

N₂ fixation rates and associated diversity (*nifH*) of microbialite and mat-forming consortia from different aquatic environments in Mexico

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ABSTRACT: The present study describes patterns and rates of N₂ fixation and associated diversity (based on the *nifH* gene) of microbialite and mat-forming bacterial consortia from different aquatic environments in Mexico. All of the communities shared a diurnal pattern of N₂ fixation with peak activity during the day while showing a unique genetic composition. Our study suggests the importance of heterocystous cyanobacteria as the main diazotrophic organisms, although *nifH* sequences were also affiliated to *Oscillatoriales*, *Chroococcales*, and several *Proteobacteria*. Genetic composition did not relate to the environmental variables analysed, although rates of nitrogenase activity associated to microbial C and N explained 11% of the variation among consortia.

KEY WORDS: N₂ fixation · Microbialites · Microbial mats · *nifH* · Diversity · Community ecology

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INTRODUCTION

Prokaryotes are key components of ecosystems because they are responsible for catalysing a vast array of metabolic reactions that define Earth's biogeochemical cycles (Ward et al. 1998, Whitman et al. 1998, Falkowski et al. 2008).

Microbial mats are benthic structures formed by the activity of complex prokaryote consortia that are organised vertically according to physicochemical gradients (Stal & Caumette 1993, Van Gernerden 1993, Paerl et al. 2000, Des Marais 2003, Dupraz & Visscher 2005). Under certain biogeochemical conditions, including the presence of carbonates, these consortia can become lithified, forming microbialites. Examples of these are thrombolites, oncolites, and stromatolites (to name the most common). Microbial

mats and microbialites are complex, self-sustainable ecosystems at a millimetric scale that are among the most productive systems of present Earth (Canfield & Des Marais 1993, Stal 2000). All of these formations share a vertical layered organisation that is often evident to the naked eye due to the coloration of pigments characteristic of each metabolic group, e.g. aerobic photoautotrophs (green), anoxygenic photoautotrophs (purple), or heterotrophs (brown) (Dupraz & Visscher 2005). The combination of different metabolisms favours the establishment of gradients, including those of O₂ and SO₄⁻² concentration, redox potential, pH, and available light (Van Gernerden 1993, Stolz 2000, Des Marais 2003).

Extant microbialites are considered direct descendants of the oldest forms of life that formed stromatolites dated back to 3500 million yr (Schopf et al.

2002). They constitute an excellent model to study microbial communities because they are sessile, have a compact organisation, and symbolise a small-scale ecosystem organised as a consortium, in which different prokaryote populations function in a coordinated and complementary way, with the result of enhancing nutrient cycling (Paerl et al. 2000).

The study of prokaryote diversity in microbialites has been conducted in different aquatic environments, as recently reviewed by Foster & Green (2011). Nevertheless, to date, we do not know if extant microbialite consortia still have a relevant impact on Earth's environment because their modern distribution is far more limited, although there is evidence that suggests they are involved in C and N cycling. In the present study, we analysed the dial rates and patterns of N_2 fixation and associated diversity (of the *nifH* gene) of microbial consortia from different geographic locations throughout Mexico that had not been characterised previously. The

goal of the present study was to describe the diazotrophic community for each study site while testing if the differences in *nifH* diversity could be associated to the variables measured, which included temperature, pH, conductivity, dissolved inorganic nutrients, and microbial C and N.

