Lawrence Berkeley National Laboratory

Recent Work

Title

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

Permalink

<https://escholarship.org/uc/item/7d86c521>

Journal

Aquatic Microbial Ecology, 78(2)

ISSN

0948-3055

Authors

Alcántara-Hernández, RJ
Valdespino-Castillo, PM
Centeno, CM
et al.

Publication Date

2016

DOI

10.3354/ame01806

Peer reviewed

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

Rocio J. Alcántara-Hernández¹, Patricia M. Valdespin-O-Castillo², Carla M. Centeno³, Javier Alcocer⁴, Martín Merino-Ibarra⁵, Luisa I. Falcón^{2,*}

¹Instituto de Geología, Universidad Nacional Autónoma de México, Ciudad de México 04510, Mexico

²Laboratorio

¹Instituto de Ecología Bacteriana, Instituto de Ecología, Universidad Nacional Autónoma de México, Ciudad de México 04510, Mexico

³Departamento de Zoología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Ciudad de México 11340, Mexico

⁴Proyecto de Investigación en Limnología Tropical, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, Tlalnepantla, Estado de México 54090, Mexico

⁵Unidad Académica de Ecología Marina, Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México, Ciudad de México 04510, Mexico

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 N₂ 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 N₂ fixation, ammonia oxidation, anammox and denitrification was present in the microbialites of Lake Alchichica, the most transcribed pathway was N₂ fixation.

KEY WORDS: Microbialites · N cycle · N₂ fixation · *Cyanobacteria* · Heterocyst

spaceINTRODU CTION

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

*Corresponding author:
falcon@ecologia.unam.mx

spaceal. 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

communities for which fossil records exist, hosting bacteria that played an essential role in atmospheric evolution and planetary biogeochemistry (van Gernerden 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, Kazmierczak 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 time-scales, fixed N has been proposed to restrict primary productivity (Falkowski 1997). The N₂ fixation process constitutes an important source of N input into biomass from atmospheric N₂ (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 N₂ 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 — Mg₅(CO₃)₄(OH)₂·4H₂O (Kazmierczak 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 N₂ 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, Kazmierczak et al. 2011).

space

space record of life (Krumbein 1983). Therefore, these benthic biostructures can be considered one of the first successfully organized

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

space

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

spaceDNA 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 sub-

spacesamples of the spongy microbialite (~5 g) were taken at 24:00, 06:00, 12:00 and 18:00 h, frozen immediately in liquid N₂ (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 N₂, 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

space

spacetubes 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 (*rpoB*1698f and *rpoB*2041r) (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.

spacePCR amplification

Selected N-cycle pathways including N₂ 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*,

space

Table 1. Primers used to survey the nitrogen cycle in crater Lake

			Alchichica microbialites.
			MgCl ₂ : magnesium chloride; T _a : annealing temperature
Gene	Sequence 5'–3'		Amplicon length (bp)
Primer			
Ammonia monooxygenase (ammonia oxidation, nitrification)			
<i>amoA</i> (Bacterial)			
amoA-1F	GGG GTT TCT ACT GGT GGT		600
amoA-2R	CCC CTC KGS AAA GCC TTC TTC		
<i>amoA</i> (Archaeal)			
Arch-amoAF	STA ATG GTC TGG CTT AGA CG		600
Arch-amoAR	GCG GCC ATC CAT CTG TAT GT		
Hydrazine oxidoreductase (anaerobic ammonium oxidation)			
<i>hzo</i>			
hzoF1	TGT GCA TGG TCA ATT GAA AG		1000
hzoR1	CAA CCT CTT CWG CAG GTG CAT G		
Dinitrogenase reductase, iron protein (nitrogen fixation)			
<i>nifH</i>			
nif4	TTY TAY GGN AAR GGN GG		456
nif3	ATR TTR TTN GCN GCR TA		
nif1	TGT GAT CCT AAA GCT GA		361
nif2	CCT CTT TAC TAC CGT AA		
Nitrite oxidoreductase subunits (nitrite oxidation, nitrification)			
<i>nxrB</i> - <i>Nitrospira</i>			
nrxBF14	TGG CAA CTG GGA CGG AAG ATG		1245
nrBR1239	TGT AGA TCG GCT CTT CGA CC		
<i>nxrA</i> - <i>Nitrobacter</i>			
F1370-F1-nxrA	CAG ACC GAC GTG TGC GAA AG		322
F2843-R2-nxrA	TCC ACA AGG AAC GGA AGG TC		
Copper-dependent nitrite reductase (nitrite reduction, denitrification)			
<i>nirK</i>			
F1aCu	ATC ATG GTS CTG CCG CG		472
R3Cu	GCC TCG ATC AGY TTG TGG TT		
Cytochrome cd₁-type nitrite reductase (nitrite reduction, denitrification)			
<i>nirS</i>			

cd3aF	GTS AAC GTS AAG GAR ACS GG
R3cd	GAS TTC GGR TGS GTC TTG
Nitrous oxide reductase (nitrous oxide reduction, denitrification)	
<i>nosZ</i>	
nosZ-F	CGY TGT TCM TCG ACA GCC AG
nosZ-R	CAT GTG CAG NGC RTG GCA GAA

space

space*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 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 cm² 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 N₂ fixation (*nifH*).

