Vol. 78: 121–133, 2017
doi: 10.3354/ame01806

AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol

Published online January 27

Genetic diversity associated with N-cycle pathways in microbialites from Lake Alchichica, Mexico

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ABSTRACT: Microbialites are an example of complex and diverse microbial assemblages where several metabolic pathways are interconnected for biomass formation coupled to mineral precipitation. Lake Alchichica (Mexico) is an oligotrophic environment where nitrogen (N) and phosphorus alternately limit productivity, and massive microbialite growths are found along the lake's perimeter. Previous studies have described the importance of N_2 fixation in these microbialites, although other pathways associated with the N cycle, including denitrification, nitrification and anaerobic ammonium oxidation (anammox), had not been evaluated. This study identified the genetic diversity associated with N cycling in both metagenomic DNA and RNA expression by targeting key genes for nitrogenase (*nifH*), ammonia monooxygenase (*amoA*), nitrite oxidoreductase (*nxrA*, *nxrB*), hydrazine oxidoreductase (*hzo*) and nitrite (*nirS* and *nirK*) and nitrous oxide (*nosZ*) reductases. While the genetic potential for N_2 fixation, ammonia oxidation, anammox and denitrification was present in the microbialites of Lake Alchichica, the most transcribed pathway was N_2 fixation.

KEY WORDS: Microbialites · N cycle · N_2 fixation · *Cyanobacteria* · Heterocyst

INTRODUCTION

Microbialites are benthic microbial communities defined as organo-sedimentary structures where microbial activity promotes lithification by trapping, binding and/or precipitating detrital or chemical sediments (Burne & Moore 1987). These biostructures can be found in freshwater environments, saline (alkaline) lakes, hypersaline ponds, tidal sand flats, shallow rock pools and hot springs (Laval et al. 2000, Berelson et al. 2011, Centeno et al. 2012, Cooper et al. 2013). Although the physicochemical environment plays a crucial role in their development, microbial activity remains the main driving force promoting accretion, with cyanobacterial photosynthate and heterotrophic respiration as the main contributors (Reid et al. 2000, Stal 2012, Cerqueda-García & Falcón 2016). These complex microbial assemblages have had a continuous presence throughout the history of life on Earth. The fossil counterparts of microbialites date back to the Archaean (~3500 million years ago) and provide the most ancient microfossil

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record of life (Krumbein 1983). Therefore, these benthic biostructures can be considered one of the first successfully organized communities for which fossil records exist, hosting bacteria that played an essential role in atmospheric evolution and planetary biogeochemistry (van Gemerden 1993, Decho et al. 2005, Paterson et al. 2008).

Several studies have described the phylogenetic diversity in microbialites (Tavera & Komárek 1996, Couradeau et al. 2011, Kaźmierczak et al. 2011, Centeno et al. 2012, Ruvindy et al. 2016). In addition, metagenomic approaches have confirmed an ample metabolic repertoire with interconnected biogeochemical pathways within millimetric scales (Breitbart et al. 2009, Khodadad & Foster 2012, Mobberley et al. 2013, Cerqueda-García & Falcón 2016). These studies have shown that *Cyanobacteria* and *Proteobacteria* in microbialites are key microorganisms with important roles for carbon (C), nitrogen (N) and sulfur cycling (Myshrall et al. 2010).

The environments where microbialites thrive are often oligotrophic and restrict microbial activity by nutrient unavailability, mostly N and/or phosphorus (P) (Pepe-Ranney et al. 2012). N is an essential element in nucleic acids and proteins and often limits marine ecosystem productivity. On geological timescales, fixed N has been proposed to restrict primary productivity (Falkowski 1997). The N_2 fixation process constitutes an important source of N input into biomass from atmospheric N_2 (Canfield et al. 2010). In contrast, denitrification and anaerobic ammonium oxidation (anammox) are biological processes that return N back to the atmosphere (Canfield et al. 2010), while nitrification connects N_2 fixation and denitrification (Klotz & Stein 2008).

Lake Alchichica (Mexico) is an oligotrophic, saline and alkaline environment with living microbialites. Both N and P have been found to limit biological productivity in the water column (Ramírez-Olvera et al. 2009), although N seems to be the limiting element most frequently, due to the very low dissolved inorganic N (DIN) concentrations found in the mixed layer (0.7–3.8 µM) during the year (Ramírez-Olvera et al. 2009, Ardiles et al. 2012). The most abundant microbialite type in the lake consists of spongy structures distributed around the entire perimeter, described as white cauliflower-like thrombolites composed mainly of hydromagnesite — $Mg_5(CO_3)_4(OH)_2{\cdot}4H_2O$ (Kaźmierczak et al. 2011) (Fig. 1). Alchichica microbialites have shown high rates of daytime nitrogenase activity (Falcón et al. 2002, 2007, Beltrán et al. 2012) associated with heterocystous cyanobacteria (Falcón et al. 2002).

In this study, we aimed to explore the genetic diversity and expression associated with N cycling in spongy microbialites from Lake Alchichica. To accomplish this, different N-cycle pathways were surveyed for N_2 fixation (*nifH*), ammonia oxidation (*amoA*), nitrite oxidation (*nxrA* and *nxrB*), anammox (*hzo*) and denitrification (*nirK*, *nirS* and *nosZ*). To encompass these results, a description of the physicochemical environment where microbialites develop was also registered. We hypothesize that N-cycle pathways including denitrification, nitrification or anaerobic ammonium oxidation (anammox) should exist in Alchichica microbialites where steep chemical–redox gradients and biogeochemical cycling occur (Tavera & Komárek 1996, Couradeau et al. 2011, Kaźmierczak et al. 2011).

