Microbial Ecology

Nitrogen Fixation in Microbial Mat and Stromatolite Communities from Cuatro Cienegas, Mexico

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Abstract

Nitrogen fixation (nitrogenase activity, NA) of a microbial mat and a living stromatolite from Cuatro Cienegas, Mexico, was examined over spring, summer, and winter of 2004. The goal of the study was to characterize the diazotrophic community through molecular analysis of the *nifH* gene and using inhibitors of sulfate reduction and oxygenic and anoxygenic photosynthesis. We also evaluated the role of ultraviolet radiation on the diazotrophic activity of the microbial communities. Both microbial communities showed patterns of NA with maximum rates during the day that decreased significantly with 3-3,4-dichlorophenyl-1′,1′-dimethylurea, suggesting the potential importance of heterocystous cyanobacteria. There is also evidence of NA by sulfur-reducing bacteria in both microbial communities suggested by the negative effect exerted by the addition of sodium molybdate. Elimination of infrared and ultraviolet radiation had no effect on NA. Both microbial communities had *nifH* sequences that related to group I, including cyanobacteria and purple sulfur and nonsulfur bacteria, as well as group II nitrogenases, including sulfur reducing and green sulfur bacteria.

Introduction

Microorganisms are commonly found forming consortia [33] in which different metabolic groups act together to function as a community. Microbial mats and stromatolites are examples of microbial communities that have a great taxonomic and metabolic dynamism [13, 40, 41, 48]. Schopf [38, 50] reported the presence of diverse bacterial flora fossilized in the 3,465-million-year-old Apex Cherts of Western Australia providing the oldest morphological evidence for life on Earth. There is ongoing debate regarding the biogenicity of such ancient fossils [8, 9]. Brassier *et al.* [8, 9] have attributed these structures to amorphous carbon formed during hydrothermal dyke vein quartz. However, recent findings by Allwood *et al.* [1] propose that there is evidence of a probable biological origin to the laminated structures (stromatolites) found in the 3,430-million-year-old Strelley Pool Chert (Australia) sediment formation in which they identify seven stromatolite morphologies that cannot be explained by abiogenic hypothesis. To date, there are examples of extant stromatolites and microbial mats that usually occur in extreme environments characterized either by their high salinity, temperature, or nutrient limitation [2, 10, 14, 19, 30, 31, 35, 34, 42]. In microbial mat and living stromatolite communities, cyanobacteria will typically be involved in organic matter production and oxygen evolution; chemotrophic and phototrophic sulfur-oxidating bacteria will form sulfur and sulfate that will be used by sulfur and sulfate-reducing bacteria (SRB). Methanogenic and methanotrophic bacteria will also be involved in the transformations of organic matter [13].

Nitrogen is usually the most limiting element in marine environments. "New" N can be obtained through N_2 fixation [15], the reaction that reduces dinitrogen molecules, which comprise 78% of the atmosphere, to ammonia [45]. It is catalyzed by the enzyme nitrogenase, which is formed out of two enzymatic subunits: dinitrogenase reductase (iron protein) and dinitrogenase (molybdenum–iron protein) [25]. Some bacteria have dinitrogenases in which molybdenum can be substituted by vanadium or iron [25]. During the electron transfer process that constitutes N_2 fixation, two ATP molecules are needed for each electron transferred [46]. Nitrogenase is an ancient and conserved enzyme that has essentially remained conserved throughout the transition from an anoxic to oxic biosphere, suggesting that it evolved in

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early Earth. It has been hypothesized that either it is a loci extremely prone to horizontal gene transfer or that it was part of the cenancestor metabolism and has been lost in most lineages [36].

It is generally assumed that cyanobacteria are the most important N_2-fixing microorganisms in most aquatic environments, although there is ample evidence suggesting that there are many other prokaryotic diazotrophs in both the bacterial and archaeal domains [45]. A recent study in microbial mats of Guerrero Negro suggests that bacteria of the phylum *Chloroflexi* formed the major percentage of the biomass throughout the mat, although cyanobacteria were the most important component in the upper millimeters [26]. Cyanobacteria have solved the conundrum between oxygen-evolving, ATP-generating metabolism and N_2 fixation in a variety of ways: (1) separating temporally both metabolisms (daytime C fixation and nighttime N_2 fixation), e.g., unicellular cyanobacteria such as *Cyanothece*, *Gloeothece*, and *Synechococcus*, as well as filamentous cyanobacteria such as *Lyngbya* [4, 43]; (2) separating spatially N_2 fixation in specialized nonoxygenic cells called heterocysts, e.g., filamentous cyanobacteria *Calothrix* and *Nostoc* that impede entry of O_2 formed in adjacent, photosynthetic cells (vegetative) [37]; (3) and by switching to diazotrophy primarily during low photosynthetic activity (e.g., filamentous cyanobacteria, *Trichodesmium*) [5–7]. Other bacteria will fix N_2 in anoxic environments that occur during organic matter decomposition, usually during the night, when O_2 evolution through photosynthesis has stopped [40].

Cuatro Cienegas Coahuila (CCC) is a valley in central Mexico located in the middle of the Chihuahuan Desert that constitutes an oasis because it contains a series of natural wells and riverine systems [28]. A striking characteristic of this environment is the presence of different microbial communities, including microbial mats and living stromatolites that are part of the trophic web sustaining a great diversity of invertebrates [21, 44]. The focus of this study was to evaluate the potential for N_2 fixation by different metabolic components of the prokaryotic community in a microbial mat and a living stromatolite from CCC. We also characterized the potential diazotrophic community through construction of DNA clone libraries for the *nifH* gene (dinitrogenase reductase).