space

spaceChemolithotrophic 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 *Polymor-*

spacephum 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 N₂ 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 N₂ 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-

space

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

DNA

Nitrogen fixation

Aerobic ammonia oxidation

Anaerobic ammonia oxidation

Denitrification (nitrite respiration)

Denitrification (nitrite respiration)

Denitrification (nitrous oxide reduction)

Total DNA sequences

RNA

Nitrogen fixation

space

amoA

space Microbialite clones Alch_nirS OTU11, KJ967609

Halomonas denitrificans DSM 18045, FJ686152*Marinobacter aquaeolei* VT8, CP000514

Microbialite clones Alch_nirS OTU1, KJ967540

Microbialite clones Alch_nirS OTU9, KJ967576

Microbialite clone Alch_nirS_3 OTU12, KJ967598 Hai River sediment clone

K46, JF966943

Microbialite clone Col_Alch_nirS60 OTU15, KJ967595

space *b nirK*, nitrite reduction (denitrification)

space Microbialite clones Alch_nirS OTU4, KJ967552

space

c nirS, nitrite reduction (denitrification)*Dinoroseobacter shibae*

Microbialite clones Alch_nirS OTU10, KJ967639

Microbialite clones Alch_nirK OTU1, KJ967601 Water column clone P7m_nirK-35, EF615341

Microbialite clone Alch_nirK46 OTU13, KJ967663

Microbialite clones Alch_nirK OTU3, KJ967641

space

d nosZ, nitrous oxide reduction (denitrification)

Microbialite clones Alch_nosZ OTU2, KJ967725

Rhodobacter sphaeroides, CP000661*Chelativorans* sp. BNC1, CP000390

Microbialite clone Alch_nirK47 OTU12, KJ967664

Microbialite clone Alch_nirK18 OTU9, KJ967644

Microbialite clone Alch_nosZ47 OTU10, KJ967750

Microbialite clones Alch_nosZ OTU1, KJ967739

Microbialite clone Alch_nosZ54 OTU08, KJ967756

Thioalkalivibrio sulfidophilus HL-EbGr7, CP001339

Activated sludge clone KRF71, DQ182218

Thioalkalivibrio nitratireducens DSM 14787, CP003989

Microbialite clones Alch_nirK OTU5, KJ967660

Bradyrhizobium japonicum, HM060301

Estuary sediment clone SF04-BC11-C12, GQ454106

Microbialite clones Alch_nirK OTU6, KJ967656

Microbialite clones Alch_nirK OTU4, KJ967655 Coastal sediment clone hbD-C8, DQ159803

space

space

space

space

space

space

space

space

space

space

space

space

space

space

space *a amoA*, aerobic ammonia oxidation

space

space Microbialite clones Alch_nirS OTU2, KJ967555

Microbialite clone Alch_nirS33 OTU14, KJ967572

space

space

space

space

space

space

space

space

space

space

space

space

space

space

space

space

space

space

space

space

space

Microbialite clones Alch_amoAOTU1, KJ967699

Lake water clone Pe37m-AOB-14, GQ342682

Lake sediment clone QLS2-52-BR17 mRNA, EU197191

Microbialite clones Alch_amoAOTU2, KJ967700

Sediment soil clone AOB-D4, JX861824

Salar de Huasco clone 17-11, EU116356

Nitrosomonas seuropaea, L08050*Nitrosomonas halophila*, AF272398*Nitrosomonas marina*, AF272405*Paracoccus denitrificans*, U05002