MATERIALS AND METHODS

Study site

Lake Alchichica is a crater lake in central Mexico (2300 m above sea level; 19° 24′ N, 97° 24′ W). This lake is the deepest crater lake in Mexico's Neovolcanic Axis (over 60 m depth) and has a diameter of ~1.8 km (Fig. 1a) (Nelson & Sánchez-Rubio 1986, Vilaclara et al. 1993). The system is classified as a soda lake (pH >8.9 with electrical conductivity ~13.39 mS cm^{-1} in the surface), formed by a phreatic explosion and mainly fed by an influx of water rich in sodium from volcanic materials and bicarbonates from Cretaceous limestone (Caballero et al. 2003). The area is arid and shows steep changes in ambient temperature from 5.5 to 30°C (mean 14.4°C), high annual evaporation rates (1590 mm) and 400 mm precipitation (García 1988, Adame et al. 2008, Armienta et al. 2008).

Sampling and nucleic acid extraction

Sampling was done in the summer of 2013, during the stratification period of the lake. The physicochemical data were measured *in situ*, and microbialite (Fig. 1b,c) and water column samples were collected.

To study the genetic diversity associated with the N cycle in microbialites, 6 sampling sites were chosen for spongy microbialites growing at <1 m depths. In all cases, the outermost layer (first 5 cm) of microbialites was sampled. For each site, 3 subsamples (each ca. 5 g) were taken, placed into sterile bags, stored at 4°C (24 h) and then frozen at –20°C until

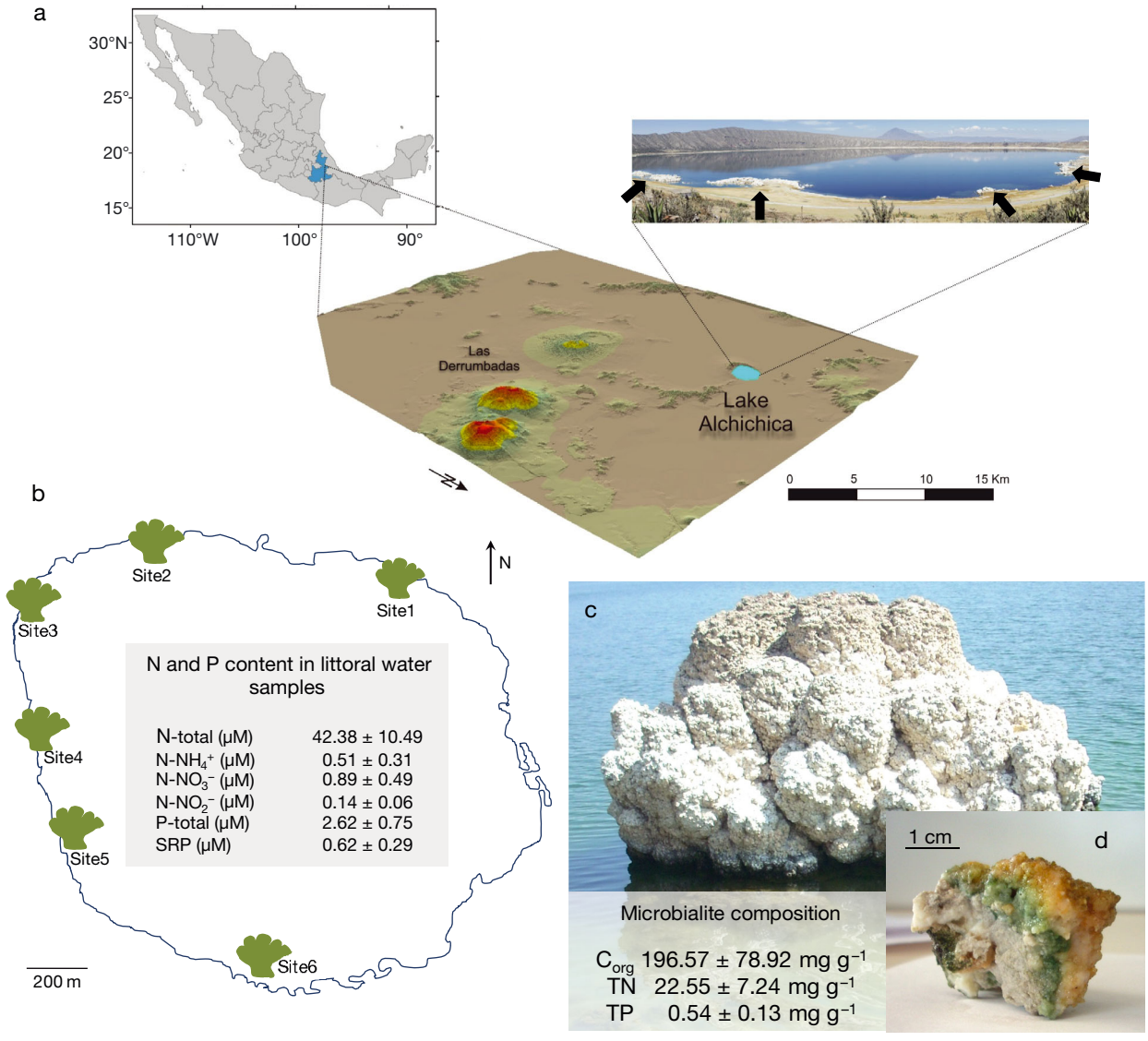

Fig. 1. (a) Geographic location of Lake Alchichica, Mexico. The topographical map shows the changes of elevation in the region. The panoramic photograph (inset) shows the semicontinuous white ring (indicated with arrows) of inactive microbialites above the water level. (b) Collection sites of spongy microbialites and mean values (± SD) of nitrogen (N) and phosphorus (P) concentrations in littoral water samples. (c) Inactive spongy microbialites exposed over the water lake level; inset indicates their mean organic carbon (C_{org}), total N (TN) and total P (TP) content. (d) Transversal section of live spongy microbialite

