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Functional-Structural Analysis of Nitrogen-Cycle Bacteria in a Hypersaline Mat from the Omani Desert

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Potential rates of ammonia oxidation, denitrification and anammox were measured in a hypersaline microbial mat. Ammonia oxidation and denitrification had potential rates of 0.8 ± 0.4 and 2.0 ± 1.0 nmol N g^{-1} h^{-1}, respectively, anammox was not detectable. The rate of N_2O production under anoxic conditions accounted for ca. 5% of total denitrification. Using qPCR, the ammonia-oxidation (amoA) genes of *gammaproteobacteria* had the highest copy number. The denitrification genes *narG* and *nirS* exhibited comparable estimates. Sequences of *nirS* gene were novel, whereas *nirK* sequences were related to sequences from the *Rhizobiales* group. Sequences of the *nosZ* gene were the most diverse and clustered with sequences from various genera. Our results demonstrate that the hypersaline mat from Oman harbors nitrifying and denitrifying bacteria with the potential to perform respective processes at detectable rates.

Keywords: acetylene inhibition assay, denitrification, functional genes, hypersaline microbial mat, nitrogen cycle, quantitative PCR

Introduction

Hypersaline microbial mats are believed to exhibit most, if not all, biogeochemical processes that exist in aquatic ecosystems, due to the presence of different physiological groups of microorganisms therein (Canfield and Des Marais 1993; Stal 1995; van Gemerden 1993). Many studies have focused on carbon and sulfur cycling in hypersaline mats (Abed et al. 2006, 2007; Kühl 1993; Revsbech and Jørgensen 1983; Wieland and Kühl 2000a, b). Although microsensors and biogeochemical techniques have been used to measure rates of respiration, areal and gross photosynthesis, sulfate reduction and sulfide oxidation and their changes with salinity and temperature, molecular tools have been employed to identify the microbes involved in these processes (Abed et al. 2006, 2007; Epping and Kühl 2000; Harris et al. 2013; Ley et al. 2006; Pringault et al. 2001; Wieland and Kühl 2000a, b). In contrast, the nitrogen cycle in hypersaline mats was rarely studied. Moreover, most of the studies on hypersaline mats were performed on samples from intertidal flats and evaporation ponds, although similar mats are also found in inland lakes and desert streams. So far, few studies examined the microbial diversity and activity in inland hypersaline microbial mats (Abed et al. 2011; Jonkers et al. 2003).

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Most progress in research of nitrogen cycling in hypersaline mats has been made with respect to nitrogen fixation and its rates under different conditions (Bolhuis et al. 2010; de Wit et al. 2005; Omoregie et al. 2004; Pinckney and Paerl 1997). In comparison to N_2 fixation, other processes in the nitrogen cycle like ammonia oxidation, denitrification and anammox have not been widely studied in hypersaline mats. A study of N-cycle pathways, using ^{15}N isotopes and inhibitors, in intertidal hypersaline mats of Camargue, France, showed that these mats acted as a nitrogen source in summer, but as a sink in winter (Bonin and Michotey 2006).

Fingerprinting based on nitrite reductase genes (*nirS* and *nirK*) revealed significant seasonal shifts of the denitrifying bacterial communities in the same mats (Desnues et al. 2007). Nevertheless, information on the identity of denitrifying bacteria in hypersaline mats is still lacking. A further hint towards the importance of N-cycle in hypersaline mats is the presence of large filamentous colorless sulfur bacteria *Beggiatoa* spp. (Garcia-Pichel 1994; Hinck et al. 2007; Jørgensen and Des Marais 1986). These microbes have the ability to store nitrate (up to 40 mM) in large intracellular vacuoles (Hinck et al. 2007) and to respire nitrate anaerobically (Beutler et al. 2012).

To understand the full N-cycle in hypersaline mats, we studied the occurrence of ammonia oxidation, denitrification and anammox in a hypersaline microbial mat from a desert stream (Wadi Muqshin) in the Sultanate of Oman, bordering dunes of the Empty Quarter (Jupp et al. 2008). We also investigated bacterial diversity using 16S rRNA

genes and selected denitrifying genes (i.e., *nirS*, *nirK*, and *nosZ*) and quantified a suite of key functional genes related to ammonia oxidation (bacterial and archaeal *amoA*) and denitrification processes (*narG*, *napA*, and *nirS*) using molecular techniques.

Materials and Methods

Sampling Site

The studied mat originated from Wadi Muqshin in southeastern Oman in the Nejd hydrogeology area of the Dhofar Region, 200 km from the Arabian Sea coast (19°35.040′N; 54°52.870′E). The Wadi contains a series of hypersaline stagnant pools harboring ca. 2 cm thick microbial mats, showing distinct lamination with 3-mm-thick photosynthetic layer and an anoxic layer below 3 mm. The mats are flooded over winter but are subjected to continuous evaporation of the overlying water during summer resulting in extremely hypersaline conditions. At the time of sampling, the salinity of the overlying water was 6% (may reach 10% in summer) as measured using a portable refractometer, the water temperature was 24.2°C (may reach 50°C in summer) and pH was 8.5 (Table 1). Mat pieces were collected in May 2008 and were cut carefully using a sterile scalpel and stored in petri dishes. Samples were shipped to the Max Planck Institute for Marine Microbiology in Bremen, Germany, where the molecular work and the process analyses were performed.

