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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 precipi- tation. Lake Alchichica (Mexico) is an oligotrophic environment where nitrogen (N) and phospho- rus 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 oxidoreduc- tase *(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

spaceINTRODUCTION

Microbialites are benthic microbial communities defined as organo-sedimentary structures where microbial activity promotes lithification by trapping, binding and/or precipitating detrital or chemical sed- iments (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

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spaceal. 2013). Although the physicochemical environ- ment plays a crucial role in their development, micro- bial activity remains the main driving force promot- ing accretion, with cyanobacterial photosynthate and heterotrophic respiration as the main contributors (Reid et al. 2000, Stal 2012, Cerqueda-García & Fal- cón 2016). These complex microbial assemblages have had a continuous presence throughout the his- tory of life on Earth. The fossil counterparts of micro- bialites date back to the Archaean (~3500 million years ago) and provide the most ancient microfossil

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spacerecord of life (Krumbein 1983). Therefore, these ben- thic biostructures can be considered one of the first successfully organized communities for which fossil records exist, hosting bacteria that played an essen- tial role in atmospheric evolution and planetary bio- geochemistry (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, Kaz´mierczak et al. 2011, Cen- teno et al. 2012, Ruvindy et al. 2016). In addition, metagenomic approaches have confirmed an ample metabolic repertoire with interconnected biogeo- chemical pathways within millimetric scales (Breit- bart et al. 2009, Khodadad & Foster 2012, Mobberley et al. 2013, Cerqueda-García & Falcón 2016). These studies have shown that *Cyanobacteria* and *Pro- teobacteria* 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 ele- ment 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 N_2 fixation pro- cess 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 pro- ductivity 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 inor- ganic 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 micro- bialite type in the lake consists of spongy structures distributed around the entire perimeter, described as white cauliflower-like thrombolites composed mainly of hydromagnesite — $Mg_5(CO_3)_4(OH)_2{\cdot}4H_2O$ (Kaz´- mierczak et al. 2011) (Fig. 1). Alchichica microbialites have shown high rates of daytime nitrogenase activity (Falcón et al. 2002, 2007, Beltrán et al. 2012) associ- ated with heterocystous cyanobacteria (Falcón et al. 2002).

spaceIn 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 physic- ochemical 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 chemi- cal−redox gradients and biogeochemical cycling occur (Tavera & Komárek 1996, Couradeau et al. 2011, Kaz´mierczak 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).

Site

Sampling and nucleic acid extraction

Sampling was done in the summer of 2013, during the stratification period of the lake. The physicochem- ical 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 micro- bialites 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

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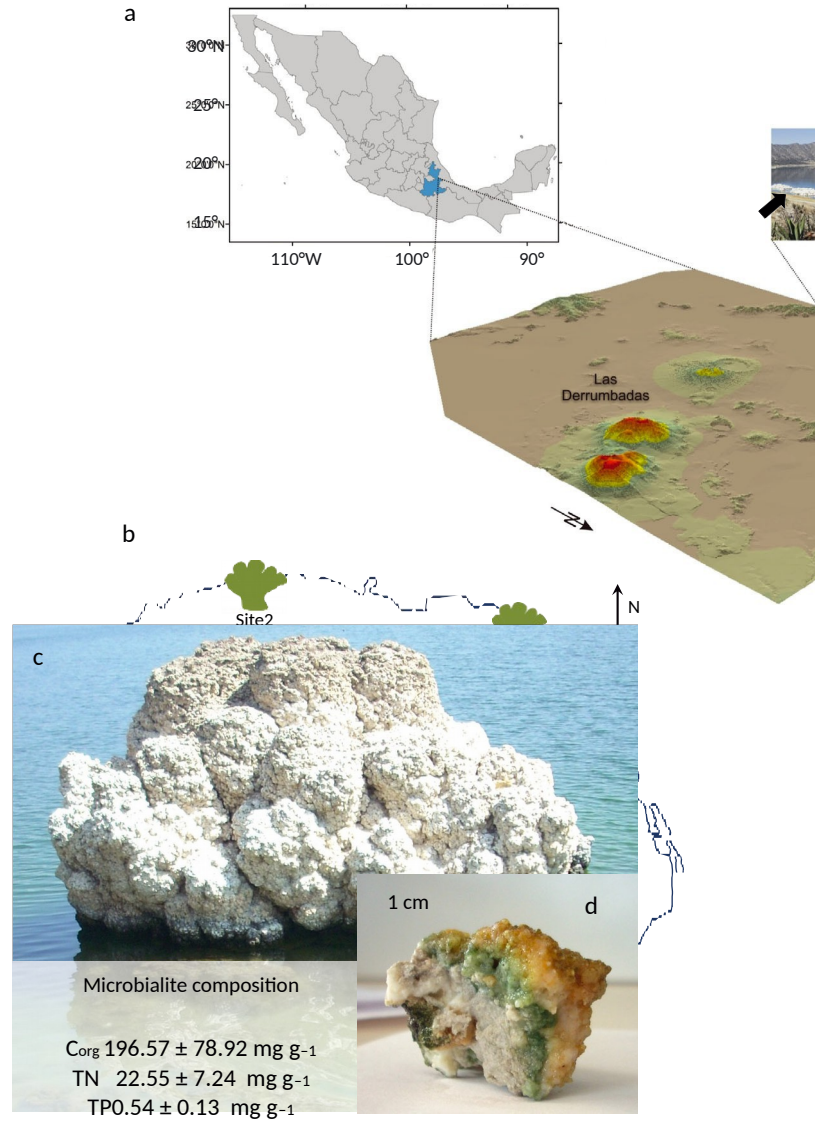

Fig. 1. (a) Geographic location of Lake Alchichica, Mexico. The topographical map shows the changes of elevation in the region. The panoramic photograph (inset) shows the semicontinuous white ring (indicated with arrows) of inactive micro- bialites above the water level. (b) Collection sites of spongy microbialites and mean values (± SD) of nitrogen (N) and phospho- rus (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 micro- bialites was used for amplifications with specific primers to explore the genetic diversity associated with N-cycling pathways.