DNA extraction. Total DNA was extracted and then purified using the method previously described for microbialites by Centeno et al. (2012). The obtained pellets were resuspended in 30 µl molecular-grade water (Sigma Aldrich). A pooled sample of microbialites was used for amplifications with specific primers to explore the genetic diversity associated with N-cycling pathways.

To analyze N-cycle gene expression in microbialites, samples were collected every 6 h setting the initial time at midnight (24:00 h). In this case, 3 subsamples of the spongy microbialite (~5 g) were taken at 24:00, 06:00, 12:00 and 18:00 h, frozen immediately in liquid N_2 (24 h) and stored at –80°C until RNA extraction. For RNA extraction, 6 g of material per site per time were disrupted by freeze–thaw cycles in liquid N_2, together with 2.5 ml of bead solution (Mo Bio Laboratories). The RNA PowerSoil® Total RNA Isolation Kit (Mo Bio Laboratories) was used following the manufacturer's instructions with slight modifications. The obtained pulverized fraction was placed into 15 ml bead

tubes and processed according to instructions. Recovered RNA was further purified using the RNA cleanup protocol of the RNeasy Mini Kit (Qiagen), including a step to remove genomic DNA with DNase I (Qiagen). After DNA hydrolysis, RNA samples per time were pooled, and residual DNA was tested using 2 µl of the eluted material as a template for PCR amplification using *rpoB*-targeted primers (rpoB1698f and rpoB2041r) (Dahllöf et al. 2000). The assay was negative for DNA; thus, cDNA was synthesized immediately by avian myeloblastosis virus reverse transcriptase (Promega) following the manufacturer's protocol. The first-strand cDNA samples were stored at −20°C until analysis.

PCR amplification

Selected N-cycle pathways including N_2 fixation, nitrification, anammox and denitrification were surveyed using *nifH*, *amoA* (bacterial and archaeal), *nxrA*, *nxrB*, *hzo*, *nirK*, *nirS* and *nosZ* genes as molecular markers (Table 1). PCR reactions contained DNA (~10 ng per reaction), 1× ViBuffer A (Vivantis), 0.4 µM each primer, 200 µM of each deoxynucleotide triphosphate, 0.5 µg $µl^{-1}$ BSA (Biolabs) and 1 U of *Taq* DNA polymerase (Vivantis). The concentration of magnesium chloride varied between amplified regions from 1.5 to 2.0 mM (Table 1). The amplification protocol was similar for *amoA*, *nxrA*, *nxrB*, *hzo*,

Table 1. Primers used to survey the nitrogen cycle in crater Lake Alchichica microbialites. $MgCl_2$: magnesium chloride; T_a: annealing temperature

Gene Primer	Sequence 5′ → 3′	Amplicon length (bp)	T_a (°C)	$MgCl_2$ (mM)	Reference
Ammonia monooxygenase (ammonia oxidation, nitrification)					
amoA (Bacterial)					
amoA-1F	GGG GTT TCT ACT GGT GGT	600	56	1.5	Rotthauwe et al. (1997)
amoA-2R	CCC CTC KGS AAA GCC TTC TTC				Rotthauwe et al. (1997)
amoA (Archaeal)					
Arch-amoAF	STA ATG GTC TGG CTT AGA CG	600	53	1.5	Francis et al. (2003)
Arch-amoAR	GCG GCC ATC CAT CTG TAT GT				Francis et al. (2003)
Hydrazine oxidoreductase (anaerobic ammonium oxidation)					
hzo					
hzoF1	TGT GCA TGG TCA ATT GAA AG	1000	53	1.5	Li et al. (2010)
hzoR1	CAA CCT CTT CWG CAG GTG CAT G				Li et al. (2010)
Dinitrogenase reductase, iron protein (nitrogen fixation)					
nifH					
nif4	TTY TAY GGN AAR GGN GG	456	55	2.0	Zani et al. (2000)
nif3	ATR TTR TTN GCN GCR TA				Zani et al. (2000)
nif1	TGT GAT CCT AAA GCT GA	361	55	2.0	Zehr & McReynolds (1989)
nif2	CCT CTT TAC TAC CGT AA				Zehr & McReynolds (1989)
Nitrite oxidoreductase subunits (nitrite oxidation, nitrification)					
nxrB-Nitrospira					
nrxBF14	TGG CAA CTG GGA CGG AAG ATG	1245	58	1.5	Pester et al. (2014)
nxrBR1239	TGT AGA TCG GCT CTT CGA CC				Pester et al. (2014)
nxrA-Nitrobacter					
F1370-F1-nxrA	CAG ACC GAC GTG TGC GAA AG	322	55	1.5	Poly et al. (2008)
F2843-R2-nxrA	TCC ACA AGG AAC GGA AGG TC				Poly et al. (2008)
Copper-dependent nitrite reductase (nitrite reduction, denitrification)					
nirK					
F1aCu	ATC ATG GTS CTG CCG CG	472	57	1.5	Hallin & Lindgren (1999)
R3Cu	GCC TCG ATC AGY TTG TGG TT				Hallin & Lindgren (1999)
Cytochrome cd_1-type nitrite reductase (nitrite reduction, denitrification)					
nirS					
cd3aF	GTS AAC GTS AAG GAR ACS GG	425	51	1.5	Michotey et al. (2000)
R3cd	GAS TTC GGR TGS GTC TTG				Throbäck et al. (2004)
Nitrous oxide reductase (nitrous oxide reduction, denitrification)					
nosZ					
nosZ-F	CGY TGT TCM TCG ACA GCC AG	700	57	1.5	Kloos et al. (2001)
nosZ-R	CAT GTG CAG NGC RTG GCA GAA				Kloos et al. (2001)