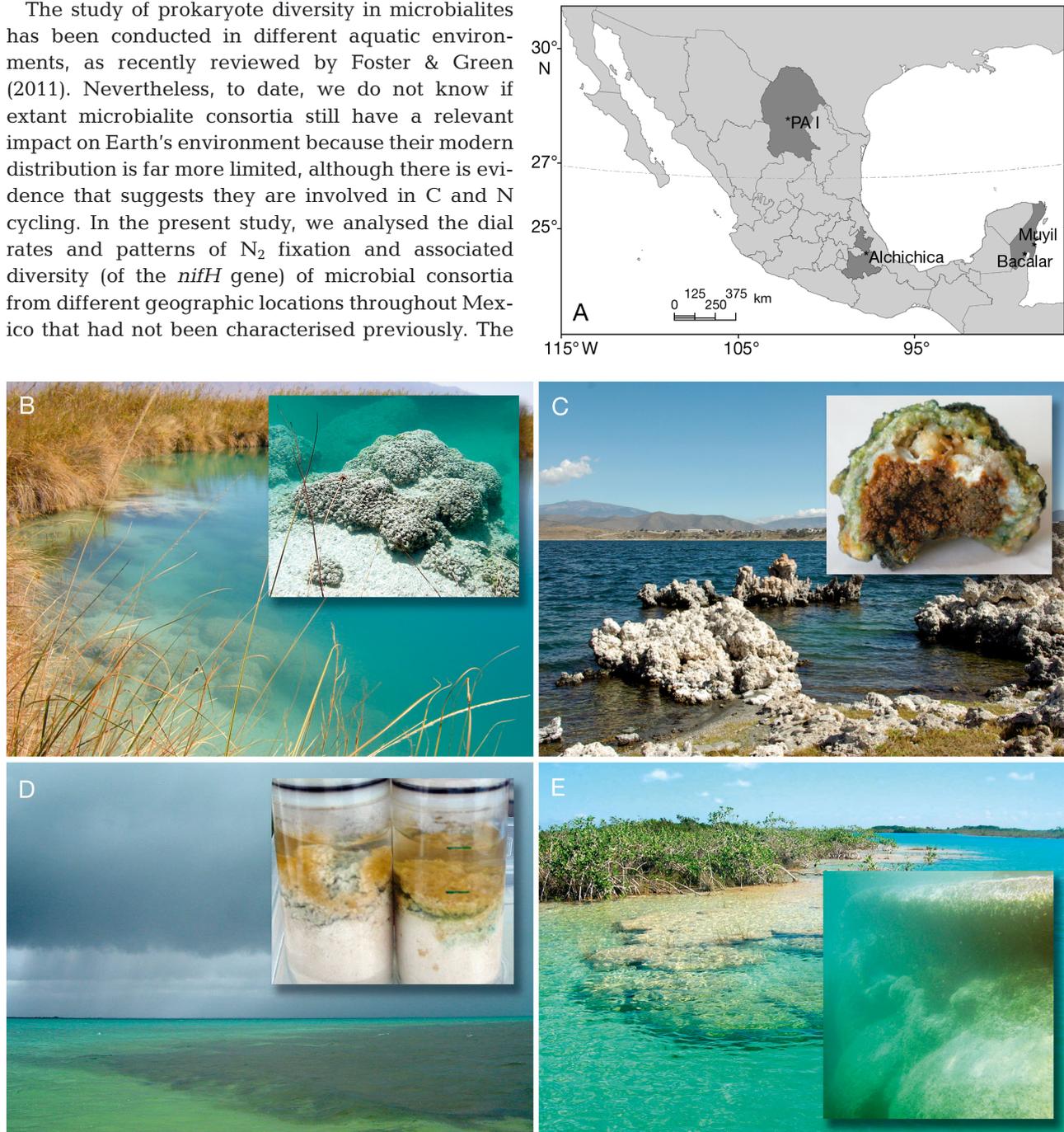


Fig. 1. (A) Map of Mexico indicating study sites; (B) microbialites (inset showing close up) from Pozas Azules I (PA I) desert pond in Cuatro Ciénegas Basin; (C) columnar and spongy (inset) microbialites from Alchichica crater-lake; (D) microbial mat from Muyil coastal lagoon (inset: sampling corers with mat); (E) microbialites (inset showing close up) from Bacalar coastal lagoon

MATERIALS AND METHODS

Sampling and N₂ fixation experiments were carried out during the summer of 2008. Study sites were chosen from locations throughout Mexico where microbial consortia had been reported. To test for differences in N₂ fixation rates and associated diversity, sites that were distant from each other and that encompassed differences in environmental variables were included.

In southern Mexico, a microbial mat was sampled from Muyil Lagoon in the Sian Ka'an Biosphere Reserve and microbialite formations were sampled from the Pirate Channel in Bacalar coastal lagoon (Gischler et al. 2008), both in the Yucatan Peninsula. In central Mexico, columnar (AC) and spongy (AS) microbialites from Alchichica crater-lake were included (Tavera & Komárek 1996, Falcón et al. 2002). In northern Mexico, massive thrombolites were sampled from the Pozas Azules I pond (Breitbart et al. 2009) within the Pozas Azules Ranch (Pronatura, Noreste AC) inside the Nature Protected Area (ANP) of Cuatro Ciénegas (Fig. 1, Table 1).

Environmental and biogeochemical characteristics

The environmental variables measured with a Hydrolab multisensor (600 QS) included temperature, conductivity, and pH of the water column surrounding the microbialites and mat. Water samples were taken in sterile polycarbonate bottles, taking care to exclude air bubbles, filtered through Whatman No. 1 and Millipore 0.45 µm filters, and taken back to the laboratory for nutrient analysis. Total inorganic phosphates (PO₄³⁻), nitrites (NO₂⁻), nitrates (NO₃⁻), ammonia (NH₄), and silicates (SiO₄⁴⁻) were determined by colorimetry with a Bran Luebbe 3 autoanalyser.

Microbial carbon (C_m) and nitrogen (N_m) were determined from all microbialites and mats by the chloroform fumigation-extraction method (Vance et al. 1987) from ~20 g collected from the surface (within

the first 5 cm). Samples were kept in dark sterile bags at 4°C until analysis. An aliquot of fresh field sample was fumigated in a chloroform-saturated atmosphere, and a negative control without fumigation was included for comparison. Samples were incubated during 24 h at 25°C and constant moisture.

C_m was extracted from fumigated and non-fumigated samples with 0.5 M K₂SO₄ and filtered through Whatman No. 42 paper (Brookes et al. 1985). The total C and inorganic C concentrations were analysed with a C analyser (UIC, model CM 5012). Organic C from extract was calculated from the difference between total C and inorganic C and was used for estimation of C_m following the equation: C_m = EC/K_{EC}, where EC is the organic C extracted from fumigated samples minus EC extracted from non-fumigated samples, and K_{EC} is the extraction efficiency of 0.45 (Joergensen 1996).