To analyze N-cycle gene expression in microbia- lites, 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 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

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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 sam- ples 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 syn- thesized immediately by avian myeloblastosis virus reverse transcriptase (Promega) following the man- ufacturer's protocol. The first-strand cDNA samples were stored at −20°C until analysis.

spacePCR 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 molec- ular 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 amplifica- tion protocol was similar for *amoA*, *nxrA*, *nxrB*, *hzo*,

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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)
Ammonia monooxygenase (ammonia oxidation, nitrification)		
amoA (Bacterial)		
amoA-1F	GGG GTT TCT ACT GGT GGT	600
amoA-2R	CCC CTC KGS AAA GCC TTC TTC	
amoA (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)		
hzo		
hzoF1	TGT GCA TGG TCA ATT GAA AG	1000
hzoR1	CAA CCT CTT CWG CAG GTG CAT G	
Dinitrogenase reductase, iron protein (nitrogen fixation)		
nifH		
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)		
nxrB-Nitrospira		
nrxBF14	TGG CAA CTG GGA CGG AAG ATG	1245
nxrBR1239	TGT AGA TCG GCT CTT CGA CC	
nxrA-Nitrobacter		
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)		
nirK		
F1aCu	ATC ATG GTS CTG CCG CG	472
R3Cu	GCC TCG ATC AGY TTG TGG TT	
Cytochrome cd_1-type nitrite reductase (nitrite reduction, denitrification)		
nirS		

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

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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 instruc- tions. Chemically competent *Escherichia coli* DH5⟨ cells were transformed with the constructed vectors, and positive clones were selected by ⟨-complemen- tation on Luria-Bertani plates containing ampicillin (50 µg ml^{-1}) and X-gal (5-bromo-4-chloro-3-indolyl- ®-D-galactopyranoside, 1.6 mg spread on the sur- face). 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 detect- ing 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 differ- ence 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 consid- ered 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.

spacePhysicochemical 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 seg- mented 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 deter- mined using a CE Instruments Flash EA 1112 ele- mental analyzer, after removal of the inorganic C (carbonate) using 1.5 M hydrochloric acid. Total P (TP) was determined by UV spectrometry as molyb- date-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), exhibit- ing a 2.5 DIN:SRP ratio. Littoral water TP and TN were more balanced (2.62 and 42.4 µM, respec- tively), 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)*.

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spaceChemolithotrophic processes such as aerobic ammo- nia oxidation and anammox showed the lowest num- ber of phylotypes (Table 2).

Ammonia oxidation

Three OTUs were detected for *amoA*. Sequences affiliated to *Nitrosomonas (Betaproteobacteria)*, dis- tributed 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 & Fran- cis 2010) and were related to *Rhodobacter sphae- roides*, *Octadecabacter antarcticus* and *Chelativo- rans* 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 *Alphaproteobac- teria* such as *Dinoroseobacter shibae* and *Polymor-*

space*phum gilvum* (78−83% similarity). The genetic diver- sity associated with nitrous oxide reduction was observed in 16 *nosZ* OTUs, with 75 to 90% identity to reported sequences mainly from coastal marine sedi- ments and isolated strains of the haloalkaliphilic *Thioalkalivibrio sulfidophilus* and the *Alphapro- teobacteria 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-

