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Diversity of nitrogen-fixing bacteria in cyanobacterial mats

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Cyanobacteria; diversity; gene expression; microbial mat; nitrogen fixation.

Abstract

The structure of the microbial community and the diversity of the functional gene for dinitrogenase reductase and its transcripts were investigated by analyzing > 1400 16S rRNA gene and *nifH* sequences from two microbial mats situated in the intertidal zone of the Dutch barrier island Schiermonnikoog. Although both microbial mat communities were dominated by *Cyanobacteria*, they differed with respect to the composition of the total bacterial community. *Proteobacteria*-related sequences were retrieved as the second most abundant group higher up in the littoral (Station I), whereas *Bacteroidetes* were the second most abundant group at the low water mark (Station II). The diazotrophic (nitrogen-fixing) communities at both stations were also different, but had more operational taxonomic units in common than the total bacterial community. Denaturing gradient gel electrophoresis also revealed differences in the total bacterial and diazotrophic community in two consecutive years. Analysis of the expression of *nifH* at Station I showed a discrepancy between the present and the active diazotrophic community. Transcript abundances of the different diazotrophs changed over a 24-h cycle and were dominated by cyanobacterial lineages in the daytime, while *Gammaproteobacteria* peaked at night. These variations might be responsible for the pattern in nitrogenase activity observed in these mats.

Introduction

Microbial mats are microscale ecosystems recognized for their large diversity and metabolic potential (reviewed by, e.g. Paerl *et al.*, 2000). These ecosystems are often found as multilayered benthic communities growing in a variety of different environments (Stal, 2000). Microbial mats are often built by filamentous *Cyanobacteria*, although in some cases, unicellular *Cyanobacteria* have been shown to be the dominant component. In addition, microbial mats may accommodate a variety of other functional groups of microorganisms. *Cyanobacteria* are oxygenic photoautotrophs and have low nutritional requirements. They utilize sunlight, H₂O and CO₂ as the sources of energy, electrons and carbon, respectively. Many *Cyanobacteria* are also capable of fixing atmospheric dinitrogen (N₂). The ability to fix N₂ confers *Cyanobacteria* with a distinctive advantage that allows them to colonize the nutrient-poor and nitrogen-depleted environments in which microbial mats often thrive. *Cyanobacteria* are therefore perfectly equipped to

form microbial mats (Stal, 2001). However, the ability to fix N₂ is not limited to *Cyanobacteria* and occurs among a variety of other members of the *Bacteria* as well as in some *Archaea*.

Microbial mats are found in a range of different environments, for example marine intertidal flats, hypersaline and alkaline environments, hot springs and deserts. Temperate intertidal microbial mats are dominated by filamentous nonheterocystous *Cyanobacteria* such as *Oscillatoria* sp., *Microcoleus chthonoplastes* and *Lyngbya aestuarii* (Stal *et al.*, 1985; Villbrandt & Stal, 1996), although heterocystous forms such as *Anabaena*, *Calothrix* and *Nodularia* have been reported as well. *Microcoleus chthonoplastes* and *L. aestuarii* are also known from tropical marine mats, together with representatives of the genera *Oscillatoria*, *Phormidium* and *Synechocystis* (Paerl *et al.*, 2000). Investigations using a variety of molecular techniques yielded a more complete picture of the genetic composition of microbial mat communities. For instance, denaturing gradient gel electrophoresis (DGGE) revealed a diverse community in a hypersaline

microbial mat comprised of the filamentous nonheterocystous cyanobacteria *Microcoleus*, *Oscillatoria*, *Leptolyngbya*, *Phormidium* and the unicellular *Pleurocapsa* and *Gloeotheca* (Fourçans *et al.*, 2004). In addition, sulfate-reducing bacteria (SRB), sulfur-oxidizing and anoxygenic phototrophic bacteria were present. Analyses of small-subunit rRNA genes of a hypersaline microbial mat revealed > 700 genotypes belonging to over 40 bacterial phyla, emphasizing the high diversity within these microecosystems (Ley *et al.*, 2006). In a number of studies, *nifH*, the gene coding for dinitrogenase reductase, which is one of the two proteins that constitute the nitrogenase complex, has been used to identify diazotrophic organisms. These studies revealed a high diversity of diazotrophic organisms in microbial mats, which was not limited to *Cyanobacteria* (e.g. Zehr *et al.*, 1995; Olson *et al.*, 1999; Steppe *et al.*, 2001; Falcón *et al.*, 2007). Sequences that were common in various microbial mats belonged to the *Cyanobacteria* as well as to purple sulfur bacteria and SRB.

In a few cases, the analysis of *nifH* was accompanied by measurements of nitrogenase activity. Nitrogenase was shown to exhibit a variety of daily activity patterns (e.g. Villbrandt *et al.*, 1990) that are difficult to interpret with respect to the potential N₂-fixing organisms (e.g. Severin & Stal, 2008). In some cases, these patterns did not correspond to what was predicted based on the dominant diazotrophs (e.g. Bauer *et al.*, 2008). The high daytime and low nighttime nitrogenase activity that was observed in a tropical microbial mat rather hinted toward an involvement of heterocystous types than the nonheterocystous filamentous and unicellular forms that were found based on the analysis of the 16S rRNA gene (Bauer *et al.*, 2008). Moreover, analyses of *nifH* in these mats showed that the major phylotypes detected did not even belong to the *Cyanobacteria*. Other studies also revealed a discrepancy between *nifH*-based clone libraries and those based on transcripts, which confirmed that abundant diazotrophs are not necessarily the key contributors to N₂ fixation (e.g. Hewson *et al.*, 2007). These results emphasize the complexity of diazotrophic microbial mat communities and the need for further studies investigating the actual diazotrophic community of microbial mats in comparison with the observed nitrogenase activity patterns.

This study aimed at the characterization of the structural as well as the functional components of two different types of microbial mats. Therefore, we assessed the microbial diversity and community structure by constructing 16S rRNA gene and *nifH* clone libraries in order to identify the major microbial taxa in both microbial mats. The analysis of functional genes such as *nifH* was performed to reveal the link between community structure and ecosystem functioning. Based on their different locations along an intertidal gradient and the dissimilar nitrogenase activity patterns that were observed for these mats (Severin & Stal, 2008), we expected to find dissimilar bacterial communities in the two

mat types, especially with regard to the diazotrophic fraction of the community. We were furthermore interested in the hitherto unknown fraction of diazotrophs that actively transcribe *nifH* and therefore potentially contribute to N₂ fixation in this system. In order to explore this question, we constructed cDNA *nifH* clone libraries, compared these with the corresponding DNA *nifH* clone libraries and evaluated the changes within the active diazotrophic community during a 24-h period in relation to the nitrogenase activity pattern.