N_m was also extracted with K₂SO₄ from fumigated and non-fumigated samples and filtered through Whatman No. 1 paper. The filtrate was digested in sulphuric acid and determined by colorimetry with a Bran Luebbe 3 autoanalyser. N_m was calculated following the same procedure as C_m, with a K_{EN} (i.e. extraction efficiency of organic nitrogen) value of 0.54 (Joergensen & Mueller 1996). C_m and N_m were expressed based on the dry weight of samples.

N₂ fixation

Estimates of N₂ fixation rates, based on nitrogenase activity, were obtained with the acetylene reduction assay using gas chromatography with a flame ionisation detector (SRI Instruments) following the protocol of Capone (1993).

Replicate samples (n = 6) of ~10 cm in diameter and 5 cm in depth were taken from each microbialite and placed individually in clear polycarbonate cylindrical chambers (30 cm height, 10 cm diameter) equipped with adjustable O-rings and sealed top and bottom lids. The chambers had serum caps adjusted to openings in the top lid for gas sampling. Once micro-

Table 1. Environmental characteristics for study sites. All samples came from surface waters (<1 m)

Parameter	Muyil	Bacalar	Alchichica columnar	Alchichica spongy	Pozas Azules I
Location	20° 04.075' N, 87° 35.817' W	18° 38.190' N, 88° 23.074' W	19° 25.081' N, 97° 24.142' W	19° 24.364' N, 97° 24.142' W	26° 49.66' N, 102° 1.46' W
Water temperature (°C)	29.3	29.0	19.5	18.8	29.3
Conductivity (mS cm ⁻¹)	1.5	2.3	13.3	13.2	2.7
pH	8.3	7.6	9.7	9.6	6.8

bialites were placed inside the chambers, they were covered with 0.22 μm filtered water collected above the microbialites up to two-thirds of the incubation chambers. The chambers were then sealed, and serum caps were placed on the top lid. The remaining volume of the chamber, consisting of the gas phase, was saturated with 20% acetylene, formed by the reaction of calcium carbide and water. To avoid over-pressure inside the chamber when saturating with acetylene, an equal gas volume was extracted from the gas phase while the acetylene was bubbled directly to the water. Chambers with samples were left *in situ*, and a volume of the gas phase (3 ml) was extracted at sunrise, midday, sunset, and midnight and stored in vacuum tubes (Becton Dickinson). Each time the gas phase was sampled, the same amount of acetylene was injected to avoid differences in pressure inside the chambers. N_2 fixation rates were calculated from ethylene (C_2H_4) concentrations following the equation of Capone & Montoya (2001):

$$(\text{C}_2\text{H}_4 \text{ produced}_{\text{sample}} / \text{C}_2\text{H}_4 \text{ produced}_{\text{standard}}) \times \text{C}_2\text{H}_4 \text{ concentration standard (nmol ml}^{-1}) \times \text{gas phase volume (ml)} \times \text{solubility factor of C}_2\text{H}_4 \text{ at 1 atmosphere pressure and 22}^\circ\text{C}$$

We used a conversion of 3:1 molecules of acetylene per molecule of N_2 (Capone & Montoya [2001]).

Molecular analysis

The total community DNA of microbialites from the same sites that were analysed for N_2 fixation rates was extracted following the protocol of Zhou et al. (1996) with modifications.

Samples of the surface layer (~2 cm) of 5 microbialites per site were taken in triplicate ($N = 15$), with sub-samples separated by ~10 cm and microbialites separated by ~2 m. Samples were frozen immediately in the field with liquid nitrogen. Back in the laboratory, the samples were macerated using liquid nitrogen and extraction buffer (100 mM Tris-HCl, 20 mM NaCl, and 100 mM EDTA) and further incubated for 30 min at 37°C with lysozyme (30 mg ml^{-1} , Sigma Aldrich) and left overnight at 55°C with proteinase K (10 mg ml^{-1} , Sigma Aldrich). Samples were centrifuged at 10 000 $\times g$, and the aqueous layer was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). DNA was precipitated with absolute isoamyl alcohol and one-tenth volume of 3 M sodium acetate and incubated for 20 min at -20°C. The DNA pellet was further purified with a DNeasy Blood & Tissue kit following the protocol of the supplier (Qiagen).

