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SHORT COMMUNICATION

Nitrogen fixation in the mucus of Red Sea corals

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ABSTRACT

Scleractinian corals are essential constituents of tropical reef ecological diversity. They live in close association with diazotrophs [dinitrogen (N_2)-fixing microbes], which can fix high rates of N_2. Whether corals benefit from this extrinsic nitrogen source is still under debate. Until now, N_2 fixation rates have been indirectly estimated using the acetylene reduction assay, which does not permit assessment of the amount of nitrogen incorporated into the different compartments of the coral holobiont. In the present study, the $^{15}N_2$ technique was applied for the first time on three Red Sea coral species. Significant ^{15}N enrichment was measured in particles released by corals to the surrounding seawater. N_2 fixation rates were species specific and as high as 1.6–2 ng N day^{-1} l^{-1}. However, no significant enrichment was measured in the symbiotic dinoflagellates or the coral host tissues, suggesting that corals do not benefit from diazotrophic N_2 fixation.

KEY WORDS: Scleractinia, Diazotrophy, Stable isotopes, Symbiosis

INTRODUCTION

Coral reef waters are oligotrophic with respect to nitrogen (D'Elia and Wiebe, 1990), thus N_2 fixation by free-living benthic or pelagic cyanobacteria and bacteria (diazotrophs) is an important source of new nitrogen in these waters (Charpy et al., 2012). Additionally, several members of the reef community, such as sponges and ascidians, have developed mutualistic associations with diazotrophs (Wilkinson et al., 1999). Scleractinian corals have also adapted to conserve nitrogen. They live in symbiosis with dinoflagellates that take up and retain dissolved inorganic nitrogen from their surrounding environment and recycle host waste metabolites (Grover et al., 2002). Scleractinian corals also live in close association with a diverse array of microbes including diazotrophs, which inhabit the skeleton, host tissue and mucus layer of corals (Olson et al., 2009; Lema et al., 2012). Coral-associated diazotrophs are capable of N_2 fixation and transformation (Shashar et al., 1994; Lesser et al., 2007), but whether corals benefit from this new nitrogen is still debated. Indeed, N_2 fixation rates in coral holobionts (the coral host and its associated dinoflagellates and microorganisms) have been estimated using the acetylene reduction assay (ARA), which consists of injecting acetylene in seawater, which is then transformed to ethylene by nitrogenase activity. While relatively easy to perform, this method does not allow spatial analysis of the amount of nitrogen incorporated into the different holobiont compartments. Furthermore, it lacks accuracy, as a theoretical conversion factor is used to transform rates of ethylene production into rates of N_2 fixation. The present study aimed to use, for the first time in coral studies, the $^{15}N_2$ gas tracer method, which measures net fixation rates in addition to net incorporation of new nitrogen into the different coral compartments as well as in seawater particles issued from the coral external layer. Our study species were *Stylophora pistillata* Esper 1797 (from deep and surface water), *Cladopsammia gracilis* (Milne Edwards and Haime 1848) and *Porites* sp.

RESULTS AND DISCUSSION

^{15}N enrichment in seawater suspended particles, collected from control bottles without corals or with corals but without ^{15}N addition, was insignificantly different from the natural levels (ANOVA, P=0.098). Following 24 h of incubation, particles collected from ^{15}N-enriched bottles, containing coral nubbins, were significantly enriched with ^{15}N (ANOVA, P=0.001). ^{15}N enrichment ranged from 0.006% to 0.0241% depending on the coral species incubated (Fig. 1A). These values correspond to 1.6–22 ng N l^{-1} day^{-1} (i.e. 0.12–1.6 nmol N l^{-1} day^{-1}) or to 0.08–0.32 ng N cm^{-2} day^{-1} when normalized to the surface area of the coral nubbins (Fig. 1B). As seawater was filtered at the beginning of the incubation, and coral nubbins had a very small fraction of apparent skeleton, this result suggests that microorganisms contained in the freshly released mucus are able to fix N_2. Our measurements are in the same range as N_2 fixation rates measured for other oligotrophic waters (from 0.1 to 4 nmol N l^{-1} day^{-1}) (reviewed in Ridame et al., 2013), suggesting that there is significant N_2 fixation, and that the enrichment technique used in this study allows an accurate measurement of this N_2 fixation. The $\%^{15}N$ atom excess values were significantly higher in particles released by *S. pistillata* from deep water and *C. gracilis* than in samples containing *S. pistillata* from surface waters and *Porites* sp. (ANOVA, P<0.01). This could be due to species-specific populations of microbes, which remain to be further studied in a larger experiment including determination of the microbial communities involved. The main finding of this study is the lack of significant nitrogen enrichment in the coral compartments, despite high rates of N_2 fixation in the incubation medium. Indeed, the $\%^{15}N$ atom excess in the coral tissue and in the symbionts was very low (between 0.0002% and 0.0015% in both compartments, Fig. 1A) and in the same range as the standard deviation of the natural samples (0.0010%). Thus, no reliable uptake could be established for corals. This observation suggests that, in our experiment, corals did not benefit from the nitrogen fixed by diazotrophs contained in their mucus layer and released into the surrounding medium, at least within 24 h. Indeed, colonies of *S. pistillata*, maintained for 3 days in $^{15}N_2$-enriched medium, presented a ^{15}N excess enrichment as high as 5% (data not shown). However, such a long incubation in a closed system may entail the death of bacterial particles and the release of ammonium and/or dissolved organic nitrogen, and abnormal release of mucus and bacteria by corals, which precludes any reliable estimations on the processes occurring on natural reefs. This study contradicts that by Lesser et al. (Lesser et al., 2007), who found some evidence that dinoflagellates in

