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research paper

Rhodolith holobionts are not sources of fixed nitrogen in a northeastern Gulf of Mexico patch reef

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ABSTRACT.—Rhodoliths provide numerous benefits to coastal ecosystems and help support high biodiversity. No study, however, has explored rhodoliths that occupy northeastern Gulf of Mexico patch reefs, and their contributions to local ecosystem function remain uncharacterized. Here, we employed the acetylene reduction assay to assess nitrogen fixation capability in rhodolith holobionts (*Lithothamnion* spp.; Rhodophyta), sediment, and surrounding seawater from a subtropical patch reef ecosystem in the northeastern Gulf of Mexico. We found no evidence for nitrogenase activity in rhodolith holobionts or seawater from our study site, while nitrogenase activity in sediment underlying rhodoliths was approximately equivalent to a nitrogen fixation rate of 0.521 (SD 0.087) nmol N_2 g dry mass^{-1} hr^{-1}. Our results suggest that rhodoliths in the northeastern Gulf of Mexico rely on sources of nitrogen from sediment nitrogen fixation or water column nutrient availability rather than the activity of symbiotic diazotrophic microorganisms. Functional analyses recognizing rhodoliths as holobionts warrant further investigation to better understand the ecology of rhodoliths.

Rhodoliths are free-living forms of nongeniculate coralline algae with global distribution (Foster 2001). Rhodoliths increase habitat complexity and support biodiverse communities by providing nursery grounds and structure for numerous benthic invertebrates and juvenile fishes, as well as epiphytic and endolithic organisms (Jackson et al. 2004, Kamenos et al. 2004a,b, Gagnon et al. 2012, Krayesky-Self et al. 2017). Studies on rhodoliths in the Gulf of Mexico (GoM) have focused on mesophotic rhodolith aggregations (beds) found at 45–90 m depth in the northwestern GoM (Fredericq et al. 2019). Shallow water rhodoliths (10–15 m depth) in the northeastern GoM remain entirely unexplored, and thus their contributions to the ecosystem biodiversity and function of the patch reefs they occupy remain unknown.

Rhodolith beds in other geographic locales can be influential contributors to nutrient cycling, especially carbon and nitrogen cycling. Though rhodolith beds can act as net sinks of NO_x^-, annual production of 486.7 g $CaCO_3$ m^{-2} has been reported in a temperate rhodolith bed (Martin et al. 2007a) alongside release of up to 260 µmol NH_4^+ m^{-2} hr^{-1} into the surrounding water column (Martin et al. 2007b). Despite a

documented contribution of associated microbes and fauna to nitrogen dynamics in other macroalgae (Bracken et al. 2007, Egan et al. 2013), the relative contributions of rhodolith-associated cryptofauna and microbes to measured nitrogen fluxes remains equivocal (McConnico et al. 2018). Consequently, nitrogen sources and net impacts of measured fluxes on total site-scale nitrogen budgets remain undescribed. Understanding contributions to nitrogen cycling, especially new nitrogen input, is particularly critical in rhodolith-bearing shallow water patch reef communities found on limestone ledges in the northeastern GoM, because nitrogen availability is a key factor frequently limiting primary productivity in pristine coastal marine environments (Karl et al. 2002). Nitrogen fixing prokaryotes (diazotrophs) associated with other benthic foundation species, such as those associated with corals and sponges in coral reef environments, make significant contributions to both host and ecosystem nitrogen budgets in shallow-water marine systems (Head and Carpenter 1975, Penhale and Capone 1981, Fiore et al. 2010). Rhodoliths similarly harbor stable microbial assemblages distinct from their environment that have the potential to support host growth and contribute to local nitrogen cycling (Cavalcanti et al. 2014, 2018). Yet, no study has experimentally assayed the potential influence of symbiotic diazotrophs on observed nitrogen fluxes associated with rhodolith beds.

To understand the ecological role of rhodoliths in northeastern GoM patch reefs, we must also characterize the functionality of the microbes comprising rhodolith holobionts. Our objective in this study was to quantify nitrogenase activity associated with rhodolith holobionts, sediment, and seawater collected from a rhodolith-bearing subtropical patch reef in the coastal northeastern GoM to determine whether rhodolith holobionts contribute new nitrogen to this patch reef system via fixation, as well as better understand benthic and pelagic nitrogen fixation at this site.

