it is the natural planktonic assemblage. t is the incubation time in hours and PN_{target}/A is the mass (µg) of particulate nitrogen of the target pool per area (cm^{-2}) of coral skeleton surface (µg N cm^{-2} h^{-1}).

The tissue was removed from the skeleton using an air-pick (Rodolfo-Metalpa et al., 2010) and homogenized with a Potter tissue grinder. Zooxanthellae and tissue were separated by centrifugation (Houlbrèque et al., 2003) and both phases were frozen, lyophilized, ground and encapsulated in tin cups for ^{15}N analysis. The GF/F filters concentrating planktonic samples and coral samples were analysed using an elemental analyser coupled to an isotopic ratio mass spectrometer (EA-IRMS, Integra CN, SerCon Ltd, Cheshire, UK).

Prey cell count

In experiments 2 and 3, the concentration of prey cells was quantified at the start and end of the incubation period to check for their ingestion by the corals. The abundance of *Cyanothece* cells was determined by epifluorescence microscopy, while the abundance of picoplankton was determined with a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) as previously described (Gasol and del Giorgio, 2000).

Statistical analyses

Significant differences in the number of *Cyanothece* cells between the start and the end of the incubation period were checked by the non-parametric Mann–Whitney test. We also checked for differences in the abundance of *Cyanothece* between containers containing coral branches (N=5) and control containers without *Cyanothece* cells (N=3). To improve the robustness of the statistical analysis, the number of cells in each control and coral sample was counted 20 times both at the start and at the end of the incubation. Statistical significance was accepted at $P<0.05$.

RESULTS AND DISCUSSION

While scleractinian corals live in close association with diverse diazotrophic communities (Grover et al., 2014; Lema et al., 2012), to the best of our knowledge, this is the first study using the new dissolved $^{15}N_2$ method (Großkopf et al., 2012; Mohr et al., 2010) to measure N_2 fixation rates in corals (experiment 1), and to quantify the amount of nitrogen incorporated through the ingestion of planktonic diazotrophs (experiments 2 and 3). Over the three experiments, significant ^{15}N enrichments were found in the zooxanthellae (Fig. 1) but never in the tissue, for which results were below the detection limit (i.e. less than three times the standard deviation of the coral tissue natural ^{15}N abundance).

For coral incubations in $^{15}N_2$-enriched seawater (experiment 1), we measured a mean (±s.d.) nitrogen assimilation of 0.33±0.14 µg N cm^{-2} h^{-1} in zooxanthellae (Fig. 1). These results confirm the presence of diazotroph communities within corals (Lema et al., 2012, 2014; Olson and Lesser, 2013; Olson et al., 2009), and reinforce previous reports that use the acetylene reduction method (Lesser et al., 2007; Shashar et al., 1994; Williams et al., 1987). Zooxanthellae loss due to thermal stress may decrease nitrogen acquisition capacity in corals (Ferrier-Pagès et al., 2010). In such

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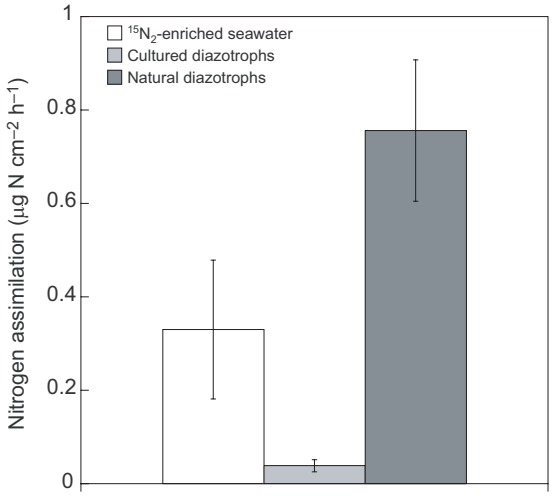

Fig. 1. Nitrogen assimilation rates in zooxanthellae. Data (N=5 for each experiment) were derived from exposure of corals to $^{15}N_2$-enriched seawater (experiment 1), cultured *Cyanothece* (experiment 2) and a natural plankton assemblage (experiment 3).

bleaching events, coral resilience can be enhanced by alternative fixed nitrogen sources such as that provided by endosymbiont diazotrophs (Cardini et al., 2014, 2016).