nirK, *nosZ* and *nirS* genes. The general PCR program consisted of an initial denaturation step at 95°C for 2 min, followed by 35 cycles of amplification at 95°C (30 s), annealing temperatures depending on the primer pairs (Table 1) (30 s) and at 72°C (60 s), and a final extension step at 72°C (2 min).

Cloning and sequencing

The obtained PCR products were inserted into the pCR®2.1 vector using the original TA Cloning Kit (Invitrogen) following the manufacturer's instructions. Chemically competent *Escherichia coli* DH5α cells were transformed with the constructed vectors, and positive clones were selected by α-complementation on Luria-Bertani plates containing ampicillin (50 µg ml^{-1}) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 1.6 mg spread on the surface). An ABI 3730xl DNA analyzer (Applied Biosystems) was used for sequencing with the M13 region.

Sequence analysis and phylogenetic reconstruction

Nucleotide sequences were translated to amino acids using SeaView software v.4.2.12 (Gouy et al. 2010), and pseudogenes were removed after detecting unexpected stop codons on all 3 reading frames. Putative open reading frames were compared with entries in databases using the standard nucleotide basic local alignment search tool (v.2.2.27) (Zhang et al. 2000). Operational taxonomic units (OTUs) were assigned with mothur (v.1.33.3) using the furthest neighbor algorithm to collapse similar sequences (Schloss et al. 2009). The cut-off level depended on the analyzed gene: 5% nucleotide sequence difference for *nifH*, *amoA*, *nirK* and *nirS* (Francis et al. 2003, Yoshida et al. 2010, Gaby & Buckley 2011); 3% for *nosZ* (Philippot et al. 2013); and 1% for *hzo* (Dang et al. 2013). Only the assigned OTUs were considered for phylogenetic analyses.

Phylogenetic reconstruction involved nucleotide sequence alignment using the translated amino acid configuration to keep the analogous codon positions lined up, using SeaView and ClustalW2 (Larkin et al. 2007, Gouy et al. 2010). Nucleotide alignments were used to construct phylogenetic trees with maximum likelihood in PhyML 3.0 (Guindon et al. 2010). Sequence data were deposited in GenBank under accession numbers KJ967530–KJ967806.

Physicochemical characterization

The physicochemical environment of microbialite-surrounding water was measured *in situ* with a YSI 6600 multiparametric probe. In addition, water samples were taken in clean polypropylene bottles to determine dissolved nutrients and total N and P. All samples were kept in the dark at 4°C (24 h) and frozen prior to analysis. Additionally, samples for nutrients determination were filtered through coupled 0.45 and 0.22 µm membranes. Dissolved N forms (ammonium, nitrate and nitrite) and soluble reactive P (SRP) were photometrically analyzed with a Skalar SanPlus segmented flow autoanalyzer (Skalar Analytical), using adapted standard methods reported by Grasshoff et al. (1983) and the circuits suggested by Kirkwood (1994). Unfiltered water samples were analyzed for total N and P as suggested by Valderrama (1981).

For total elemental analysis in microbialites, a subsample of 1 cm^2 area of each microbialite was excised, lyophilized and ground in an agate mortar. Organic C and total N (TN) contents were determined using a CE Instruments Flash EA 1112 elemental analyzer, after removal of the inorganic C (carbonate) using 1.5 M hydrochloric acid. Total P (TP) was determined by UV spectrometry as molybdate-reactive P, after high-temperature persulfate oxidation.

RESULTS

The littoral water surrounding the microbialites showed low nutrient concentrations in all cases (SRP, 0.62 µM), but particularly for DIN (1.54 µM), exhibiting a 2.5 DIN:SRP ratio. Littoral water TP and TN were more balanced (2.62 and 42.4 µM, respectively), showing a 16.2 TN:TP ratio. Microbialite composition (Fig. 1c) also showed very low N and P contents relative to C (C:N ratio = 8.7, C:P ratio = 364 and N:P ratio = 41.8 in mass).

Genetic diversity associated with the N cycle in Lake Alchichica microbialites

A total of 364 sequences were obtained from metagenomic DNA samples, including *nifH*, bacterial *amoA*, *hzo*, *nirK*, *nirS* and *nosZ* amplicons (Table 2). Archaeal *amoA* and nitrite oxidation genes (*nxrA* and *nxrB*) were not detected in our survey. The largest number of OTUs recovered was for denitrification (*nirK*, *nirS* and *nosZ*), followed by N_2 fixation (*nifH*).

Chemolithotrophic processes such as aerobic ammonia oxidation and anammox showed the lowest number of phylotypes (Table 2).