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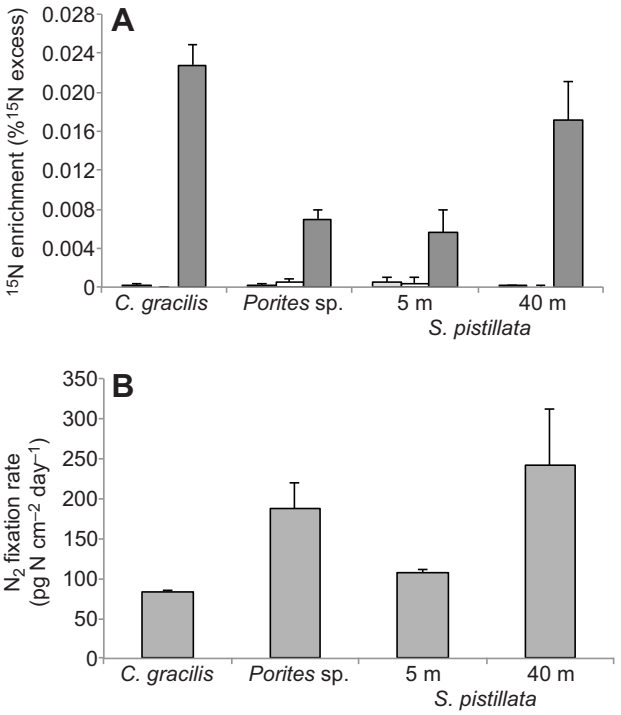

Fig. 1. ^{15}N enrichment and N_2 fixation rates in Red Sea corals. (A) ^{15}N enrichment in coral tissue [light grey bars (left)], in symbionts [white bars (middle)] and in seawater particles [dark grey bars (right)] of the three coral species: *Cladopsammia gracilis*, *Porites* sp. and *Stylophora pistillata* [collected at 5 m depth (shallow) and 40 m depth (deep)]. Values were multiplied by 100 for clarity. (B) N_2 fixation rates by seawater particles.

symbiosis with the coral *Montastrea cavernosa* were able to acquire nitrogen from N_2 fixation by diazotroph symbionts. The nature and location of these symbionts within the coral is, however, subject to debate (Oswald et al., 2007). Interpretations/conclusions from ARA experiments should be taken with care, because ARA measures total N_2 fixation without tracing the fate of the new nitrogen into the organic matter. Nutrients can sometimes be acquired from skeletal endoliths (Fine and Loya, 2002). Taken together, these observations suggest that the microbial community in coral tissues and mucus is highly dynamic and can change rapidly (Lema et al., 2012), which in turn may alter its capacity to transfer nutrients to the host.

The use of $^{15}N_2$ tracer for the first time with corals has clearly revealed that diazotrophs released with the mucus of scleractinian tropical corals can present high rates of N_2 fixation. These rates are equivalent to those previously measured in other oligotrophic environments. This study, however, clearly demonstrates the lack of rapid uptake of new nitrogen by the coral host and its symbionts, although N_2 fixation occurs in their immediate surroundings. This observation thus contradicts previous interpretation from ARA experiments on corals.

MATERIALS AND METHODS

Experiments were performed with three species (three large nubbins per species) of the corals *S. pistillata*, *C. gracilis* and *Porites* sp., collected at 5 m depth from the reef of Eilat (Gulf of Aqaba, Red Sea), at a temperature of 25°C. Three nubbins of *S. pistillata* were also collected at 40 m depth, at a temperature of 24.7°C. Care was taken to avoid having a large surface of apparent skeleton, which might have contained diazotrophs. Rates of N_2 fixation were measured using $^{15}N_2$ gas (98%, Eurisotop) as outlined elsewhere (Montoya et al., 1996). Sealed 500 ml glass bottles, equipped with a Vacutainer syringe valve, were completely filled with 0.45 µm filtered oligotrophic seawater, prior to the introduction of the coral colonies. The water contained nanomolar concentrations of nitrate, ammonium and phosphate, as measured monthly by the National Monitoring Program of the Gulf of Aqaba (NMP), Eilat. The $^{15}N_2$ gas was then injected through the tap with a syringe, after removing the same seawater volume, for a final enrichment of ca. 80 atom% excess. Bottles were incubated in an outdoor seawater flow-through system for 24 h, at the seawater temperature and under natural irradiance, shaded to the level received by the corals at 5 or 40 m depth. Controls were also performed with only seawater enriched with $^{15}N_2$ or with corals without ^{15}N enrichment. At the end of the incubations, seawater was filtered through GF/F filters, pre-combusted for 5 h at 450°C, and filters were freeze-dried. Corals were rinsed for 15 min with filtered and unenriched seawater. Coral tissue was completely removed from the skeleton with an air brush and homogenized with a Potter tissue grinder. Homogenates were separated into animal and algal fractions according to Grover et al. (Grover et al., 2002), and each fraction was freeze-dried. Organic nitrogen content (PON) and ^{15}N enrichment in the particulate matter were quantified with a mass spectrometer (Delta Plus, ThermoFisher Scientific, Germany) coupled via a type III interface with a C/N analyser (Flash EA, ThermoFisher Scientific). The enrichment of the samples with ^{15}N was recorded as atom % excess. Nitrogen uptake rates were calculated according to Grover et al. (Grover et al., 2002) and considered significant when ^{15}N excess enrichment in the PON was greater than three times the standard deviation obtained with natural samples.

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Competing interests

The authors declare no competing financial interests.

Author contributions

All authors performed the experiments. R.G. and C.F.-P. developed the concepts, J.-F.M. and L.E. performed the analytical measurements and analyzed the data, and R.G. and C.F.-P. prepared and edited the manuscript.

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