In a similar experiment, Grover et al. (2014) did not find significant ^{15}N enrichment either in the zooxanthellae or in the tissues of Red Sea corals after 24 h of incubation. They reported ^{15}N enrichment ranging between 0.0002% and 0.0015% (considerably lower than those measured here in experiment 1: 0.012–0.219%; data not shown). Their lack of significant enrichment might be due to (i) the use of the $^{15}N_2$ bubble method (Montoya et al., 1996), which may significantly underestimate N_2 fixation rates (Mohr et al., 2010; Großkopf et al., 2012), (ii) longer incubation periods than ours (24 versus 4 h), leading to increased oxygen concentrations in the incubation containers and inhibiting N_2 fixation (oxygen deactivates the nitrogenase enzyme; Postgate, 1982) or (iii) Red Sea corals harbour fewer or less active diazotrophs than those of New Caledonia. Our results probably indicate a significant heterogeneity in the ability of the coral versus the zooxanthellae to assimilate nitrogen. While both bear the enzymatic machinery to assimilate ammonium (the primary by-product of N_2 fixation), zooxanthellae account for most of the uptake of dissolved inorganic nitrogen from the environment and can fix 14–23 times more nitrogen than the host (Pernice et al., 2012).

When corals were incubated with *Cyanothece* cells (experiment 2), the average nitrogen assimilation rate in zooxanthellae was 0.04±0.01 μg N cm^{-2} h^{-1} (Fig. 1), revealing for the first time that one scleractinian coral feeds on planktonic diazotrophs. The consumption of *Cyanothece* was confirmed by a significant decrease in their concentration while corals were present (1.27×10^4 to 1.04×10^4 cells ml^{-1}, N=5, P=0.02; Fig. 2). No significant changes in *Cyanothece* concentration were measured over the same incubation period in control containers without corals (N=3, P=0.70). Considering that corals feed half the time, our nitrogen assimilation rates (0.48 μg N cm^{-2} day^{-1}) represent a third of the daily nitrogen input associated with the ingestion of non-diazotrophic picoplankton and nanoplankton estimated for the same coral species (1.4 μg N cm^{-2} day^{-1}; Houlbrèque et al., 2004). The lack of significant ^{15}N enrichment within the tissue in experiment 2 is consistent with the preferential transfer of prey nitrogen to the zooxanthellae (Piniak and Lipschultz, 2004). The high ^{15}N enrichment in zooxanthellae

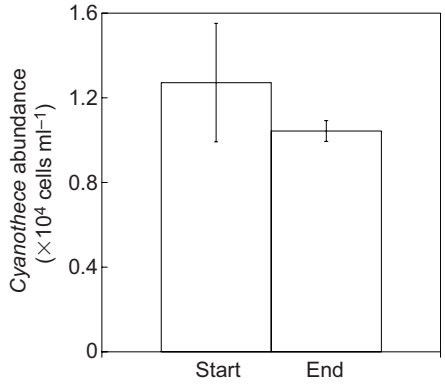

Fig. 2. *Cyanothece* cell abundance at the start and end of the incubation period. Data were obtained during experiment 2.

measured in our short incubation period (4 h) might be due to direct uptake of prey nitrogen digested within the coelenteron, probably as ammonium, rather than host assimilation. However, other studies have observed complete prey digestion after even shorter incubations (e.g. 3 h in *Galaxea fascicularis*; Hii et al., 2009), suggesting that at least part of the *Cyanothece* cells could have already been digested after 4 h when nitrogen recycling (i.e. synthesis into host macromolecules, catabolism, excretion and uptake via zooxanthellae) should have started. It is likely that digestion had only begun when our incubations were stopped, impeding detectable ^{15}N enrichment in the tissue.

Nitrogen assimilation rates in zooxanthellae measured when corals were fed with natural plankton (0.76±0.15 μg N cm^{-2} h^{-1}, experiment 3; Fig. 1) were ∼20-fold higher than those measured when corals were fed with *Cyanothece* (experiment 2). Despite the fact that *Cyanothece* is a common diazotroph in the lagoon (Turk-Kubo et al., 2015), and hence is likely to be present in the planktonic assemblage used in experiment 3, we did not perform quantitative analyses to infer their abundance from the number of *nifH* gene copies. During the experiment, the concentration of all plankton groups decreased (from 0.11×10^4 to 0.04×10^4 cells ml^{-1}, 1.88×10^4 to 1.22×10^4 cells ml^{-1} and 0.20×10^4 to 0.08×10^4 cells ml^{-1}, respectively, for *Synechococcus*, *Prochlorococcus* and picoeukaryotes; Fig. 3), except for bacteria, being statistically significant for *Prochlorococcus* and picoeukaryotes (P<0.05). In terms of the number of prey ingested (normalized to surface and polyp