Ammonia oxidation

Three OTUs were detected for *amoA*. Sequences affiliated to *Nitrosomonas* (*Betaproteobacteria*), distributed within the *Nitrosomonas europaea/mobilis* lineage and the *N. marina* lineage (Fig. 2a). Anammox genetic diversity in Alchichica microbialites was represented by 1 *hzo* OTU, which showed 99% similarity to sequences detected in marine sediments.

Denitrification

Nitrite respiration (*nirK* and *nirS*) recovered 13 and 15 OTUs, respectively. The *nirK* amplicons showed 70 to 85% similarity to environmental sequences from estuaries, water column samples from eutrophic freshwater lakes and lake sediments (Mosier & Francis 2010) and were related to *Rhodobacter sphaeroides*, *Octadecabacter antarcticus* and *Chelativorans* sp. BNC (Fig. 2b,c). The *nirS* sequences from Alchichica microbialites showed 73 to 93% similarity to environmental sequences from sediments and soils and 71 to 85% similarity to isolated strains (Fig. 2c). The OTU with more clones was nirS_OTU1 (44.4%) and sequences that clustered within the same group related to *Marinobacter aquaeolei* (Fig. 2c). Phylotypes nirS_OTU2 and nirS_OTU3 contributed with 31% of the total sequences in the spongy microbialite samples, closely related to isolated *Alphaproteobacteria* such as *Dinoroseobacter shibae* and *Polymorphum gilvum* (78–83% similarity). The genetic diversity associated with nitrous oxide reduction was observed in 16 *nosZ* OTUs, with 75 to 90% identity to reported sequences mainly from coastal marine sediments and isolated strains of the haloalkaliphilic *Thioalkalivibrio sulfidophilus* and the *Alphaproteobacteria D. shibae* and *P. gilvum* (Fig. 2d). OTU nosZ_OTU1 contained almost 51% of the *nosZ* sequences detected.

N fixation

The genetic diversity associated with N_2 fixation (*nifH*) was predominantly from *Cyanobacteria*, with a minor representation of *Proteobacteria* and *Clostridia* (Fig. 3). OTU nifH_OTU1 was the most abundant (50% clones), showing 96% identity with a clone from a periphyton mat affiliated to *Nostocales*. Phylotype nifH_OTU3 was the second most abundant and related to *Alphaproteobacteria*, i.e. *Rhizobium* sp. TJ171 (81% identity). The *nifH* sequences detected also related to environmental clones reported from microbialites of Laguna Bacalar, in the Yucatan Peninsula, Mexico (Beltrán et al. 2012). Only N_2 fixation (*nifH*) RNA transcripts were recovered (Table 2). The OTUs found in the diel expression experiment, shown in Fig. 3, were mostly affiliated to *Nostocales* cyanobacteria.

DISCUSSION

Microbialites have been described as a plethora of microbial metabolisms with large functional diversity supported by autotrophy and diazotrophy (Viss-

Table 2. Number of sequences obtained from different nitrogen-cycle pathways in microbialites of crater Lake Alchichica. DNA amplifications were done from metagenomic DNA extracted; RNA amplifications were done from synthesized cDNA. OTUs: operational taxonomic units

Molecule of study Nitrogen-cycle process	Gene	Sequenced clones	Pseudogenes and chimeras detected	Analyzed sequences	Number of OTUs
DNA					
Nitrogen fixation	*nifH*	37	8	29	8
Aerobic ammonia oxidation	*amoA*	37	0	37	3
Anaerobic ammonia oxidation	*hzo*	40	0	40	1
Denitrification (nitrite respiration)	*nirK*	92	5	87	13
Denitrification (nitrite respiration)	*nirS*	99	0	99	15
Denitrification (nitrous oxide reduction)	*nosZ*	76	4	72	16
Total DNA sequences		381	17	364	
RNA					
Nitrogen fixation	*nifH*	25	4	21	4