Polymerase chain reaction (PCR) was used to amplify a 359 base pair region of the *nifH* gene (dinitrogenase reductase) following the protocol of Zani et al. (2000). Amplification reactions consisted of 30 cycles of 95°C (1 min), 55°C (1 min), and 72°C (1 min), with a final extension of 72°C (30 min). The DNA from each site ($n = 3$) was pooled together in equal concentrations as PCR template in replicated reactions ($n = 5$). Amplified fragments were gel purified together using the QIAquick gel extraction kit (Qiagen) and ligated with pCR[®]2.1-TOPO vector (Invitrogen). *Escherichia coli* competent cells were transformed with plasmids. The Montage Plasmid Miniprep₉₆ system (Millipore) was used to extract plasmids from positive transformants. Sequences were obtained with the Sanger protocol, and the plasmid fragments were edited using BioEdit Sequence Alignment Editor v.7.0.5.3 (Hall 1999). The resulting sequences were aligned with Sequencher v.3.1.1 (Gene Codes) and arranged in phylotypes considering 95% nucleotidic similarity (Ben-Porath & Zehr 1994) of total genetic distance using a neighbour-joining approach. Topologies were verified with neighbour-joining, maximum parsimony and maximum likelihood algorithms supported with bootstrap, implemented with PAUP* v.4.0b10 (Swofford 2002). Phylogenetic analysis considered 104 sequences, including 56 reference sequences obtained from GenBank and a representative of each phylotype (48) obtained in the present study. Reference sequences came from cultivated organisms and environmental studies of microbial mat and microbialite consortia. The archaeon *Methanosarcina mazei* was included as an outgroup because the recovered sequences in the present study pertained to *Bacteria*.

Richness was estimated for each site using the SChao1 and Good's C coverage indices (Kemp & Aller 2004). Rarefaction and estimates of diversity using the Shannon and Simpson indices were computed in the R language using the 'rarefy' function from the 'vegan' package (Legendre & Legendre 1998).

Sequences generated in the present study were deposited in the GenBank database under accession numbers HQ397724 to HQ398104.

Statistical analysis

To determine if there was significant variation between N_2 fixation rates among sites and activity

periods for each season, a 2-way ANOVA was computed with the 'ANOVA.2way.R' function (Legendre 2007).

A redundancy analysis (RDA) was used to analyse the relationship between the phylotype frequency data at all of the sites and the explanatory environmental variables. A community composition matrix (Sites × Phylotypes [5 × 48]) was built and transformed using the chi-square transformation (Legendre & Gallagher 2001). The variables included C_m, N_m, NH₄, NO₃⁻, NO₂⁻, PO₄³⁻, SiO₄⁴⁻, PO₄³⁻, temperature, pH, conductivity, and N₂ fixation rates. All analyses were computed using R-language functions. The RDA to study the relationships between the microbial community composition and the envi-

ronmental variables were computed with the 'rdaTest' function (Legendre & Durand 2010). A forward selection procedure was used to select significant variables that would affect genetic composition with a determination coefficient >0.05 computed with the 'forward.sel' function of the 'packfor' library (Dray et al. 2009).

RESULTS

Environmental variables

The abiotic variables showed variation among the sites (Table 1). Alchichica crater-lake had the lowest temperature (18.8°C), with the rest of the sites oscillating between 29°C and 29.3°C. Alchichica had the highest conductivity and pH of all of the sites. Available forms of inorganic nitrogen (NH₄ + NO₃⁻ + NO₂⁻) were below detection levels in all sites except for Alchichica crater-lake and Pozas Azules I desert pond (Fig. 2). All sites showed meso-oligotrophic conditions ranging between 0.01 and 0.1 mg l⁻¹ (Mueller & Helsel 1999) (Fig. 2). Dissolved silicates were most abundant in Bacalar and Muyil coastal lagoons in the Yucatan Peninsula and in Pozas Azules I desert pond in Cuatro Ciénegas, while Alchichica crater-lake had the lowest concentrations (Fig. 2). Microbialites from Alchichica (AC and AS) had the highest concentrations of C_m and N_m (Table 2).

N₂ fixation rates

Microbialites and mat-forming communities showed the same pattern of N₂ fixation, with significantly greater (p = 0.05) rates during the day (06:00 to 12:00 h or 12:00 to 18:00 h) compared to night periods (Fig. 3). The microbialites of crater-lake Alchichica had the highest rates of N₂ fixation of all sites (Fig. 3).

Table 2. Microbial carbon (C_m), nitrogen (N_m) (μg g⁻¹) and proportions (C_m:N_m) for all sites

	C _m	N _m	C _m :N _m
Muyil	134	37	4
Bacalar	102	25	4
Alchichica columnar	263	43	6
Alchichica spongy	183	22	8
Pozas Azules I	581	17	34