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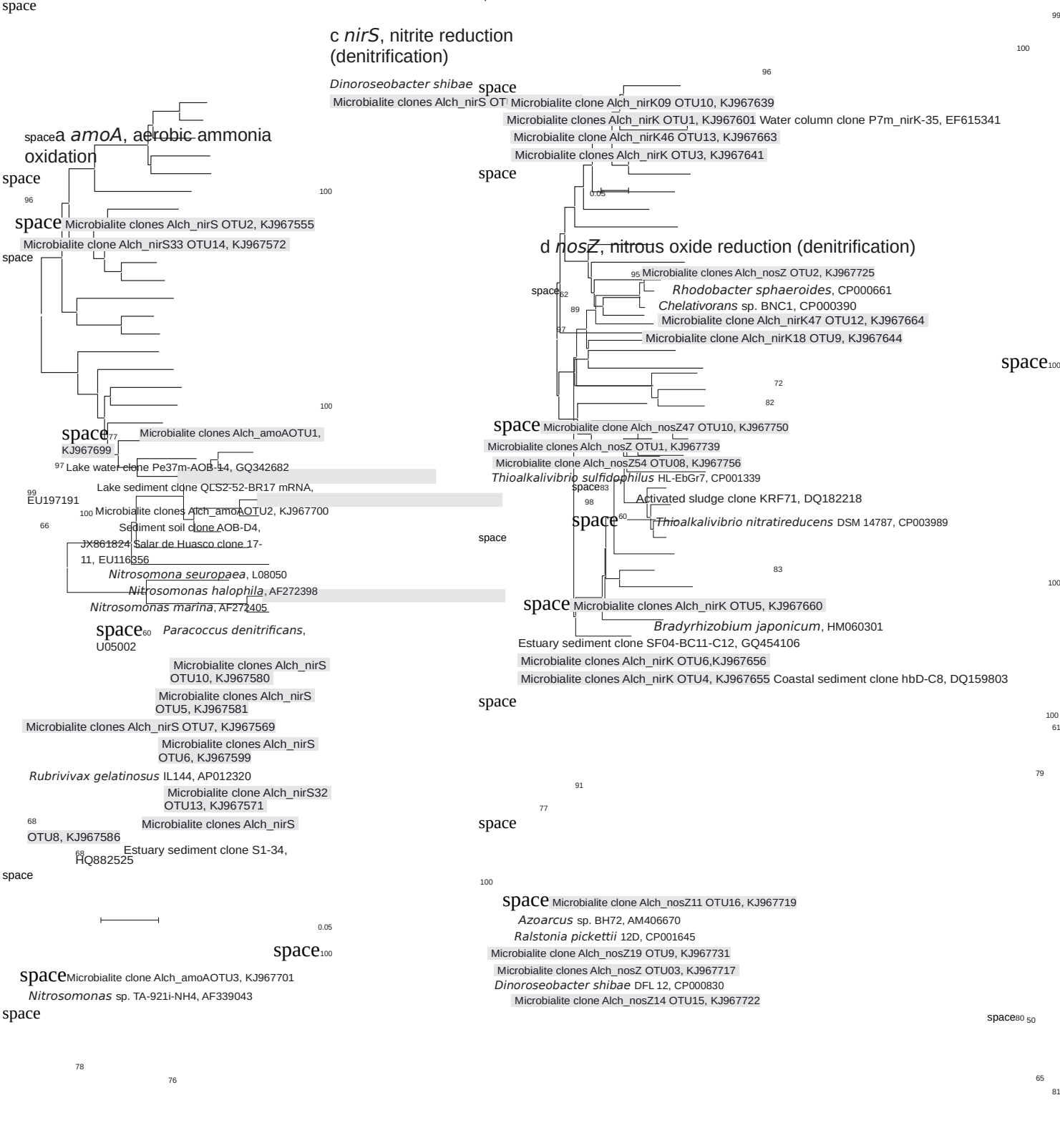
Table 2. Number of sequences obtained from different nitrogen-cycle pathways in microbialites of crater Lake Alchichica. DNA amplifications were done from metagenomic DNA extracted; RNA amplifications were done from synthesized cDNA. OTUs: operational taxonomic units

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



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 denitri- fication were the most abundant. The relative abun- dance of phylotypes associated with each N path- way 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-