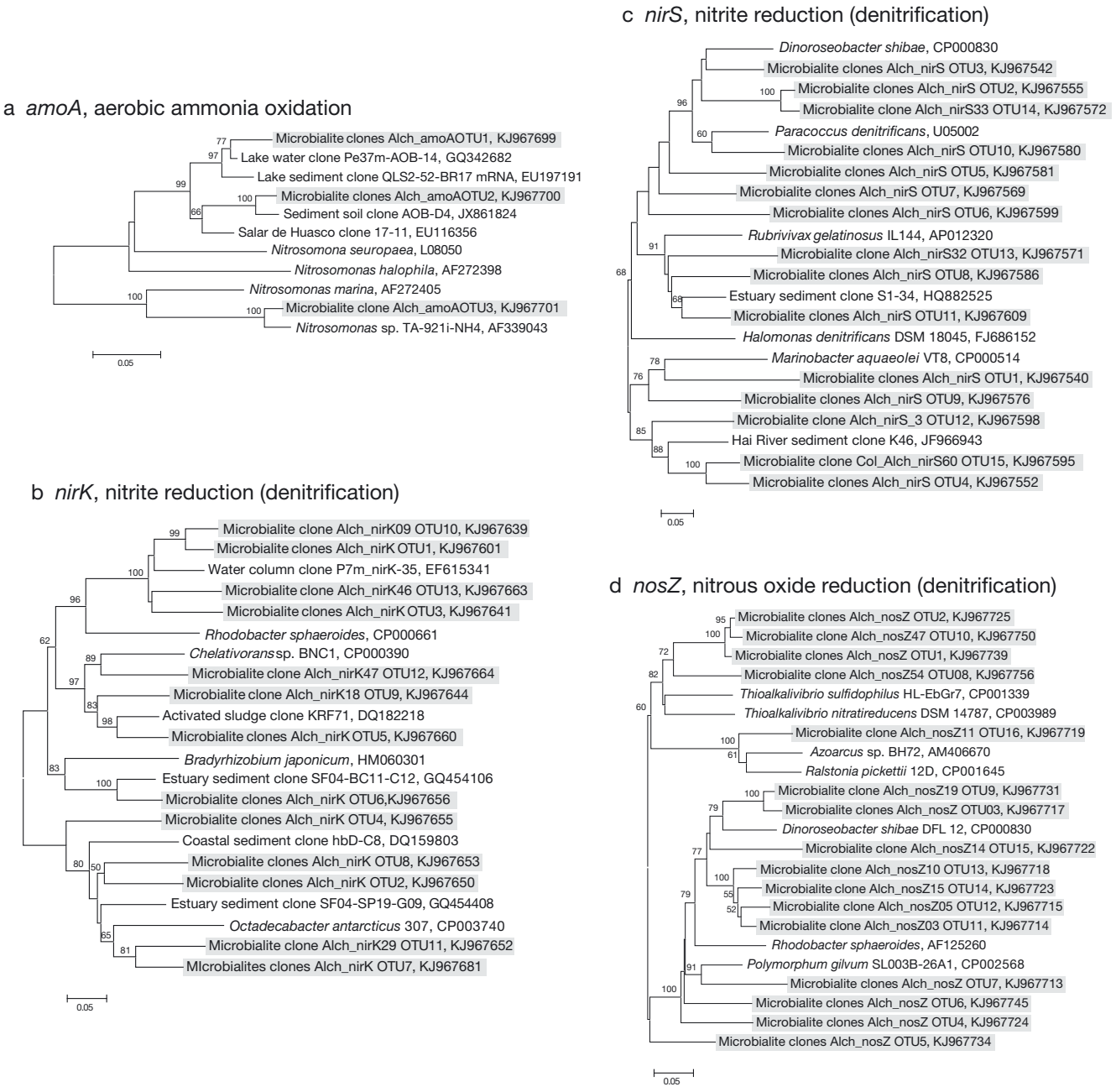

Fig. 2. Maximum likelihood phylogenetic tree for genes involved in nitrogen-cycle pathways in Alchichica microbialites: (a) bacterial *amoA* (378 nucleotides), (b) *nirK* (435 nucleotides), (c) *nirS* (412 nucleotides), (d) *nosZ* (649 nucleotides). Grey-shaded operational taxonomic units represent those obtained in this study. Midpointed maximum likelihood trees with bootstrap values ≥50% are shown (1000 replicates). Divergence is represented by each scale bar

cher & Stolz 2005, Stal 2012). This study represents the overall genetic diversity associated with N cycling in microbialites. As we hypothesized, genes involved in N_2 fixation, ammonia oxidation and denitrification were present in the environmental DNA analyzed, where phylotypes related to denitrification were the most abundant. The relative abundance of phylotypes associated with each N pathway was similar to that reported in previous metagenomic studies on microbialites from Cuatro Cienegas, Mexico (Breitbart et al. 2009); however, these authors did not find genes associated with nitrification or anammox.

The ability to use N oxides as electron acceptors is a widely spread feature in *Bacteria* and *Archaea* and has been detected as a major functional capacity in microbialites (Breitbart et al. 2009, Mobberley et al. 2013) and microbial mats (Desnues et al. 2007, Peim-