Materials and methods

Sampling

The study site was located on the Dutch barrier island Schiermonnikoog, which is situated in the Wadden Sea close to the Dutch mainland. The geographic coordinates are 53°29'N and 6°08'E. Microbial mats were found at the sandy beach covering the north bank of the island facing the North Sea (Fig. 1). The beaches supporting microbial mats eventually turn into salt marshes as a result of mats being overgrown by higher plants (green beaches). Because of this succession and the gradually increasing profile of the beach, different mat types develop along the littoral gradient.

The two sampling sites within this area were chosen based on microscopic observations of the cyanobacterial community composition as well as the position along the littoral gradient. Station I is located near the dunes and influenced by freshwater (rain and upwelling groundwater) and irregularly inundated by seawater, depending on the tide and the wind direction. The mats at this station were covered by sand that was deposited by wind transport. These mats revealed a high cyanobacterial diversity as judged by microscopic examinations and contained heterocystous as well as nonheterocystous filamentous *Cyanobacteria* and also unicellular forms. Diatoms were the second major group of oxygenic phototrophic organisms. Measurements of nitrogenase activity at this station showed strong daily patterns, which has been attributed to shifts in the active diazotrophic community (Severin & Stal, 2008). Station II is situated near the low water mark. The regular tidal inundation of this station made the influence of seawater far more important than was the case at Station I. The *Cyanobacteria* of the mats at Station II were mostly nonheterocystous forms and dominated by *L. aestuarii*. Diatoms were also abundant. Occasionally, heterocystous *Cyanobacteria* were found, but they were not a structural part of this community. The daily pattern of nitrogenase activity agreed with those recorded for nonheterocystous *Cyanobacteria* in which most of the activity was confined to the night (Severin & Stal, 2008).

The two stations were sampled over a 24-h period in summer (May/June) 2006 and 2007. In 2006, the mats were sampled at intervals of 4 h, and in 2007, samples were taken

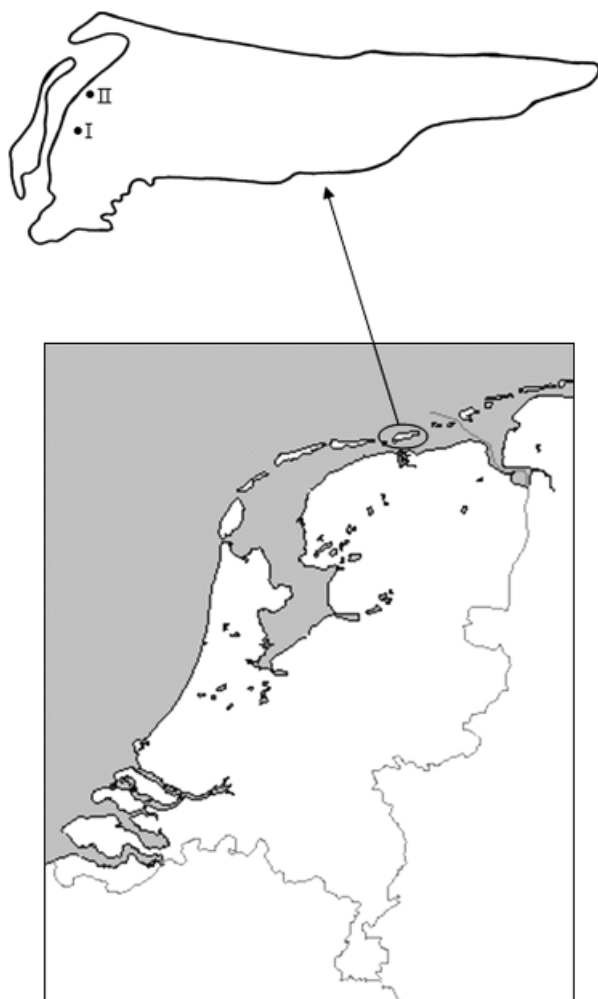


Fig. 1. Geographical location of Stations I and II on the island Schiermonnikoog.

every 2 h. At each of the time points, three cores were taken from different spots within the mat surface area. The samples were taken using disposable 10-mL syringes, of which the top was removed to obtain a corer with a diameter of 1.5 cm. The top 2–3 mm of the mat was sampled and divided into four quarters using a scalpel. Each quarter was transferred into a separate cryovial (Simport Plastics, Beloeil, QC, Canada) and immediately frozen and stored in liquid nitrogen (dry shipper). Samples for the construction of clone libraries based on the 16S rRNA gene and *nifH* and its transcripts were taken in 2006. To compare the total bacterial as well as the diazotrophic communities in two subsequent years, the same locations were sampled again in 2007.

Nucleic acid extraction

DNA was extracted from one sample per time point and station using the MO-BIO UltraClean Soil DNA Isolation

kit (MO-BIO Laboratories Inc., Carlsbad, CA) according to the manufacturer's instructions. The quality and quantity of extracted DNA were checked on a 1% agarose gel and using the NanoDrop ND 1000 (NanoDrop Technologies Inc., Wilmington, DE). All DNA extracts from one station were combined and stored at -20°C until used.

RNA was extracted from triplicate samples (three randomly chosen samples per time point and station) using the Qiagen RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions, but replacing the enzyme digest by a bead-beating step (Vortex Genie[®]2 from Scientific Industries Inc., Bohemia, NY; equipped with a Vortex Adapter for 1.5–2.0-mL tubes from MO-BIO Laboratories Inc.; using 0.1-mm glass beads, Sartorius AG, Göttingen, Germany, at maximum speed for 2 min). The quality and quantity of the extracted RNA was checked on a 1% agarose gel. The triplicates of one time point were combined per station and treated with DNase (DNase I, Invitrogen Corporation, Carlsbad, CA) following the manufacturer's instructions. DNA-free RNA was immediately used for reversed transcription using random primers (Superscript II Reverse Transcriptase and Random Primers, Invitrogen Corporation). The resulting cDNA was checked on a 1% agarose gel and stored at -20°C .