Methods

Site. Cuatro Cienegas is a valley of approximately 30×40 km located at ~740 m above sea level and surrounded by high mountains (>3,000 m) (Fig. 1). This enclosed evaporitic basin receives ~150 mm of annual

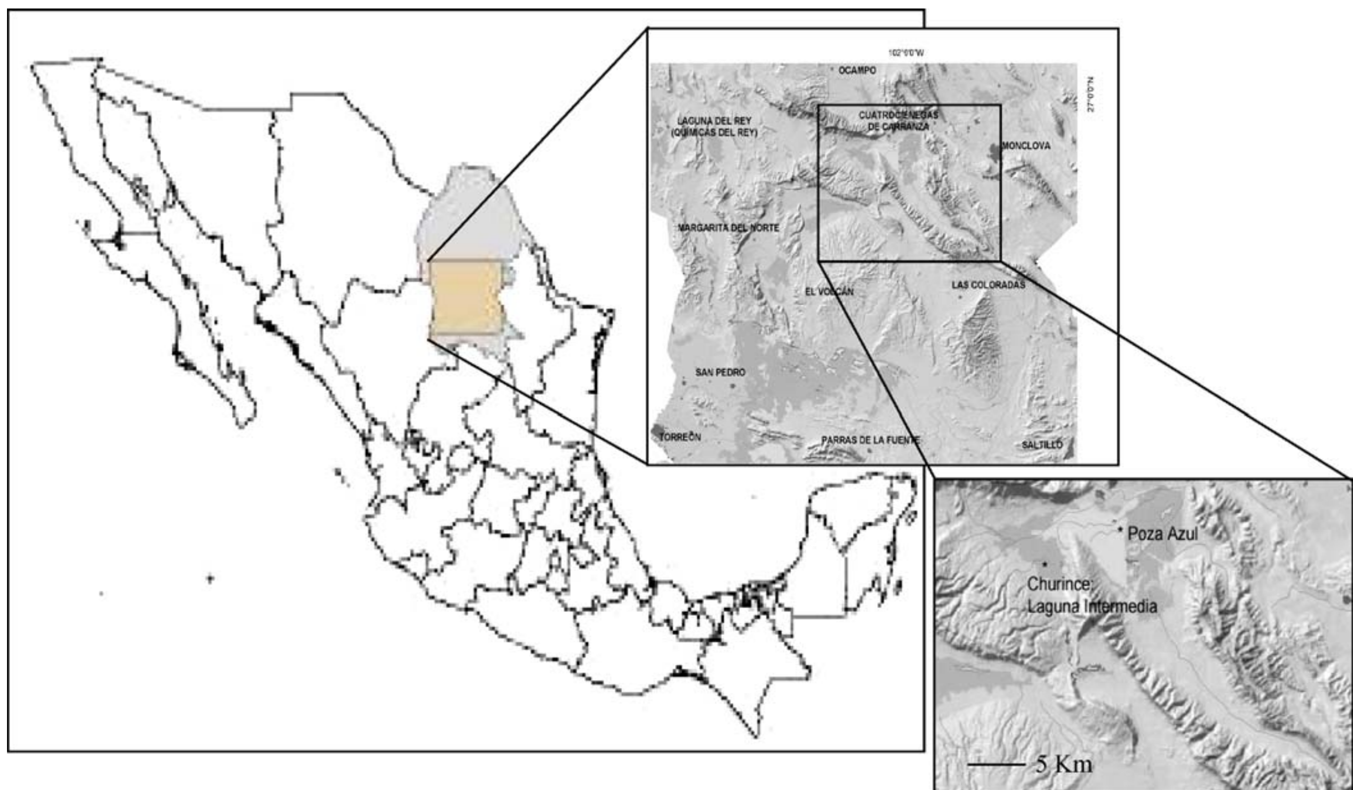

Figure 1. Map of Cuatro Cienegas, Coahuila, showing location of our study sites: Churince and Poza Azul.

precipitation. Despite the arid climate, CCC harbors an extensive system of natural springs, streams, and pools [44]. This study focused on two contrasting systems: Poza Azul and Churince. Poza Azul is a pool formed by a spring that nurtures the larger river "Rio Mesquites." The cretaceous karst landscape that surrounds this pool is rich in carbonates, making possible the formation of massive living stromatolites (Fig. 2A, B). Stromatolites are microbial mats that also have layers of precipitated minerals. This pool remains at a constant temperature (30–35°C) throughout the year. Meanwhile, the western part of the valley is dominated by Jurassic gypsum soil with an hydrological system called Churince that starts at a spring that forms a small river that flows toward a shallow (20–60 cm) lake (Laguna Intermedia) and continues to a desiccation pond (Laguna Grande).