Table 1. Physicochemical characteristics of the sampling site

Parameter	Unit	
Lat/Long coordinates		N 19° 35.05′; E 54° 52.90′
UTM coordinates		277792 E; 2166842 N
pH		8.5
Temperature	°C	24.2
Conductivity	(mS cm^{-1})	77.6
Salinity	%	6
Sodium*	mg l^{-1}	18,679
Potassium*	mg l^{-1}	619
Magnesium*	mg l^{-1}	3,289
Calcium*	mg l^{-1}	948
Manganese*	mg l^{-1}	<0.05
Iron*	mg l^{-1}	<0.05
Chloride*	mg l^{-1}	30,493
Sulphate*	mg l^{-1}	11,854
Bicarbonate (HCO_3^-)*	mg l^{-1}	284
NO_3-N (0–3 mm layer)§	μmol l^{-1}	6 ± 2
NO_3-N (3–10 mm layer)§	μmol l^{-1}	2.4 ± 0.7
NH_4 (0–3 mm layer)§	μmol l^{-1}	170 ± 5
NH_4 (3–10) mm layer)§	μmol l^{-1}	129 ± 12

*Data obtained in February 2004 (from JUPP et al. 2008)

§determined in thawed mat samples incubated for 1 day in water containing 7 μmol l^{-1} NO_3^- and <1 μmol l^{-1} $NH_4^{\pm}$.

Potential Ammonia Oxidation Rates

Potential rate of ammonia oxidation (i.e., the maximum rates under fully oxic conditions without limitation by ammonium availability and diffusive solute transport), the first step in nitrification, in the mat was measured as previously described (Stief and de Beer 2006). Briefly, a slurry was made of preweighed whole mat pieces (2–3 g each) in 30 ml sterile seawater (salinity 6%) in 110-ml glass bottles. For the measurement of ammonia oxidation, the medium contained 25 μM ammonium sulfate ($(NH_4)_2SO_4$) and 20 mM sodium chlorate ($NaClO_3$) that inhibits nitrite oxidation (i.e., the second step of nitrification) (Belser and Mays 1980).

The rate of ammonium oxidation can then be inferred from the linear increase in nitrite (NO_2^-) concentration during the incubation. The bottles were stirred gently at 100 rpm throughout the incubation. The medium was purged with air using a glass pipette connected to an air pump. Subsamples of the slurry (2 ml each) were collected after 5 minutes, 2 h, 4 h, 6 h, 8 h, and 24 h and were immediately frozen at −20°C until analysis. At the end of the experiment, all samples were thawed and centrifuged at 5000 rpm for 10 min to remove soil particles. In the particle-free water samples, NO_2^- was analyzed using the sodium iodide (NaI) reduction assay (Yang et al. 1997) on a NO_x analyzer (CLD 86; Eco-Physics). All incubations were run in triplicates at 26°C, and autoclaved mat pieces served as negative controls.

Potential Denitrification Rates

Potential denitrification rates (i.e., the maximum rates under fully anoxic conditions without limitation by nitrate availability and diffusive solute transport) were measured in slurry incubations of whole mats (i.e., oxic and anoxic layers) by using the acetylene inhibition technique (Sørensen 1978). The addition of acetylene inhibits the last step of denitrification (i.e., the conversion of N_2O to N_2) and thus results in the accumulation of N_2O. Mat samples (2–3 g each) were incubated in 110-ml glass bottles filled with 30 ml sterile anoxic seawater (bubbled with helium gas for 15 minutes), thus leaving a headspace volume of 80 ml. Because acetylene also inhibits nitrification, the seawater was amended with 25 μM NO_3^-.

Each bottle was wrapped with aluminum foil, to prevent O_2 production by photosynthesis, and sealed gas-tight with a rubber stopper, through which it was purged with helium gas for 10 min. Ten percent of the headspace volume was replaced by acetylene and the bottles were shaken on a plate shaker at 150 rpm. Gas samples (1 ml each) were drawn from the headspace using a gas-tight syringe at different time intervals (i.e., 5 min, 2 h, 4 h, 6 h, and 8 h) and injected into gas-tight sample vials (3 ml exetainers, Labco, UK) preflushed with helium. N_2O concentrations in the exetainers were then measured on a gas chromatograph equipped with a ^{63}Ni electron-capture detector (GC 7890; Agilent Technologies). The linear increase in N_2O concentration was used to calculate the denitrification rate per gram of mat. An equivalent series of mat pieces was incubated without the addition of acetylene to quantify the potential rate of N_2O production

under anoxic conditions. Additional incubations without nitrate and with autoclaved mat pieces served as negative controls.

Anammox Rates

Mat samples were incubated in 30 ml of sterile seawater amended with 25 μM $^{15}NH_4Cl$ and 25 μM $Na^{14}NO_2^-$ in 110-ml glass bottles. The bottles were flushed with helium for 10 minutes to create completely anoxic conditions and to reduce the N_2 background, wrapped with aluminum foil to prevent O_2 production by photosynthesis, and incubated at 26°C on a plate shaker (100 rpm). Gas samples (2.5 ml each) from the headspace were collected at different time intervals and transferred into 6 ml exetainers filled completely with He-saturated water. After injection of the gas sample into the exetainer, 100 μl 50% $ZnCl_2$ was added to stop any biological activity. The excess concentration of $^{29}N_2$ in the headspace was determined from the $^{29}N_2$:$^{28}N_2$ ratio by GC-IRMS. Autoclaved mat pieces served as negative controls.

