MICROBIAL ECOLOGY

Microb Ecol (2001) 41:36–44
DOI: 10.1007/s002480000066

Diazotrophy in Modern Marine Bahamian Stromatolites

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Received: 29 February 2000; Accepted: 6 July 2000; Online Publication: 7 November 2000

ABSTRACT

N_2 fixation (nitrogenase activity), primary production, and diazotrophic community composition of stromatolite mats from Highborne Cay, Exuma, Bahamas, were examined over a 2-year period (1997–1998). The purpose of the study was to characterize the ecophysiology of N_2 fixation in modern marine stromatolites. Microbial mats are an integral surface component of these stromatolites and are hypothesized to have a major role in stromatolite formation and growth. The stromatolite mats contained active photosynthetic and diazotrophic assemblages that exhibited temporal separation of nitrogenase activity (NA) and photosynthesis. Maximal NA was detected at night. Seasonal differences in NA and net O_2 production were observed. Photosynthetic activity and the availability of reduced organic carbon appear to be the key determinants of NA. Additions of the *de novo* protein synthesis inhibitor chloramphenicol did not inhibit NA in March 1998, but greatly inhibited NA in August 1998. Partial sequence analysis of the *nifH* gene indicates that a broad diversity of diazotrophs may be responsible for NA in the stromatolites.

Introduction

Modern-day microbial mats are laminated, self-sustaining ecosystems analogous to Earth's first extant biotic communities, stromatolites [19]. These first complex communities are thought to have developed in shallow lagoons and beach environments [37]. Stromatolites may be defined in several ways [12], but they may generally be characterized as sedimentary structures containing lithified laminae potentially produced by an associated microbial mat. Accretion of lithified laminae over time provides stromatolites with a layered cross-sectional appearance. Whereas soft microbial mats (without lithified laminae) are ubiquitous coastal features, marine stromatolites are relatively rare [7]. The paucity of marine stromatolites has hampered efforts to understand how ancient stromatolites formed and grew. Recently, actively accreting marine stromatolites inhabiting normal marine salinity (32–38 ppt) waters were discovered near several of the Exuma islands in the Bahamas, including Highborne Cay (Fig. 1) [9, 13, 34]. Microbial mat metabolic activities (primary production, respiration) are hypothesized to have an active role in producing the lithified laminae within the

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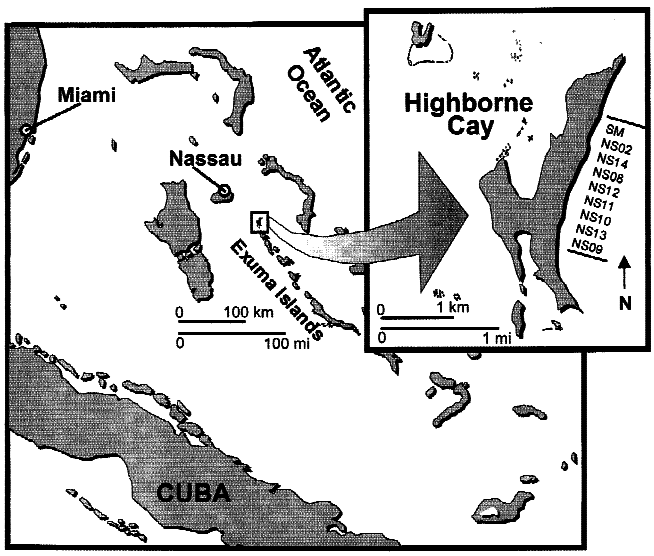

Fig. 1. Map showing location of Highborne Cay, Bahamas. Inset shows enlarged picture of Highborne Cay, the approximate location of the stromatolite field, and the approximate distribution of reference locations.

Highborne Cay stromatolites [42] (Reid et al., in press). As a result, the Highborne Cay stromatolites provide an excellent opportunity to investigate biogeochemical cycling within modern stromatolite mats and the role nutrient cycling plays in stromatolite formation and growth.

Microbial mats, including stromatolites, are often found in environments that are chronically limited by nutrients, e.g., P, N, Fe, and other trace nutrients [24]. The ability to generate, efficiently retain, and recycle nutrients allows mats to grow and proliferate in these environments [2, 5]. Generally, N is the most consistent limiting nutrient in marine microbial mats [17, 29, 30]. Mats have three sources of N they may exploit to meet N demands: (1) mineralized N that is recycled within the system, (2) combined N (e.g., NH_4^+, NO_3^-, organic N) supplied externally (from the water column, atmospheric deposition, runoff), and (3) N supplied through biological N_2 fixation. The latter two sources represent "new" N that is obtained from outside the system. On a daily basis, N_2 fixation and/or N uptake from the water column may provide only 2–20% of the daily N requirements of gross primary production [2]. Thus, remineralized N, recycled and sequestered within the system, represents the greatest N source. However, diffusion, erosion, advection, denitrification, grazing, and/or burial represent N sinks that must be replenished through new N acquisition [17]. Therefore, N_2 fixation represents a biogeochemical process that may be essential for the growth and maintenance of the Highborne Cay stromatolite mats.