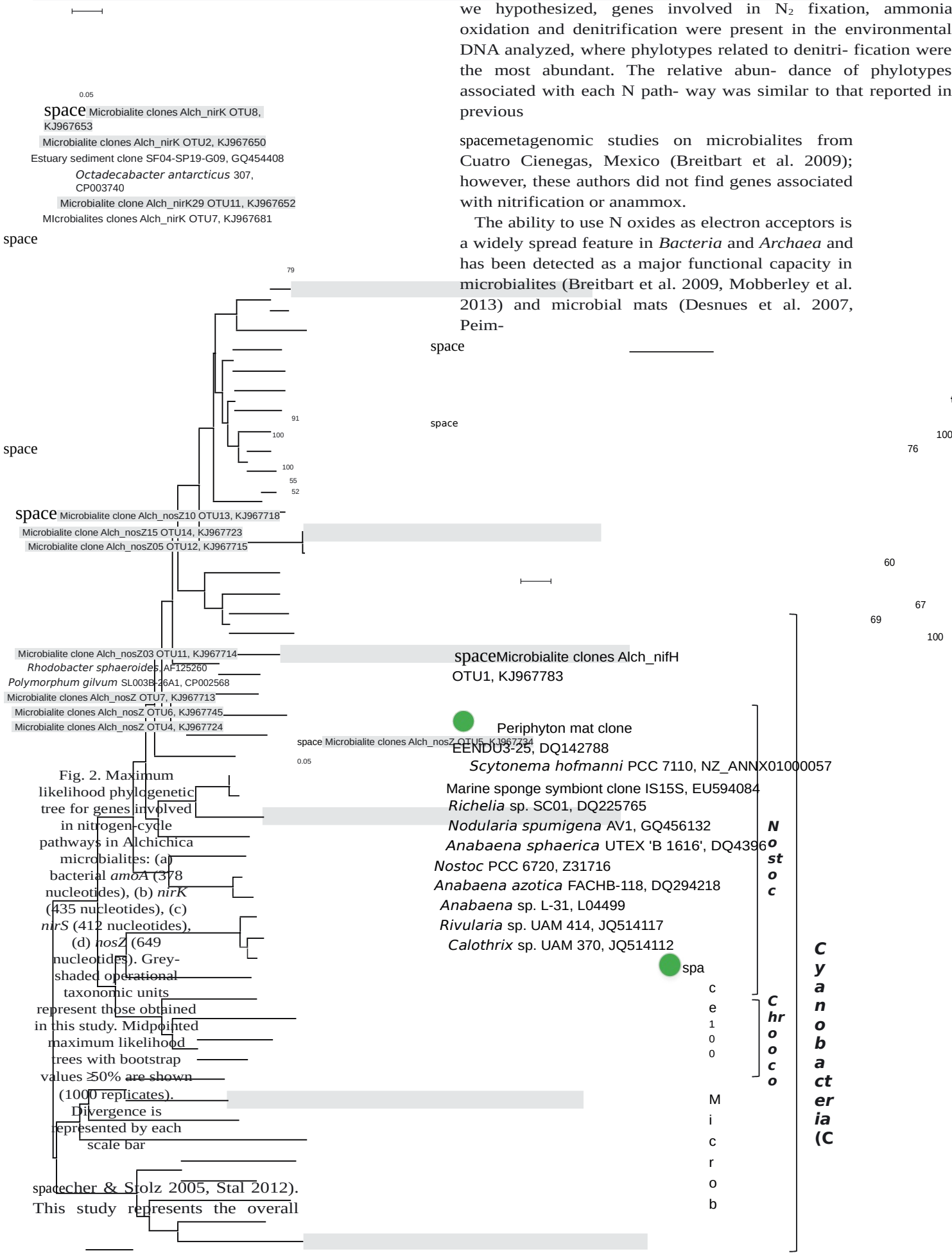
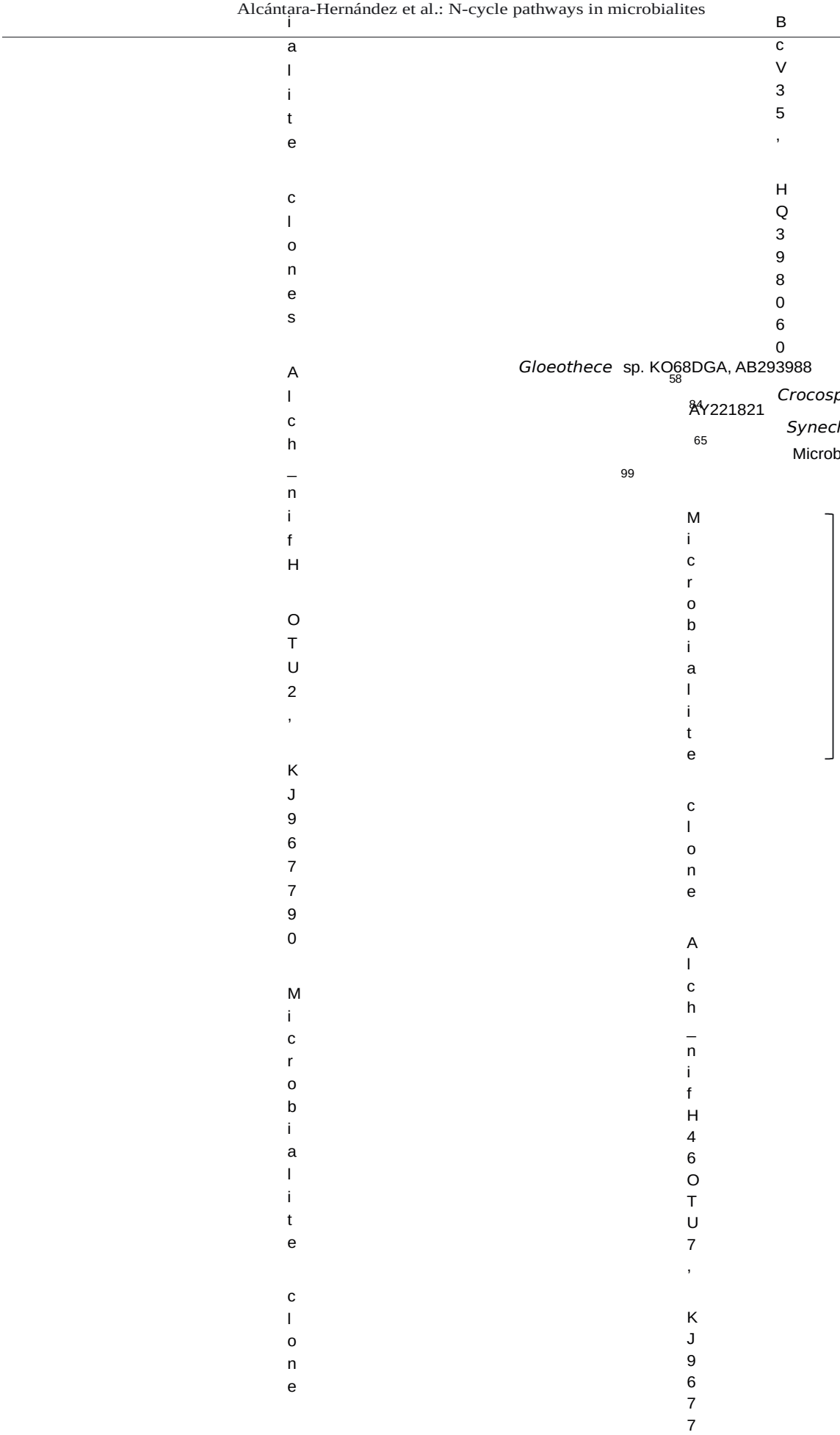