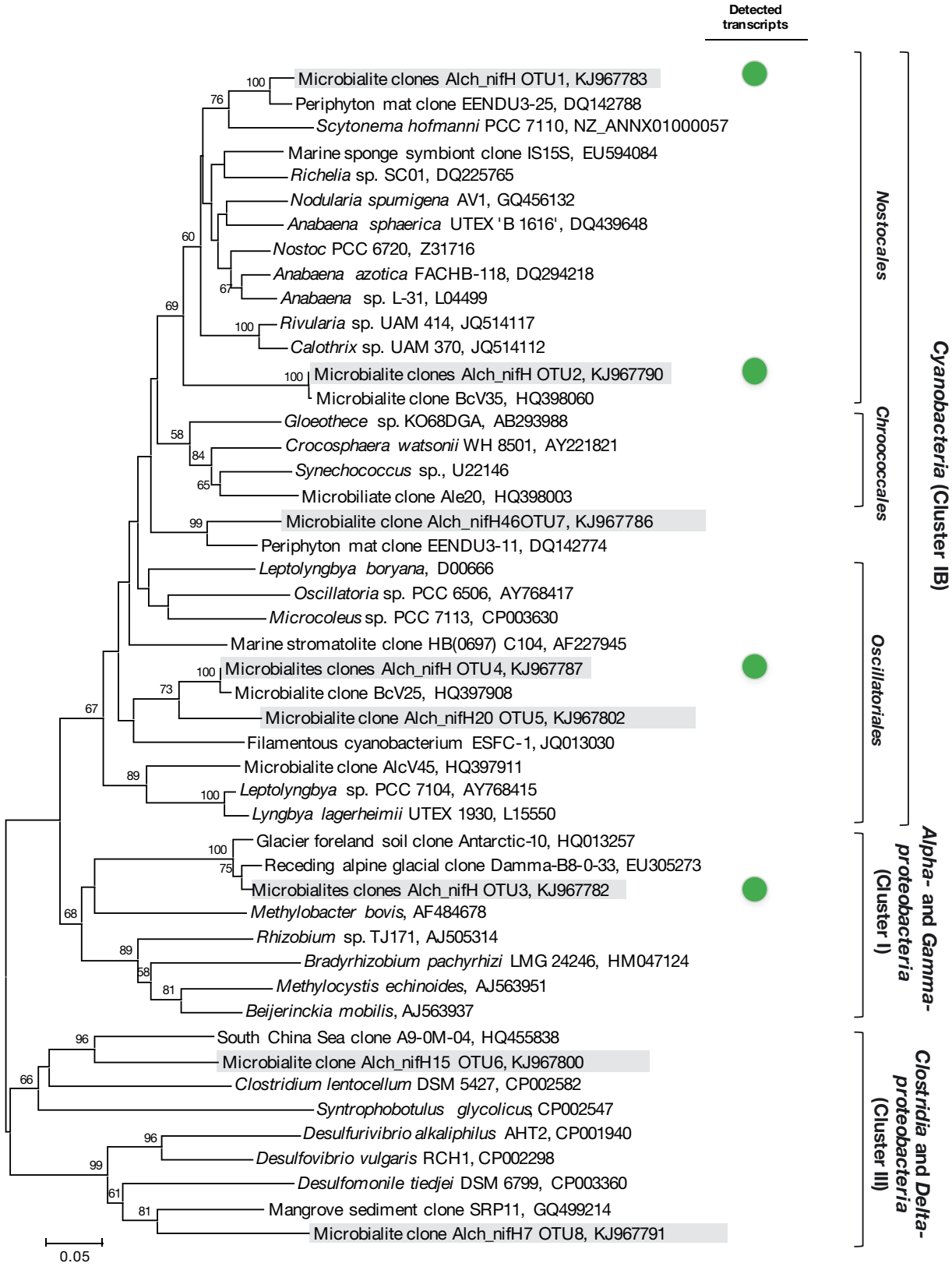

Fig. 3. Midpointed neighbor-joining (Kimura 2-parameter) tree based on *nifH* gene partial sequences (315 nucleotides); bootstrap values >50% are shown (1000 replicates). The scale bar represents 5% divergence. Phylotypes recovered from DNA are highlighted in grey, and the green circles indicate those recovered in the gene expression (RNA) diel experiment

bert et al. 2012, Alcántara-Hernández et al. 2014, Fan et al. 2015). Denitrification occurs mainly under microaerophilic or anaerobic conditions, such as those present in the lower portions of the microbialite structure, but was not detectable with transcripts for either nitrite respiration (*nirK* and *nirS*) or nitrous oxide reduction (*nosZ*). Visscher & Stolz (2005) have suggested the coupling of sulfide oxidization to denitrification during nighttime. However, in microbial mats under N-limited systems, low denitrification rates have been observed, which can nevertheless increase significantly if nitrate is added (Joye & Paerl 1994, Stal 2003, Joye & Lee 2004, Fan et al. 2015). In Alchichica microbialites, the N-limited conditions and the physicochemical environment might favor the assimilation of N oxides including nitrate and nitrite despite the large genetic diversity associated with denitrification.

The aerobic and anaerobic oxidation of ammonia pathways had the lowest diversity. Previous metagenomic studies had reported the lack of nitrification genes in oncolites from Cuatro Cienegas Basin in northern Mexico (Breitbart et al. 2009), while a few nitrification genes were detected in thrombolitic mats from Highborne Cay, Bahamas (Mobberley et al. 2013). The low diversity and abundance associated with ammonia oxidation in microbialites from Lake Alchichica could relate to long replication times of chemolithotrophs and limitation in saline (alkaline) environments due to bioenergetic constraints (Sorokin & Kuenen 2005, Oren 2011). In other studies, small numbers of ammonia-oxidizing phylotypes have been found in shallow soda pools of eastern Austria (Hornek et al. 2006) and in water column samples from Mono Lake (Ward et al. 2000). Aerobic ammonia oxidation by *Thaumarchaeota* was also verified using *amoA*-targeted primers (Francis et al. 2005), but no PCR product was detected for any sample with the methodology here employed, possibly since microbialites are known to harbor low amounts of *Archaea* (Centeno et al. 2012). Ammonia monooxygenase genes from *Candidatus* Nitrosopumilus maritimus have been detected in other thrombolites (Breitbart et al. 2009, Couradeau et al. 2011, Centeno et al. 2012, Khodadad & Foster 2012, Mobberley et al. 2013).

Alchichica microbialite *hzo* sequences related to environmental sequences from sediments of the Bohai and South China seas (Dang et al. 2013, Li et al. 2013) and clustered within the *Scalindua*-like cluster I, a clade composed exclusively of environmental sequences detected in bay and oceanic sediments (Dang et al. 2013, Li et al. 2013). The detection of an anammox phylotype was possible with the *hzo* gene-targeted method here employed, although further studies must be done to understand the relevance of this process in microbialites.