N_2 fixation is a complex, energy-demanding process [31]. Many environmental factors within mats may regulate N_2 fixation. These factors include pH, pO_2, NH_4^+, organic carbon availability, H_2S, and water availability [31, 32]. These dynamic controls exhibit steep spatial gradients that fluctuate over a diel cycle within the Highborne Cay mats, as in other mat systems [42]. Therefore, the diazotrophic community within mats must optimize N_2 fixation in the presence of oscillating, often inhibitory, environmental conditions. Accordingly, various strategies have evolved (e.g., microbial consortia, metabolic diversity, temporal partitioning) that allow a diazotrophic community to optimize N_2 fixation [26, 38, 40, 45].

The primary goals of this investigation were to gain insight into how Highborne Cay stromatolite mats optimize their input of new N via N_2 fixation and to better understand the role N_2 fixation plays in modern marine stromatolite primary production and nutrient cycling. The diel patterns of N_2 fixation (acetylene reduction), primary factors controlling N_2 fixation (e.g., photosynthesis, organic and inorganic nutrients), impact of seasonality, and the diazotrophic microbial community composition (*nifH* DNA analysis) were examined.

Methods

Site

The stromatolites examined in the study are located on the east facing beach of Highborne Cay, Bahamas (24° 43′N, 76° 49′ W) (Fig. 1). They occur in a discrete zone in the shallow sub-tidal as variably sized short domes and ridges ranging up to 0.4 m long and 0.2 m high. See Reid et al. [13] for a more detailed site description. The cyanobacterial community is diverse and includes *Schizothrix* sp., *Microcoleus* sp., *Oscillatoria* sp., *Phormidium* sp., *Lyngbya* sp., unicellular *Solentia* sp., and diatoms (L. Prufert-Bebout, personal communication). The stromatolite region was partitioned into different areas to aid location and referencing. The investigation was carried out during four different trips to Highborne Cay: January 1997, June 1997, March 1998, and August 1998. Samples taken for experimentation were identified by their field location (Fig. 1). Samples were collected the previous afternoon or the morning the experiments were conducted. The experiments were performed in plastic swimming pools with running seawater pumped from an intake positioned ~20 m off the Highborne Cay dock, with the exception of January 1997. During this trip the seawater intake connected to the RV *Calanus* was utilized. The RV *Calanus* was moored alongside the Highborne Cay dock. These conditions yielded a close simulation of *in situ* temperature and irradiance conditions.

N_2 Fixation (Acetylene Reduction)

Nitrogen (N*2*) fixation was assayed by nitrogenase activity (NA). Nitrogenase is the enzyme complex responsible for reducing dinitrogen [31]. NA was determined using the acetylene reduction assay [41]. Cores (1.13 $cm^2 \times 0.5$ cm) were placed in 37-ml glass serum vials, 20 ml of seawater was added, and the vials were capped with rubber stoppers. Five ml of acetylene, generated from calcium carbide, was injected into each serum vial. After 4-h incubations the serum vials ($n = 3$) were shaken vigorously for 30 s to stop NA and equilibrate acetylene and ethylene. Three ml of gas in the headspace was transferred to evacuated 3-ml serum vials. Upon return to the Institute of Marine Sciences (IMS), Morehead City, NC, 2 ml from the 3-ml serum vials was transferred to evacuated 2-ml autosampler vials. Ethylene concentrations were quantified using a Shimadzu model GC9A gas chromatograph with flame ionization detector and a 2-m Porapak-T column at 80°C. Negative controls included stromatolite cores incubated without acetylene and water (no cores) injected with acetylene.

Oxygen Production

Stromatolite cores (0.3 $cm^2 \times 0.3$ cm) were collected and placed into drinking straw pieces cut to the core dimension in order to seal the sides of the cores. Modeling clay was used to cover the bottom of the straw core tubes so that only the top of the core was exposed to the water. Cores were then placed in 11-ml glass serum vials. Vials were sealed underwater using rubber stoppers through which a 25 gauge needle was inserted. The needle provided a means of pressure relief and helped eliminate air bubbles. Once the vial was capped, the needle was gently removed. Dissolved O_2 partial pressure (pO_2) was measured before and after incubations using 1 ml of vial water (Cameron Instruments BC202 gas cell using a Clarke style electrode). Vials were gently hand-mixed periodically during and at the end of the incubation. Net primary production was defined as an increase in O_2 concentration per unit time. Incubations lasted 2–4 h ($n = 4$ or 5).