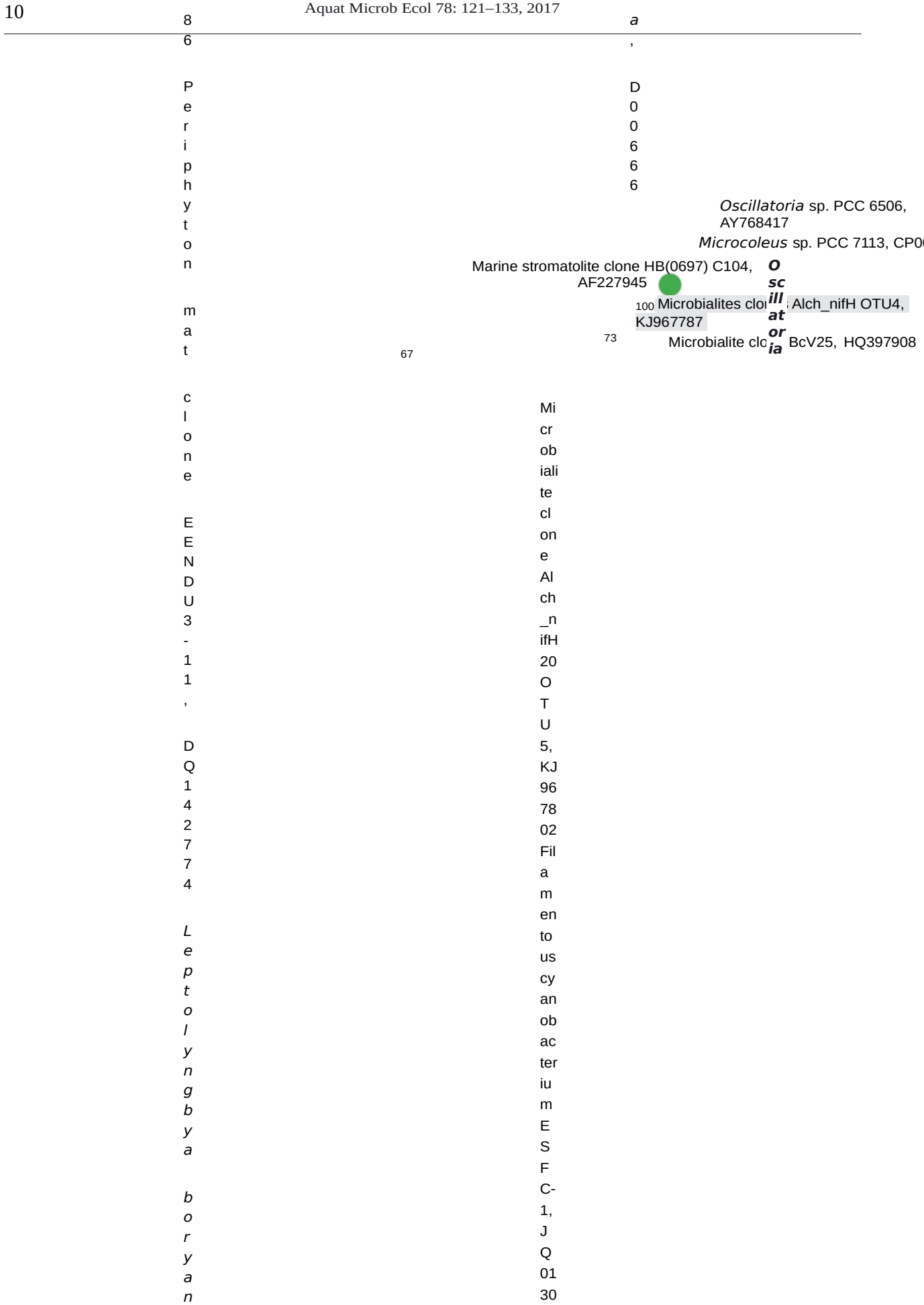
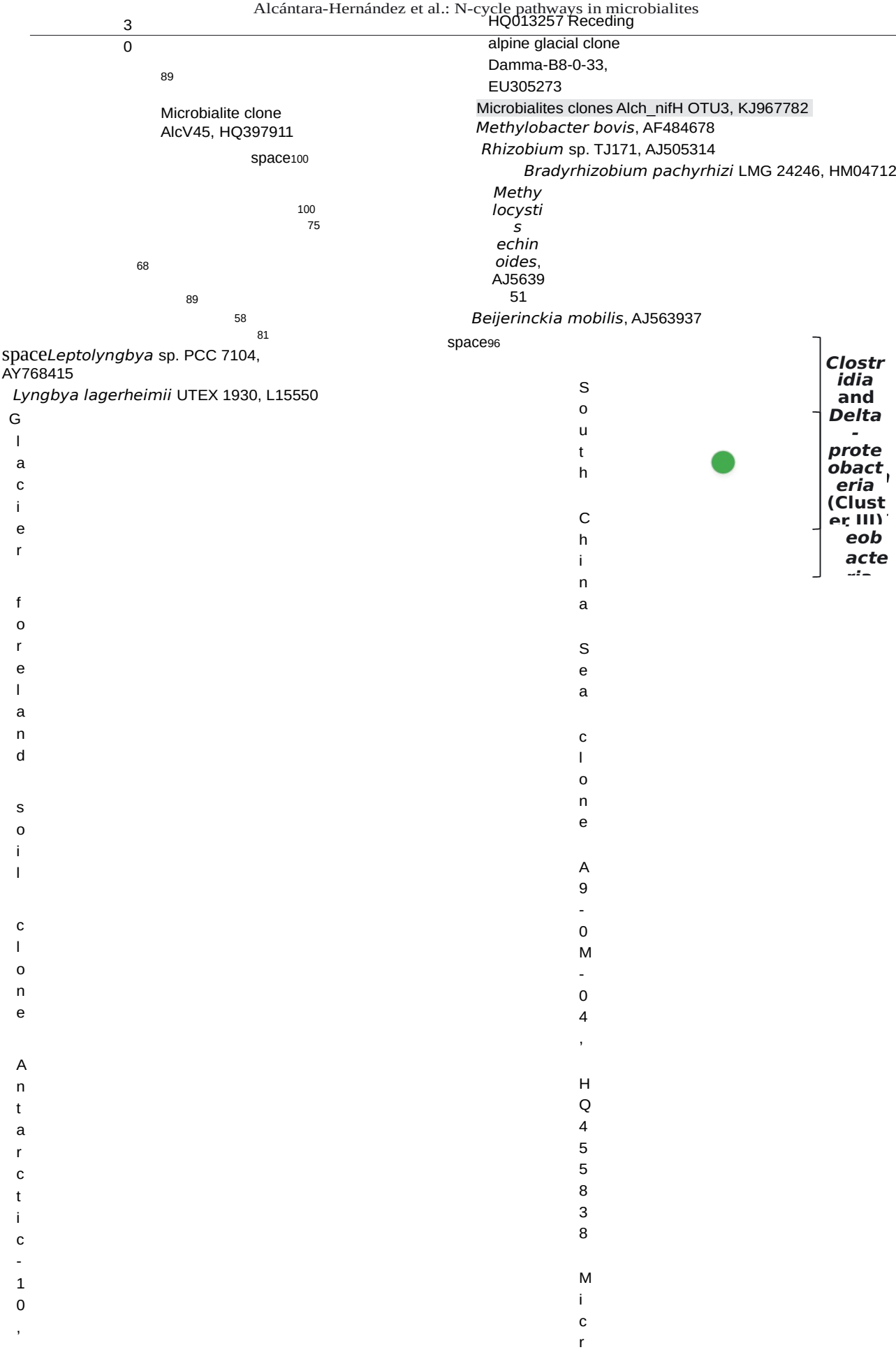