Materials and Methods

Study Site and Sampling.—Samples of rhodoliths, sediment, and seawater were collected on SCUBA at an offshore patch reef (Turtle Towers, 29°43′0.09″N, 84°30′0.10″W) in the northeastern GoM located on the West Florida Shelf near Carrabelle, Florida (Fig. 1). Rhodolith and sediment samples were collected on 10 September, 2018, and seawater and additional rhodolith samples were collected on 7 January, 2019. This study site consists of an elevated limestone ledge (about 1–2 m of relief) that is covered with 0–3 cm of sediment. Rhodoliths can be found on top of the sediment, as well as aggregated in lower-lying crevices in the ledge. The rhodoliths at this site are small, and the size class we targeted [mean (SD) = 11.8 (2.96) mm length; measured at the longest point of each rhodolith including branched protuberances] was representative of the average size found at this site. We targeted *Lithothamnion* spp. (Rhodophyta), identified based on habit using the macroscopic morphological properties provided in Richards et al. (2016). Rhodolith specimens (n = 98) were haphazardly collected by hand between 13 and 14 m depth. The top 2 cm of sediment from areas underlying sampled rhodoliths was collected and homogenized in a single container. Additionally, we collected water from 13 m depth in 18.9 L lidded buckets. Seawater samples (n = 3) were immediately filtered at the surface using Whatman 0.6 µm nuclepore filters and stored in 50 ml Falcon tubes for later nitrite (NO_2^-) and nitrate (NO_3^{2-}) concentration analysis. Both the sediment and rhodoliths were contained in the sample buckets filled with seawater for immediate transportation to Florida State University to begin each Acetylene Reduction Assay (ARA; approximately 4 hrs later).

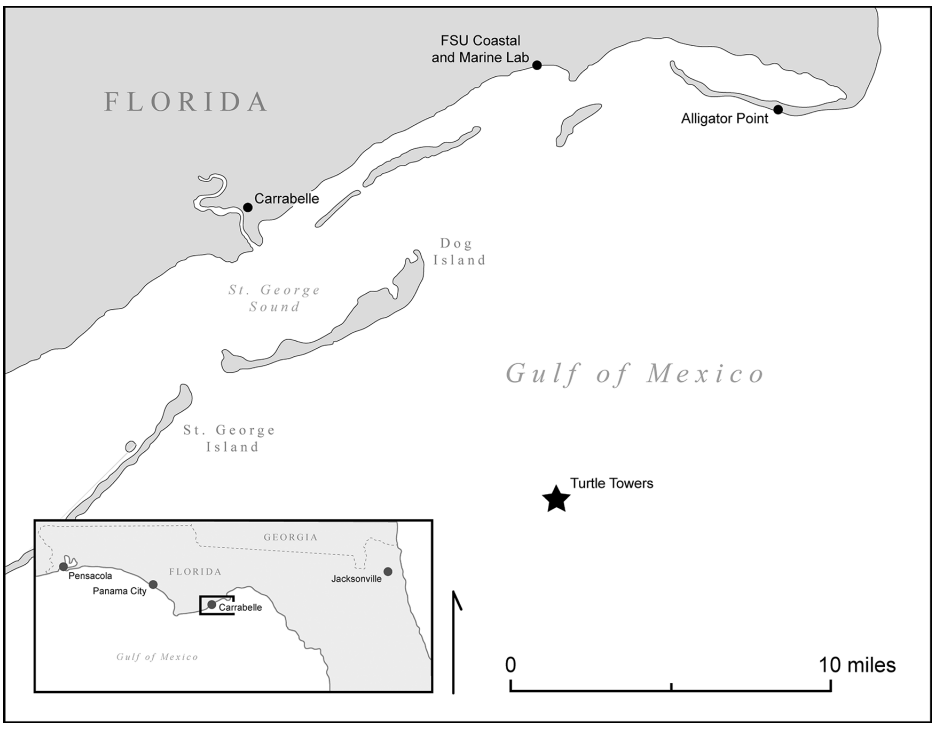

Figure 1. Map showing location of our study site, Turtle Towers (marked with star), in relation to the Florida coastline. Inset: location of broader study area (marked with box) in relation to the northwestern coast of Florida. Map made by JR Cissell.