Abundance of Functional Genes as Revealed by qPCR

Quantitative PCR was performed to quantify key functional genes for both archaeal and bacterial ammonia oxidation (*amoA* encoding the ammonia monooxygenase subunit A) as well as genes in the first two steps in denitrification: nitrate reduction (*narG* and *napA* encoding membrane-bound and periplasmic nitrate reductases, respectively) and nitrite reduction (*nirS* encoding cytochrome cd_1-containting nitrite reductase). A mat sample was subjected to DNA extraction using the PowerBiofilm DNA isolation kit (MOBIO laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Quantitative PCR was performed using previously described primers (seen later in Table 3) and protocols (Lam et al. 2009). All quantitative PCR reactions were run on an iCycler iQ Real-Time PCR Detection System equipped with iQ^{TM}5 software (Bio-Rad, Germany) and with the PowerSYBR Green PCR Master Mix (Applied Biosystems). The copy number of the genes was calculated after correcting for the concentration and dilution factors and presented as copy number per g of mat (Table 2).

Table 2. Primers used in the quantification of denitrifying and ammonia oxidizing (AO) genes and the estimated copy number g^{-1} of mat

Functional group	Target gene	Primer	Sequence (5′-3′)	Reference	Copy g^{-1} mat
β-proteobacteria (AO)	*amoA*	amoA1F	GGG GTT TCT ACT GGT GG	Rotthauwe et al. 1997	6.7 ± 1.72E + 06
		amoA2R	CCT CKG SAA AGC CTT CTT C	Rotthauwe et al. 1997	
γ-proteobacteria (AO)	*amoA*	amoA3F	GGT GAG TGG GYT AAC MG	Purkhold et al. 2000	7.2 ± 2.23E + 07
		amoB4R	GCT AGC CAC TTT CTG G	Purkhold et al. 2000	
Archaeal (AO)	*amoA*	Arch-amoAF	STA ATG GTC TGG CTT AGA CG	Francis et al. 2005	0.1 ± 0.10E + 07
		Arch-amoAR	GCG GCC ATC CAT CTG TAT GT	Francis et al. 2005	
Nitrate reducers	*narG*	narG1960F	TAY GTS GGS CAR GAR AA	Philippot et al. 2002	8.5 ± 0.7E + 06
		narG2650R	TYT CRT ACC ABG TBG C	Philippot et al. 2002	
Denitrifiers	*nirS*	cd3aF	GTS AAC GTS AAG GAR ACS GG	Michotey et al. 2000	3.9 ± 1.5E + 06
		R3cd	GAS TTC GGR TGS GTC TTG A	Michotey et al. 2000	
Denitrifiers	*napA*	v66	TAY TTY YTN HSN AAR ATH ATG TAY GG	Flanagan et al. 1999	9.3 ± 0.7E + 07
		v67	DAT NGG RTG CAT YTC NGC CAT RTT	Flanagan et al. 1999	

Table 3. Pyrosequencing and diversity estimators of 16S rRNA, *nirS*, *nirK*, and *nosZ* genes from the microbial mat from Oman

	No. of sequences		At 97% cut-off					
Target gene	Before cleaning	After cleaning	No. of OTUs	Chao 1 (lower/upper)	ACE	Jackknife	Shannon	Simpson
16S rRNA	19420	7467	1065	1985 (1786/2240)	38682	27340	7.78	0.01
nirS	19487	8836	20	29 (22/56)	34	31	0.07	0.98
nirK	42724	1004	57	107 (76/189)	153	116	2.16	0.20
nosZ	8584	4148	259	438 (365/562)	553	502	4.08	0.04

Pyrosequencing of 16S rRNA and Functional Genes

Purified DNA extracts from the mat were submitted to the Research and Testing Laboratory (RTL, Lubbock, TX, USA) for tag-pyrosequencing of amplified gene fragments of bacterial 16S rRNA, *nirS*, *nirK*, as well as *nosZ* (nitrous oxide reductase encoding gene) that is a biomarker gene for the last step of denitrification. Tag-encoded FLX amplicon pyrosequencing (bTEFAP) was performed as described before (Dowd et al. 2008a,b) using the GS FLX titanium sequencing kit XLR70. One-step PCR was conducted with a mixture of hot-start and hot-start high-fidelity *Taq* polymerases along with specific primers.

The primers used for the amplification of 16S rRNA were 28F (GAGTTTGATCNTGGCTCAG) and 519R (GTNTTACNGCGGCKGCTG), generating a fragment size of ca. 491 bp. The primers used for the amplification of functional genes were *nirS*372F: TGTAGCCAGCATTGTAGCGT and *nirS*845R: TCAAGCCAGACCCATTTGCT for *nirS* genes, *nirK*1F: GGMATGGTKCCSTGGCA and *nirK*5R: GCCTCGATCAGRTTRTGG for *nirk* genes and *nosZ*F: CGYTGTTCMTCGACAGCCAG and *nosZ*R: CGSACCTTSTTGCCRTYGCG for *nosZ* genes.

The bTEFAP sequencing was performed according to the RTL protocols (www.researchandtesting.com). Obtained sequences were trimmed, low quality ends and tags were removed and were checked for chimeras using custom software (Dowd et al. 2008b) and the Black Box Chimera Check software B2C2 (http://www.researchandtesting.com/B2C2.html). Sequences shorter than 250 bp were excluded from further analysis. The program MUSCLE was used for unstructured multiple sequence alignment using non-default parameters –maxiters 2, -diags, which were shown to minimize alignment expansion in short hypervariable tags (Edgar 2004).