Microbialite clones Alch_nirS OTU10, KJ967580

Microbialite clones Alch_nirS OTU5, KJ967581

Microbialite clones Alch_nirS OTU7, KJ967569

Microbialite clones Alch_nirS OTU6, KJ967599

Rubrivivax gelatinosus IL144, AP012320

Microbialite clone Alch_nirS32 OTU13, KJ967571

Microbialite clones Alch_nirS OTU8, KJ967586

Estuary sediment clone S1-34, HQ882525

Microbialite clone Alch_amoAOTU3, KJ967701

Nitrosomonas sp. TA-921i-NH4, AF339043

Microbialite clone Alch_nosZ11 OTU16, KJ967719

Azoarcus sp. BH72, AM406670*Ralstonia pickettii* 12D, CP001645

Microbialite clone Alch_nosZ19 OTU9, KJ967731

Microbialite clones Alch_nosZ OTU03, KJ967717

Dinoroseobacter shibae DFL 12, CP000830

Microbialite clone Alch_nosZ14 OTU15, KJ967722

Microbialite clone Alch_nosZ11 OTU16, KJ967719

Azoarcus sp. BH72, AM406670*Ralstonia pickettii* 12D, CP001645

Microbialite clone Alch_nosZ19 OTU9, KJ967731

Microbialite clones Alch_nosZ OTU03, KJ967717

Dinoroseobacter shibae DFL 12, CP000830

Microbialite clone Alch_nosZ14 OTU15, KJ967722

Microbialite clone Alch_nosZ11 OTU16, KJ967719

Azoarcus sp. BH72, AM406670*Ralstonia pickettii* 12D, CP001645

Microbialite clone Alch_nosZ19 OTU9, KJ967731

Microbialite clones Alch_nosZ OTU03, KJ967717

Dinoroseobacter shibae DFL 12, CP000830

Microbialite clone Alch_nosZ14 OTU15, KJ967722

Microbialite clone Alch_nosZ11 OTU16, KJ967719

Azoarcus sp. BH72, AM406670*Ralstonia pickettii* 12D, CP001645

Microbialite clone Alch_nosZ19 OTU9, KJ967731

Microbialite clones Alch_nosZ OTU03, KJ967717

Dinoroseobacter shibae DFL 12, CP000830

Microbialite clone Alch_nosZ14 OTU15, KJ967722

Microbialite clone Alch_nosZ11 OTU16, KJ967719

Azoarcus sp. BH72, AM406670*Ralstonia pickettii* 12D, CP001645

Microbialite clone Alch_nosZ19 OTU9, KJ967731

Microbialite clones Alch_nosZ OTU03, KJ967717

Dinoroseobacter shibae DFL 12, CP000830

Microbialite clone Alch_nosZ14 OTU15, KJ967722

Microbialite clone Alch_nosZ11 OTU16, KJ967719

Azoarcus sp. BH72, AM406670*Ralstonia pickettii* 12D, CP001645

Microbialite clone Alch_nosZ19 OTU9, KJ967731

space

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 trees with bootstrap values $\geq 50\%$ are shown (1000 replicates). Divergence is represented by each scale bar

spacecher & 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

spacemetagenomic 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-

space

space

spaceMicrobialite clones Alch_nifH OTU1, KJ967783

Periphyton mat clone

EENDU3-25, DQ142788

Scytonema hofmanni PCC 7110, NZ_ANNX01000057

Marine sponge symbiont clone IS15S, EU594084

Richelia sp. SC01, DQ225765

Nodularia spumigena AV1, GQ456132

Anabaena sphaerica UTEX 'B 1616', DQ4396

Nostoc PCC 6720, Z31716

Anabaena azotica FACHB-118, DQ294218

Anabaena sp. L-31, L04499

Rivularia sp. UAM 414, JQ514117

Calothrix sp. UAM 370, JQ514112

spa

c

e

1

0

0

M

i

c

r

o

b

N
o
s
t
o
cC
h
r
o
c
oC
y
a
n
o
b
a
c
t
e
r
i
a
(C

		B
a		c
l		V
i		3
t		5
e		,
c		H
l		Q
o		3
n		9
e		8
s		0
		6
		0
A	<i>Gloeotheca</i> sp. KO68DGA, AB293988	
l	⁵⁸	
c	⁸⁴ AY221821	<i>Crocospaera watsonii</i> WH 8501,
h	⁶⁵	<i>Synechococcus</i> sp., U22146
–	99	Microbiliate clone Ale20, HQ398003
n		
i		
f		
H		
O		
T		
U		
2		
,		
K		
J		
9		
6		
7		
7		
9		
0		
M		
i		
c		
r		
o		
b		
i		
a		
l		
i		
t		
e		
c		
l		
o		
n		
e		