Acetylene Reduction Assay.—To estimate potential sources of, and rate differences in, nitrogen fixation, rhodoliths, sediment, and unfiltered seawater were assessed for diazotrophic activity indirectly using the ARA technique. We performed the following four different experiments: (1) ARA on benthic sediment samples (n = 7); (2) ARA on rhodoliths (n = 10); (3) ARA on natural seawater (n = 4); and (4) ARA on rhodoliths incubated in artificial, inorganic nitrogen-free Aquil seawater (n = 4). The specific methods that differed among experiments are detailed in their respective subheadings below (experiments 3 and 4 under combined subheading; *see* Fig. 2); general methods are described here.

Samples were contained in individual 20 ml gas-tight bottles sealed with crimp-seals equipped with rubber septa. Using gas-tight syringes (Hamilton), 2 ml of the gas phase was removed followed by injecting 2 ml of pure acetylene gas (generated from reacting CaC_2 with milliQ H_2O and capturing the resulting gas in inflatable tedlar bags), resulting in a 20% acetylene headspace for each sample bottle. Bottles were randomly and evenly divided into two incubation groups for each experiment: Time 0 and Time 12 (experiment 2 only) or Time 24 (experiments 1, 3, and 4). The Time 0 incubation groups served as control groups for any potential initial ethylene concentration and were assayed for ethylene production immediately after spiking the headspace. We incubated the Time 12 groups for 12 hrs with either 12 hrs of light or 12 hrs of dark depending on the treatment, kept in an incubation chamber held

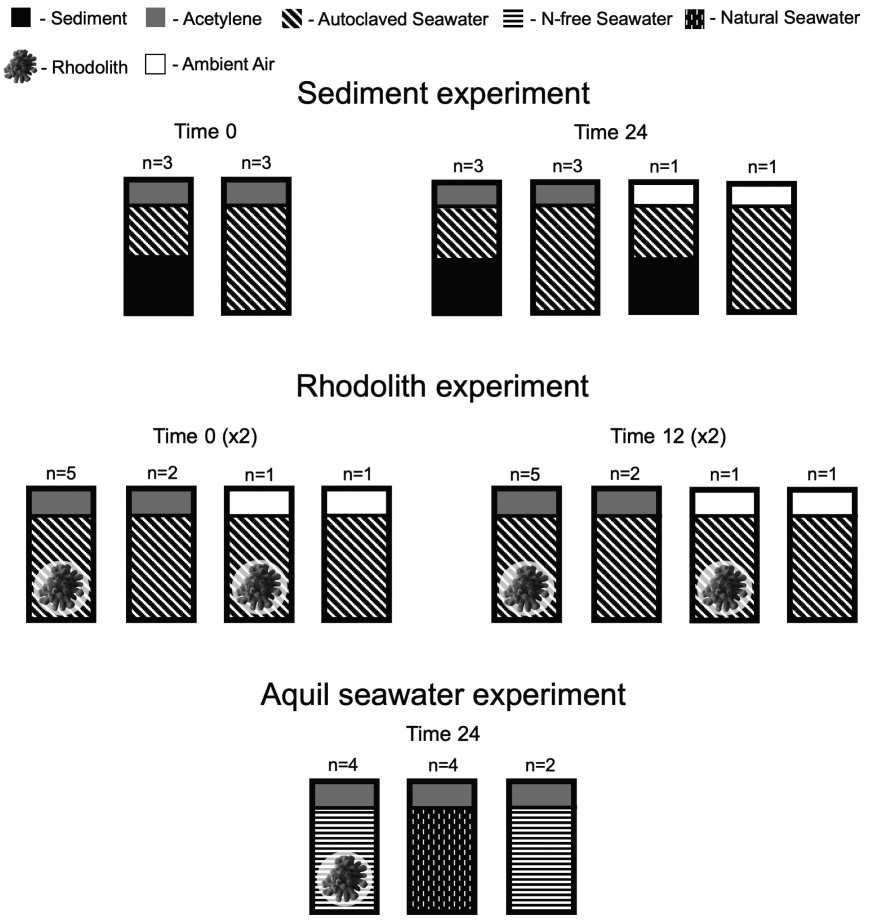

Figure 2. Conceptual representation of different experiments, different incubation groups within each experiment, and composition of incubation bottles within each group.

at a constant 24 °C. Light incubations were performed at 100 µmol photons m^{-2} s^{-1}, consistent with levels measured at depth at this site, ranging from 50 to 150 µmol photons m^{-2} s^{-1}. Time 12 groups were assayed for ethylene production immediately after the 12-hr incubation. The Time 24 groups were incubated for 24 hrs, with 12 hrs of constant light and 12 hrs of constant dark in an incubation chamber held at a constant temperature of 24 °C. Time 24 group bottles were assayed for ethylene immediately following the 24-hr incubation.