Fig. 2. Maximum likelihood phylogenetic tree for genes involved in nitrogen-cycle pathways in Alchichica microbialites: (a) bacterial *amoA* (378 nucleotides), (b) *nirK* (435 nucleotides), (c) *nirS* (412 nucleotides), (d) *nosZ* (649 nucleotides). Grey-shaded operational taxonomic units represent those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

spacecher & Srolz 2005, Stal 2012). This study represents the overall


Microbialite clone AlchnifH46OTU7, KJ967790
Microbialite clone AlchnifHOTU2, KJ967790
Alchnif clones
BcV35, HQ398060
Gloeothece sp. KO68DGA, AB293988
Crocosphaera watsonii WH 8501, AY221821
Synechococcus sp., U22146
Microbiliate clone Ale20, HQ398003
58
84
65
99


86
Periphyton mat clone ENDU3-11, DQ142774 Leptolyngbya boryana
a, D00666
Oscillatoria sp. PCC 6506, AY768417
Microcoleus sp. PCC 7113, CP003630
Marine stromatolite clone HB(0697) C104, AF227945
100
Microbialites clone Alch_nifH OTU4, KJ967787
73
Microbialite clone BcV25, HQ397908
67
Oscillatoria
Microbialite clone Alch_nifH 20 OTU 5, KJ967802 Filamentous cyanobacterium ESFC-1, JQ0130


3
0
89
Microbialite clone AlcV45, HQ397911
space100
100
75
68
89
58
81
spaceLeptolyngbya sp. PCC 7104, AY768415
Lyngbya lagerheimii UTEX 1930, L15550
Glacier foreland soil clone Antarctic-10,
HQ013257 Receding alpine glacial clone Damma-B8-0-33, EU305273
Microbialites clones Alch_nifH OTU3, KJ967782
Methylobacter bovis, AF484678
Rhizobium sp. TJ171, AJ505314
Bradyrhizobium pachyrhizi LMG 24246, HM047124
Methylocystis echinoides, AJ563951
Beijerinckia mobilis, AJ563937
space96
South China Sea clone A9-0M-04, HQ455838
Micr
Clostridia and Delta-proteobacteria (Cluster III)
eob
acte