M
i
c
r
o
b
i
a
l
i
t
ec
l
o
n
eA
l
c
h
–
n
i
f
H
4
6
O
T
U
7
,K
J
9
6
7
7

8		a
6		,
P		D
e		0
r		0
i		6
p		6
h		6
y		
t		<i>Oscillatoria</i> sp. PCC 6506,
o		AY768417
n		<i>Microcoleus</i> sp. PCC 7113, CP003630
	Marine stromatolite clone HB(0697) C104,	O
	AF227945	sc
m		100 Microbialites clon
a		KJ967787
t	67	73
		Microbialite clon
		ill
		at
		or
		ia
		BcV25, HQ397908
c		
l		Mi
o		cr
n		ob
e		iali
		te
E		cl
E		on
N		e
D		Al
U		ch
3		_n
-		ifH
1		20
1		O
,		T
		U
D		5,
Q		KJ
1		96
4		78
2		02
7		Fil
7		a
4		m
		en
L		to
e		us
p		cy
t		an
o		ob
l		ac
y		ter
n		iu
g		m
b		E
y		S
a		F
		C-
b		1,
o		J
r		Q
y		01
a		30
n

o
b
i
a
l
i
t
e

clone

A
l
c
h
-
n
i
f
H
1
5

O
T
U
6
,

K
J
9
6
7
8
0
0

66

Clostridium lentocellum DSM
5427, CP002582

Syntróp

96
AHT2, CP001940

99
CP002298

61
CP003360

81

M
a
n
g
r
o
v
e

sedimentation

SRP 11, GQ 499214

Microbiolite

C
I

hobotulus glycolicus,
CP002547

Desulfurivibrio alkaliphilus

Desulfovibrio vulgaris RCH1,

Desulfomonile tiedjei DSM 6799,

Fig. 3. Midpointed neighbor-joining (Kimura 2-parameter) tree based on *nifH* gene partial sequences (315 nucleotides); boot-strap 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

space

spacebert 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 Ciénegas 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

space 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.

N₂ 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 N₂ 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, Kazmierczak 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

space

space 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.

spacebloom 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 N₂ 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 N₂ fixation, ammonia oxidation, anammox and denitrification is present in Lake Alchichica microbialites. The most active pathway is N₂ fixation, where heterocystous cyanobacteria play an important role.

Acknowledgements. We thank L. A. Oseguera and the Proyecto de Investigación en Limnología Tropical (FES Izta- cala, 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.

LITERATURE CITED

- Adame MF, Alcocer J, Escobar E (2008) Size fractionated phytoplankton biomass and its implications for the dynamics of an oligotrophic tropical lake. *Freshw Biol* 53: 22–31
- Alcántara-Hernández RJ, Centeno CM, Ponce-Mendoza A, Batista S, Merino-Ibarra M, Campo J, Falcón LI (2014) Characterization and comparison of potential denitrifiers in microbial mats from King George Island, Maritime Antarctica. *Polar Biol* 37:403–416
- Ardiles V, Alcocer J, Vilaclara G, Oseguera LA, Velasco L (2012) Diatom fluxes in a tropical, oligotrophic lake dominated by large-sized phytoplankton. *Hydrobiologia* 679: 77–90
- Armienta MA, Vilaclara G, la Cruz-Reyna De S, Ramos S and others (2008) Water chemistry of lakes related to active and inactive Mexican volcanoes. *J Volcanol Geotherm Res* 178:249–258
- Beltrán Y, Centeno CM, García-Oliva F, Legendre P, Falcón LI (2012) N₂ fixation rates and associated diversity (*nifH*) of microbialite and mat-forming consortia from different aquatic environments in Mexico. *Aquat Microb Ecol* 67: 15–24
- Berelson WM, Corsetti FA, Pepe-Ranney C, Hammond DE, Beaumont W, Spear JR (2011) Hot spring siliceous stromatolites from Yellowstone National Park: assessing growth rate and laminae formation. *Geobiology* 9: 411–424
- Breitbart M, Hoare A, Nitti A, Siefert J and others (2009) Metagenomic and stable isotopic analyses of modern freshwater microbialites in Cuatro Ciénegas, Mexico. *Environ Microbiol* 11:16–34
- Burne RV, Moore LS (1987) Microbialites: organosedimentary deposits of benthic microbial communities. *Palaos* 2:241–254
- Caballero M, Vilaclara G, Rodríguez A, Juárez D (2003) Short-term climatic change in lake sediments from lake Alchichica, Oriental, Mexico. *Geofis Int* 42:529–537
- Canfield DE, Glazer AN, Falkowski PG (2010) The evolution and future of Earth's nitrogen cycle. *Science* 330: 192–196
- Centeno CM, Legendre P, Beltrán Y, Alcántara-Hernández RJ, Lidstroem UE, Ashby MN, Falcón LI (2012) Microbialite genetic diversity and composition relate to environmental variables. *FEMS Microbiol Ecol* 82:724–735
- Cerqueda-García D, Falcón LI (2016) Metabolic potential of microbial mats and microbialites: autotrophic capabilities described by an in silico stoichiometric approach from shared genomic resources. *J Bioinform Comput Biol* 14:1650020
- Charpy L, Alliod R, Rodier M, Golubic S (2007) Benthic nitrogen fixation in the SW New Caledonia lagoon. *Aquat Microb Ecol* 47:73–81
- Charpy L, Palinska KA, Casareto B, Langlade MJ, Suzuki Y, Abed RMM, Golubic S (2010) Dinitrogen-fixing cyanobacteria in microbial mats of two shallow coral reef ecosystems. *Microb Ecol* 59:174–186
- Cooper JAG, Smith AM, Arnscheidt J (2013) Contemporary stromatolite formation in high intertidal rock pools, Giant's Causeway, Northern Ireland: preliminary observations. *J Coast Res* 65:1675–1680
- Couradeau E, Benzerara K, Moreira D, Gérard E, Kazmierczak J, Tavera R, López-García P (2011) Prokaryotic and eukaryotic community structure in field and cultured microbialites from the alkaline Lake Alchichica (Mexico). *PLOS ONE* 6:e28767
- Dahllöf I, Baillie H, Kjelleberg S (2000) *rpoB*-based microbial community analysis avoids limitations inherent in 16S rRNA gene intraspecific heterogeneity. *Appl Environ Microbiol* 66:3376–3380