PCR, cloning and sequencing

From the DNA extracted from the mat samples in 2006, the nearly complete 16S rRNA gene was amplified using the primer pair 8F (5'-AGA GTT TGA TCM TGG CTC AG-3')/1492R (5'-GGT TAC CTT GTT ACG ACT T-3') (Weisburg *et al.*, 1991). Each 25- μL PCR mix contained 2.5 pmol of each primer, 0.2 mM dNTPs, 1 \times reaction buffer and 0.625 U Taq DNA Polymerase (New England BioLabs, Ipswich, MA) as well as 10–15 ng DNA. Cycling conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 2 min, and a final extension period of 7 min at 72°C . PCR products were checked on a 1% agarose gel.

For the amplification of *nifH* and its transcripts from the DNA and RNA extracted from the mat samples in 2006, a nested PCR with the internal primer pair *nifH* 1 (5'-TGY GAY CCN AAR GCN GA-3') and *nifH* 2 (5'-ADN GCC ATC ATY TCN C-3') (Zehr & McReynolds, 1989) and the external primers *nifH* 3 (5'-ATR TTR TTN GCN GCR TA-3') and *nifH* 4 (5'-TTY TAY GGN AAR GGN GG-3') was performed (Zani *et al.*, 2000). For the first amplification, the PCR mixture was identical to the one used for the 16S rRNA gene amplification, except for the primers and the use of the Qiagen PCR buffer and the Qiagen HotStar Taq Polymerase (Qiagen GmbH). The use of degenerated primers required 25 pmol of each primer per reaction. For the nested reaction, 2 μL of the PCR product from the first reaction was used as a

template. Cycling conditions for both PCR steps included 94 °C for 15 min, 35 cycles of 94 °C for 1 min, 54 °C (first reaction) or 57 °C (nested reaction) for 1 min and 72 min for 1 min, followed by an extension period of 72 °C for 10 min. PCR products were checked on a 1% agarose gel. To analyze community shifts at Station I, the combined cDNA for each time point was processed in the same way.

The fresh PCR products were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen Corporation) following the manufacturer's instructions. The white transformants were used for amplification with the plasmid M13-primer pair (F: 5'-GTA AAA CGA CGG CCA G-3' and R: 5'-CAG GAA ACA GCT ATG AC-3') and checked by gel electrophoresis. Cycling conditions were 95 °C for 2 min, followed by 40 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min and a final extension step at 72 °C for 19 min. For each station, a total of 96 16S rRNA gene and 188 *nifH* clones were sequenced for DNA analyses and 188 clones per time point were sequenced for the analysis of *nifH* transcripts at Station I. PCR products were purified with the Sephadex G-50 Superfine-powder (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and 45- μ L Millipore Multi-Screen-plates (Millipore Corporation, Billerica, MA). After determining the quantity of the purified PCR product, a sequencing reaction was performed using the BigDye Terminator chemistry (BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems Inc., Foster City, CA). For bacterial 16S rRNA gene clones, four overlapping pieces were sequenced using the primers 8F, 907RM (5'-CCG TCA ATT CMT TTG AGT TT-3') (Muyzer *et al.*, 1997), 1346R (5'-TAG CGA TTC CGA CTT CA-3') (Nübel *et al.*, 1996) and 1492R and assembled during the alignment. *NifH* was sequenced using the M13 reverse primer.

DGGE

PCR-DGGE of the bacterial 16S rRNA gene and *nifH* was performed on samples from both stations in 2006 and 2007. For the amplification of the 16S rRNA gene, the primers GC-358F (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG-3') and 907 RM (Muyzer *et al.*, 1997) were used. PCR and DGGE were performed as described by Muyzer *et al.* (1997). *NifH* was amplified and analyzed by DGGE using a semi-nested approach (Widmer *et al.*, 1999). The primers were *nifH*-forA (5'-GCI WTI TAY GGN AAR GGN GG-3'), GC-*nifH*-forB (5'-GCC CGC CGC GCG CGG CGG GCG GGG CGG GGG CAC GGG GGG GGI TGY GAY CCN AAV GCN GA-3') and *nifH*-rev (5'-GCR TAI ABN GCC ATC ATY T-3'). *Cyanobacteria*-selective primers to amplify *nifH* (GC-CNF: 5'-CGC CCG CCG CGC CCC GCG CCG GTC CCG CCG CCC CCG CCC GCG TAG GTT GCC ACC CTA AGG CTG A-3', CNR: 5'-GCA TAC ATC GCC ATC ATT

TCA CC-3') (Olson *et al.*, 1998) were used for a higher resolution of this group of diazotrophs. DGGE conditions were those described by Diez *et al.* (2007). DGGE was performed using the CBS system (DGGE 2000-model, CBS Scientific Company, Salona Beach, CA). Dominant *nifH* bands were excised using sterile razor blades, suspended in 20 μ L of MilliQ and stored at 4 °C overnight. An aliquot of 2 μ L was used for reamplification with the PCR primers (without GC-clamp), cloning (four clones per band) and sequencing.

Sequence analysis

Sequences were aligned in BIOEDIT (Ibis Biosciences, Carlsbad, CA), corrected by manual inspection and analyzed for similarity in BLASTN (Basic Local Alignment Search Tool, National Center for Biotechnology Information, Bethesda). Additionally, the 16S rRNA gene sequences were classified using the RDP classifier (Ribosomal Database Project, Michigan State University, Michigan; Wang *et al.*, 2007) and confirmed the taxonomic ascription of the sequences by BLASTN.

α - and β -diversity of the total microbial and of the diazotrophic community at both stations was estimated using the DOTUR and SONS software programs (Schloss & Handelsman, 2005, 2006) based on the alignment files for the 16S rRNA gene and *nifH* clone libraries. Clustering analyses were performed using the CLUSTERER software (<http://www.comcen.com.au/~journals/clusterabs2006.htm>, Klepac-Ceraj *et al.*, 2006).

All sequences obtained in this study are deposited at the NCBI GenBank database under the accession numbers GQ441193–GQ442612.