Ethylene production in each sample was measured by injecting 1 ml of headspace gas into an SRI 310 gas chromatograph equipped with a flame ionization detector (Stewart et al. 1967; H_2 carrier gas flow rate 9 ml min^{-1}; detector temperature 155 °C; column temperature 45 °C). The amount of ethylene produced was quantified relative to an ethylene standard. Samples were dried at 60 °C for 48 hrs to measure dry mass. To obtain the mass of organic matter in our samples, we oxidized samples at 500 °C for 4 hrs. All results are reported normalized to the dry mass (g) of each sample unless otherwise stated. To facilitate comparison to existing literature data on rates of nitrogen fixation, we converted our total ethylene production to

approximate nitrogen production using a conversion ratio of 4:1 (Peterson and Burris 1976, Capone and Montoya 2001, Brocke et al. 2018).

Sediment Experiment.—To determine the amount of nitrogen fixation activity in sediment underlying rhodoliths at Turtle Towers, we performed an ARA experiment on sediment on 10 September, 2018. We placed collected sediment into 7 individual bottles (n = 7) until the bottom 2 cm of each bottle contained sediment (to replicate in situ sediment depth). Autoclaved seawater from the study site was then added to bring each individual bottle to a total volume of 10 ml, leaving a 10 ml headspace in each bottle. The Time 0 group consisted of 3 replicate sediment bottles (n = 3), and 3 replicate seawater bottles (n = 3). The Time 24 incubation group consisted of 3 replicate sediment bottles (n = 3), 3 replicate seawater bottles (n = 3), and 2 control types lacking acetylene addition [sediment (n = 1), autoclaved seawater (n = 1)], incubated with 12 hrs of dark (18:00–6:00 hr), and 12 hrs of light (6:00–18:00 hr). Ethylene production was measured as described above.

Rhodolith Experiment.—Rhodolith holobionts were assayed for nitrogen fixation activity on 12 September, 2018, following a 2-d acclimation period in the lab in a large bucket filled with seawater from Turtle Towers kept aerated and flowing with an aquarium pump. We only used rhodoliths able to fit into the 1 cm wide opening of the 20 ml gas-tight bottles without fracturing in this experiment to prevent any potential effects of fracturing from influencing our results (<5 rhodoliths excluded from incubations based on size). To prepare the rhodoliths for the incubation, we manually removed fouling macro-epiphytes prior to the ARA using sterile forceps. We then randomly placed 2–5 individual rhodoliths into each sample bottle, or until the bottom approximately 2 cm of each bottle was filled with rhodoliths. To obtain separate measures of ethylene production in the dark and light, two different experimental groups were used. For both experimental groups, the Time 0 group and the Time 12 incubation group each consisted of 5 replicate rhodolith bottles (n = 5), 2 replicate autoclaved seawater bottles (n = 2), and 2 control bottles, one with rhodoliths and one with autoclaved seawater, both having no acetylene addition. The first experimental group was incubated for 12 hrs in darkness (19:00–7:00 hr). The second experimental group was incubated for 12 hrs in light (8:00–20:00 hr) immediately following the dark incubation. Ethylene production was measured as described above.