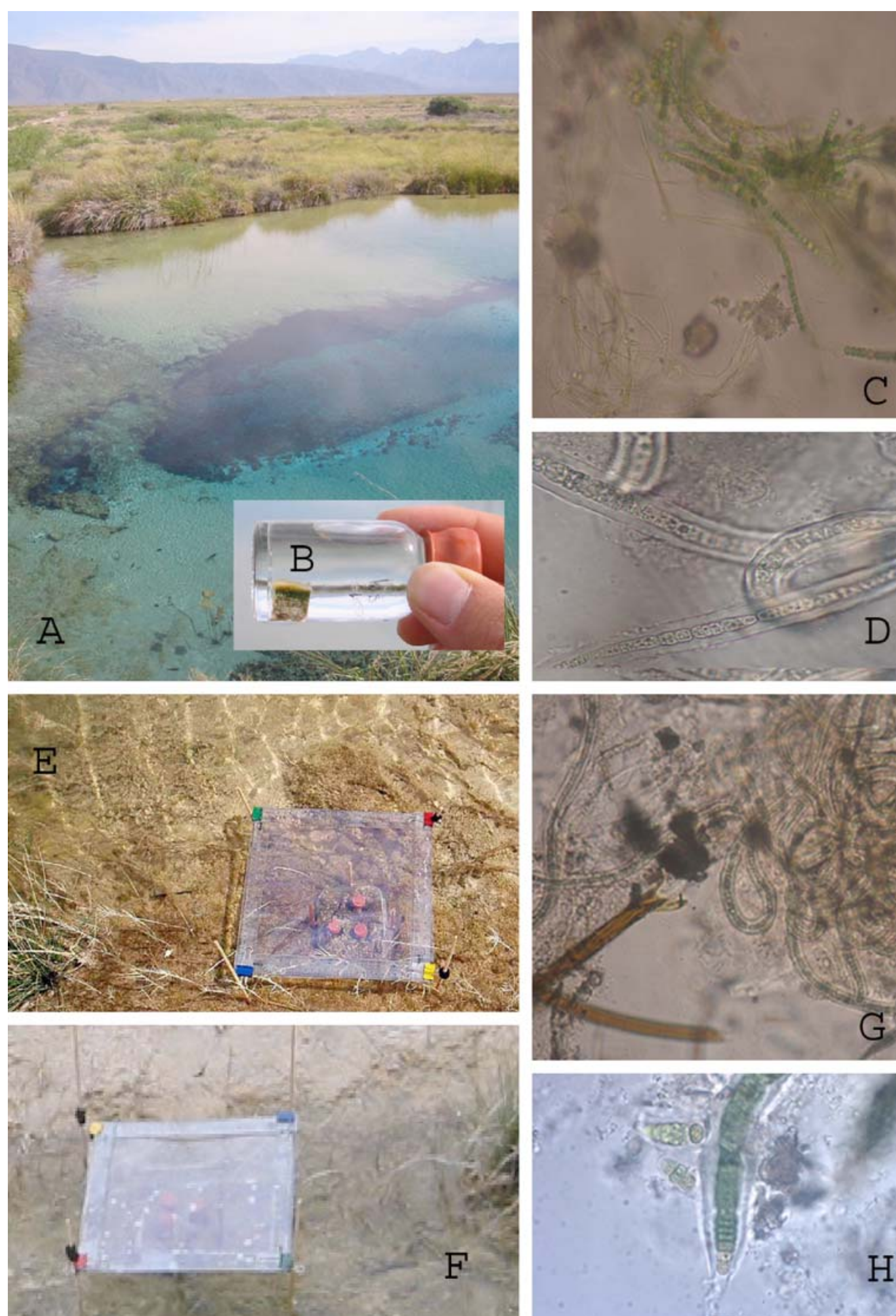

Figure 2. A Overview of Poza Azul living stromatolite. B Stromatolite section of ~1 cm^3 used in ARA. C, D Microphotographs showing different filamentous cyanobacteria from the Poza Azul living stromatolite (×400). E, F Overview of ARA set up in Churince microbial mat. G, H Microphotographs showing different cyanobacteria from the Churince microbial mat (×400).

Microbial mats that do not form liteous stroma (Fig. 2E, F) are present in this system. Both microbial communities studied have a strong component of cyanobacteria, filamentous (with and without heterocysts) and unicellular as evidenced by microscopic observations (Fig. 2C, D, G, H). The major solutes that have been reported for CCC aquatic systems are SO_4^{2-}, Mg^+, Cl^-, Na^+, Ca^{++}, and K^+ in order of importance [23].

N_2 Fixation (Acetylene Reduction Assay). Nitrogenase activity (NA) was measured with the acetylene reduction assay (ARA) [11] *in situ* in a microbial mat from the Churince system and a living stromatolite from Poza Azul, both in CCC. Serum vials (14 mL) with the bottom cut off were gently inserted in the microbial mat (Fig. 2E, F). Samples of the living stromatolite ($n=3$, treatment) were collected and cut to 1 cm^3 that were placed inside whole serum vials (Fig. 2B). Vials were closed with rubber stoppers and samples were covered with 0.2 μm of filtered water (4 mL) collected from the same sites as the mat and stromatolite, leaving a gas phase (3 mL) in all treatments [14, 17]. Acetylene reduction assay started with saturation of the atmosphere to 20% by replacing the gas phase with acetylene that was formed from the reaction of calcium carbide and water. NA was followed during 24-h cycles in 6-h intervals when gas samples (1 mL) were taken from the gas phase of the serum vials and collected in vacuum vials ($n=3$, serum vial; $n=9$, treatment/time point) (Vacutainer® 3-mL red cap, with no additives, Becton, Dickinson, Franklin Lakes, NJ, USA) for the latter analysis by gas chromatography. To maintain gas volume after sampling, we replaced the volume extracted at each time point by injecting acetylene. Time zero samples were taken immediately after saturation of the atmosphere with acetylene to verify base levels of ethylene. Experiments were ended after 24 h when incubated samples were stored in liquid N_2 for further genetic characterization. Acetylene reduction assays were designed to evaluate the potential contribution to N_2 fixation patterns of different bacterial metabolic groups of the microbial communities. We carried out these experiments during spring, summer, and winter of 2004 using four different metabolic treatments and filtered water as control.