High-quality reads were then clustered based on $\geq$ 95% sequence identity into operational taxonomic units (OTUs) by using the Mothur software (Schloss et al. 2009). Due to the large number of sequences, Mothur was used to create a set of unique sequences, representative of each OTU (at 5% cutoff) via the getotu.rep command. These sequences were searched against public databases with BLAST (Altschul et al. 1997) to verify their affiliation with the respective genes, and detailed sequence analyses were conducted with the ARB software package (Ludwig et al. 1998). Based on amino acids translated from *nirS* and *nosZ* genes, these sequences were aligned to our own databases that consist of all publicly available sequences for both genes as of July 2012 (except for few environmental metagenomes).

Sequences that were of low quality and could not be aligned properly were omitted. Phylogenetic affiliations of the mat sequences were determined via maximum parsimony with the quick-add search function in ARB against a consensus tree that has been constructed with all sequences in the database based on maximum likelihood, parsimony and distance matrix algorithms. For clarity reasons, only the mat sequences along with their immediate relatives are shown.

Results

Ammonia Oxidation, Denitrification and Anammox Rates

Measurements of the potential rate of ammonia oxidation showed a continuous increase in concentration of NO_2^- with time (Figure 1A). The maximum concentration of NO_2^- was reached after 6h of incubation, after which it remained unchanged. The calculated potential rate of ammonia oxidation was 0.79 ± 0.43 nmol N g^{-1} h^{-1}. The killed controls did not produce NO_2^- (Figure 1A).

The potential rate measurements of denitrification showed a progressive increase in the accumulation of N_2O in the incubation bottles, indicative of denitrification activity, within the 24 h of incubation (Figure 1B). The potential rate of total denitrification in the mat (i.e., including the production of both N_2 and N_2O) calculated for the 2–6-h time interval was 2 ± 1.1 nmol N g^{-1} h^{-1}. N_2O production in the absence of acetylene inhibition showed also a slight increase with time and reached its maximum after 6 h of incubation (Figure 1C).

The calculated rate of spontaneous N_2O production during the 2–6-h interval reached 0.09 ± 0.07 nmol N g^{-1} h^{-1}. From these rates, it is deduced that the relative share of N_2O production from the total production of nitrogenous gases by denitrification was $4.7 \pm 0.9\%$ under anoxic conditions. In the killed controls, neither denitrification activity nor N_2O emission was observed (Figures 1B and C). The anammox process was not detectable in the mat using $^{15}NH_4^+$ incubations, since the production of $^{29}N_2$ was not significantly different from zero for both living and killed mats (data not shown).

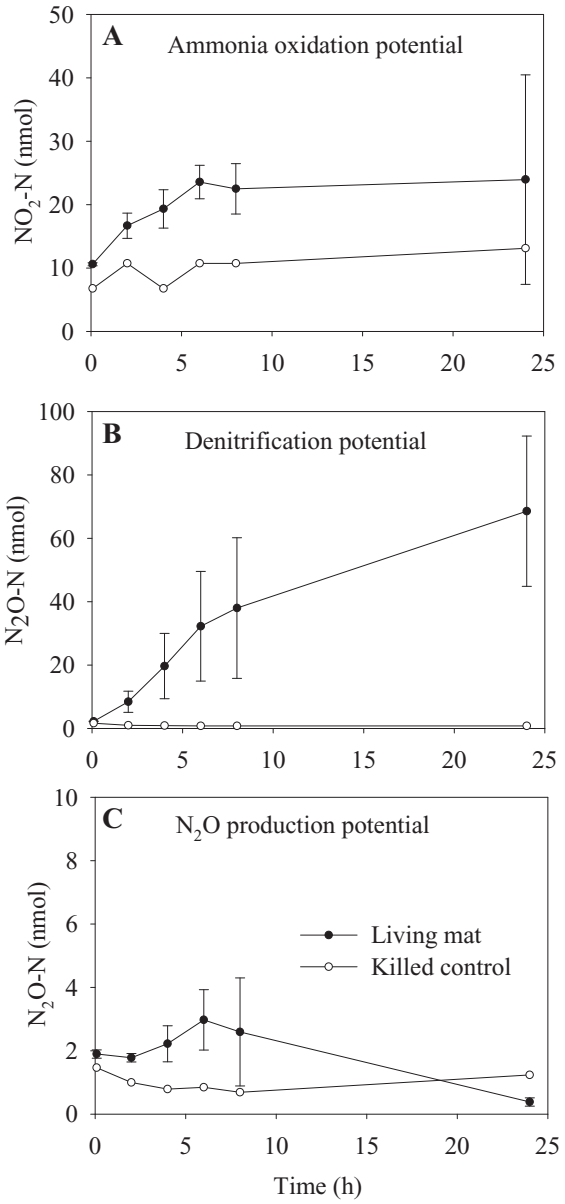

Fig. 1. Potential ammonia oxidation measured as NO_2-N production over time (A), and potential denitrification activity, measured as N_2O-N production over time, in slurry incubations of microbial mat amended with 25 μM NO_3^- and with (B) or without (C) acetylene inhibition of the last step of denitrification. The linear increase in N_2O concentration was used to calculate the rates of complete (B) and incomplete (C) denitrification per g of mat. Error bars represent ± standard deviation ($n = 6$). Additional incubations without nitrate and with autoclaved mat pieces served as negative controls.