Results

Species richness

The diversity of the 16S rRNA gene and of *nifH*, based on the Chao1 richness estimator, varied between the two stations (Supporting Information, Table S1). At a 99% similarity cut-off, the Chao1 diversity richness estimator based on 16S rRNA gene sequences predicted a higher bacterial diversity for Station II than for Station I, even though more sequences were analyzed from Station I. The opposite was the case when diazotrophic diversity based on *nifH* DNA sequences was explored. Diversity estimations of diazotrophic taxa were twice as high for Station I as for Station II, even though more sequences from Station II were analyzed.

Rarefaction curves for 16S rRNA gene did not reach an asymptote at 99% or 97% similarity cut-off, whereas the rarefaction curves of *nifH* leveled off at the 99% similarity cut-off (Fig. S1a and b).

Clustering analyses using the 16S rRNA gene and *nifH* sequences were performed in order to compare the general

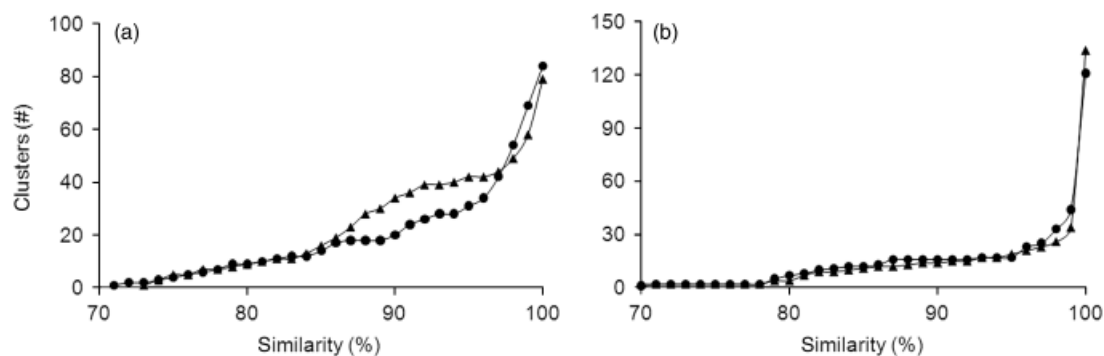


Fig. 2. Clustering analyses of clone libraries based on the 16S rRNA gene (a) and *nifH* (b) at Station I (●) and Station II (▲) in 2006 showing the number of clusters at a certain sequence similarity (%). A steep increase in the number of clusters from 99 to 100% similarity hints toward a high degree of microdiversity.

structure of the abundant bacterial and diazotrophic taxa in both stations (Fig. 2a and b). We found considerable 16S rRNA gene microdiversity at Station II, with a higher proportion of clusters within the 99% similarity cut-off than at Station I, where the operational taxonomic units (OTUs) collapsed more gradually (Fig. 2a). The degree of microdiversity was even higher within the *nifH* gene clusters (Fig. 2b).

Community composition

We used nonparametric richness diversity estimators to estimate the similarity between both microbial mats (Stations I and II) based on OTU membership. The results are shown for a similarity cut-off of 97%, 99% and 100% to allow a comparison of the diversity (and the similarity of the stations) at the three most common cut-off levels (Table 1, Fig. S2). In the following, however, we concentrated on a similarity cut-off of 99%.

Bacterial OTUs based on analysis of the 16S rRNA gene sequences and shared between Stations I and II increased significantly (threefold) on decreasing the similarity cut-off (Table 1, Fig. S2). This pattern was even more evident from the *nifH* analysis. At the 99% cut-off, the similarity between the two stations based on the Jaccard as well as the Sørensen index was higher for the diazotrophic than for the total bacterial community (Table S2). Likewise, the structure of the diazotrophic community at both stations (θ) was more similar than the structure of the total bacterial community (Table S2).

We were also interested in the shared OTUs within the cDNA and DNA clone libraries of the diazotrophic taxa at Station I in order to identify the active fraction of the present diazotrophic community (Table 1, Fig. S2). Despite the different sampling sizes for both fractions (867 vs. 127 sequences for cDNA and DNA, respectively), we observed a substantial proportion of identical sequences among the

Table 1. Summary of the diversity comparison between Stations I and II in 2006 based on shared OTUs

Target	Similarity cut-off (%)	Shared OTUs	Sequences in shared OTUs (%)	
			I	II
16S rRNA gene	100	4	14.3	15.2
	99	9	40.5	27.9
	97	9	51.2	39.2
<i>nifH</i>	100	9	11.8	8.4
	99	19	48.0	43.5
	97	8	73.2	66.9
<i>nifH</i> cDNA-DNA			cDNA	DNA
	100	21	34.9	55.9
	99	23	66.4	86.6
	97	21	83.6	92.9

shared OTUs at the 99% similarity cut-off. Less than 10% of the OTUs found in the *nifH* cDNA clone libraries of Station I were identical to OTUs in the corresponding *nifH* DNA libraries, but these 10% harbored 66% of the sequences within the shared OTUs. The similarity between the present and the active diazotrophic community based on the Jaccard as well as the Sørensen index was high (Table S2).

Diazotrophic and nondiazotrophic *Cyanobacteria*

In order to investigate the role of *Cyanobacteria* and other potential diazotrophs in microbial mat N_2 fixation, we compared the composition of abundant bacterial and diazotrophic taxa by constructing clone libraries based on the 16S rRNA gene and *nifH* for both stations (Table 2).

At both stations, clone libraries of the 16S rRNA gene were dominated by *Cyanobacteria*-related sequences (Fig. 3a and b). *Lyngbya* and *Microcoleus* sequences were more prominent at Station I than at Station II. Clone libraries of *nifH* also revealed a dominance of *Cyanobacteria*-related

Table 2. Phylogenetic summary based on analyses of bacterial 16S rRNA gene (A) and *nifH* (B) clone libraries