Chlorophyll *a* (Chl *a*) concentration was obtained for all samples assayed for acetylene reduction after overnight extraction of ~1 g of each microbial community using 90% aqueous methanol. The extract was filtered through a GF Whatman filter and absorbance of extract at 665 nm was read in a spectrophotometer. The procedure was repeated approximately three times for each sample until no more pigment was extracted. Temperature was measured in the overlying water of both microbial communities around noon for all seasons. Microscopic observations of bacterial communities from both the microbial mat and stromatolite was done back in the laboratory with a Zeiss Axioskop 40 microscope.

Metabolic Inhibitors. 3-3,4-Dichlorophenyl-1′,1′-dimethylurea (DCMU) was used at 2×10^{-5} M [2, 3] because it inhibits linear electron transport flow on the acceptor side of photosystem II (PSII) [6]. Dichlorophenyl-1′,1′-dimethylurea will block quinone A (Q_A) oxidation [6] and thus will affect oxygenic phototrophs that have PSII (e.g., cyanobacteria). Heterocystous cells lack PSII and most ATP used in N_2 fixation comes from PSI and from vegetative cells. However, nonheterocystous cyanobacteria are affected by DCMU addition. Because the O_2-forming PSII is inhibited in the presence of DCMU, microbial communities submitted to this treatment will show a decrease in O_2 levels. Nitrogenase is irreversibly inhibited by oxygen [5–7], thus, DCMU blockage can facilitate N_2 fixation by nonheterocystous cyanobacteria and anoxygenic phototrophs or potentially inhibit NA by heterocystous cyanobacteria during the day, depending on the carbohydrate storage present in vegetative cells and the length of exposure to this inhibitor [2, 3, 5–7]. Sodium molybdate (Na_2MoO_4) at 20 mM, a structural analogue to sulfate, will inhibit sulfate reduction by SRB, which occurs during anoxic conditions [14, 42]. We also submitted the samples to two light treatments: absence of IR or UV wavelengths [14] using Rosco filters (Rosco Laboratories, Stamford, CT, USA) (IR thermashield filter transmits 80% of visible light and reflects infrared wavelengths, UV filter allows less than 10% of transmission below 390 nm). Anoxygenic photoautotrophs have bacteriochlorophylls that have absorption spectra in the infrared and are visible, especially bacteriochlorophylls c, d, and e, as well as light-harvesting carotenoids in the blue to green region, which are employed for photosynthesis [27, 32]. The deleterious effect of ultraviolet radiation on microorganisms has been well documented, including damages to DNA, protein, and cellular structures [12].

DNA Extraction, nifH Amplification, Sequencing, and Phylogenic Analysis. Total environmental DNA was extracted from mat and living stromatolite samples used for ARA following the protocol of Zhou *et al.* [53] with modifications. Microbial communities were pulverized on ceramic mortars with liquid nitrogen and ~3 g of the microbial mat and ~2 g of the stromatolite were placed in 9 mL of extraction buffer (100 mM Tris–HCl, 1.5 M NaCl, 100 mM EDTA, 100 mM Na_3PO_4, pH 8) with 1% cetyl trimethylammonium bromide. Samples were frozen at −20°C and left to thaw at 65°C three times, incubated with lysozyme (1 mg/mL, Invitrogen, Carlsbad, CA, USA) for 30 min at 37°C, and left overnight with proteinase K (0.1 mg/mL, Invitrogen) and sodium

dodecyl sulfate (3%) at 60°C. Supernatant was collected with centrifugation (5000×*g*, 10 min) and cleaned twice with phenol/chloroform/isoamyl alcohol (25:24:1). DNA was precipitated with isopropanol and sodium acetate (3 M) at room temperature. A 359-basepair region of the *nifH* gene was amplified with nested polymerase chain reaction (PCR) following the protocol of Zani *et al.* [51] with addition of 5% bovine serum albumin (Invitrogen) of the total PCR reaction volume (50 μl). The thermal cycle for *nifH* amplification was as follows: 93°C, 1 min 20 s; 50°C, 1 min; and 70°C, 1 min 30 s for 30 cycles. Thermal cycles had an initial denaturation step of 94°C for 10 min and a final extension of 72°C for 30 min. Negative controls using PCR water were run with nested *nifH* PCR. Amplified products were separated by cloning using *Escherichia coli*-competent cells and pCR®2.1 TOPO® plasmid (Invitrogen). Positive clones were sequenced in both directions in an automated capillary sequencer (ABI PRISM® 3100-*Avant* Genetic Analyzer, Applied Biosystems, Foster City, CA, USA) using T7 and Sp6 primers [18]. Sequences were aligned and edited with Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA). Each sequence was translated to amino acid and screened in all six reading frames for stop codons. Sequences with stop codons were considered nonfunctional pseudogenes and were left out of the phylogenetic analysis. Potentially functional sequences were searched against the NCBI dataset using BLASTx, and best-hit sequences were downloaded and included in the phylogenetic analysis. Phylogenetic inferences (parsimony) were done with PAUP*4.0b8 (Sinauer Associates, Inc., Sunderland, MA, USA). We performed 500 bootstrap replicates for a dataset consisting of 368 characters, of which 235 were parsimoniously informative. The branch-swapping algorithm was tree bisection reconnection. The data matrix had 73 taxa.