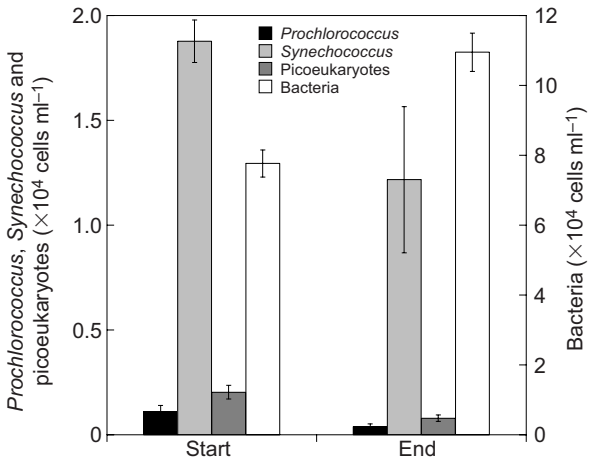

Fig. 3. Picoplankton cell abundance at the start and end of the incubation period. Cell abundance was recorded during experiment 3 for *Prochlorococcus*, *Synechococcus* and picoeukaryotes, and for bacteria.

considering 50 polyps cm^{-2} for *S. pistillata*), *Cyanothece* (experiment 2) were quantitatively the major group [19.7 (±6.31)×10^4 cells cm^{-2} h^{-1} or 3.93(±1.26)×10^3 cells $polyp^{-1}$ h^{-1}], followed by picoeukaryotes [7.37(±2.86)×10^4 cells cm^{-2} h^{-1} or 1.47 (±0.57)×10^3 cells $polyp^{-1}$ h^{-1}] and *Prochlorococcus* [4.29 (±1.54)×10^4 cells cm^{-2} h^{-1} or 0.86(±0.31)×10^3 cells $polyp^{-1}$ h^{-1}].

The rates of ingestion for *Cyanothece* are in the same range as those obtained for other cyanobacteria on the same coral species (0.3×10^4 cells $polyp^{-1}$ h^{-1}; Houlbrèque et al., 2004), but natural plankton nitrogen assimilation rates were ∼20-fold higher. Under natural conditions, corals extend their tentacles and swell their coenosarc to prepare for feeding as a result of their ability to sense the diffuse cloud of metabolites that surrounds plankton swarms (Hellebust, 1965). The release of dissolved organic compounds by *Cyanothece* is known to be negligible (Benavides et al., 2013a; Berthelot et al., 2015), and thus may not provide the metabolite cloud needed to chemically trigger feeding in *S. pistillata*. Under this scenario, in experiment 2, *Cyanothece* might have been only 'mistakenly' trapped by adhesion on polyps and mucus. Conversely, interactions between the different plankton groups included in the natural planktonic assemblage of experiment 3 may provide a greater release of dissolved organic compounds (estimated to represent ∼20% of primary production; Marañón et al., 2004), providing a chemical signal similar to the one experienced by corals *in situ* and favouring the ingestion of diazotrophs. While the significant decrease of *Cyanothece* cells during experiment 2 renders their ingestion by the coral unequivocal, the ^{15}N enrichment measured in experiment 3 could be due to different processes: (i) direct uptake of diazotrophs digested within the coelenteron (as in experiment 2), (ii) uptake of ^{15}N-enriched dissolved nitrogen compounds fixed by the diazotrophs and released extracellularly (Benavides et al., 2013b; also as in experiment 2) and/or (iii) ingestion of non-diazotrophic plankton enriched in ^{15}N as a result of diazotroph-derived nitrogen transfer (Bonnet et al., 2016).

In experiments 2 and 3, planktonic cells were concentrated to monitor as quickly as possible a significant change in cell concentrations due to coral ingestion without enclosing corals for too long. While the increased concentration of cells could enhance prey ingestion rates (Anthony, 1999; Ribes et al., 2003), the concentrations used here are in the range of those usually found in the lagoon (Conan et al., 2008; Jacquet et al., 2006; Turk-Kubo et al., 2015). Moreover, the use of short incubations also minimized possible changes in the planktonic community (death due to enclosure or grazing of picoplankton by nanoplankton).

Conclusions

We show evidence of ^{15}N enrichment in zooxanthellae by direct N_2 fixation and verify the use of widespread diazotrophic cells like *Cyanothece* in coral lagoons by one major coral species: *S. pistillata*. We estimate that the uptake of natural plankton (<100 µm) including diazotrophic cells would bring 0.76±0.15 µg N cm^{-2} h^{-1}, which is six times the daily nitrogen input calculated for the picoplankton and nanoplankton in Houlbrèque and Ferrier-Pagès (2009). The high abundance and activity of diazotrophs in the New Caledonian lagoon suggests that these cells represent a non-negligible source of nitrogen for corals. Additional experiments are needed to decipher through which pathways and mechanisms planktonic diazotrophs transfer nitrogen to corals.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceived and designed the experiments: M.B., F.H., S.B. and A.L. Performed the experiments: F.H., M.C. and M.B. Analysed the data: M.B. and M.C. Contributed reagents/materials/analysis tools: F.H., A.L. and O.G. Wrote the paper: M.B. and F.H., with comments and suggestions from all authors.

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