16S rRNA gene	No. of clones	Station I	No. of clones	Station II
		Closest relative in GenBank (identity)		Closest relative in GenBank (identity)
(A)				
Presumed Cyanobacteria	38		28	
	16	<i>Lyngbya</i> sp. (90–99%)	9	<i>Leptolyngbya</i> sp./ <i>L. antarctica</i> (96–99%)
	11	<i>Microcoleus chthonoplastes</i> (93–99%)	8	<i>Phormidium animale</i> / <i>P. pseudopristleyi</i> (95–98%)
	6	<i>Leptolyngbya</i> sp. (88–98%)	4	<i>Microcoleus chthonoplastes</i> (97%)
	2	<i>Hydrocoleum lyngbyaceum</i> (93–94%)	3	<i>Lyngbya</i> sp. (95–96%)
	2	<i>Spirulina</i> sp. (97%)	2	<i>Nodularia harveyana</i> (98–99%)
	1	<i>Calothrix</i> sp. (97%)	1	<i>Calothrix</i> sp. (96%)
			1	<i>Spirulina</i> sp. (97%)
Presumed Proteobacteria	20		10	
Alpha-Subdivision	7	<i>Thalassobaculum</i> sp. (90%)	3	<i>Caulobacter</i> sp. (95%)
		<i>Maritimibacter</i> sp. (91%)		<i>Phyllobacterium</i> sp. (90%)
		<i>Agrobacterium sanguineum</i> (97–98%)		<i>Amaricoccus tamworthensis</i> (93%)
		<i>Roseobacter</i> sp. (97%)		
		<i>Sulfitobacter</i> sp. (97%)		
Beta-Subdivision	1	<i>Rubrivivax gelatinosus</i> (91%)	1	<i>Hydrogenophaga taeniospiralis</i> (98%)
Delta-Subdivision	3	<i>Haliangium ochraceum</i> (93–94%)	0	
Gamma-Subdivision	9	<i>Arhodomonas</i> sp. (93–98%)	6	<i>Aeromonas salmonicida</i> (99%)
		<i>Silanimonas lenta</i> (97%)		<i>Thermomonas brevis</i> (94%)
		<i>Alkalilimnicola ehrlichei</i> (93%)		<i>Haliea</i> sp. (95–96%)
		<i>Lysobacter</i> sp. (92%)		
Presumed CFB	2		16*	
		<i>Algoriphagus</i> sp. (98%)		<i>Maribacter</i> sp. (91%)
				<i>Lewinella cohaerens</i> (95–96%)
				<i>Algoriphagus</i> sp. (96%)
Chloroplasts	29		20	
Unidentified clones	5		11	
(B)				
Presumed Cyanobacteria	81		100	
	73	<i>Lyngbya aestuarii</i> (97–99%)	74	<i>Lyngbya aestuarii</i> (97–99%)
	5	<i>Oscillatoria</i> sp. (88%)	23	<i>Gloeotheca</i> sp. (83–84%)
	2	<i>Phormidium</i> sp. (90%)	2	<i>Phormidium</i> sp. (90%)
	1	<i>Gloeotheca</i> sp. (83%)	1	<i>Oscillatoria</i> sp. (96%)
Presumed Proteobacteria	43		49	
Alpha-Subdivision	0		0	
Beta-Subdivision	0		1	<i>Burkholderia xenovorans</i> (81%)
Delta-Subdivision	6	<i>Geoalkalibacter ferrihydriticus</i> (80–82%)	42	<i>Geoalkalibacter ferrihydriticus</i> (78–82%)
				<i>Desulfovibrio dechloracetivorans</i> (78–80%)
				<i>Desulfovibrio vulgaris</i> (79–82%)
Gamma-Subdivision	37	<i>Ectothiorhodospira shaposhnikovii</i> (82–86%)	6	<i>Pseudomonas azotifigens</i> (86%)
		<i>Ectothiorhodospira mobilis</i> (86%)		<i>Methylomonas methanica</i> (79–82%)
		<i>Pseudomonas stutzeri</i> (86–87%)		<i>Ectothiorhodospira mobilis</i> (89%)
		<i>Thiorhodospira sibirica</i> (81%)		<i>Ectothiorhodospira shaposhnikovii</i> (84%)
		<i>Methylomonas</i> sp. (79%)		
Presumed Chlorobi	0		9	<i>Chlorobaculum macestae</i> (77–80%)
Others	38		27	
(none of the groups above or unidentified)				

* > 90% similarity shown.

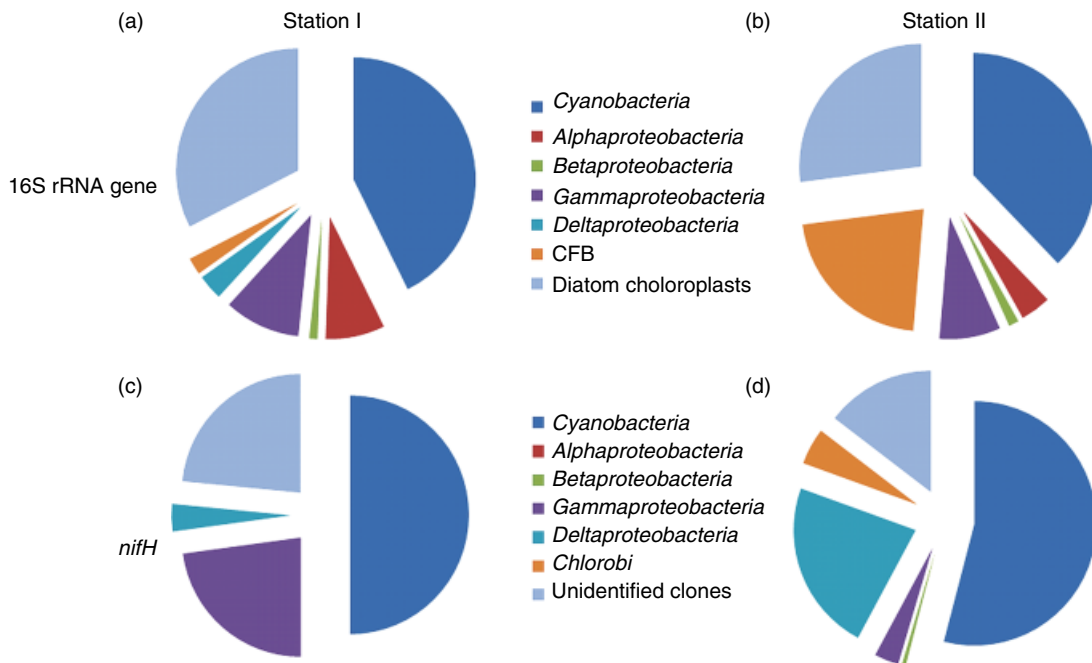


Fig. 3. Relative contributions of major bacterial groups to the 16S rRNA gene and *nifH* clone libraries in 2006. (a) 16S rRNA gene, Station I; (b) 16S rRNA gene, Station II; (c) *nifH*, Station I; and (d) *nifH*, Station II.

sequences (Fig. 3c and d). *Lyngbya aestuarii* was the most common cyanobacterial diazotroph at both stations. *Oscillatoria*-related sequences were also found at both stations, but were more common at Station I. Sequences related to unicellular *Cyanobacteria* were hardly found at Station I, but comprised the second most abundant cyanobacterial cluster at Station II.