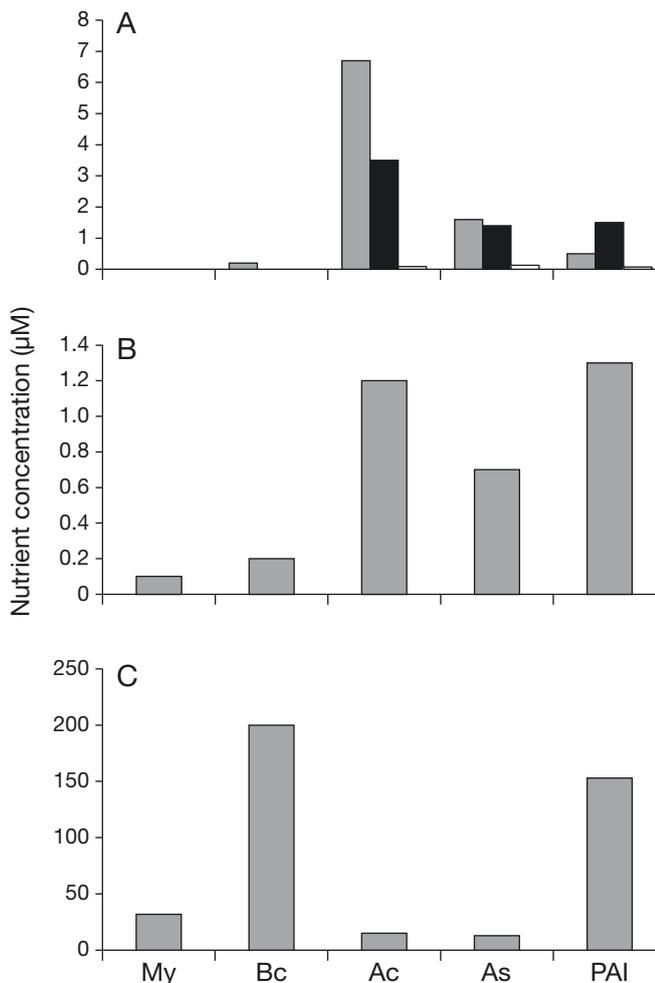


Fig. 2. Concentration (μM) of available nutrients in water column surrounding the analysed microbialites and mat. Dissolved inorganic nutrients: (A) NO₂⁻ (grey), NO₃⁻ (black), and NH₃ (white, 3rd column), (B) PO₃⁴⁻, (C) SiO₄⁴⁻. My: Muyil microbial mat; Bc: Bacalar microbialites; Ac: Alchichica columnar microbialites; As: Alchichica spongy microbialites; PAI: Pozas Azules I microbialites

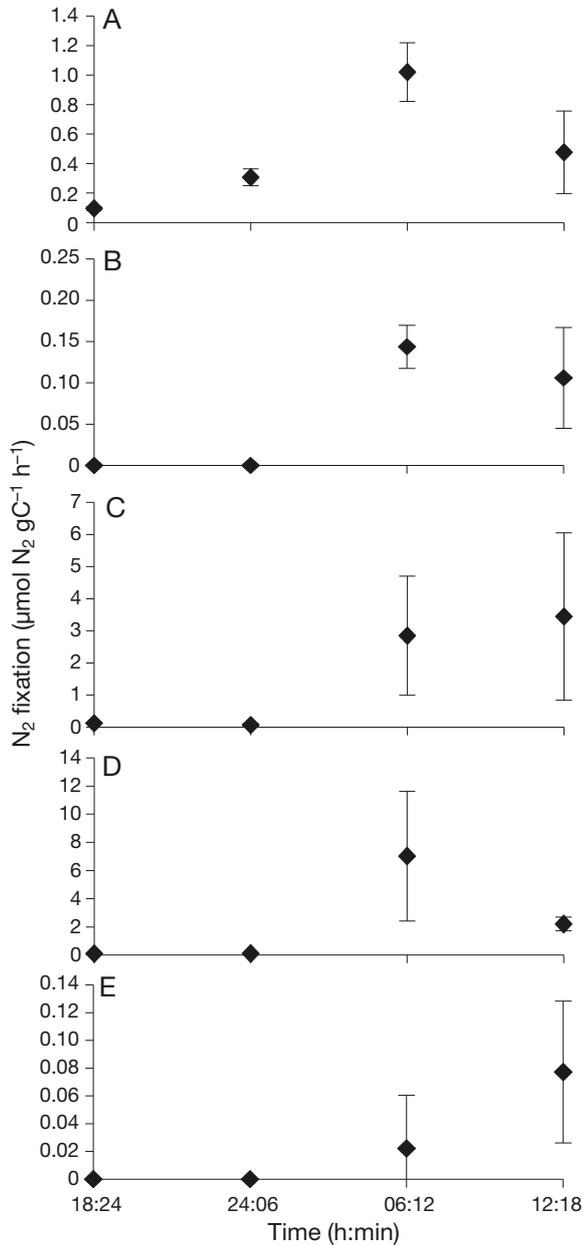


Fig. 3. Pattern of N_2 fixation rates (mean \pm SD) during daily experiments; activity was measured at the indicated times. (A) Muyil, (B) Bacalar, (C) Alchichica columnar, (D) Alchichica spongy, (E) Pozas Azules I

Diversity associated with the dinitrogenase reductase (*nifH*) gene

In total, 381 sequences of *nifH* gene were obtained from the 5 study sites: 83 in Muyil, 64 in Bacalar, 79 in AS, 85 in AC, and 70 in Pozas Azules I. Sequences were clustered into 48 phylotypes considering 95% nucleotidic similarity (Ben-Porath & Zehr 1994). Coverage of *nifH* phylotypes based on Good's C, which gives the proportion of the population represented by the phylotypes in the sample, ranged between 70 and 90% and did not differ from predictions (S_{chao1}) (Kemp & Aller 2004). The Shannon and Simpson diversity indices identify the microbial mat in Muyil coastal lagoon as having the highest diversity of potential N_2 -fixers, followed by the columnar microbialite of Alchichica crater-lake (Table 3, Fig. 4).