Carbon Fixation

Carbon (CO_2) fixation was assayed by placing cores (1.13 $cm^2 \times 0.5$ cm) in 20-ml glass scintillation vials with 20 ml of seawater and 0.2 ml of $NaH^{14}CO_3$ (3.5 μCi; 58 μCi μmol^{-1}). Incubations lasted 3–4 h ($n = 3$). To correct for any dark CO_2 fixation and/or non-biotic uptake, three vials were wrapped in foil. After incubation, cores were placed on paper and allowed to dry in the dark. Once dry, the cores and 15 ml of concentrated HCl (12N) in a 25 ml beaker were placed in a 2 L Rubbermaid storage container. The container was sealed and the cores exposed to the concentrated HCl fumes for 16 h (overnight) in order to remove excess inorganic carbon. Afterwards, cores were placed in 7-ml scintillation vials and stored in the dark. Upon return to IMS, 5 ml of scintillation cocktail (Cytoscint [ICN Inc.]) was added and each sample was stored in the dark for another 48 h before being counted. Counts (Beckman TD 5000 Liquid Scintillation Counter) were converted to disintegrations per minute using a quench curve based on calibrated [^{14}C] hexadecane (NEN Inc.). The dissolved inorganic carbon content was measured by infrared gas analysis (Licor Model Li6252).

Diel Experiments

To examine the patterns of NA and $H^{14}CO_3^-$ uptake over a 24-h cycle, two experiments were performed in June 1997. NA measurements were made every 4 h for 24–26 h, and $H^{14}CO_3^-$ uptake measurements were made every 4 h from 0600 to 0800 h (2 h for the last time point). For all diel incubations, stromatolite material recently (<16 h) collected from the field site was used. Subsamples were taken just prior to the incubation periods and incubations were carried out in the plastic pools.

Metabolic Inhibitors

The influence of total and oxygenic photosynthesis on day and night NA was investigated using metabolic inhibitors. Oxygenic photosynthesis was inhibited by addition of the photosystem II inhibitor 3-3,4-dichlorophenyl-1,1-dimethylurea (DCMU) (2×10^{-5} M final) [4]. *De novo* protein synthesis was inhibited by addition of chloramphenicol (CAP) (150 μg L^{-1} final) [21]. Metabolic inhibitors were added to serum vials just prior to incubation. When investigating the effect of inhibiting photosynthesis for the entire light cycle on nighttime NA, stromatolite samples were incubated in the presence of DCMU from 0500 to 2000 h.

Nutrient Additions

Nutrient addition bioassays were conducted to examine nutrient limitation of N_2 fixation and primary production as measured by NA and $H^{14}CO_3^-$ uptake. Stromatolites were collected and transported to the pools. Stromatolite pieces were placed in 500-ml plastic containers rinsed with 0.1 N HCl and dH_2O. Each container was filled with 350 ml of seawater and placed on elevated stands in the pools. The stands allowed the tops of the plastic containers to extend above the water line, yet maintained ambient temperature and irradiance conditions. Incubations lasted 7 to 11 days. Each morning the water in the containers was replaced with fresh seawater and nutrients were added. To limit the effects of evaporation, small amounts of seawater from the incubation pools were added to the containers periodically through the day. Nutrient additions (final concentrations) consisted of nitrate (10 μM $NaNO_3$), phosphate (5 μM H_2PO_4), and $FeCl_3$ + sodium EDTA (200 nM each).

DNA Extraction, PCR, Sequencing, and Phylogenetic Analysis

Stromatolite cores (1.6 $cm^2 \times 0.5$ cm) for DNA extraction were placed in 15-ml culture tubes with 5 ml "high" TE buffer [100 mM Tris-Cl, 100 mM EDTA (pH 8.0)] and stored at −10°C until transport. During transport to IMS (~10 h), the cores were placed in coolers with ice packs and then stored at −80°C until extraction. Cores were thawed in a 30°C water bath. The cores were washed

twice with 10 ml "high" TE buffer for 15 min at 24°C. The "high" TE washes were performed in an effort to remove some of the exopolymeric material commonly associated with microbial mats that can inhibit enzymatic reactions. Cores were extracted using the Qiagen (Chatsworth, CA) DNeasy Plant Kit. Using 400 µl of the kit lysis buffer per 1.0 g wet sediment weight, cores were manually ground with sterile disposable plastic grinders (one grinder per tube) and incubated at 75°C for 3 h. Cores were ground and then vortexed several times during the 75°C incubation. Periodic microscopic examinations revealed this to be an effective lysis method. After lysis, tubes were centrifuged at 3000 *g* for 5 min and the supernatant removed to a 2.0 ml screw cap tube. The manufacturer's instructions were followed for the remainder of the extraction. After the final elution step, the DNA suspension was concentrated using NaCl and isopropanol and resuspended in a 100 µl final volume of "low" TE buffer [10 mM Tris-Cl, 1 mM EDTA (pH 8.0)]. Amplification of dinitrogenase reductase (*nifH*) partial sequences was done using oligonucleotide primer sets "Z" [44], "Ar" [20], or "Cy" [20]. Cloning, sequencing, and phylogenetic analysis were conducted according to Olson et al. [20].