- ✦ [Dang H, Zhou H, Zhang Z, Yu Z, Hua E, Liu X, Jiao N \(2013\)](#) Molecular detection of *Candidatus Scalindua pacifica* and environmental responses of sediment anammox bacterial community in the Bohai Sea, China. *PLOS ONE* 8:e61330
- ✦ [Decho AW, Visscher PT, Reid RP \(2005\)](#) Production and cycling of natural microbial exopolymers (EPS) within a marine stromatolite. *Palaeogeogr Palaeoclimatol Palaeoecol* 219:71–86
- ✦ [Desnues C, Michotey VD, Wieland A, Zhizang C, Fourcans A, Duran R, Bonin PC \(2007\)](#) Seasonal and diel distributions of denitrifying and bacterial communities in a hypersaline microbial mat (Camargue, France). *Water Res* 41:3407–3419
- ✦ [Falcón LI, Escobar-Briones E, Romero D \(2002\)](#) Nitrogen fixation patterns displayed by cyanobacterial consortia in Alchichica crater-lake, Mexico. *Hydrobiologia* 467:71–78
- ✦ [Falcón LI, Cerritos R, Eguiarte LE, Souza V \(2007\)](#) Nitrogen fixation in microbial mat and stromatolite communities from Cuatro Ciénegas, Mexico. *Microb Ecol* 54:363–373
- ✦ [Falkowski PG \(1997\)](#) Evolution of the nitrogen cycle and its influence on the biological sequestration of CO₂ in the ocean. *Nature* 387:272–275
- space ✦ [Fan H, Bolhuis H, Stal LJ \(2015\)](#) Denitrification and the denitrifier community in coastal microbial mats. *FEMS Microbiol Ecol* 91:fiu033
- ✦ [Francis CA, O'Mullan GD, Ward BB \(2003\)](#) Diversity of ammonia monooxygenase *amoA* genes across environmental gradients in Chesapeake Bay sediments. *Geobiology* 1:129–140
- ✦ [Francis CA, Roberts KJ, Beman JM, Santoro AE, Oakley BB \(2005\)](#) Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proc Natl Acad Sci USA* 102:14683–14688
- ✦ [Gaby JC, Buckley DH \(2011\)](#) A global census of nitrogenase diversity. *Environ Microbiol* 13:1790–1799

García E (1988) Distribución de los grupos climáticos de Köppen en México. Instituto de Geografía, Universidad Nacional Autónoma de México