Abundance of Functional Genes

The *amoA* genes of ammonia-oxidizing bacteria and archaea as well as the functional genes *narG*, *napA* and *nirS*, key to nitrate-reducing and denitrifying bacteria were detectable at the DNA level in the mat (Table 2). The copy numbers of the gammaproteobacterial *amoA* genes were one order of magnitude higher than the betaproteobacterial *amoA* genes (Table 2). The copy number of archaeal amoA genes were much lower than both the *amoA* genes of *Beta-* and *Gammaproteobacteria* (5X and 60X less, respectively). The genes *narG* and *nirS* exhibited comparable estimates in the mat, with a slightly higher abundance of *narG*. The copy number of *napA* gene was around 10 times higher than both *narG* and *nirS* and reached $9.3 \pm 0.7 \times 10^7$ copy g^{-1} of mat.

Diversity of 16S rRNA Genes in the Mat

A total of 7467 sequences of 16S rRNA gene (out of 19,420 sequences before trimming) were obtained after discarding low quality reads and primer sequences (Table 3). The sequences were distributed among 20 different major bacterial groups, with *Cyanobacteria*, *Proteobacteria*, *Spriro-chaete*s, *Clostridium* class, *Chloroflexi*, and *Flavobacteria* encompassing approximately 70% of the total number of sequences (Figure 2A). The number of detected OTUs at the 97% sequence similarity threshold was 1065 (Table 3) and the calculated diversity indices, based on this cut-off, were 1985 (Chao 1), 38682 (ACE), 27340 (Jackknife), 7.78 (Shannon) and 0.01 (Simpson).

Cyanobacteria constituted about 8% of the total number of obtained sequences, with *Oscillatoria* as the most frequently occurring genus (Figure 2B). Then, 15% of the cyanobacterial sequences were affiliated with sequences of the nitrogen-fixing *Nostocales* and *Stigonematales* groups. The remaining sequences were distributed among the genera *Microcoleus*, *Geitlerinema*, *Halothece*, *Cyanothece*, *Lepto-lyngbya*, *Spirulina*, *Lyngbya*, and *Halomicronema*. The proteobacterial sequences belonged mainly to *Alpha-*, *Delta-*, and *Gammaproteobacteria* (28% of total sequences) and few sequences of the *Beta-* and *Epsilonproteobacteria* (≤1.5%).

Alphaproteobacteria constituted 13% of the total number of sequences, of which 38% and 36% belonged to the orders *Rhodobacterales* and *Rhizobiales*, respectively. Although *Rhodobacterales* order contained sequences that fell phylogenetically into the genera *Paracoccus*, *Rhodovulum*, *Hyphomonas*, *Roseobacter*, and *Rhodobacter*, the *Rhizobiales* order had sequences that were related to the genera *Rhodomicrobium*, *Pravibaculum*, *Rhodobium*, *Rhizobium*, *Mesorhizobium*, and *Nitrobacter* (Figure 2B).

The majority of the deltaproteobacterial sequences belonged to around 12 different genera of sulfate-reducing bacteria. *Gammaproteobacteria* constituted 4% of the total sequences and contained sequences related to *Haliea*, *Pseudomonas*, *Alteromonas*, *Beggiatoa*, and *Halochromatium*. Sequences of the *chloroflexi* group were mainly affiliated to the genera *Levilinea, Anaerolineae, Chloroflexus, Oscillochloris*, and *Chlorothrix* (Figure 2B). The remaining less dominant groups were distributed among *Spirochaetes*, *Flavobacteria*, *Cytophaga*, *Sphingobacteria*, *Planctomycetes*, *Acidobacteria*, *Acidobacteria*, *Deinococci*, and *Nitrospira*.

Diversity of Functional Genes

A total of 8836, 1004 and 4148 sequences of *nirS*, *nirK*, and *nosZ*, respectively, were generated after discarding

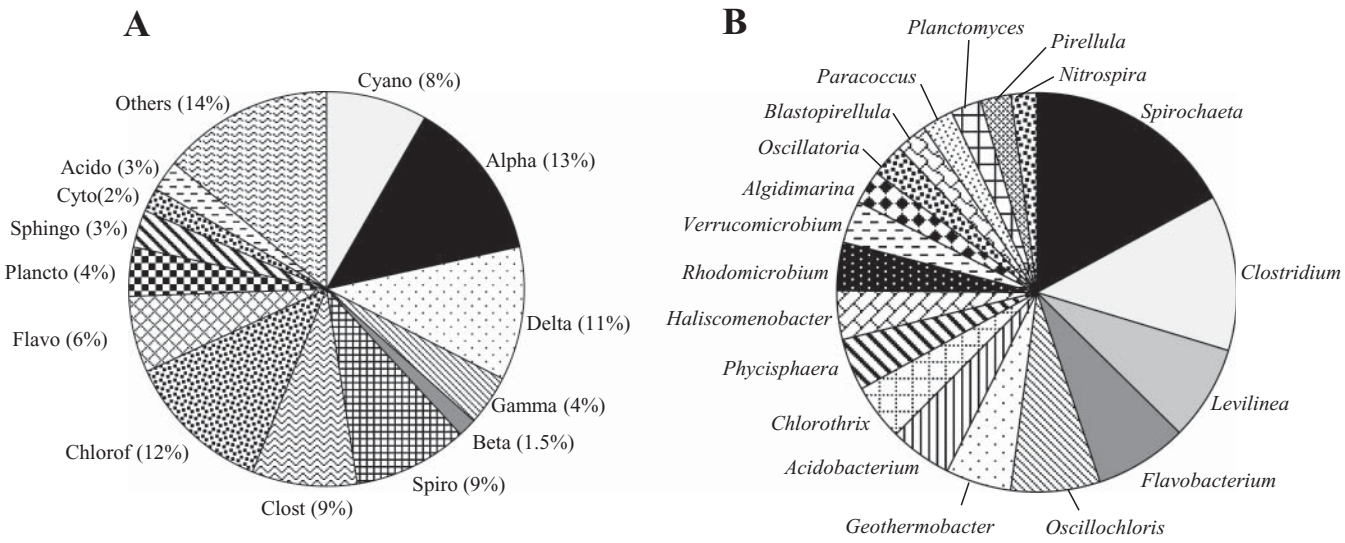

Fig. 2. Relative abundance of the most common bacterial classes (A) and genera (B) encountered by 16S rRNA genes pyrosequencing of the mat samples.