Other bacterial taxa

The contribution of other bacterial groups to the 16S rRNA gene clone libraries varied between the stations (Fig. 3a and b). At Station I, *Proteobacteria*-related sequences were more important than at Station II, whereas members of the *Cytophaga–Flavobacter–Bacteroidetes* group were hardly detected at Station I, but were present in higher numbers than *Proteobacteria* at Station II. The contribution of subdivisions to the proteobacterial sequences was similar for both stations and was dominated by *Alpha*- and *Gammaproteobacteria*. Sequences belonging to the *Deltaproteobacteria* were only found at Station I.

Diazotrophs other than *Cyanobacteria* were also abundant. At both stations, > 25% of the *nifH* sequences were closely related to members of the *Proteobacteria*, which were the second most prominent group in the clone libraries (Fig. 3c and d). *Gammaproteobacteria* were present at both stations, but were more prominent at Station I, whereas

Deltaproteobacteria prevailed at Station II. At Station II, *nifH* sequences belonging to the *Chlorobi* were also found.

Spatial and temporal variations of abundant bacterial and diazotrophic taxa

The abundant bacterial and diazotrophic taxa at both stations in two consecutive years were compared by performing DGGE on the bacterial 16S rRNA gene and *nifH* (Fig. 4a and b). Furthermore, the cyanobacterial fraction of the diazotrophic community was analyzed (Fig. 4c).

The bacterial community structure varied between stations of the same year, but also between subsequent years at the same station. A band at the same position in the gel was only detected for Station I (2006+2007, arrow 1, Fig. 4a). The diazotrophic community structure also varied considerably between samples. Some similarities between both years were found for Station I (arrow 2, Fig. 4b) as well as for Station II (arrow 3, Fig. 4b) and also for the 2006 samples of Stations I and II (arrows 4 and 5, Fig. 4b). One of the bands appearing at the same position at Station I in 2006 and 2007 (arrow 2) represented a sequence of a filamentous nonheterocystous cyanobacterium, most closely related to *Oscillatoria sancta*. The other sequence appearing at the same position at Stations I and II in 2006 (arrow 4, Fig. 4b) did not show a close relationship with any known diazotroph. Both stations and years also showed

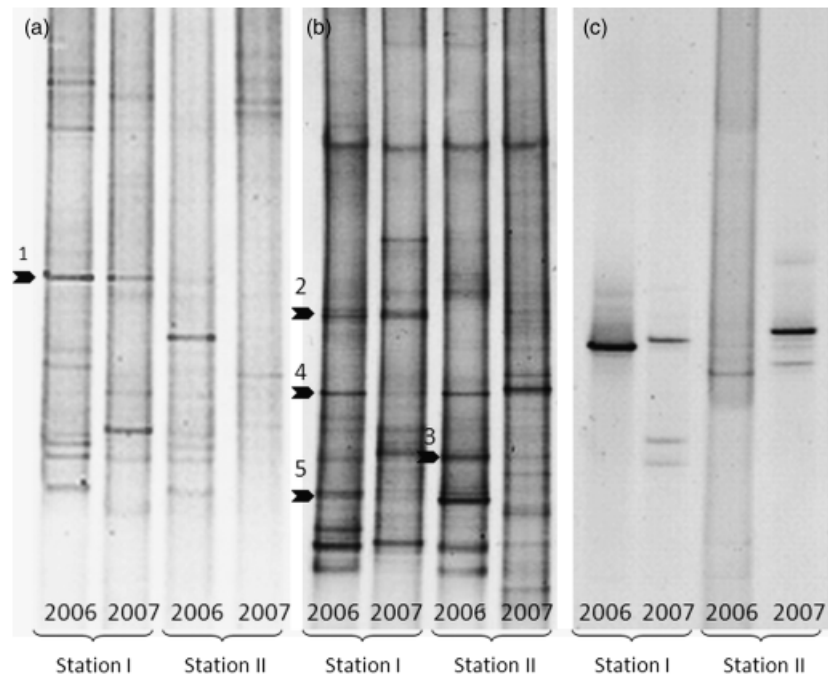


Fig. 4. DGGE profiles of the bacterial 16S rRNA gene (a), bacterial *nifH* (b) and cyanobacterial *nifH* fragments (c) in 2006 and 2007.

considerable differences in the structure of the cyanobacterial diazotrophic community. Most bands sequenced of the cyanobacterial diazotrophic community belonged to *O. sancta*, but sequences belonging to heterocystous *Cyanobacteria*, *Anabaena* and possibly *Nodularia* were also retrieved.

Daily activity patterns of diazotrophic taxa

We were interested in understanding how the high diversity of diazotrophs shaped the daily pattern of nitrogenase activity. Therefore, we examined *nifH* expression in the microbial mat of Station I in more detail. In 2006, samples for the extraction of RNA were taken at regular time intervals during a full day–night cycle. The clone libraries of the *nifH* transcripts revealed the dominance of cyanobacterial and proteobacterial sequences. Averaged over the whole 24-h period, cyanobacterial *nifH* transcripts made up almost half of the clone library. Gammaproteobacterial *nifH* transcripts accounted for the other half.