Non-axenic, cyanobacterial cultures initially derived from Highborne Cay stromatolites were kindly provided by L. Prufert-Bebout. All cultures except "Bahamian Heterocystous Cyano" were initially inoculated using Highborne Cay stromatolite material. The "Bahamian Heterocystous Cyano" culture was inoculated using a non-lithified mat from Shroud Cay, which is also part of the Exuma Cays. *D. Salexigens* and *D. Orientis* were kindly provided by R. Deveraux.

Statistical Analysis

Statistical analyses consisted of a one- or two-way analysis of variance using SPSS software. A posteriori multiple comparisons of means was achieved using the Bonferonni procedure with $\alpha = 0.05$.

Results

In January 1997, nitrogenase activity (NA) during daylight (not shown) was negligible. In June 1997, NA and photosynthetic CO_2 incorporation measured over a diel cycle proved highly variable, but exhibited a distinct diel NA variation with the highest NA occurring at night during the 2200 to 0200 h period (Fig. 2). During these experiments, substantial NA occurred during the early morning, rapidly declined to low or undetectable levels after 1000 h, and then increased at night. Addition of DCMU did not stimulate or inhibit NA by a statistically significant margin. In March and August 1998, NA was measured during two periods (1000 to 1400 h and 2200 to 0200 h) for stromatolite samples collected from several different sites. Figure 3A shows the combined results of all the locations assayed ($n = 6$ in March and $n = 9$ in August). The combined results show that nighttime

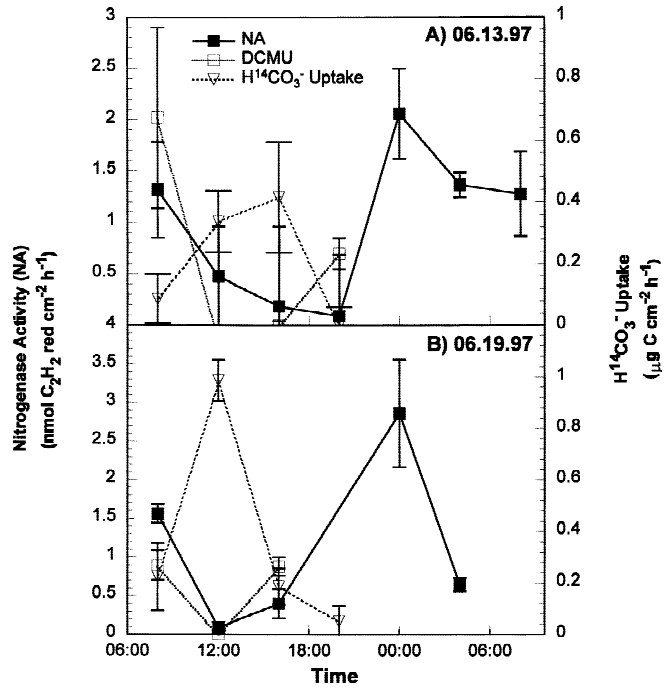

Fig. 2. Diel measurements of nitrogenase activity (acetylene reduction) and $H^{14}CO_3^-$ uptake for 06/13/97 (A) and 06/19/97 (B). --(■)--, NA; ··(□)·· DCMU NA; --(▽)-- $H^{14}CO_3^-$ uptake. Samples collected from the NS-8 region were used for each diel experiment. Error bars = 1 ± standard error (s.e.).

NA was significantly greater than daytime NA for both March and August. However, there was a great deal of variability among individual stromatolite locations, and three of the six locations sampled in March and one of the nine locations sampled in August did not exhibit significantly greater nighttime NA ($\alpha < 0.05$). Net O_2 production was also higher in August (Fig. 3A). In March, CAP additions did not inhibit nighttime NA, but in August CAP additions almost entirely inhibited nighttime NA (Fig. 3B). These results indicate that NA in August was dependent upon protein synthesis during the incubation period. In contrast, the lack of CAP inhibition in March indicates that *de novo* protein synthesis was not required for nighttime NA.