low-quality reads. The sequences of *nirS* genes in the mat were assigned to 20 OTUs based on a 97% nucleic acid sequence similarity cutoff. Then, 99% of these sequences (15 OTUs) formed one cluster that was phylogenetically affiliated to uncultured denitrifying bacteria from sediments of Pearl River Estuary (HQ882515) and San Francisco Bay Estuary (GQ453987), with a maximum sequence similarity of 80% (Figure 3A).

The remaining five OTUs, were also affiliated to sequences of unknown denitrifying bacteria obtained from Chinese soils (JF261041 and 42). Sequences of the *nirK* gene were assigned to a total of 57 OTUs and formed two clusters of 31 and 26 OTUs each (Figure 3B).

All *nirK* sequences were phylogenetically related to sequences from the *Rhizobiales* group of *Alphaproteobacteria*. Sequences of the *nosZ* gene generated the highest number of OTUs (i.e., 259) among the three studied denitrifying genes (Table 3). And, 94% of these sequences, representing 159 OTUs clustered with *nosZ* sequences from *Halmonas koreensis* (FJ686170) and *Rhodanobacter* sp. (GQ404523) but the sequences similarity was $\leq$ 40% (Figure 3C). The second cluster, containing 99 OTUs and representing 6% of the sequences, was phylogenetically affiliated to *nosZ* sequences from *Pseudomonas* species (Figure 3C). A single *nosZ* sequence from our mat was related to a *nosZ* sequence belonging to *Marinobacter hydrocarbonoclasticus*.

Discussion

Denitrification in the Hypersaline Mat of Oman

The potential rates of N-cycle pathways suggest that nitrogen loss of fixed nitrogen occurs mainly by denitrification, and not by anammox, in the hypersaline mat of Oman. Microbial mats, in general, offer a suitable niche for denitrifying bacteria, mainly because of the presence of a suboxic layer, the continuous supply of organic matter produced by phototrophs and the supply of nitrate from the water column or through the nitrifying microbes in the mat. Indeed, several marine microbial mats have been previously shown to exhibit denitrification activity such as the mats from Camargue, France and mats from intertidal flats of Tomales Bay and Elkhorn Slough, California, USA (Desnues et al. 2007; Golet and Ward 2001; Joye and Paerl 1993).

The denitrification rates measured in these mats were comparable to the potential rate measured in our mat. Although the denitrification rate in the mat from Oman was equivalent to 1.2 ± 1.1 mmol N m^{-2} d^{-1}, converted to units that match those used in other studies, others measured rates as high as 0.88, 0.2 and 3.14 mmol N m^{-2} d^{-1} in the mats from Camarague, Tomales Bay and Elkhorn Slough, respectively (Desnues et al. 2007; Golet and Ward 2001; Joye and Paerl 1993). Potential rate measurements with and without acetylene inhibition showed that N_2O gas constituted around 5% of the totally produced gases during denitrification under anoxic conditions. N_2O emission due to incomplete denitrification was also found to be minimal in other mat systems (Golet and Ward 2001).

However, since N_2O can be produced by both nitrification and denitrification (Meyer et al. 2008), we have no evidence that the produced N_2O originates entirely from incomplete denitrification. The potential rate of N_2O production corresponded to around 11.4% of the potential nitrification rate, suggesting that some of the N_2O might also be produced by the nitrification process.

When the potential rates of denitrification and nitrification in our mats are compared, it becomes evident that the nitrate supplied by the nitrifying bacteria might constitute a major fraction of nitrate reduced by denitrification. In fact, the pore water content of NO_3^-, which is readily available for denitrifying bacteria, was around 6 ± 2 μmol/l (Table 1) and this would sustain denitrification activity at the measured rates for only about 1.6 h, if there is no replenishment either from