Of special interest for the analysis of the active diazotrophic community was the change in the contribution of different bacterial groups over a 24-h cycle (Fig. 5). Between noon and midnight, *Cyanobacteria*-related sequences accounted for 50–75% of the total. *Oscillatoria*-related sequences dominated during the early morning (4–8 am), whereas *L. aestuarii* sequences were only found to be of some importance in the afternoon (4 pm). Sequences related to unicellular *Cyanobacteria* were dominant at all other time points, although they were a minor component in the *nifH*

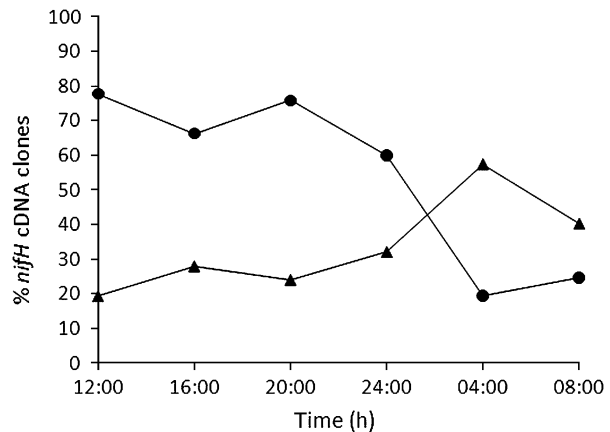


Fig. 5. Relative contribution of *Cyanobacteria*-related (●) and *Gammaproteobacteria*-related (▲) sequences to the *nifH* cDNA clone libraries at six time points over a 24-h cycle in 2006.

clone libraries. After midnight, the proportion of cyanobacterial *nifH* transcripts declined to only 25% and *Gammaproteobacteria*-related sequences became much more important (Fig. 5). *Ectothiorhodospira*-related sequences were the dominant *Gammaproteobacteria* at all times.

Discussion

In microbiology, the characterization of an ecosystem or the comparison of microbial populations within an ecosystem often includes diversity estimations based on the expected

numbers of OTUs at a certain similarity level. We used a 99% similarity cut-off to distinguish different OTUs taking into account that most Taq polymerase errors and variation within paralogous 16S rRNA gene copies are constrained at that level (Acinas *et al.*, 2004b) and, most importantly, to not overlook the fraction of microdiversity observed in the majority of marine microbial communities (Acinas *et al.*, 2004a; Klepac-Ceraj *et al.*, 2004; Haverkamp *et al.*, 2008). Assuming that *Cyanobacteria* were a major component of the diazotrophic community at both stations, the higher diazotrophic diversity at Station I agreed with our expectations that were based on microscopic observations, which revealed a diverse cyanobacterial community. Rarefaction curves for the 16S rRNA gene sequences indicated that the existing diversity was not fully covered by the clone libraries, but that the high-ranking phylogenetic groups were represented in our dataset. Most of the *nifH* diversity was found within the 100–99% similarity cluster. This hinted toward a predominance of microdiverse clusters (Acinas *et al.*, 2004a), but it is unclear whether the high degree of microdiversity would add to the functional diversity of the diazotrophic community. The results also demonstrated that the diazotrophic taxa targeted by our primers were dominated by closely related microorganisms and fairly well represented with our sampling efforts. The analysis of OTUs shared between the two microbial mats revealed that the diazotrophic community of both stations was more similar to each other than the total bacterial community. The lower similarity of the bacterial community might be explained by the different habitats along the littoral gradient. Nevertheless, the set of environmental conditions that determines the presence of the diazotrophic fraction of the community at each of the stations may be comparable, giving rise to a similar diazotrophic community.

Microbial mats worldwide have been shown to harbor diazotrophic organisms and exhibit nitrogenase activity (e.g. Steppe *et al.*, 1996; Olson *et al.*, 1999; Omeregic *et al.*, 2004; Falcón *et al.*, 2007; for an overview, see Severin & Stal, 2010). N₂ fixation in microbial mats has often been attributed to diazotrophic *Cyanobacteria* because they are the major structural element and often also pioneers in these nitrogen-depleted environments. However, an increasing number of studies have questioned the role of *Cyanobacteria* in N₂ fixation in microbial mats and suggested that other groups might contribute as well (e.g. Zehr *et al.*, 1995; Steppe *et al.*, 1996; Steppe & Paerl, 2002; Ley *et al.*, 2006). Analyses of the bacterial community by the two molecular markers (the 16S rRNA gene and *nifH*) revealed that *Cyanobacteria* were the dominant component of both mats. They prevailed even more at Station I. *Cyanobacteria* are often the main structural component of microbial mats, but their community composition may vary as a result of changing environmental conditions. The two stations

investigated here differed with regard to their location within the intertidal area of the sandy North Sea beach. This difference in the location results in differences in environmental conditions such as water availability, salinity and temperature. It also affects the sedimentation patterns, oxygen concentration and oxygen penetration depth. The higher freshwater input at Station I selected for typical freshwater species such as the heterocystous *Cyanobacteria*. Microscopic observations confirmed the presence of heterocystous *Cyanobacteria* and revealed a mixed cyanobacterial community at Station I. At Station II, the filamentous nonheterocystous *L. aestuarii* was the dominant species. Curiously, 16S rRNA gene clone libraries and DGGE analyses did not identify *L. aestuarii* as the dominant form at Station II, but did at Station I. That the structurally dominant cyanobacterium *Lyngbya* was not seen as a dominant DGGE band was probably caused by the limited amount of sequence data obtained from the DGGE gels. The other cyanobacterial sequences retrieved from some of the DGGE bands corresponded to the *Cyanobacteria* found in the mats. Apart from the differences in the community structure at both stations in the same year, the DGGE banding pattern also revealed a change in the total bacterial community and in the diazotrophic community from 2006 to 2007. Climatic differences between the years, especially with respect to temperature, light intensity and water availability during the onset of the mat development, but also at the time of the investigation, probably caused these changes. The spring preceding the sampling in 2006 was sunny, but wet and allowed strong mat development. In 2007, spring was relatively dry, which might have had an impact on the degree of mat development as well as on the types of organisms contributing to the microbial mat community.