N_2 fixation is an important process carried out in microbial mats and microbialites, as many of them inhabit oligotrophic systems (Severin et al. 2010, Stal 2012). Beltrán et al. (2012) suggested that cyanobacteria were the most relevant diazotrophs in Alchichica microbialites, followed in minor proportion by *Alphaproteobacteria*, sharing an analogous composition to marine microbialites (Steppe et al. 2001) and microbial mats (Zehr et al. 1995). The detection of *nifH* mRNA sequences in this study is consistent with previous observations of high daytime nitrogenase activity in Alchichica's microbialites (Falcón et al. 2002, Beltrán et al. 2012). A minor number of *nifH* OTUs were recovered from transcripts (RNA), suggesting only a small set of active N_2 fixers (Fig. 3); this pattern has also been observed in microbial mats (Moisander et al. 2006, Woebken et al. 2012). The most active nifH_OTU2 and nifH_OTU1 phylotypes clustered within *Nostocales* and showed respectively 85 and 89% similarity with *Anabaena* spp., while phylotype nifH_OTU4 had 84% identity with *Leptolyngbya* sp. PCC 7104 and with Elkhorn Slough Filamentous Cyanobacterium-1 (ESFC-1) (Woebken et al. 2012). *Nostocales* (filamentous heterocystous) and *Oscillatoriales* (filamentous nonheterocystous) have been described as the most abundant cyanobacteria in Alchichica microbialites (Tavera & Komárek 1996, Kaźmierczak et al. 2011) and in other nonlithifying and lithifying mats (Severin et al. 2010, Khodadad & Foster 2012). Furthermore, microbial mats dominated by heterocystous cyanobacteria have shown high nitrogenase activity during daytime (Falcón et al. 2002, Charpy et al. 2007, Severin et al. 2010), since nitrogenase activity can be coupled to photosynthesis, avoiding oxygen inhibition through spatial separation of N_2 fixation in the heterocysts (Stal 1995, Staal et al. 2002).

Lake Alchichica microbialites contain a large genetic diversity associated with N cycling, where N_2 fixation is the most active pathway and is mainly driven by heterocystous cyanobacteria. Cyanobacteria play an important role in microbialites for N acquisition, in addition to CO_2 drawdown via photosynthesis for biomass, extracellular polymeric substances formation and carbonate mineral precipitation. Tavera & Komárek (1996) identified heterocystous and unicellular cyanobacteria in microbialites of Lake Alchichica including *Aphanocapsa* sp., *Calothrix* sp., *Chroococcus* sp. and *Rivularia* sp. There are other microorganisms

in charge of transforming different N forms by nitrification, anammox and denitrification, but these processes might be occurring at low rates.

Our nutrient data are consistent with previous observations of N limitation in the water column (Ramírez-Olvera et al. 2009, Ardiles et al. 2012), and the microbialite composition (C:N ratio = 8.7) we found suggests that N also limits microbial activity in Alchichica's microbialites. It is reasonable to consider that nitrate concentration in the water surrounding the microbialites differs from that in the microsites of the biogenic structure, making denitrification possible in the microoxic–anoxic interfaces. However, mineralization might be small compared to N_2 fixation, driven by cyanobacterial photosynthetic activity and the N demands of the system. This study might not reflect effectively all denitrifiers in the system since the genetic region amplified misrepresents *Deltaproteobacteria*, *Epsilonproteobacteria* and *Verrucomicrobia* (Sanford et al. 2012).

Another relevant feature of the N-cycling dynamics in the water column of Lake Alchichica is the seasonal — and regular — bloom of *Nodularia* spp. by the onset of the stratification period (June–October) (Oliva et al. 2009). The massive growth of this cyanobacterium has been reported as an important source of fixed N to the system, diminishing N_2 fixation rates of microbialites after the bloom period (Falcón et al. 2002). Notwithstanding this N relief, the diazotrophic activity of microbialites is detected before, during and after the bloom. It might be possible that other pathways of the N cycle, such as nitrification, anammox and denitrification, also exhibit seasonal variations, changing N-cycling genetic diversity in time (as was described for P utilization genes in microbialites and bacterioplankton within the system, see Valdespino-Castillo et al. 2016). However, further studies must be done to address this issue.

The low relative content of both N and P found in the microbialites (C:N ratio = 8.7, C:P ratio = 364) is likely due to intense internal recycling (Valdespino-Castillo et al. 2016) of these 2 elements within the microbial community as compared to C, which may be left behind to contribute to the accretion of these organo-sedimentary structures. Therefore, the much higher N:P ratios found within the microbialites (N:P ratio = 41.8 and 92.5 for mass and molar ratios, respectively) relative to both the TN:TP ratio (16.2) and DIN:SRP ratio (2.5) in the water column could simply be the result of a much higher effectiveness of N_2 fixation within the microbialite community as compared to the water column community, which would help explain the seasonal *Nodularia* spp. bloom in Lake Alchichica (Falcón et al. 2002). Although genes for the entire N cycle were present in Alchichica microbialites, in this study we only found the expression of the N_2 fixation pathway.

CONCLUSIONS

In this study, we analyzed microbialites from Lake Alchichica to understand the genetic diversity associated with N cycling. It was apparent that the potential for N_2 fixation, ammonia oxidation, anammox and denitrification is present in Lake Alchichica microbialites. The most active pathway is N_2 fixation, where heterocystous cyanobacteria play an important role.

Acknowledgements. We thank L. A. Oseguera and the Proyecto de Investigación en Limnología Tropical (FES Iztacala, UNAM) for fieldwork support in Lake Alchichica. We also gratefully acknowledge O. Gaona, A. Cruz-Peralta and F. S. Castillo-Sandoval for valuable technical support. C.M.C. and P.M.V.C. received postdoctoral scholarships from Ciencias Biológicas, IPN, and UC MEXUS. All samples were collected under collector permit No. PPF/DGOPA.033/2013 (L.I.F.). This work was supported by grants awarded to L.I.F. (SEP-CONACyT No. 0151796 and PAPIIT-UNAM No. IN202016) and R.J.A.H. (PAPIIT-UNAM No. IA209516). The authors state no conflicts of interest.

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

Submitted: May 24, 2016; Accepted: November 4, 2016
Proofs received from author(s): January 19, 2017