All sites had *nifH* phylotypes that related to *Cyanobacteria* and *Proteobacteria*. Cyanobacterial *nifH* sequences clustered within *Nostocales* (heterocystous), *Chroococcales* (N_2 -fixing unicellular), and *Oscillatoriales* (non-heterocystous filamentous) (Fig. 5). Proteobacterial *nifH* sequences pertained to classes *Alpha*- and *Gammaproteobacteria* (Fig. 5). Cyanobacterial sequences recovered in the present

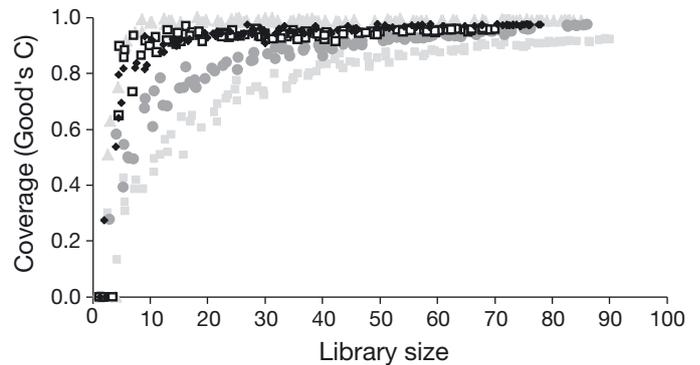


Fig. 4. Coverage (Good's C) for each clone library (size, in no. of different clones). (■) Muyil microbial mat, (□) microbialites from Bacalar, (●) Alchichica columnar, (▲) Alchichica spongy, (◆) Pozas Azules I

Table 3. Coverage, predicted number of phylotypes (*nifH*), and diversity indices for all sites

Site	No. clones	Observed phylotypes	Predicted value of S_{chao1}	% coverage (Good's C)	Rarefaction (N = 70)	Shannon index (<i>H</i>)	Simpson index (<i>D</i>)
Muyil	83	22	28	81	20	2.66	10.09
Bacalar	64	6	8	75	6	0.94	2.05
Alchichica columnar	85	13	13	93	12	1.95	4.78
Alchichica spongy	79	3	3	85	3	0.70	1.84
Pozas Azules I	70	6	7	86	6	1.0	1.98