- ✦ [Gouy M, Guindon S, Gascuel O \(2010\)](#) SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol Biol Evol* 27:221–224
- Grasshoff K, Ehrhardt M, Kremling K (1983) Methods of seawater analysis, 2nd edn. Verlag Chemie, Weinheim
- ✦ [Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O \(2010\)](#) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol* 59:307–321
- ✦ [Hallin S, Lindgren PE \(1999\)](#) PCR detection of genes encoding nitrite reductase in denitrifying bacteria. *Appl Environ Microbiol* 65:1652–1657
- ✦ [Hornek R, Pommerening-Röser A, Koops HP, Farnleitner AH, Kreuzinger N, Kirschner A, Mach RL \(2006\)](#) Primers containing universal bases reduce multiple *amoA* gene specific DGGE band patterns when analysing the diversity of beta-ammonia oxidizers in the environment. *J Microbiol Methods* 66:147–155
- Joye SB, Lee RY (2004) Benthic microbial mats: important sources of fixed nitrogen and carbon to the Twin Cays, Belize ecosystem. *Atoll Res Bull* 528:1–24
- ✦ [Joye SB, Paerl HW \(1994\)](#) Nitrogen cycling in microbial mats: rates and patterns of denitrification and nitrogen fixation. *Mar Biol* 119:285–295
- ✦ [Kazmierczak J, Kempe S, Kremer B, López-García P, Moreira D, Tavera R \(2011\)](#) Hydrochemistry and microbialites of the alkaline crater lake Alchichica, Mexico. *Facies* 57:543–570
- ✦ [Khodadad CLM, Foster JS \(2012\)](#) Metagenomic and metabolic profiling of nonlithifying and lithifying stromatolitic mats of Highborne Cay, the Bahamas. *PLOS ONE* 7: e38229
- Kirkwood DS (1994) SanPlus segmented flow analyzer and its applications. Seawater analysis. Skalar, Amsterdam
- ✦ [Kloos K, Mergel A, Rösch C, Bothe H \(2001\)](#) Denitrification within the genus *Azospirillum* and other associative bacteria. *Funct Plant Biol* 28:991–998
- ✦ [Klotz MG, Stein LY \(2008\)](#) Nitrifier genomics and evolution of the nitrogen cycle. *FEMS Microbiol Lett* 278: 146–156
- ✦ [Krumbein WE \(1983\)](#) Stromatolites — the challenge of a term in space and time. *Dev Precambrian Geol* 7:385–423
- ✦ [Larkin MA, Blackshields G, Brown NP, Chenna R and others \(2007\)](#) Clustal W and Clustal X version 2.0. *Bioinformatics* 23:2947–2948
- ✦ [Laval B, Cady SL, Pollack JC, McKay CP and others \(2000\)](#) Modern freshwater microbialite analogues for ancient dendritic reef structures. *Nature* 407:626–629

space

- space ✦ [Li M, Hong Y, Klotz MG, Gu JD \(2010\)](#) A comparison of primer sets for detecting 16S rRNA and hydrazine oxidoreductase genes of anaerobic ammonium-oxidizing bacteria in marine sediments. *Appl Microbiol Biotechnol* 86: 781–790
- ✦ [Li M, Hong Y, Cao H, Gu JD \(2013\)](#) Community structures and distribution of anaerobic ammonium oxidizing and *nirS*-encoding nitrite-reducing bacteria in surface sediments of the South China Sea. *Microb Ecol* 66:281–296
- ✦ [Michotey V, Méjean V, Bonin P \(2000\)](#) Comparison of methods for quantification of cytochrome *cd1*-denitrifying bacteria in environmental marine samples. *Appl Environ Microbiol* 66:1564–1571
- ✦ [Mobberley JM, Khodadad CL, Foster JS \(2013\)](#) Metabolic potential of lithifying cyanobacteria-dominated stromatolitic mats. *Photosynth Res* 118:125–140