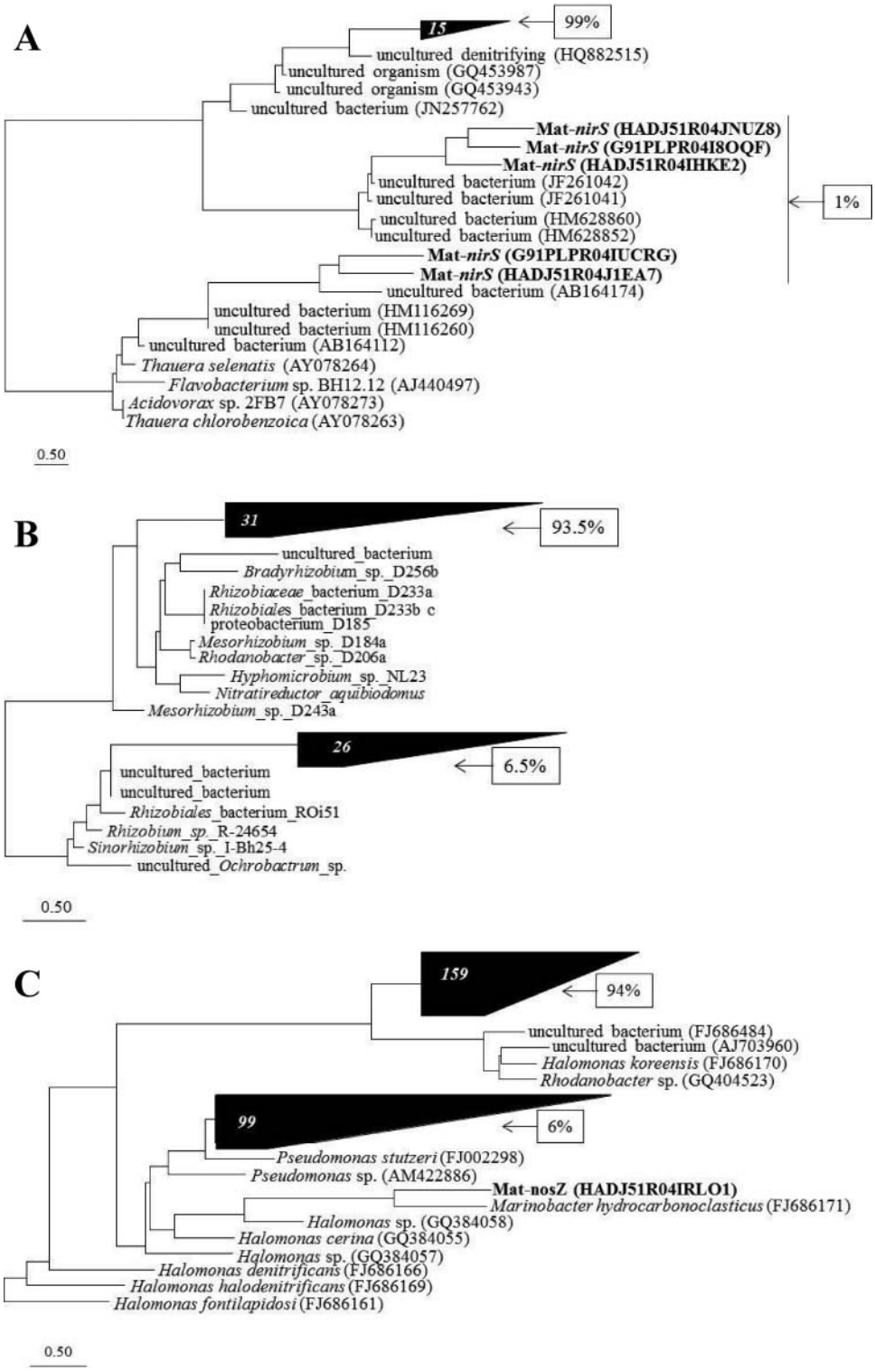

Fig. 3. Phylogeny of *nirS* (A), *nirK* (B), and *nosZ* (C) genes in the microbial mat. Phylogenetic affiliations of the mat sequences were determined via maximum parsimony with the quick-add search function in ARB against a consensus tree that has been constructed with all sequences in the database based on maximum likelihood, parsimony and distance matrix algorithms. For clarity, only the mat sequences along with their immediate relatives are shown.

nitrification activity in the mat, or from the water column. The limitation of NO_3^- in both the top and bottom layers of the mat under *in situ* conditions (see Table 1) may be due to the competition of mat microorganisms and the uptake of a major fraction by cyanobacteria and photosynthetic bacteria at the mat surface. The intracellular content of NO_3^- in the mat was also very small (data not shown), suggesting a minimal role of *Beggiatoa* spp. in the nitrogen cycle in this mat. It

can thus be assumed that during the summer draught, denitrification is supplied with NO_3^- by nitrification in the mat, while during the winter floods, the water column can be additional source of NO_3^- to denitrifying bacteria in the mat.

16S rRNA Bacterial Diversity of the Hypersaline Mat of Oman

The hypersaline mat from Oman had a typical bacterial community composition of previously studied hypersaline microbial mats from different geographical locations (Abed and Garcia-Pichel 2001; Abed et al. 2007a; Ferris et al. 1996; Nübel et al. 1999; Schneider et al. 2013). The detected genera of *Microcoleus*, *Halothece Halomicronema* and *Deinococci* are known to contain species that are tolerant to extreme conditions of salinity, temperature and UV radiation (Abed et al. 2002; Garcia-Pichel et al. 1998; Rainey et al. 1997), which is consistent with the environmental conditions of the sampling site. The retrieved sequences from cyanobacteria, *Chloroflexi*, *Gammaproteobacteria* and *Deltaproteobacteria* suggest an active carbon and sulfur cycle in the mat. Similarly, sequences indicative of the nitrogen cycle were also obtained from the same mat. For example, the detection of 15% of the cyanobacterial sequences belonging to the *Nostocales* and *Stigonematales* groups indicates the potential of this mat to fix atmospheric nitrogen. Even the mat-building *Microcoleus chthonoplastes*, which was not previously assigned as a diazotroph, was shown to possess a complete *nif*-gene cluster and to express these genes only under environmental conditions but not in cultures (Bolhuis et al. 2010). Sequences belonging to bacteria with a known role in the nitrogen cycle such as *Beggiatoa*, *Paracoccus*, *Roseobacter*, *Rhodomicrobium*, *Mesorhizobium*, and *Nitrobacter* were also detected.