The relationship between photosynthesis and NA was further investigated (Fig. 4). When stromatolite mat photosynthesis was inhibited for an entire day, either through incubation with DCMU or by sand burial (a commonly observed occurrence *in situ*), the nighttime NA was substantially reduced as compared to untreated controls (Fig. 4A). Plotting nighttime NA and net O_2 production for those samples that were assayed for both on the same day further indicates the dependence of nighttime NA upon reduced

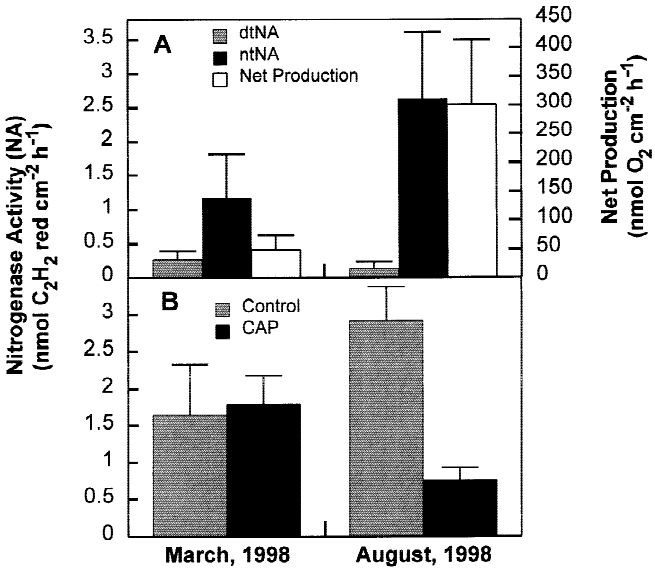

Fig. 3. Seasonal differences in nitrogenase activity and net O_2 production. (A) Average daytime (dt) and nighttime (nt) NA and net O_2 production for March and August 1998. The average NA and net primary production measurements for stromatolite mats from different locations were pooled and averaged for six experiments in March and nine experiments in August. (B) Nighttime NA response to chloramphenicol (CAP) additions for March and August. Results shown are for experiments done with samples from the NS-10 region. Error bars = 1 ± s.e.

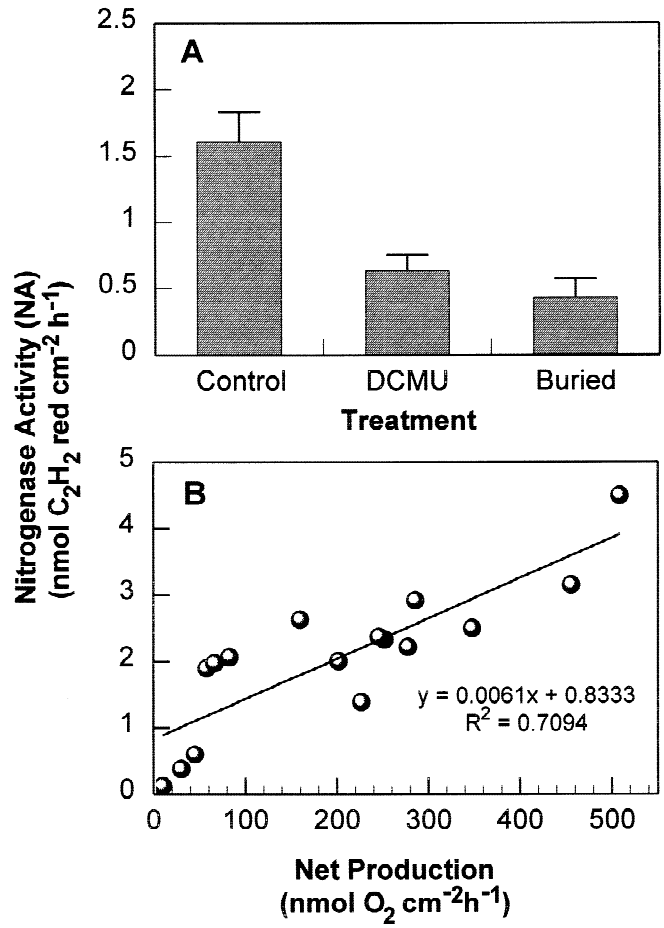

Fig. 4. Dependence of nitrogenase activity on primary production. (A) Effect of inhibiting photosynthesis by DCMU additions or burial under sand on nighttime NA. Results shown are representative of experiments conducted in March 1998, using samples from the NS-2 region. (B) Linear regression of NA plotted versus net photosynthesis (R^2 = 0.71, P < .01). Each point is the averaged result of n = 3 for NA and n = 5 for net primary production. Error bars = 1 ± s.e.

organic carbon and/or reductant generated by photosynthesis earlier in the day (R^2 = 0.71) (Fig. 4B). This supports the earlier findings of Bebout et al. [4] for non-lithified mats.