Phylotype Richness Estimation. To assess if libraries were large enough to represent the diversity present in the studied sites, we analyzed our data with the software tool provided by Kemp and Aller [24] that uses the S_{Chao1} and S_{ACE} estimators.

Statistical Analysis. Statistical analyses were done to test for differences between peaks of nitrogen fixation using Statistica-advanced software. We ran *t* tests for independent samples after evaluating that the distribution of the variables was normal and verifying the equality of variances with the Levene test.

Nucleotide Sequence Accession Numbers. The sequences generated in this study have been deposited in the GenBank database under the following accession numbers: DQ485349–DQ485403.

Results

N_2 Fixation

Churince Microbial Mat. All treatments, except when DCMU was added, showed the same trend of acetylene reduction activity with ethylene production peaks during both daytime measurements (sunrise–noon and noon–sunset) during all seasons sampled (Fig. 3A–C). The control treatment had maximum NA from noon (12 h) to sunset (18 h) and sunrise (6 h) to noon with acetylene reduction rates of 571 ± 101 and 811 ± 84 nmol C_2H_4 m^{-2} h^{-1} in the spring and 569 ± 11 and 904 ± 95 nmol C_2H_4 m^{-2} h^{-1} in the summer, respectively. Winter samples were significantly lower ($p < 0.0140$) with maximum acetylene reduction rates in control samples of 169 ± 11 nmol C_2H_4 m^{-2} h^{-1}. Samples exposed to DCMU had a significant ($p < 0.0237$) drop in ethylene production during the daytime (06:12 and 12:18) of 90% in average for spring and summer, and of only 4% in the winter. Sodium-molybdate-exposed samples showed an average decrease of 57% in spring, 67% in summer, and 94% in the winter, during dusk or night (18:24 and 24:06); daytime samples showed no effect in NA for this treatment. Samples treated for absence of infrared radiation showed no significant difference in relation to the control. Although we observed higher rates of NA when UV radiation was eliminated, these were not significantly different from the control. There was no significant difference between Chl *a* values for the mat in spring and summer (29.9 ± 4.8 and 27.2 ± 3.3 μg Chl *a* cm^{-2}, respectively) although winter values were significantly ($p < 0.0210$) lower (14 ± 0.9 μg Chl *a* cm^{-2}). Water temperature overlying the mat was of 23°C in spring, 27°C in summer, and 12°C in winter.

Poza Azul Stromatolite. Nitrogenase activity was not significantly different between all three seasons and showed its maximum rates during the midnight to sunrise (average for all three seasons: 153 ± 5 nmol C_2H_4 m^{-2} h^{-1}) and sunrise to noon (average for all three seasons: 248 ± 20 nmol C_2H_4 m^{-2} h^{-1} time points (Fig. 4A–C). Nitrogenase activity was statistically lower ($p < 0.0133$) to that measured in the microbial mat for spring and summer. Samples exposed to DCMU showed a significant decrease of NA during daytime points ranging from 67% in spring and summer to 91% in the winter. The stromatolite showed a peak of NA during the night that decreased 59% when exposed to Na_2MoO_4. There was no difference between the control and the IR treatment and no negative effect of UV radiation was evidenced in NA. There was no significant difference between Chl *a* values for the stromatolite during all sampling seasons (12.6 ± 1.6, 13.1 ± 1.3, and 13.4 ± 0.6 μg Chl *a* cm^{-2}, respectively, for spring, summer and winter).