obialite clone Alch_nifH15 OTU6, KJ967800

Clostridium lentocellum DSM 5427, CP002582

Syntrop

Mangrove sediment clone SRP11, GQ499214

Microbialite cl

hobotulus glycolicus, CP002547

Desulfurivibrio alkaliphilus AHT2, CP001940

Desulfovibrio vulgaris RCH1, CP002298

Desulfomonile tiedjei DSM 6799, CP003360

66

96

99

61

81

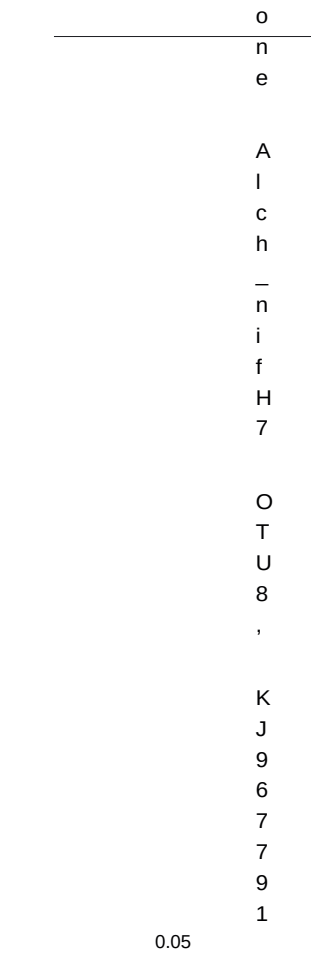


Fig. 3. Midpointed neighbor-joining (Kimura 2-parameter) tree based on *nifH* gene partial sequences (315 nucleotides); boot- strap values > 50% are shown (1000 replicates). The scale bar represents 5% divergence. Phylotypes recovered from DNA are highlighted in grey, and the green circles indicate those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

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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 deni- trification 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 metage- nomic 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 associ- ated with ammonia oxidation in microbialites from Lake Alchichica could relate to long replication times of chemolithotrophs and limitation in saline (alka- line) environments due to bioenergetic constraints (Sorokin & Kuenen 2005, Oren 2011). In other stud- ies, 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 sam- ple with the methodology here employed, possibly since microbialites are known to harbor low amounts of *Archaea* (Centeno et al. 2012). Ammonia mono- oxygenase 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 environ-mental sequences detected in bay and oceanic sedi- ments (Dang et al. 2013, Li et al. 2013). The detection

spaceof an anammox phylotype was possible with the *hzo* gene-targeted method here employed, although fur- ther studies must be done to understand the rele-vance 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 cyanobac- teria were the most relevant diazotrophs in Alchi- chica microbialites, followed in minor proportion by *Alphaproteobacteria*, sharing an analogous composi- tion 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), sug- gesting 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 *Lepto- lyngbya* sp. PCC 7104 and with Elkhorn Slough Fila- mentous 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, Kaz ´mierczak et al. 2011) and in other nonlithifying and lithifying mats (Severin et al. 2010, Khodadad & Foster 2012). Furthermore, microbial mats domi- nated 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 photosynthe- sis, avoiding oxygen inhibition through spatial sepa- ration of N_2 fixation in the heterocysts (Stal 1995, Staal et al. 2002).

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

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spacein charge of transforming different N forms by nitrifi- cation, anammox and denitrification, but these pro- cesses 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 possi- ble in the microoxic−anoxic interfaces. However, min- eralization 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 *Deltapro- teobacteria*, *Epsilonproteobacteria* and *Verrucomi- crobia* (Sanford et al. 2012).

Another relevant feature of the N-cycling dynam- ics in the water column of Lake Alchichica is the sea- sonal — and regular — bloom of *Nodularia* spp. by the onset of the stratification period (June−October) (Oliva et al. 2009). The massive growth of this cyano- bacterium 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, anam- mox and denitrification, also exhibit seasonal varia- tions, changing N-cycling genetic diversity in time (as was described for P utilization genes in micro- bialites 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). Al- though 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 associ- ated with N cycling. It was apparent that the poten- tial 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 impor- tant role.

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

Gene Primer	Sequence 5′ → 3′
Ammonia monooxygenase (ammonia oxidation, nitri	
amoA (Bacterial)	
amoA-1F	GGG GTT TCT ACT GGT GGT
amoA-2R	CCC CTC KGS AAA GCC TTC
amoA (Archaeal)	
Arch-amoAF	STA ATG GTC TGG CTT AGA
Arch-amoAR	GCG GCC ATC CAT CTG TAT
Hydrazine oxidoreductase (anaerobic ammonium ox	
hzo	
hzoF1	TGT GCA TGG TCA ATT GAA
hzoR1	CAA CCT CTT CWG CAG GTG
Dinitrogenase reductase, iron protein (nitrogen fixat	
nifH	
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, ni	
nxrB-Nitrospira	
nrxBF14	TGG CAA CTG GGA CGG AAC
nxrBR1239	TGT AGA TCG GCT CTT CGA
nxrA-Nitrobacter	
F1370-F1-nxrA	CAG ACC GAC GTG TGC GAA
F2843-R2-nxrA	TCC ACA AGG AAC GGA AGC
Copper-dependent nitrite reductase (nitrite reductio	
nirK	
F1aCu	ATC ATG GTS CTG CCG CG
R3Cu	GCC TCG ATC AGY TTG TGG
Cytochrome cd_1-type nitrite reductase (nitrite reduct	
nirS	
cd3aF	GTS AAC GTS AAG GAR ACS
R3cd	GAS TTC GGR TGS GTC TTG
Nitrous oxide reductase (nitrous oxide reduction, de	
nosZ	
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 reducti	
Total DNA sequences	
RNA	
Nitrogen fixation	