Other important members of the diazotrophic community of both stations were *Gamma*- and *Deltaproteobacteria*, although the contribution of the subdivisions of the *Proteobacteria* to the *nifH* clone libraries was strikingly different between both stations. The difference in the contribution of *Bacteria* to microbial mat communities may be caused by the presence of dissimilar habitats due to different prevailing environmental conditions, for example desiccation (Rothrock & Garcia-Pichel, 2005) or temperature (Ward *et al.*, 1998). *Gammaproteobacteria*, which prevailed at Station I, include anoxygenic phototrophic purple sulfur bacteria, which are prominently present in microbial mats, where they occupy the illuminated anoxic zone below the *Cyanobacteria*, sometimes visible as a distinct pink layer. The majority of these sequences were related to *Ectothiorhodospira*. The genus *Ectothiorhodospira* comprises anoxygenic phototrophs that use H₂S as an electron donor and deposit elemental sulfur outside the cell. The possession of *nifH* has been reported for most of the members of the

Ectothiorhodospiraceae (Tourova *et al.*, 2007). *Deltaproteobacteria*, being the predominant *Proteobacteria* at Station II, consist mostly of SRB, which are known to be an important functional group in intertidal microbial mats (Steppe & Paerl, 2002). However, the predominance of *Deltaproteobacteria* at Station II (23% of all clones) should be interpreted with caution because it has recently been shown that *nifH* of *M. chthonoplastes* also groups with *Deltaproteobacteria* rather than with *Cyanobacteria* (Bolhuis *et al.*, 2010). It is therefore possible that some of the *nifH* sequences previously assigned to *Deltaproteobacteria* are of a different origin. This possibility was further supported by the fact that *Deltaproteobacteria* were not found in the corresponding 16S rRNA gene clone library. However, prominent members of the diazotrophic community may represent a minor component of the total bacterial community. Such a discrepancy has been observed for a variety of microbial mats dominated by nonheterocystous *Cyanobacteria* (e.g. Fernández-Valiente *et al.*, 2001; Omoregie *et al.*, 2004; Bauer *et al.*, 2008). Hence, this study confirms previous observations that *Cyanobacteria* may not be the only contributors to N₂ fixation in microbial mats even when they are the dominant structural component.

Because the existence of a functional gene demonstrates the presence, but not necessarily the expression of that gene, we also investigated the *nifH* transcripts at Station I. Averaged over the entire 24-h period, cyanobacterial *nifH* transcripts made up almost half of the clone library. This was in agreement with the results from the *nifH* gene clone libraries. However, in contradiction with the observations based on *nifH* sequences, gammaproteobacterial *nifH* transcripts were as important as cyanobacterial sequences. *Ectothiorhodospira*-related sequences were the dominant *Gammaproteobacteria* expressing *nifH* at all times, although *nifH* copies belonging to this phylotype were virtually absent in the DNA clone library. This discrepancy between the contribution to *nifH* DNA and cDNA clone libraries indicated that *Gammaproteobacteria* showed a higher expression of *nifH* compared with other (active) diazotrophs. *Gammaproteobacteria* and *Cyanobacteria* were therefore assumed to be key contributors to N₂ fixation throughout the day–night cycle. Our results reveal that the extent of *nifH* expression by different diazotrophic organisms changed over the daily cycle. The reason for this shift is not precisely known, but is most likely connected to the prevailing light and oxygen conditions. Nitrogenase activity at Station I revealed peaks at sunset and sunrise, which hinted toward the activity of nonheterocystous *Cyanobacteria* (Severin & Stal, 2008). This pattern has also been reported for a similar mat dominated by filamentous nonheterocystous *Cyanobacteria* (Villbrandt *et al.*, 1990), although our Station I also exhibited some nitrogenase activity at night (Severin & Stal, 2008). We explained the daily variations of nitrogenase activity in

terms of (1) an adaptation of the active diazotrophs to changing conditions, (2) to shifts in the active community during the day–night cycle or (3) to a combination of both. The discrepancy between the number of gene copies and transcripts in clone libraries can be explained by assuming that not every diazotroph expresses *nifH* under the changing conditions during a 24-h cycle. Some might not be fixing N₂ at all. A discrepancy between present and active diazotrophs has been shown in several studies and may be more pronounced in coastal environments than offshore (e.g. Man-Aharonovich *et al.*, 2007). The analysis of OTUs shared between the present and the active diazotrophic community indicates that the active diazotrophic taxa were genetically redundant and in general well represented by the DNA genotypes, although unique active diazotrophic taxa were found in the cDNA libraries. For instance, some of the overlapping OTUs were present in the DNA library at least once, but overrepresented in the cDNA *nifH* library. Three of these OTUs of the cDNA *nifH* clone library, which contained 135, 197 and 41 sequences, corresponded to cyanobacterial lineages distantly related to *Oscillatoria* sp. PCC 6506 (88% similarity) and *Gloeothece* sp. KO68DGA (85%) and to the gammaproteobacterium *Ectothiorhodospira mobilis* strain DSM 237 (81%). This indicates that these organisms were not only present and presumably active; they also showed a higher expression of *nifH* compared with other (active) diazotrophs and, hence, might be major contributors to N₂ fixation in this mat. There were OTUs in the *nifH* cDNA library that were absent in the *nifH* DNA library, such as the uncultured bacterium clone CB914H5, which is distantly related to the alphaproteobacterium *Rhodobacter capsulatus* (89%). This type of diazotroph is probably not sufficiently abundant to be retrieved by PCR of the *nifH* DNA. However, because *nifH* is expressed, it is presumably an active diazotroph. We also found OTUs overrepresented in the DNA *nifH* library, such as an uncultured microorganism distantly related to the gammaproteobacterium *Pseudomonas stutzeri* strain A15101 (85%), which comprised almost three times as many sequences in the DNA as compared with the cDNA *nifH* library. Hence, this organism contributed to the pool of potential diazotrophs, but seemed not to contribute to the fixation of N₂ in this mat.

In conclusion, the two mats represent highly diverse microecosystems that occur at different locations along the intertidal gradient. The bacterial communities of the two mat types were conspicuously dissimilar. The differences between the two diazotrophic communities are likely the reason for the distinctly different nitrogenase activity patterns in these mats (Severin & Stal, 2008). We noted a discrepancy between the diazotrophic organisms that were present and those that actually transcribed *nifH*. Shifts in the active diazotrophic community reflected changes in the

environmental conditions and underline the highly dynamic nature of these microbial ecosystems.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Rarefaction curves at 100%, 99% and 97% similarity cut-offs generated from 16S rRNA gene (a) and *nifH* (b) sequences from Station I and Station II in 2006.

Fig. S2. Venn diagrams representing the overlap of OTUs at 100%, 99% and 97% similarity cut-offs.

Table S1. Number of sequences and OTUs per clone library and Chao1 diversity estimates at 97%, 99% and 100% similarity cut-offs on the basis of the 16S rRNA gene and *nifH* clone libraries for Station I and II in 2006.

Table S2. Abundance-based Jaccard and Sørensen similarity indices and the estimation of community structure similarity (θ) for Station I and II in 2006.

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