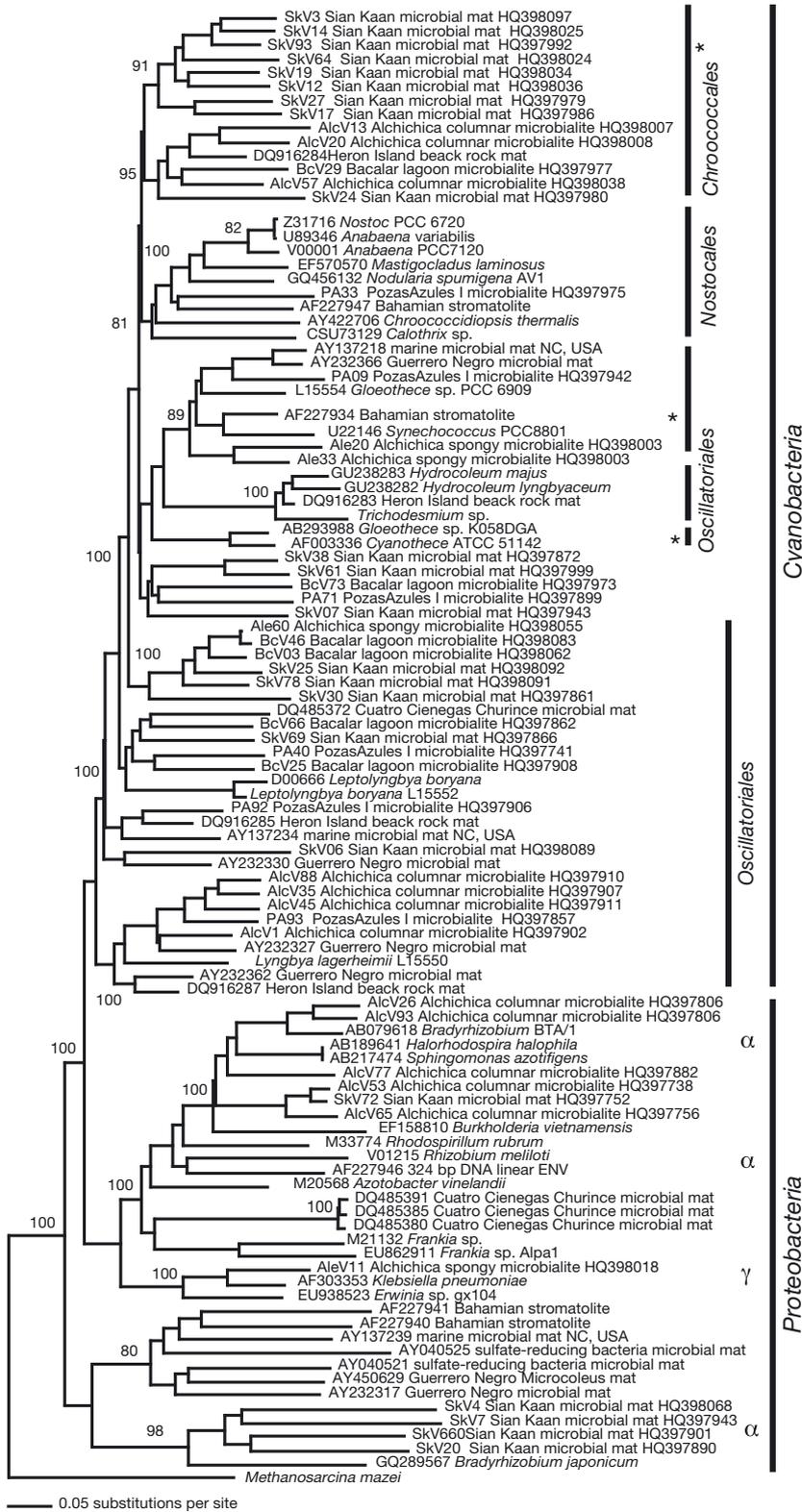


Fig. 5. Phylogenetic reconstruction using neighbour-joining, showing *nifH* diversity recovered from all sites (mat and microbialites). The archaeon *Methanosarcina mazei* was included in the analysis as the outgroup. Numbers in nodes indicate bootstrap support values. GenBank accession numbers are provided for each clone included in the analysis

study interspersed with those from other tropical and temperate microbial mats and microbialites (Steppe et al. 2001, Omoregie et al. 2004, Steppe & Paerl 2005, Yannarell et al. 2006, Díez et al. 2007, Falcón et al. 2007). Some sites, for example, Sian Ka'an microbial mat, formed single clusters within *Chroococcales*, and Alchichica crater-lake columnar microbialite grouped within *Oscillatoriales*, while other clusters encompassed sequences from several study sites. Sequences affiliated to *Nostocales* formed a single cluster.

Variable analysis

No environmental variable included in the present study could explain community composition based on *nifH* differences among sites. Nevertheless, the redundancy analysis showed that N₂ fixation rates, associated to C_m and N_m, explained 11% of the variation in microbial community composition among all of the microbialites and mats analysed (R^2 adj. = 0.11, $p = 0.049$) (Fig. 6).

The biplot ordinated the phylo-type distribution according to sampling site, as expected considering the low abundance of shared types between localities. Only 1 phylo-type shared an ordination axis between a pair of localities: Bacalar lagoon in the Yucatan Peninsula and Alchichica crater-lake in central Mexico. The above suggests that the community of N₂-fixers is site-specific in genetic composition and shares the same diurnal trend of nitrogenase activity.

DISCUSSION

The microbial communities analysed were found along heterogeneous environmental conditions

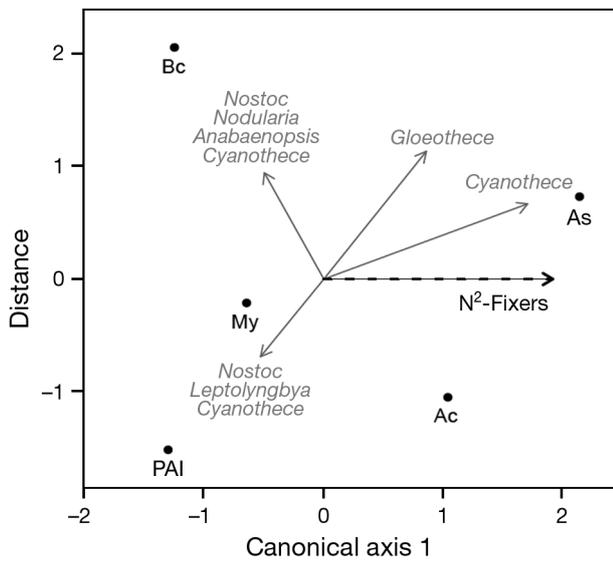


Fig. 6. Redundancy analysis biplot illustrating the effect of N_2 fixation on microbial community composition for the 5 sites under study. Abscissa: canonical axis associated with N_2 fixation rate (N_2 fixation rate variable in the graph); the ordinate is the first principal component of the residual variation used to produce a 2-dimensional graph. Dashed line: microbial communities, solid lines: registered phylotypes. My: Muyil microbial mat; Bc: Bacalar microbialites; Ac: Alchichica columnar microbialites; As: Alchichica spongy microbialites; PAI: Pozas Azules I microbialites

that coincided in meso-oligotrophic status. To date, we do not know if the microbial communities that form microbialites and mats can resist large inputs of inorganic nutrients, but we can predict that their role as active carbon and N_2 -fixers could be affected under such scenarios.