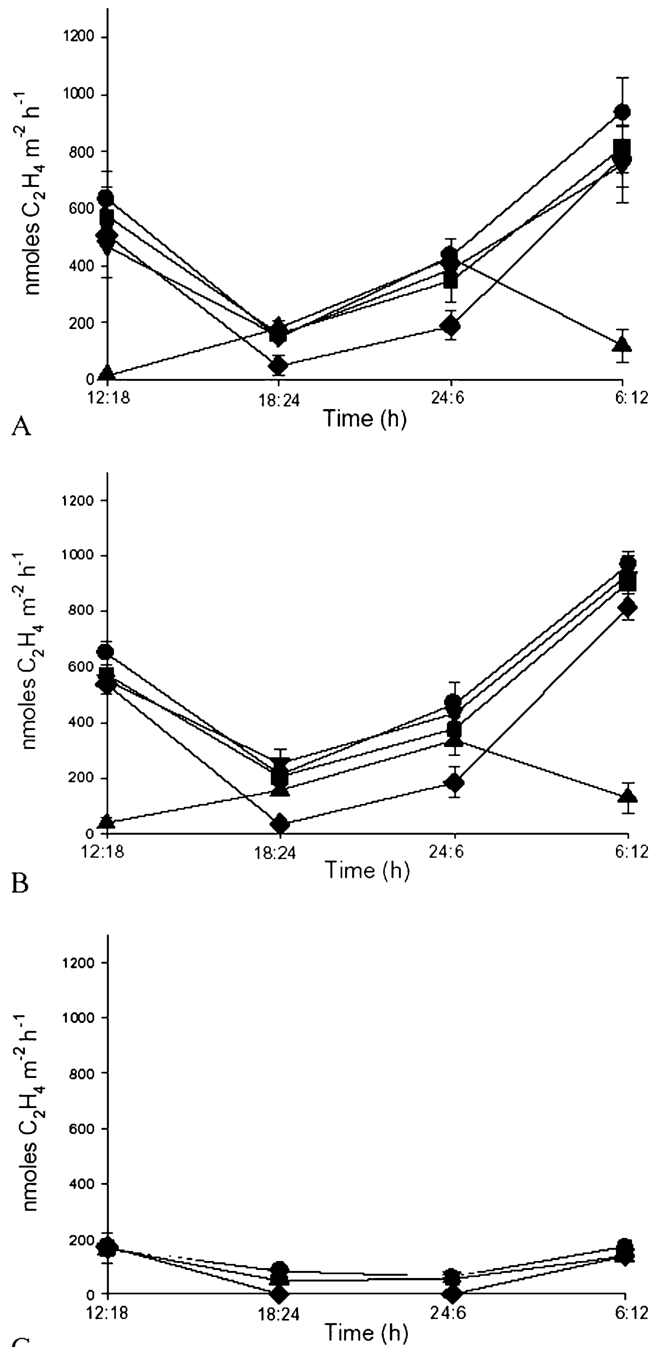

Figure 3. Results of simultaneous measurements of NA in Churince microbial mat. Control (square), +DCMU (upright triangle), +Na_2MoO_4 (diamond), −UV (circle), −IR (inverted triangle). A Spring, B summer, and C winter. Metabolic inhibitors were added at the beginning of the 24-h cycle.

Water temperature overlying the stromatolite was of 30°C in spring, 31°C in summer, and 30°C in winter.

Community Composition. A total of 96 *nifH* sequences were recovered, 39 from the microbial mat, and 57 from the stromatolite. Out of the total sequences, 14 from the Churince microbial mat and 42 from the Poza Azul stromatolite were potentially functional because they lacked stop codons in all six reading

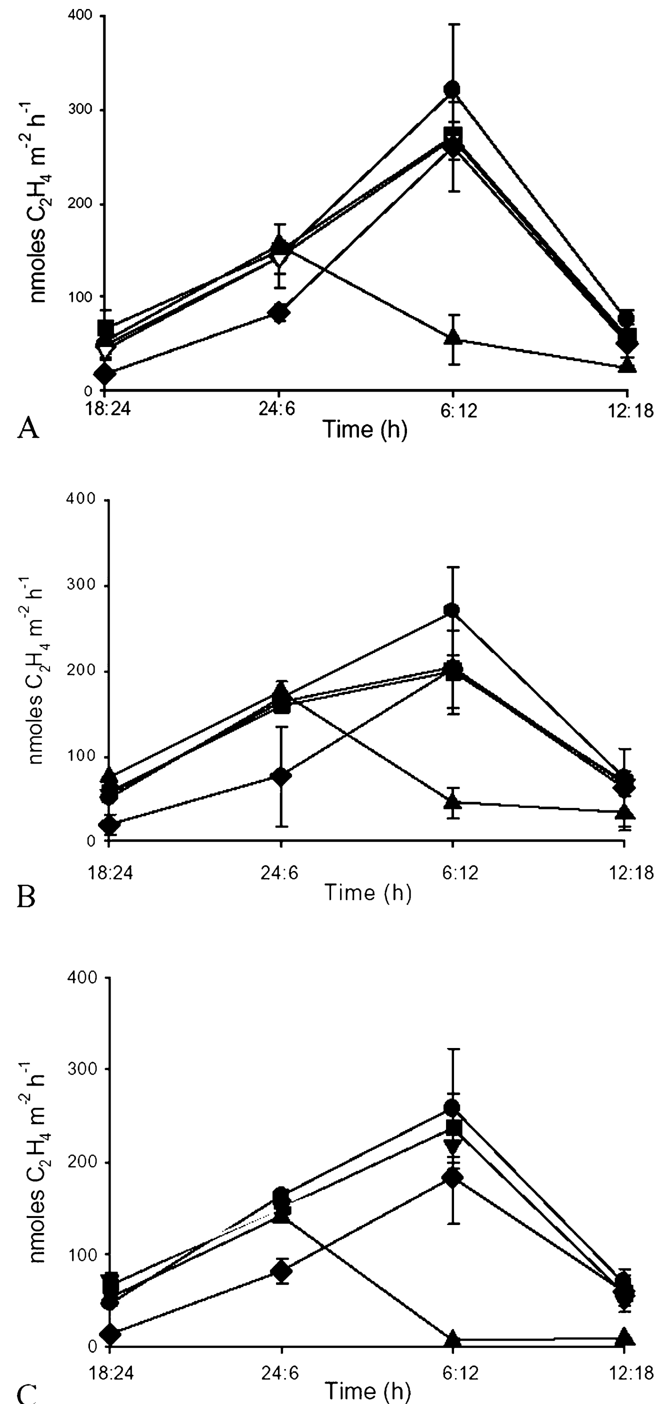

Figure 4. Results of simultaneous measurements of NA in the Poza Azul living stromatolite. Control (square), +DCMU (upright triangle), +Na_2MoO_4 (diamond), −UV (circle), −IR (inverted triangle). A Spring, B summer, and C winter. Metabolic inhibitors were added at the beginning of the 24-h cycle.

