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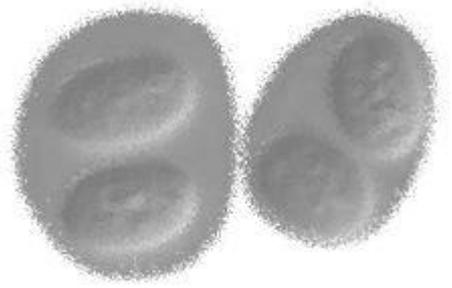
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Chapter 4

Active vs. present nitrogen-fixing microorganisms in a diverse and dynamic cyanobacterial mat community

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Abstract

The structure of the microbial community and the diversity of the functional gene for dinitrogenase reductase and its transcripts were investigated by analyzing more than 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 OTUs (operational taxonomic units) in common than the total bacterial community. The filamentous non-heterocystous cyanobacterium *Lyngbya aestuarii* was the dominant diazotroph at both sites. Based on *nifH* sequences, unicellular *Cyanobacteria* were the second-most important group at Station II, but they only represented a small fraction at Station I. Furthermore, *Gammaproteobacteria* dominated at Station I, whereas *Deltaproteobacteria* were more important at Station II. DGGE (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 active diazotrophic community. Transcript-abundances of the different diazotrophs changed over a 24h cycle and were dominated by cyanobacterial lineages at 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 micro-scale 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 provides *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, such as marine intertidal flats, hypersaline and alkaline environments, hot springs and deserts. Temperate intertidal microbial mats are dominated by filamentous non-heterocystous *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. *M. 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, DGGE (Denaturing Gradient Gel Electrophoresis) revealed a diverse community in a hypersaline microbial mat comprised of the filamentous non-heterocystous cyanobacteria *Microcoleus*, *Oscillatoria*, *Leptolyngbya*, *Phormidium* and the unicellular *Pleurocapsa* and *Gloeotheca* (Fourçans et al., 2004). In addition, sulfate reducing bacteria, sulfur oxidizing and anoxygenic phototrophic bacteria were present. Analyses of small-subunit rRNA genes of a hypersaline microbial mat revealed more than 700 genotypes belonging to over 40 bacterial phyla, emphasizing the high diversity within these micro-ecosystems (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 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 sulfate reducing bacteria.

In a few cases, the analysis of *nifH* was accompanied by measurements of nitrogenase activity (e.g., Bauer et al., 2008). The results were surprising and unexpected because the observed nitrogenase activity did not correspond to what was predicted based on the

dominant diazotrophs. The high daytime and low nighttime nitrogenase activity that was observed in these mats rather hinted to an involvement of heterocystous types than to the non-heterocystous filamentous and unicellular forms that were found based on the analysis of the 16S rRNA gene. 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.

Because isolation and cultivation techniques retrieve only a small fraction of the microbial community (often said to be less than 1%) cultivation-independent molecular techniques have greatly increased our knowledge of the genetic and physiological diversity among microorganisms. Assessing the microbial diversity is crucial in order to understand the ecology, to identify the metabolic processes, and to assess the resilience towards disturbances and external forcing of an ecosystem. However, exploring microbial diversity is challenging. One approach is to construct 16S rRNA gene clone libraries in order to obtain an inventory of the present microorganisms. Despite the potential biases of this approach (von Witzingerode et al., 1997) it is used to generate lists of 'operational taxonomic units' (OTUs) that identify the major microbial constituents of an ecosystem. Based on the sample size and the number of retrieved OTUs, a range of diversity indices (e.g., Chao1) estimating the total number of present OTUs can be calculated. Furthermore, the amount of common OTUs shared by different samples can be estimated when comparing the species composition of several samples. Abundance-based indices like 'Jaccard' or 'Sørensen' can be used to estimate the similarity between certain sets of samples. The analysis of functional genes is of interest when the link between community structure and ecosystem functioning is to be investigated. However, the presence of a certain functional gene is not indicative for the presence of the enzyme it encodes. An alternative approach is to reverse transcribe extracted mRNA and subsequently use this for the construction of clone libraries and for quantitative real-time PCR. However, gene expression does not necessarily coincide with the presence of an active enzyme but is indicative for the metabolic activity of the organism.

This study aimed at the characterization of the structural as well as the functional components of two different types of microbial mats. The two mats represent highly diverse micro-ecosystems which differ in location along an intertidal gradient and therefore experience a different set of environmental conditions. We analyzed clone libraries based on the 16S rRNA gene as well as on *nifH* and its transcripts. This allowed for the comparison of organisms that actively transcribed *nifH* with present diazotrophs that did not. It also allowed for the estimation of the degree of similarity between the two different stations. Furthermore, we followed the changes in the community composition over two years by using DGGE. The results are discussed with respect to the nitrogenase activity patterns previously observed for these mats (Severin & Stal 2008).