Aquil Seawater Experiment.—We performed a follow-up ARA on 8 January, 2019 to determine if incubation in nitrogen-free artificial seawater encourages nitrogenase activity in any diazotrophs present in our samples. This experiment was performed prior to our quantification of NO_2^- and NO_3^{2-} concentrations in natural seawater from this site. Rhodoliths were acclimated in seawater from the collection site for one day in a large, shaded bucket in the lab kept aerated by an aquarium pump prior to the ARA. Four replicate sample bottles for rhodoliths (n = 4) were prepped as described above, but using N-free artificial Aquil seawater media (Sunda et al. 2005; salinity 35, pH 7.43) to bring each bottle to a solid/liquid phase volume of 10 ml. Additionally, we added 10 ml of natural seawater collected from our study site to each of 4 replicate bottles (n = 4), and 10 ml of artificial Aquil seawater media to each of 2 replicate bottles (n = 2). All sample bottles were measured after a 24-hr

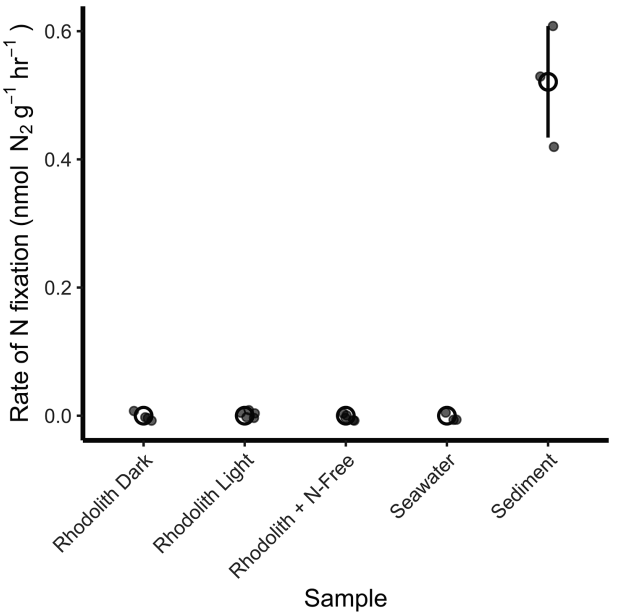

Figure 3. Rate of nitrogen fixation in nmol N_2 g dry mass^{-1} hr^{-1} for each sample type. Unfilled circles denote mean (SD). Small gray points denote individual sample values. All samples had a rate of 0 except sediment, which had a mean rate of 0.521 (SD 0.087) nmol N_2 g dry mass^{-1} hr^{-1}.

incubation in a controlled environmental chamber with 12 hrs of dark (18:00–6:00 hr), and 12 hrs of light (6:00–18:00 hr). We measured pH in each sample bottle before and after the 24-hr incubation.

Nutrient Analysis.—Nitrate and nitrite were measured from the water samples collected on 7 January, 2019, using a colorimetric vanadium reduction method described in García-Robledo et al. (2014). Briefly, nitrite was measured following a colorimetric reaction with a Griess solution in a UV-VIS spectrophotometer. Nitrate was first reduced to nitrite using vanadium chloride and subsequently measured as nitrite using the Griess reaction. All reagents and standards (nitrite and nitrate standard) were freshly prepared. The method was able to measure a minimum of 0.2 uM NO_2^- or NO_3^- with a precision of 0.2 uM.

Results

Rates of Nitrogen Fixation.—We performed four different ARA experiments to determine nitrogenase activity in rhodolith holobionts, sediment, and seawater collected from Turtle Towers, Gulf of Mexico, Florida. Sediment samples (n = 3) collected were the only tested samples to exhibit ethylene production in any of our experiments. Sediment samples produced nitrogen at a rate of 0.521 (0.087) nmol N_2 g dry mass^{-1} hr^{-1} [mean (SD); Fig. 3], or 33.4 (7.92) nmol N_2 g organic mass^{-1} hr^{-1}. Rhodolith holobionts incubated in both natural seawater (n = 5 dark; n = 5 light) and artificial N-free Aquil seawater (n = 4) did not produce any ethylene (Fig. 3). Water sampled from our site (n = 3) and the artificial nitrogen-free seawater similarly did

not exhibit any ethylene production during our experiment (Fig. 3). The pH in the bottles containing rhodoliths and artificial N-free seawater increased an average of 1.47 (0.14) during our 24-hr incubation, with a maximal pH of 9.09. The pH of natural seawater changed an average of 0.03 (0.05) over the 24-hr incubation. The pH of artificial N-free seawater without rhodoliths changed 0.31 (0.01) over the 24-hr incubation. It should be noted that the artificial seawater may not have been fully equilibrated with respect to CO_2. During the acetylene incubation both biological activity and headspace/water equilibration likely led to a larger change in pH in the artificial seawater compared to the natural seawater. While this pH difference could affect N_2-fixation rates, our data clearly indicate that N_2 fixation did not occur. All control samples from the Time 0 incubation groups showed no ethylene concentration. Our results indicate that seawater and rhodolith holobionts do not contribute fixed nitrogen into this system, while the underlying sediment does contribute nitrogen via the activity of diazotrophic prokaryotes.