Figure 5. Maximum parsimony phylogram of *nifH* showing bootstrap values greater than 60% for translated DNA sequences. Sequences obtained in this study are denoted with a asterisks for Churince microbial mat and ovals for Poza Azul living stromatolite.

frames. A group of 15 sequences from the Poza Azul stromatolite shared highest similarity to sequences reported in the GenBank database that related to purple nonsulfur bacteria, nine sequences from the Poza Azul stromatolite, and four from the Churince microbial mat related to cyanobacteria. A separate group of sequences (17 from the Poza Azul stromatolite and 10 from the Churince microbial mat) affiliated with SRB. A sequence from the Poza Azul stromatolite showed closest affiliation to a previously reported sequence of an unknown bacteria, recovered from a denaturing gradient gel electrophoresis study in periphyton, as to the *nifH* sequence used as outgroup pertaining to *Methanosarcina barkeri*. The sequences obtained clustered in groups I and II of nitrogenases, as proposed by Raymond *et al.* [36] (Fig. 5). Group I sequences contained the purple sulfur and cyanobacteria, whereas group II had the anaerobic SRB and green sulfur bacteria. Sequences here reported clustered among sites and the only *nifH* sequence that was common to the Churince microbial mat and Poza Azul stromatolite related to a *Calothrix* spp. that came from Yellowstone National Park (USA) microbial mats (Fig. 5).

The number and identity of sequences recovered in this study were not enough to represent the total community of diazotrophs (*nifH*) covering a mere 20 to 30% of the expected richness in the microbial mat and stromatolite, respectively, as estimated with S_{Chao1} and S_{ACE} (data not shown).

Discussion

Cuatro Cienegas is an endangered ecosystem suffering from damaging human impact due mainly to the agriculture of alfalfa in the Mexican desert, which in turn is causing the collapse of the few aquatic environments in the region [44]. We believe that it is very interesting to have living microbial communities forming mats and stromatolites in desert springs that are quite far away from coastal regions (~800 km). The microbial communities reported here seem to function similarly to bacterial communities from coastal regions, e.g., Guerrero Negro microbial mats, Bahamas stromatolites, and North Carolina microbial mats.

The sequences recovered in this study were clearly more related among them than to any sequence previously deposited in the public database. The above suggests that there is a great diversity of potentially

important N_2 fixers in the pools of Cuatro Cienegas that have been isolated from other aquatic ecosystems. The phylogeny reported here clearly shows that the *nifH* sequences recovered from the Churince microbial mat and the Poza Azul stromatolite form separate clusters for groups I and II nitrogenases, as well as for different bacterial lineages. Nevertheless, although there are strong lineage affinities, sequences from this study can only be related at the genus level to *Calothrix* spp.

The CCC microbial mat and stromatolite showed highest N_2 fixation rates (as measured by NA) during the day, which follows the pattern of heterocystous diazotrophic cyanobacteria [13, 33, 40, 41, 45, 47, 48]. Heterocysts, the specialized cells for N_2 fixation, lack PSII activity and thus the oxygen-evolving capacity that characterizes vegetative cells. Because nitrogenase is inactivated by O_2, heterocyst-forming colonies have the capacity to fix N_2-coupled to photosynthetic activity by spatially separating both processes [5–7, 40, 41]. Molecular clock analyses have suggested that heterocysts evolved approximately after 2,450–2,320 Ma, when partial pressure of O_2, formed as a by-product of photosynthesis, reached levels to inhibit NA [47]. Because DCMU will inhibit PSII, there can be a stimulation of N_2 fixation because the community goes anoxic. Nevertheless, when heterocystous cyanobacteria are responsible for N_2 fixation, DCMU-exposed samples will show a decrease in NA because the electron transfer pathway from PSII to PSI is blocked, and thus ATP formed in PSI, which is necessary to reduce N_2, will stop being generated in the vegetative cells [5–7]. Our results suggest that heterocystous cyanobacteria could be important in both the microbial mat because of the 90% decrease of daytime NA when exposed to DCMU, and living stromatolite that had a decrease in daytime NA of 67% in spring and summer, and 90% in winter. The microbial mat NA data suggests that heterocystous cyanobacteria could be major components of the diazotrophic community in the Churince mat in spring and summer, whereas in the winter, Na_2MoO_4-exposed samples showed a decrease of 94%, suggesting that SRB could be the main diazotrophs for this season. Sulfur-reducing bacteria will utilize free sulfur as SO_4 or elemental S, and thus when the atmosphere is saturated in Na_2MoO_4, an analogue to SO_4, SRB metabolism will be affected [14]. The Poza Azul stromatolite had NA patterns that suggest that heterocystous cyanobacteria could be more important during the winter although they are still major components of NA in spring and summer. These experiments also point at the potential importance of SRB as diazotrophs in the stromatolite during all three seasons because Na_2MoO_4-exposed samples decreased by an average of 58% during the nighttime points. The Poza Azul stromatolite had a second NA peak from midnight to sun dawn, which suggests activity of nonheterocystous cyanobacteria, as well as of other anaerobic bacteria, for example, purple sulfur and nonsulfur, green sulfur, and SRB. Although we also found evidence of nonheterocystous cyanobacteria as potential diazotrophs in both microbial communities, our metabolic-oriented NA experiments point to the potential activity of heterocystous cyanobacteria as major diazotrophs. Nevertheless, our results showed no significant effect of lack of UV radiation in the NA of both microbial communities. Previous research has shown that *Calothrix* has a UV-protective pigment, scytonemin [10, 20], that shows *in vivo* absorption maximum at 370 nm [20]. Because both our molecular and metabolic data suggest that heterocystous cyanobacteria, possibly *Calothrix*, could be responsible for at least some of the N_2 fixation in the microbial mat and living stromatolite, we infer that this cyanobacterium may be protected against the deleterious effects of UV radiation via scytonemin. Furthermore, Sheridan [39] hypothesizes that UVR will maintain normal three-dimensional community composition in microbial mats because most UVR-resistant bacteria, such as scytonemin-bearing cyanobacteria, will occupy the surface levels, protecting non-UVR-resistant cyanobacteria. Although our cloning effort was of only 20–30% for the total estimated richness (S_{Chao1} and S_{ACE}) of diazotrophs in the microbial mat and living stromatolite, we were able to identify groups of *nifH* affiliated to green sulfur, purple sulfur, purple nonsulfur, SRB, cyanobacteria, and archaea. This coincides with the proposed metabolisms that could be held responsible for NA in both microbial communities suggested from the metabolic inhibitor NA experiments. Nevertheless, our NA experiments were not able to detect a negative effect when blocking IR radiation. This could be because of the fact that although most bacteriochlorophylls have absorption spectra in the IR, some (bacteriochlorophylls c, d, and e) also have absorption peaks in the visible, thus anoxygenic phototrophs that also have carotenoids could have obtained the energy necessary for ATP production and NA in the visible spectrum [27, 32].