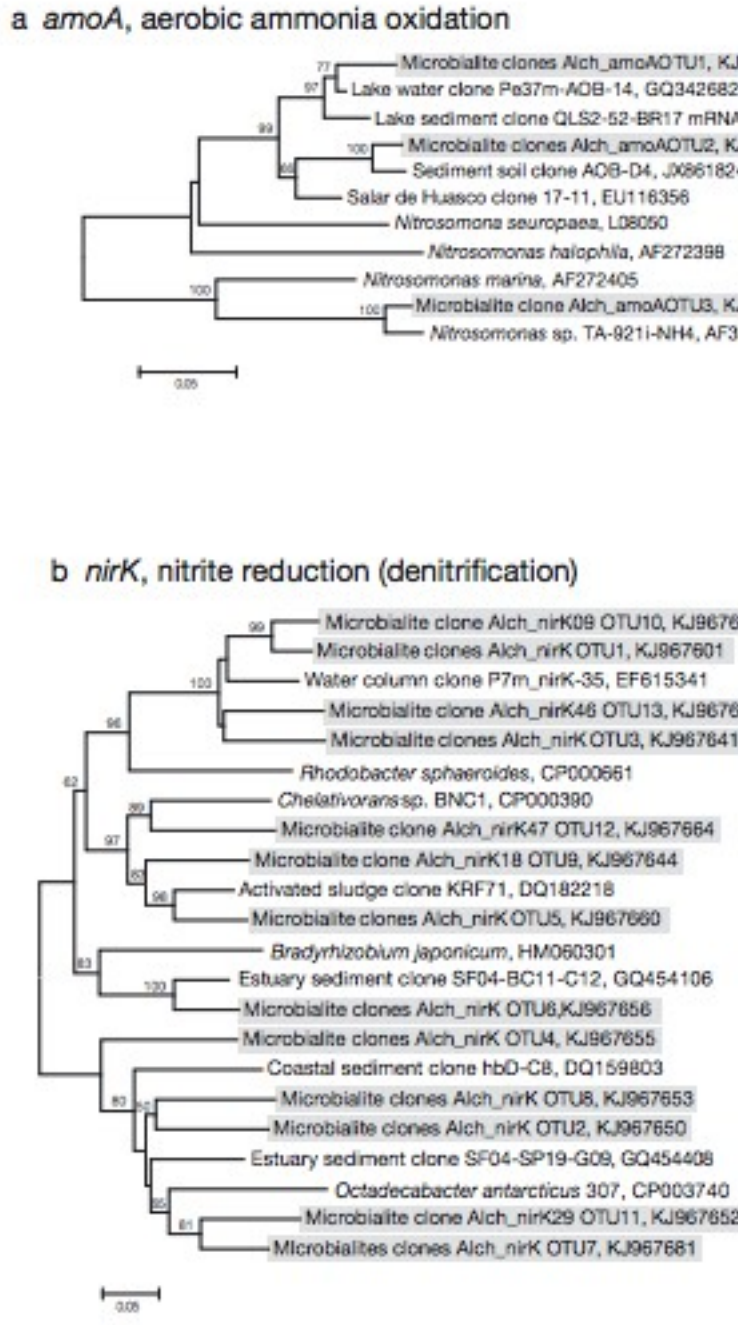
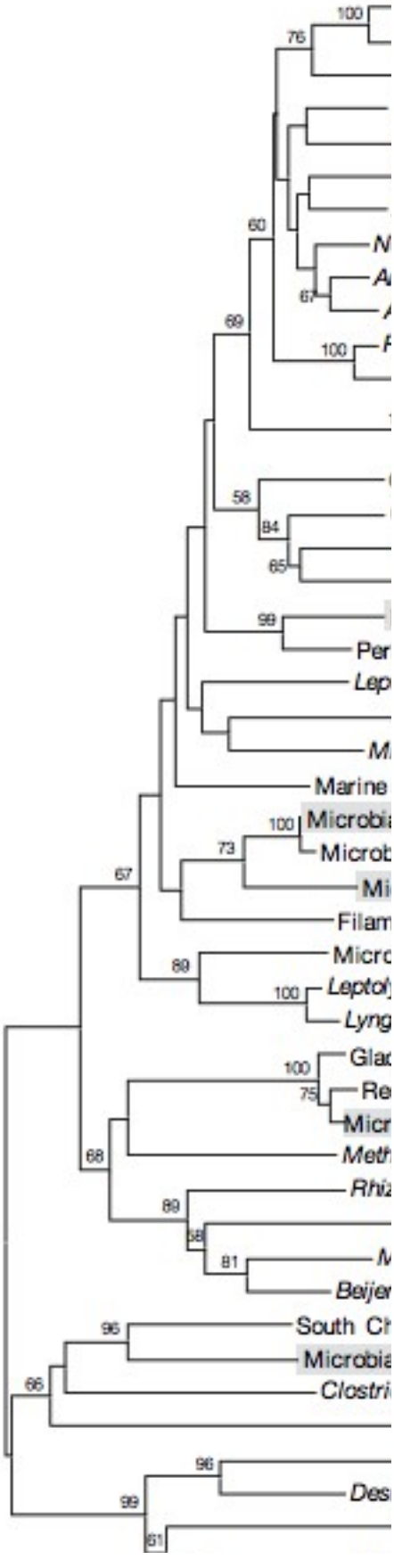


Fig. 2. Maximum likelihood phylogenetic tree for genes i
bacterial *amoA* (378 nucleotides), (b) *nirK* (435 nucleotides
operational taxonomic units represent those obtained in
values ≥50 % are shown (1000 replicate


100
76
60
67
69
100
58
84
65
99
Per
Lep
M
Marine
100
Microbi
73
Microb
67
Mi
Filam
Micro
89
100
Leptol
Lyng
100
Glac
75
Re
Micr
68
Meth
Rhiz
89
88
81
Beije
96
South Ch
Microbia
66
Clostri
96
Des
99
81