Water Column Nutrient Analysis.—All seawater samples had nondetectable nitrate or nitrite concentrations, indicating that less than 0.2 uM NO_2^- and NO_3^{2-} was present in the samples.

Discussion

Our finding of no nitrogen fixation activity associated with shallow water northeastern GoM rhodolith holobionts under both ambient and nitrogen-starved conditions is surprising given the general prevalence of macroalgal–diazotroph associations with high fixation rates (42.8–686 nmol N_2 g^{-1} hr^{-1}; Head and Carpenter 1975, Capone et al. 1977, Phlips and Zeman 1990, de Oliveira et al. 2012, Egan et al. 2013), as well as the identity of nitrogen fluxes previously documented with other rhodolith beds (Martin et al. 2007b). The species richness and abundance of organisms supported by rhodolith beds are positively correlated with both the complexity and density of beds, with more structurally complex rhodolith beds supporting greater biodiversity (Steller et al. 2003, Gagnon et al. 2012, Teichert 2015, Gabara et al. 2018). Rhodolith diameter is similarly positively correlated with associated cryptofaunal biomass (McConnico et al 2018, Veras et al. 2020). Hence, factors that influence rhodolith productivity and growth, such as nitrogen availability, have a direct influence on the habitat building ability of rhodoliths, and the richness of the communities rhodoliths support (Karl et al. 2002, Carvalho et al. 2020). Rhodolith-bearing patch reefs in the northeastern GoM are species rich, marked by the intrusion of tropical taxa including numerous species of gorgonian octocorals (predominantly *Leptogorgia virgulata*) and scleractinian corals, and support a highly diverse mobile invertebrate and fish community with high endemism (Livingston 1984, Gotelli 1988, Rowe 2017). However, the small size of rhodoliths at this site, potentially constrained by nitrogen limitation, could limit the benefits their structure confers to this site, as well as limit reciprocal nutrient benefits from associated invertebrates and fishes (McConnico et al. 2018). A better understanding of nitrogen dynamics at this site is therefore critical for a holistic categorization of any benefits these rhodoliths confer to support the high observed biodiversity at this site, or if nitrogen availability limits the potential contributions these rhodoliths can make.

Inorganic nutrient analysis (NO_2^- and NO_3^{2-}) on seawater from our study site showed dissolved inorganic nitrogen (DIN) levels below detectable limits. Data on nutrient dynamics for this particular area are limited; however, our data are concordant with those reported in Qian et al. (2003) who reported a severe drop of surface (about 1–3 m depth) nitrate concentrations to undetectable levels from concentrations exceeding 80 μM along an increasing salinity gradient from estuarine to offshore in the northeastern GoM. Mortazavi et al. (2000) found that DIN export from the nearby Apalachicola Bay [which receives substantial fluvial nutrient input from the Apalachicola River (distance of 44.65 km from Turtle Towers)] to the GoM was lowest from May–September [average 0.64 (SE 0.10) mmol N m^{-2} d^{-1}], and highest between the months of October and February [average 6.2 (SE 0.39) mmol N m^{-2} d^{-1}]. Our measurements of DIN being below detectable limits during a season when DIN should have been near its maximum (Mortazavi et al. 2000, 2001) could indicate that this site is persistently oligotrophic or exhibits rapid uptake and assimilation of water column DIN. This geographic region had among the highest tested concentrations of planktonic chlorophyll *a* in the northeastern GoM (0.3–0.5 μg L^{-1}), indicative of high water column productivity that could rapidly assimilate any water column nitrate (Qian et al. 2003). Rates of dissolved organic nitrogen (DON) export from Apalachicola Bay, however, are high and range from 7.0 (SE 0.5) to 33.1 (SE 7.2) t N d^{-1} (Mortazavi et al. 2001). Via this route, the rhodolith community at this site, similar to Pacific rhodolith beds, may in part rely on subsidies of organic nitrogen from nearby sources to support productivity (Gabara 2020).