All of the communities shared N_2 fixation patterns, with maximum rates during daytime. The same patterns had been reported from Alchichica crater-lake (Falcón et al. 2002), mats and microbialites from Cuatro Ciénegas (Falcón et al. 2007), and distant sites to those here, such as Antarctic lakes (Olson et al. 1998), lakes in Belize (Rejmánková & Komárková 2000), Tomales Bay (Stal 1995), and the North Sea (Stal et al. 1984), among others. Although nitrogenase was active both day and night, explaining the diversity of *nifH* sequences related to non-heterocystous *Cyanobacteria* and *Proteobacteria*, maximum rates occurred during the day. The diurnal increase in N_2 fixation rates coincides with a spatial separation with oxygenic photosynthesis because nitrogenase is inhibited in the presence of free oxygen (Berman-Frank et al. 2001). Previous studies based on morphologic characterisations (Tavera & Komárek 1996) had reported the presence of diverse *Cyanobacteria* in

microbialites from crater-lake Alchichica, including *Nostocales*, *Chroococcales*, *Oscillatoriales*, and *Pleurocapsales*, and based on a metagenomic approach, Breitbart et al. (2009) confirmed the importance of *Nostocales* and *Chroococcales* in a microbialite from Pozas Azules I in Cuatro Ciénegas. Further, based on morphologic and molecular characterisations, Gischler et al. (2008) indicated the importance of *Oscillatoriales* as components of the microbialite community in Bacalar lagoon. Although microbialites analysed shared the presence of *Cyanobacteria* and *Proteobacteria* as potential diazotrophs (*nifH*), sequences arranged in phylotypes were mostly related to geographic origin, in agreement with other studies (Olson et al. 1998, 1999, Omoregie et al. 2004, Falcón et al. 2007). Out of the 381 sequences obtained, 48 unique phylotypes were defined, of which only 2 were shared between pairs of localities: the mat from Muyil coastal lagoon shared 1 phylotype with the columnar microbialite from Achichica crater-lake, and the microbialites from Bacalar coastal lagoon and Alchichica crater-lake spongy type shared another. Overall, no single phylotype was common to all microbialites and mats analysed. The above suggests a high site-specific diversity above 95% genetic similarity. It is noteworthy that all sites shared major phylogenetic groups, including *Chroococcales*, *Oscillatoriales*, and *Nostocales Cyanobacteria* plus *Alpha*- and *Gammaproteobacteria*.

High N_2 fixation rates of microbialites dominated by *Cyanobacteria* have been attributed to increased growth rates (Stal 1995, Pinckney et al. 1995, Rejmánková & Komárková 2000), which implies larger nitrogen demand (Sterner & Elser 2002). Correspondingly, our data indicate an inverse relation between the availability of nitrogen and N_2 fixation rates, in agreement with Rejmánková & Komárková (2000). Biomass N requirements cannot be obtained solely from external sources, and N_2 fixation has the potential to decrease N limitation (Steppe et al. 1996, Olson et al. 1999). Collectively, nitrogenase activity and molecular data suggest the overall importance of *Cyanobacteria* and heterotrophic *Proteobacteria* as components of the microbialite and mat-forming consortia studied in terms of their N_2 fixation potential. Nitrogenase activity coupled to C_m and N_m was identified in the present study as a key component that structures a fraction (11%) of the community composition (based on *nifH*) from geographically distant consortia. It is clear that other environmental factors are driving the diversity of N_2 -fixers, which is interesting because all of the consortia shared the same pattern in nitrogenase activity but were different in

genetic composition. As in other studies, *Cyanobacteria* and *Proteobacteria* are acknowledged as major contributors to fixed N₂.

Acknowledgements. Funding was provided by SEP-CONACyT No. 56045 (L.I.F.) and PAPIIT No. IN225709-3 (L.I.F.). A graduate student travel UNAM-PAEP fellowship was awarded to Y.B. to conduct statistical analysis with P.L. Graduate student fellowships CONACyT were awarded to Y.B. and C.M.C. Facilities for molecular analysis were provided by LIPA laboratory (UNAM). R. Pérez-Ishiwara provided technical assistance. Special thanks to V. Souza and L. Eguiarte for gas chromatography facilities (LEMyE laboratory, UNAM). Technical assistance is acknowledged from C. Martínez and R. Velázquez for all physicochemical and biogeochemical parameters, which were analysed in the Soil Biogeochemistry Laboratory (CIECO, UNAM). J. Domínguez, A. Sandoval, C. Belman, and C. Ezcurdia provided field assistance. Field support in Cuatro Ciénegas was provided by PRONATURA Noreste, AC, inside the Pozas Azules Ranch. Field support in Sian Ka'an Nature Preserve was provided by Community Tours SA de CV. All samples were collected under SEMARNAT permits FAUT.0112 and DGOPA.09392.301109.4119.

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Editorial responsibility: Douglas Capone, Los Angeles, California, USA

*Submitted: March 11, 2011; Accepted: May 3, 2012
Proofs received from author(s): June 25, 2012*