In the January and June 1997 nutrient addition bioassays (phosphate, nitrate, and Fe-EDTA additions), only the combined addition of Fe-EDTA and nitrate significantly stimulated photosynthetic $H^{14}CO_3^-$ incorporation above the untreated controls (not shown). At no time did an individual nutrient addition increase photosynthetic $H^{14}CO_3^-$ incorporation. This suggests that, at times, photosynthetic production may be limited by combined, as opposed to single, nutrients. The only significant increase in NA resulted from the addition of glucose. However, NA was only measured during the day for these experiments. A similar experiment in March 1998 in which stromatolite samples were incubated with an organic carbon mix (final concentrations: 20 mM glucose, 20 mM lactate, and 20 mM acetate) for 11 days resulted in a greater than twofold increase in nighttime NA compared to the untreated control (Fig. 5). These results provide further evidence for the importance of reduced organic carbon availability in supporting nighttime NA.

Partial dinitrogenase reductase (*nifH*) sequences were obtained from genomic DNA extracted from stromatolite cores taken in June 1997 and August 1998. *NifH* sequences were also obtained from an enrichment culture selective for sulfate-reducing bacteria (SRB) and several non-axenic cyanobacterial cultures that were originally inoculated with Highborne Cay stromatolite mat material. The diazotrophic community contains a diverse assemblage (Fig. 6). The *nifH* sequences obtained from the stromatolite cores clustered into one of three groups: cyanobacterial, alpha proteobacterial, and obligately anaerobic bacterial (e.g., SRB) sequences [45]. Several sequences derived from both the natural samples and cultures exhibited a high degree of similarity (>85% at the amino acid level) to unicellular cyanobacteria

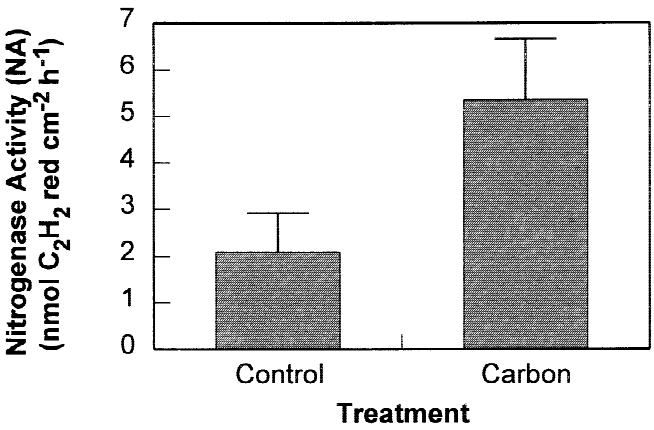

Fig. 5. Effect of organic carbon additions on NA in March 1998. Final concentrations: glucose, 20 mM; lactate, 20 mM; and acetate, 20 mM. Results shown are from experiments conducted in March 1998 with samples collected from the NS-2 region. Error bars = 1 ± s.e.

such as *Xenococcus* sp. and *Synechococcus* sp. Two sequences, one derived from a stromatolite core and the other from a cyanobacterial culture, showed a great deal of similarity (>92% at the amino acid level) to the filamentous, non-heterocystous mat cyanobacteria *Lyngbya lagerhemmii* and a North Carolina intertidal microbial mat-derived *nifH* sequence, BSCyano (Fig. 6). The cyanobacterial-like *nifH* sequences obtained from the non-axenic cultures did not match any derived directly from the cores, but served to further demonstrate the relatively high diversity of diazotrophs within the community.

Discussion

The Highborne Cay stromatolite mats showed temporal separation of N_2 fixation (as measured by NA) and oxygenic photosynthesis (Fig. 2). This phenomenon has commonly been observed in mats dominated by filamentous, non-heterocystous cyanobacteria [1, 4, 39], but there are exceptions [3, 16]. N_2 fixation is O_2 sensitive and hence requires O_2-free conditions. Confining N_2 fixation to periods of low or no photosynthesis allows the diazotrophic community to circumvent O_2 inhibition. Addition of the photosystem II (PSII) inhibitor DCMU to mats frequently leads to increased