- ✚ [Moisander PH, Shiue L, Steward GE, Jenkins BD, Bebout BM, Zehr JP \(2006\) Application of a *nifH* oligonucleotide microarray for profiling diversity of N₂-fixing micro-organisms in marine microbial mats. Environ Microbiol 8:1721–1735](#)
- ✚ [Mosier AC, Francis CA \(2010\) Denitrifier abundance and activity across the San Francisco Bay estuary. Environ Microbiol Rep 2:667–676](#)
- ✚ [Myshrall KL, Mobberley JM, Green SJ, Visscher PT, Havemann SA, Reid RP, Foster JS \(2010\) Biogeochemical cycling and microbial diversity in the thrombolitic microbialites of Highborne Cay, Bahamas. Geobiology 8: 337–354](#)
- Nelson SA, Sánchez-Rubio G (1986) Trans Mexican Volcanic Belt field guide. Volcanology Division, Geological Association of Canada and Instituto de Geología, Universidad Nacional Autónoma de México
- Oliva MG, Lugo A, Alcocer J, Peralta L (2009) Planktonic bloom-forming *Nodularia* in the saline Lake Alchichica, Mexico. Nat Resour Environ 15:22
- ✚ [Oren A \(2011\) Thermodynamic limits to microbial life at high salt concentrations. Environ Microbiol 13:1908–1923](#)
- ✚ [Paterson DM, Aspdén RJ, Visscher PT, Consalvey M and others \(2008\) Light-dependant biostabilisation of sediments by stromatolite assemblages. PLOS ONE 3: e3176](#)
- ✚ [Peimbert M, Alcaraz LD, Bonilla-Rosso G, Olmedo-Alvarez G and others \(2012\) Comparative metagenomics of two microbial mats at Cuatro Ciénegas basin I: ancient lessons on how to cope with an environment under severe nutrient stress. Astrobiology 12:648–658](#)
- ✚ [Pepe-Ranney C, Berelson WM, Corsetti FA, Treants M, Spear JR \(2012\) Cyanobacterial construction of hot spring siliceous stromatolites in Yellowstone National Park. Environ Microbiol 14:1182–1197](#)
- ✚ [Pester M, Maixner F, Berry D, Rattei T and others \(2014\) *nxrB* encoding the beta subunit of nitrite oxidoreductase as functional and phylogenetic marker for nitrite-oxidizing *Nitrospira*. Environ Microbiol 16:3055–3071](#)
- ✚ [Philippot L, Spor A, Henault C, Bru D and others \(2013\) Loss in microbial diversity affects nitrogen cycling in soil. ISME J 7:1609–1619](#)
- ✚ [Poly F, Wertz S, Brothier E, Degrange VR \(2008\) First exploration of *Nitrobacter* diversity in soils by a PCR cloning-sequencing approach targeting functional gene *nxrA*. FEMS Microbiol Ecol 63:132–140](#)
- ✚ [Ramírez-Olvera MA, Alcocer J, Merino-Ibarra M, Lugo A \(2009\) Nutrient limitation in a tropical saline lake: a microcosm experiment. Hydrobiologia 626:5–13](#)
- space ✚ [Reid RP, Visscher PT, Decho AW, Stolz JF and others \(2000\) The role of microbes in accretion, lamination and early lithification of modern marine stromatolites. Nature 406: 989–992](#)
- ✚ [Rotthauwe JH, Witzel KP, Liesack W \(1997\) The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl Environ Microbiol 63: 4704–4712](#)
- ✚ [Ruvindy R, White RA III, Neilan BA, Burns BP \(2016\) Unravelling core microbial metabolisms in the hypersaline microbial mats of Shark Bay using high-throughput metagenomics. ISME J 10:183–196](#)
- ✚ [Sanford RA, Wagner DD, Wu Q, Chee-Sanford JC and others \(2012\) Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. Proc Natl Acad Sci USA 109:19709–19714](#)
- ✚ [Schloss PD, Westcott SL, Ryabin T, Hall JR and others \(2009\) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537–7541](#)
- ✚ [Severin I, Acinas SG, Stal LJ \(2010\) Diversity of nitrogen-fixing bacteria in cyanobacterial mats. FEMS Microbiol Ecol 73:514–525](#)
- ✚ [Sorokin DY, Kuenen JG \(2005\) Chemolithotrophic haloalkaliphiles from soda lakes. FEMS Microbiol Ecol 52:287–295](#)
- ✚ [Staal M, te Lintelo H, Hekkert S, Herman P, Stal LJ \(2002\) Comparison of models describing light dependence of N₂ fixation in heterocystous cyanobacteria. Appl Environ Microbiol 68:4679–4683](#)
- ✚ [Stal LJ \(1995\) Physiological ecology of cyanobacteria in microbial mats and other communities. New Phytol 131: 1–32](#)
- Stal LJ (2003) Nitrogen cycling in marine cyanobacterial mats. In: Krumbein WE, Paterson DM, Zavarzin GA (eds) Fossil and recent biofilms: a natural history of life on Earth. Springer, Dordrecht, p 119–140
- Stal LJ (2012) Cyanobacterial mats and stromatolites. In: Whitton BA (ed) Ecology of Cyanobacteria II: their diversity in space and time. Springer, Dordrecht, p 65–125
- ✚ [Steppe TF, Pinckney JL, Dyble J, Paerl HW \(2001\) Diazotrophy in modern marine Bahamian stromatolites. Microb Ecol 41:36–44](#)
- Tavera R, Komárek J (1996) Cyanoprokaryotes in the volcanic lake of Alchichica, Puebla State, Mexico. Algal Stud 83:511–538
- ✚ [Throbäck IN, Enwall K, Jarvis Ö, Hallin S \(2004\) Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiol Ecol 49:401–417](#)
- ✚ [Valderrama JC \(1981\) The simultaneous analysis of total nitrogen and total phosphorus in natural waters. Mar Chem 10:109–122](#)
- ✚ [Valdespino-Castillo PM, Alcántara-Hernández RJ, Merino-Ibarra M, Alcocer J, Macek M, Moreno-Guillén OA, Falcón LI \(2016\) Phylotype dynamics of bacterial P utilization genes in microbialites and bacterioplankton of a monomictic endorheic lake. Microb Ecol, doi:10.1007/s00248-016-0862-1](#)
- ✚ [van Gemerden H \(1993\) Microbial mats: a joint venture. Mar Geol 113:3–25](#)
- Vilaclara G, Chávez M, Lugo A, González H, Martha G (1993) Comparative description of crater-lakes basic

space

space

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

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

spaceLimnol 25:435–440

✦ [Visscher PT, Stolz JF \(2005\) Microbial
mats as bioreactors: populations,
processes, and products.
Palaeogeogr Pal-
aeoclimatol
Palaeoecol 219:87–100](#)