Our molecular data also suggest that we have a potentially important purple sulfur and nonsulfur diazotrophic bacterial community in both microbial communities studied. We found n*ifH* sequences that related to group II of nitrogenases [36] that are characterized for being of anaerobic organisms. In this group we obtained sequences from the mat and stromatolite that related to SRB, *Desulfovibrio gigas*, *Desulfovibrio salexigens* [35], and green sulfur bacteria *Chlorobium tepidum* [16]. *Chlorobium tepidum* is capable of using both sulfide and thiosulfate as sources of sulfur and electron donors for photosynthesis [16]. The rest of the sequences clustered with nitrogenases of group I [36], which are typical Mo–Fe nitrogenases. In this group, sequences from the stromatolite clustered to *Rhodopseudomonas palustris*, a highly energetically versatile bacterium capable of both

aerobic and anaerobic metabolisms, including photoautotrophy, photoheterotrophy, chemoautotrophy, and chemoheterotrophy [29, 49]. *Rhodospirillum rubrum* is a purple nonsulfur bacterium capable of living under aerobic or anaerobic conditions, making it capable of obtaining energy from a great array of substrates [52]. *Heliobacterium modesticaldum* [22] is another bacterium to which sequences from the stromatolite related. *Halorhodospira halophila*, a purple sulfur bacteria that can utilize elemental sulfur or H_2S as electron donor, clustered to *R. palustris* and *R. rubrum*, forming a basal cluster to a group of sequences from the stromatolite. Our NA experiments were not able to fully identify all the metabolic bacterial groups involved in N_2 fixation in both microbial communities. Previous research has suggested the potential role of archaeal methanogens and methanotrophs as part of microbial, diazotrophic ensembles [14]. Further, a n*ifH* sequence from the stromatolite clustered to an uncultured clone obtained from periphyton and together they were more closely related to the archaeal outgroup, *M. barkeri*, suggesting that a greater cloning effort is needed to characterize these communities. Molecular data also evidenced the differences in composition of diazotrophs between the microbial mat from Churince and the living stromatolite from Poza Azul. Sequences from both microbial communities clustered to each other, suggesting biogeographic relationships. Poza Azul is separated in approximately 5 km from Laguna Intermedia and previous hydrological studies have proposed that the springs that feed both systems are different [23].

Although NA rates were significantly lower in the living stromatolite during spring and summer compared to the microbial mat, they were constant throughout all seasons, whereas NA rates in the microbial mat had major oscillations between spring–summer and winter. Previous research in CCC [44] has shown that temperature varies significantly in the region of the Churince system where we carried out the microbial mat experiments, whereas it remains constant (30–35°C) throughout the year in Poza Azul. Nevertheless, to date, there are no published studies in CCC that follow environmental variables such as salinity, pH, and nutrients in the different aquatic systems of CCC. Integrating our data on a yearly basis, and assuming that our spring–summer data are representative of 8 months, while our winter data represent 4 months, we can calculate a yearly NA rate of 530 mmol C_2H_4 m^{-2} h^{-1} for the microbial mat. Our yearly NA estimate for the stromatolite is of 192 mmol C_2H_4 m^{-2} h^{-1}. Rates of NA from both microbial communities are comparable to those from other microbial mats and living stromatolites [14, 34], although they are much lower than NA observed in microbial mats from Guerrero Negro, Baja California, Mexico [3, 30, 31], and the McMurdo Ice Shelf, Antarctica [19].

Cuatro Cienegas Coahuila has been reported to sustain highly diverse microbial communities that have large affinities to marine bacteria [44]. In their study, Souza *et al.* [44] hypothesize that CCC maintained Mesozoic marine bacterial communities in the interstitial pore water and that present day microbial mats and stromatolites originated back in the Jurassic. Our study cannot shed light on the potential time of formation of the microbial mat and living stromatolite analyzed, although we should mention that previous research on modern marine stromatolites from Bahamas [34, 35] and microbial mats from North Carolina, USA [42] had already proposed the role of cyanobacteria and SRB as potential N_2 fixers. Further research is needed to open the "black box" of microbial dynamics that are responsible for the biogeochemical interactions that coexist in microbial mats and living stromatolites.

Acknowledgements

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