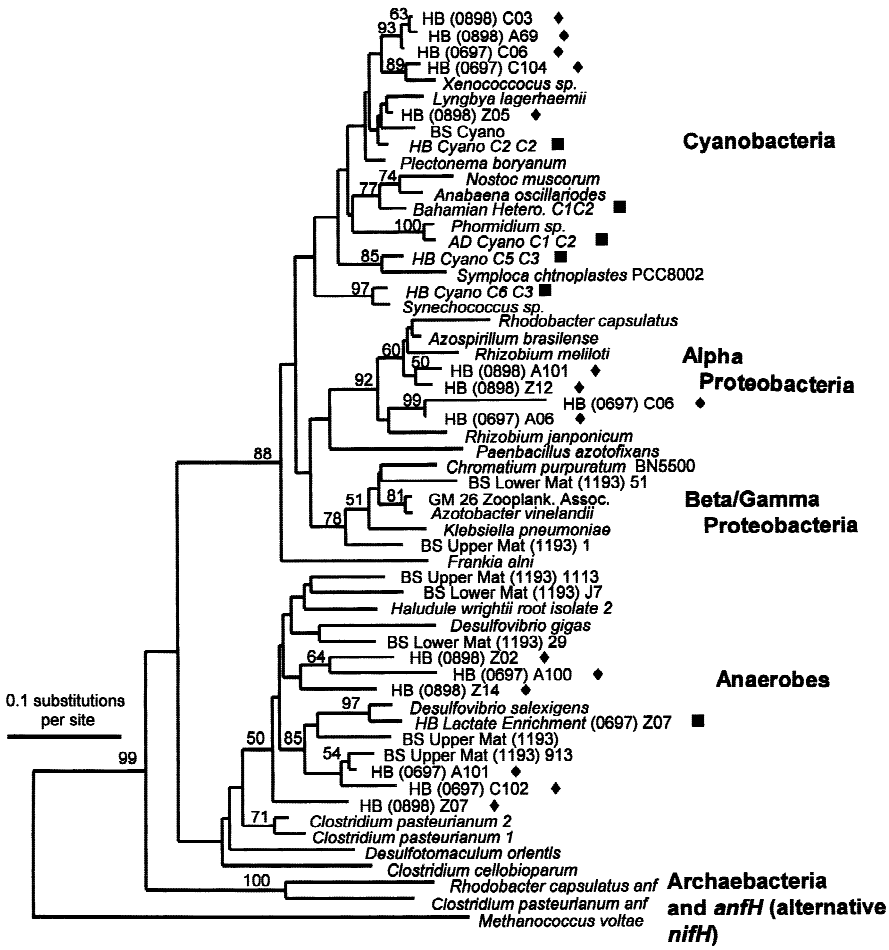

Fig. 6. *NifH* phylogenetic tree. Tree topology was obtained using Dayhoff PAM matrix and Neighbor Joining in Phylip utilizing translations of an ~327 bp section of the dinitrogenase reductase gene (*nifH*). The "◆" and the "□" symbols denote sequences derived from Highborne Cay stromatolite cores and Highborne Cay stromatolite cultures during this study, respectively. The numbers in the parantheses indicate the month and year the stromatolite core was taken. A, C, or Z denote the primer set that produced the amplification product. Bootstrap values >50 that support a particular branching are listed above the branches. Accession numbers: AF227925–AF227947.

NA during the day [4, 39]. However, DCMU additions did not stimulate NA in the Highborne Cay stromatolite mats. In addition to anoxic conditions, N_2 fixation requires ample ATP and reductant. Sixteen ATP and eight electrons are required to reduce one N_2 molecule [31]. Therefore, failure of DCMU to stimulate NA may have indicated that the diazotrophic community simply did not possess sufficient stores of reduced energy equivalents [4, 33].

On an areal basis, rates of NA were relatively low when compared to other non-lithified mat and stromatolitic mat systems [4, 26, 28, 30]. Since photosynthesis [4, 6, 23], DOC additions [3], P [29], temperature [15], pore water NH_4^+ [8], and trace metals (Fe and Mo) [14] have all been proposed and/or found to regulate N_2 fixation in marine systems, several of these factors were tested to determine which most influenced NA. Of the variables tested, only photosynthetic activity and DOC additions had a significant effect upon NA. This indicates that, similar to other mat systems [4, 6, 23], NA in the Highborne Cay stromatolites is most influenced by reduced organic carbon provided by photosynthesis. However, failure to stimulate NA by the addition of potentially limiting inorganic nutrients does not necessarily exclude the possibility that the availability of inorganic nutrients such as P or Fe may have limited NA in some fashion. The inorganic nutrient (P and Fe) concentrations that were added in our experiments might have been insufficiently low, other essential nutrients may not have been tested, and/or the length of the incubations in the presence of the nutrients may have been too short to yield a detectable response.