Abundance and Diversity of N-Cycle Functional Genes

The measured denitrification activity in the mat from Oman was further confirmed by the detection of denitrification-related functional genes either by qPCR and/or pyrosequencing. The first step of denitrification, namely the reduction of nitrate to nitrite, is performed with the help of *narG* and *napA* genes, which were both detectable in the mats. The detection of higher abundance of *napA* than *narG* gene suggests that most of the nitrate reduction is carried out by periplasmic nitrate reductase-containing microorganisms. It is well known that more organisms are capable of nitrate reduction than of complete denitrification (Zumft 1997), thus it is not surprising to detect a greater abundance of *narG* and *napA* than *nirS* genes.

The activity of *narG* and *napA* genes result in the formation of nitrite, which is then converted to nitric oxide with the help of the detected genes *nirS* and *nirK*. However, nitrite can also be reduced to ammonium via the process of dissimilatory nitrate reduction to ammonium (DNRA). Although we have no direct evidence that this process occurs in our mats, conditions in other hypersaline mats have been shown favorable for its occurrence (Bonin and Michotey 2006). DNRA, along with ammonification, mineralization and nitrogen fixation, could contribute to the pool of ammonium, which was measured at high concentrations in the mats (see Table 1). On the other hand, the removal of ammonium by nitrifiers in the mats was confirmed by measuring ammonia oxidation at detectable rates as well as by the detection of *amoA* genes by qPCR.

Although qPCR showed the presence of both bacterial and archaeal *amoA* genes, the copy numbers suggested a more important role of bacterial than archaeal ammonia oxidizers in these mats. Previous results showed the coexistence of these two group in the same mats (Sobolev et al. 2013), although those organisms seem to require different chemical conditions. Although archaeal ammonia oxidizers outnumbered bacterial ammonia oxidizers in many environments (e.g., Abell et al. 2010; Wuchter 2006), the opposite was observed in others (e.g., Wankel et al. 2011). This was shown to be influenced by different environmental parameters such as pH, temperature and ammonium (He et al. 2007; Nicol et al. 2008; Tourna et al. 2008). For instance, archaeal ammonia oxidizers were detected at high abundance in hot spring microbial mats, where temperatures exceed 40°C, whereas bacterial ammonia oxidizers were absent (Zhang et al. 2008; Zhao et al. 2011). Therefore, a comprehensive study is needed to identify the environmental determinants of the distribution and abundance of ammonia oxidizing communities in our mats.

The detection of *nirS* and *nirK* in the same mat suggests the presence of two microbial populations capable of nitrite reduction, since these two genes have never been found in the same cell (Zumft 1997). Indeed, the phylogeny of the *nirS*- and *nirK* sequences retrieved from our mat supports this assumption. The *nirS* sequences did not branch with known denitrifying bacteria and only had $\leq$ 80% similarity to the closest relatives, indicating their novelty. In contrast, the *nirK* sequences fell within the *Rhizobiales* group of *Alphaproteobacteria*. The detection of this group in the mat was further supported by the detection of 36% of the 16S rRNA alphaproteobacterial sequences belonging to the same group.

The *Rhizobiales* group was also shown to be present in other hypersaline microbial mats based on 16S rRNA sequences (Schneider et al. 2013; Sørensen et al. 2005). An interesting feature of the obtained sequences of *nirS* and *nirK* in our mat was the detection of higher number of OTUs in *nirK* than in *nirS* sequences (57 vs. 20), although the total number of sequences was around 8 times higher in the case of *nirS* gene. Previous studies in different ecosystems demonstrated variable patterns of predominance of these two genes, with *nirS* exhibiting higher diversity than *nirK* in some cases but the opposite trend in others (Braker et al. 2000; Liu et al. 2003; Priemé et al. 2002; Smith and Ogram 2008; Yoshida et al. 2010).

In hypersaline microbial mats, *nirS* bacteria were found predominant in the deep anoxic zone while *nirK* bacteria were detected in all layers (Desnues et al. 2007; Golet and Ward 2001). This could explain the higher diversity of *nirK* bacteria in mats, since these microorganisms are apparently better adapted to the fluctuating conditions of oxygen, sulfide and pH within mats. Indeed, a *nirK*-containing bacterium belonging to the genus *Mesorhizobium* was shown to perform

aerobic denitrification (Okada et al. 2005). Both 16S rRNA and *nirK* sequences related to this genus were detected in our mat.

Complete denitrification through the conversion of N_2O to N_2 was also confirmed by the detection of *nosZ* genes. The high diversity of this gene, as indicated by the number of OTUs and the phylogeny of sequences, suggested that there are many bacteria in this mat capable of performing this step. This could be the reason why complete denitrification was the dominant process in the mats and N_2O emission contributed to a minor fraction (i.e., <5%) of the total produced nitrogenous gases. Most of the *nosZ* sequences were related to the genera *Halomonas*, *Rhodanobacter*, *Pseudomonas*, and *Marinobacter*. Bacteria belonging to these genera were also encountered in the same mat via pyrosequencing analysis of the 16S rRNA gene as well as in other hypersaline microbial mats using cultivation and molecular techniques (Abed et al. 2007b; Abed 2010). So far there is only very limited information available about *nosZ* sequences from microbial mats to make comparisons with. A study on *Beggiatoa* mats from Guaymas Basin, Gulf of California reported 20 *nosZ* sequences, but all of them were most closely associated with environmental clones (Bowles et al. 2012).

In conclusion, nitrification and denitrification is likely a general phenomenon for hypersaline mats due to the favorable conditions therein. The hypersaline mats of Oman harbor novel microorganisms with the potential to perform nitrification and denitrification at detectable rates. The denitrifying bacteria within this mat belong to novel species, besides other species related to the *Rhizobiales* group, *Pseudomonas*, *Halomonas* and *Marinobacter*.

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