Nitrogen requirements of macroalgae in other systems can be fully met by association with fauna and diazotrophs (Bracken et al. 2007, Egan et al. 2013). However, the small size of rhodoliths at this site likely excludes stable faunal associations. Further, the seeming lack of active symbiotic diazotrophs and low water column NO_2^- and NO_3^{2-} availability may make sediment associated nitrogen input critical to support rhodolith growth at this site. The rate of fixation we documented in sediment at this site [0.521 (SD 0.087) nmol N_2 g dry mass^{-1} hr^{-1}] is comparable to those documented in coastal estuarine sediments (0.0107–7.85 nmol N_2 g^{-1} hr^{-1}; Seitzinger and Garber 1987, Hou et al. 2018), but higher than those documented in tropical sediments (0.00250–0.250 nmol N_2 g^{-1} hr^{-1}; O'Neil and Capone 1989). Physical characteristics of sediment, such as porosity (positive correlation) and median grain size (negative correlation), can influence rates of nitrogen fixation (Andersson et al. 2014). The sediment at this site is primarily carbonate (Davis 2017), with a median grain size and porosity likely similar to that documented in the nearby St. Marks National Wildlife Refuge (30°4′27.1″N, 84°10′49.6″W) of 177.2 μm and 42.3% respectively (Lisle and Comer 2011). Sediments with similar porosity and grain size from brackish water along coastal Sweden frequently exhibited low nitrogenase activity (<0.01 mmol C_2H_4 m^{-2} d^{-1}), indicating that physical sediment characteristics at our tested site could constrain benthic fixation, in addition to other biophysical site characteristics, such as frequency of sediment disturbance and salinity (Andersson et al. 2014).

Given the lack of any nitrogenase activity we documented in water column samples, benthic-pelagic coupling of fixation may be a significant component of the nitrogen budget of this patch-reef community and in support of rhodolith growth (Fig. 3). Indeed, sediment-associated bacterial communities can be dominant sources of fixed nitrogen to other nearshore ecosystems, producing as high as 64% of total nitrogen requirements on coral reefs (Cardini et al. 2016). Further work must

be performed to characterize the physiology of these rhodoliths, as well as the trophic structure at this site, to understand the relative contribution sediment fixation makes to total nitrogen demand in this system.

Though our study is an important first step toward parsing nitrogen sources in rhodolith bed communities and understanding nitrogen fixation in patch reefs in the northeastern GoM, future work should expand on the limited spatiotemporal scale and sample size of this study. Similar experiments in a system harboring larger rhodoliths would help elucidate if the small sizes of rhodoliths at this site [mean 11.8 (SD 2.96) mm length] limit microbial associations. The diffusive barrier of acetylene and any ethylene produced by any intra-thalli diazotrophs also may not have had time to diffuse through the thalli over a 12-hr or 24-hr incubation, potentially influencing our results. However, appreciable ethylene production was observed in a similar experimental setup utilizing another calcified organism, the coral *Acropora variabilis*, after only 60 min of acetylene exposure (Williams et al. 1987). Given our results, we recommend that studies of rhodolith bed nitrogen cycling should increasingly focus on parsing the relative contributions of host-associated microbes and fauna, as well as underlying sediment microbial communities, to determine the still unresolved sources of nitrogen compounds, especially NH_4^+, in observed nitrogen fluxes with rhodolith beds (Martin et al. 2007b, McConnico et al. 2018). This will help elucidate the relative importance of microbial vs faunal associations to rhodolith growth and persistence (McConnico et al. 2018).

In conclusion, we found that rhodolith holobionts we sampled from the northeastern GoM are not appreciable sources of fixed nitrogen to the shallow-water patch reefs they occupy. The nitrogen budget at this site is potentially controlled via diffusive sediment flux where nitrogen fixation was measured, or from organic nitrogen introduced by tidal changes which could create spatiotemporally heterogenous nutrient availability (Mortazavi et al. 2001). A better understanding of the functionality of microorganisms found in association with rhodoliths, as well as nutrient limitations and trophic structure of this site, is necessary to characterize the ecological and biogeochemical role of these critical benthic foundation species in this understudied geographic region.

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