Photosynthesis, as measured by net O_2 production and $H^{14}CO_3^-$ fixation, and biomass, as measured by chlorophyll *a* concentrations, were also relatively low when compared to other mat systems [4] (Pinckney et al., in preparation). Lower production rates, on an areal basis, may be attributed to relatively low biomass per unit area [39]. One factor that may contribute to the relatively lower biomass is the physical hydrologic conditions under which the Highborne Cay stromatolites develop. The influence that hydrodynamic factors, such as wave action, have on microbial mat establishment and proliferation is difficult to quantify [11]. Microbial mats generally develop in lagoons, ponds, or tidal flats that are sheltered from high tidal flows or wave energy and are rarely found on beaches that experience substantial wave action [7, 19]. Even though the formation of lithified laminae may provide structural protection to the Highborne Cay stromatolites against wave action [11], wave action may still be a prohibitive factor for the establishment and growth of the cyanobacteria. Another factor that may limit production and biomass development in microbial mats is inorganic nutrient availability [35]. Although dissolved inorganic nitrogen (DIN) and phosphate concentrations in the waters around Highborne Cay were below 2 μM and 1 μM, respectively, addition of these and other nutrients failed to stimulate primary production. This may be due to the same causes as mentioned above for NA. An additional explanation as to why nutrient additions failed to stimulate production may be that the community was incapable of assimilating additional nutrients more rapidly because it has adapted to the nutrient-depleted conditions through slow but efficient growth.

As indicated by the *nifH* sequence data, the Highborne Cay stromatolite mats contain a diazotrophic assemblage possessing diverse metabolic capabilities including non-heterocystous filamentous and unicellular cyanobacteria, alpha-proteobacteria, and obligately anaerobic proteobacteria such as SRB. The Highborne Cay stromatolite mats exhibited several interesting features that may be linked to their diazotrophic community composition. Firstly, although NA rates appear to be linked to seasonal changes, maximal NA is still maintained at night. This differs somewhat from previous findings in a temperate intertidal mat in North Carolina that also showed lower rates during the winter, but exhibited maximal NA during the day [23]. Secondly, the effect of CAP appeared to vary between seasons. This indicates that different mechanisms of protein expression within the diazotrophic community may yield the same diel NA patterns. The nitrogenase system is highly conserved among prokaryotes [31], yet diazotrophs have evolved varying physiological strategies that allow them to optimize the process [32]. As a consequence, different patterns and responses of NA to *in situ* experimental manipulations may be linked to differences in community composition [16, 22, 32]. The PCR methodology we used in this study was not quantitative. In future work, quantitative application of methods such as denaturing gradient gel electrophoresis (DGGE) [27] and reverse transcriptase PCR (RT-PCR) [18] will allow a more accurate determination of community abundances and elucidation of those organisms that are actively expressing the nitrogenase genes while concurrently assessing potential long- and short-term changes.

To what extent modern stromatolites may be used to interpret how Precambrian and Phanerozoic stromatolites developed and grew is still unresolved [12]. The confirmation that N_2 fixation occurs in modern marine stromatolites, therefore, is not necessarily conclusive evidence that diaz-

otrophs were essential microbial components of ancient stromatolites. There are separate and distinct lines of evidence, however, suggesting that diazotrophs might have been a key community within Precambrian stromatolites. N_2 fixation is believed to be an ancient process that arose during the Archaen before an oxic atmosphere developed [10]. The sensitivity of N_2 fixation to O_2 [31], the high degree of structural and functional conservation within the nitrogenase system [45], and the broad distribution through the bacterial and archaeal kingdoms [43] all support the idea of its antiquity. Diazotrophs appear to be ubiquitous members in modern-day microbial mat communities inhabiting disparate extreme environments that are potentially representative of Precambrian environments such as hypersaline lagoons and Antarctic lakes [25]. Additionally, microfossils resembling present-day filamentous microorganisms that have the ability to fix N_2 have been found in stromatolites dating to the early Archaen, >2.5 BYA [36]. This discovery further suggests the possibility that diazotrophs and ancient stromatolites may have coincided in time and space. Given these observations and the possibility that ancient stromatolites, like their modern counterparts, developed in N-depleted waters [10], we suggest that diazotrophs composed a key functional community within these earliest ecosystems.

In summary, these results suggest that possessing a diazotrophic assemblage with a high degree of metabolic diversity helps ensure the input of new N through N_2 fixation for the Highborne Cay stromatolites. This diversity allows N_2 fixation to be temporally separated from potentially inhibitory O_2 conditions created by photosynthesis. Because N_2 fixation within the stromatolites occurs predominantly at night, it is likely driven by consumption of organic carbon that is largely supplied by photosynthesis. In this regard, whether the NA is attributable to the primary source of the organic carbon, the cyanobacteria, or heterotrophic bacteria associated with the cyanobacteria cannot be definitively concluded.

Acknowledgments

We would like to thank G.G. Steppe and M. Go for technical assistance and the RIBS group for helpful discussions. We also thank the crew of the RV *Calanus* for their superb efforts and logistical support. The manuscript has greatly benefited from the comments of two anonymous reviewers. This work was supported by NSF LEXEN 9808959 and NSF OCE 9530215 (Research Initiative of Bahamian Stromatolites). JD was supported by an NSF Graduate Research Fellowship. This is RIBS contribution #11.

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