✦ [Ward BB, Martino DP, Diaz MC, Joye
SB \(2000\) Analysis of ammonia-
oxidizing bacteria from hypersaline
Mono Lake, California, on the basis
of 16S rRNA sequences. Appl
Environ Microbiol 66:2873–2881](#)

✦ [Woeckjen D, Burow LC, Prufert-Bebout
L, Bebout BM and others \(2012\)
Identification of a novel
cyanobacterial group as active
diazotrophs in a coastal microbial
mat using NanoSIMS analysis.
ISME J 6:1427–1439](#)

✦ [Yoshida M, Ishii S, Otsuka S, Senoo K
\(2010\) *nirK*-harboring denitrifiers
are more responsive to
denitrification- inducing conditions
in rice paddy soil than *nirS*-harbor-](#)

spaceing bacteria. Microbes
Environ 25:45–48

✦ [Zani S, Mellon MT,
Collier JL, Zehr JP
\(2000\) Expression of
nifH genes in natural
microbial
assemblages in Lake
George, New York,
detected by reverse
transcriptase PCR.
Appl Environ
Microbiol
66:3119–3124](#)

✦ [Zehr JP, McReynolds
LA \(1989\) Use of
degenerate oligo-
nucleotides for
amplification of the
nifH gene from the
marine
cyanobacterium
*Trichodesmium
thiebautii*. Appl
Environ Microbiol
55:2522–2526](#)

✦ [Zehr JP, Mellon M,
Braun S, Litaker W,
Steppe T, Paerl HW
\(1995\) Diversity of
heterotrophic
nitrogen-fixation
genes in a marine
cyanobacterial mat.
Appl Environ
Microbiol
61:2527–2532](#)

✦ [Zhang Z, Schwartz S,
Miller W \(2000\) A](#)

Table 1. Primers used to survey the nitrogen cycle in c
anno

Gene Primer	Sequence 5' → 3'
Ammonia monooxygenase (ammonia oxidation, nitrification)	
<i>amoA</i> (Bacterial)	
amoA-1F	GGG GTT TCT ACT GGT GGT
amoA-2R	CCC CTC KGS AAA GCC TTC
<i>amoA</i> (Archaeal)	
Arch-amoAF	STA ATG GTC TGG CTT AGA
Arch-amoAR	GCG GCC ATC CAT CTG TAT
Hydrazine oxidoreductase (anaerobic ammonium oxidation)	
<i>hzo</i>	
hzoF1	TGT GCA TGG TCA ATT GAA
hzoR1	CAA CCT CTT CWG CAG GTG
Dinitrogenase reductase, iron protein (nitrogen fixation)	
<i>nifH</i>	
nif4	TTY TAY GGN AAR GGN GG
nif3	ATR TTR TTN GCN GCR TA
nif1	TGT GAT CCT AAA GCT GA
nif2	CCT CTT TAC TAC CGT AA
Nitrite oxidoreductase subunits (nitrite oxidation, nitrification)	
<i>nrxB</i> -Nitrospira	
nrxBF14	TGG CAA CTG GGA CGG AAC
nrxBR1239	TGT AGA TCG GCT CTT CGA
<i>nrxA</i> -Nitrobacter	
F1370-F1-nrxA	CAG ACC GAC GTG TGC GAA
F2843-R2-nrxA	TCC ACA AGG AAC GGA AGC
Copper-dependent nitrite reductase (nitrite reduction)	
<i>nirK</i>	
F1aCu	ATC ATG GTS CTG CCG CG
R3Cu	GCC TCG ATC AGY TTG TGG
Cytochrome cd₁-type nitrite reductase (nitrite reduction)	
<i>nirS</i>	
cd3aF	GTS AAC GTS AAG GAR ACS
R3cd	GAS TTC GGR TGS GTC TTG
Nitrous oxide reductase (nitrous oxide reduction, denitrification)	
<i>nosZ</i>	
nosZ-F	CGY TGT TCM TCG ACA GCC
nosZ-R	CAT GTG CAG NGC RTG GCA

Table 2. Number of sequences obtained
DNA amplifications were done from met

Molecule of study
Nitrogen-cycle process
DNA
Nitrogen fixation
Aerobic ammonia oxidation
Anaerobic ammonia oxidation
Denitrification (nitrite respiration)
Denitrification (nitrite respiration)
Denitrification (nitrous oxide reductase)
Total DNA sequences
RNA
Nitrogen fixation

a *amoA*, aerobic ammonia oxidation

b *nirK*, nitrite reduction (denitrification)

Fig. 2. Maximum likelihood phylogenetic tree for genes in
bacterial *amoA* (378 nucleotides), (b) *nirK* (435 nucleotides)
operational taxonomic units represent those obtained in
values $\geq 50\%$ are shown (1000 replicates)