Materials and Methods

Sampling

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

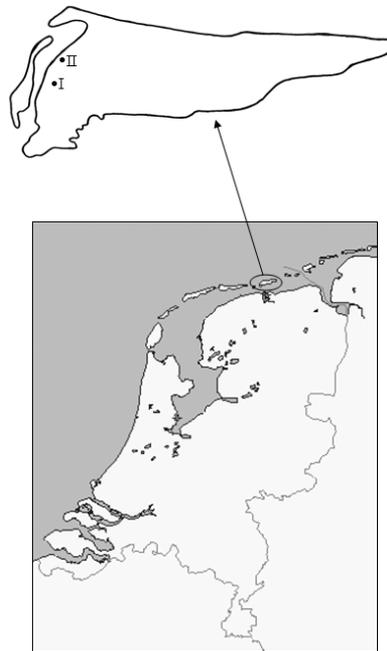


Figure 1. Geographical location of Station I and II on the island Schiermonnikoog

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 wind direction. The mats at this station were covered by sand that was deposited by wind transport. These mats revealed high cyanobacterial diversity as judged by microscopic examinations and contained heterocystous as well as non-heterocystous 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 non-heterocystous 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 non-heterocystous *Cyanobacteria* in which most of the activity was confined to the night (Severin & Stal 2008). The two stations were sampled in summer (May / June) 2006 and 2007.

The mats were sampled by 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 in four quarters by using a scalpel. Each quarter was transferred into a separate cryovial (Simport Plastics, Beloeil, Qc J3G 4S5, Canada) and immediately frozen and stored in liquid nitrogen (dry shipper). Samples were taken over a 24h period at intervals of 4h (2006) and 2h (2007).

Nucleic acid extraction

DNA was extracted using the MO-BIO UltraClean Soil DNA Isolation-kit (MO-BIO Laboratories, Inc., Carlsbad, CA 92010, USA) according to the manufacturer's instructions. Quality and quantity of extracted DNA were checked on a 1% agarose gel and with the NanoDrop ND 1000 (NanoDrop Technologies, Inc., Wilmington, DE 19810, USA). All DNA extracts from one station were combined and stored at -20 °C until used.

RNA was extracted from triplicate samples using the Qiagen RNeasy Mini-kit (Qiagen GmbH, 40724 Hilden, Germany) following the manufacturer's instructions but replacing the enzyme-digest by a bead-beating step (0.1 mm glass-beads, maximum speed for 2 min) (Sartorius AG, 37075 Göttingen, Germany). Quality and quantity of the extracted RNA was checked on a 1% agarose gel. The triplicates were combined and treated with DNase (Deoxyribonuclease I, Invitrogen Corporation, Carlsbad, CA 92008, USA) 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, Carlsbad, CA 92008, USA). 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 reaction mix

contained 2.5 pmol of each primer, 0.2 mM dNTPs, 1x reaction buffer and 0.625 U Taq DNA Polymerase (New England BioLabs, Ipswich, MA 01938, USA) 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') *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, 40724 Hilden, Germany). 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 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. For analyzing community shifts at Station I, the combined cDNA for each time point was processed the same way.

The fresh PCR products were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen Corporation, Carlsbad, CA 92008, USA) 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. A total of 96 16S rRNA gene- and 188 *nifH*-clones were sequenced and analyzed. PCR products were purified with the Sephadex G-50 Superfine-powder (GE Healthcare Bio-Sciences AB, 751 84 Uppsala, Sweden) and 45 µl Millipore MultiScreen-plates (Millipore Corporation, Billerica, MA 01821, USA). 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 94404, USA). 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 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). Primers were *nifH*-forA (5'-GCI WTI TAY GGN AAR GGN GG-3'), GC-*nifH*-forB (5'-GCC CGC CGC GCG 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 with the CBS system (DGGE 2000-model, C.B.S. Scientific Company 420 South Cedros, Salona Beach, CA 92075, USA). Dominant *nifH* bands were excised using sterile razor blades, suspended in 20 µl of MilliQ and stored at 4°C overnight. An aliquot of 2 µl was used for re-amplification with the PCR-primers (without GC-clamp), cloning (4 clones per band) and sequencing.

Sequence analysis

Sequences were aligned in BioEdit (Ibis Biosciences, Carlsbad, CA 92008, USA), corrected by manual inspection and analyzed for similarity in BLASTn (Basic Local Alignment Search Tool, National Center for Biotechnology Information, 8600 Rockville Pike, Bethesda, USA). Alpha- and beta-diversity of the total microbial and of the diazotrophic community at both stations was estimated by the DOTUR and SONS software programs (Schloss & Handelsman 2005 and 2006) based on the alignment files for the 16S rRNA gene and *nifH* clone libraries. Clustering analyses was performed with the Clusterer-software (<http://www.comcen.com.au/~journals/clusterabs2006.htm>, Klepac-Ceraj et al., 2006) and normalized (% cluster).

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

Results

Species richness

Diversity of the 16S rRNA gene and of *nifH*, based on the Chao1 richness estimator, varied between the two stations (Table 1). At a 99% similarity cut-off the Chao1 diversity richness estimator based on 16S rRNA gene sequences predicted 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.

Table 1. Number of sequences per clone library, Chao1 diversity estimates and OTUs 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

	# Seqs	97 % cut-off		99 % cut-off		100 % cut-off	
		Chao 1	OTUs	Chao 1	OTUs	Chao 1	OTUs
Station I							
16S rRNA	84	51	34	106	47	431	73
<i>nifH</i>	127	48	28	147	60	555	108
Station II							
16S rRNA	79	109	43	155	52	525	67
<i>nifH</i>	154	40	23	71	55	553	131

Rarefaction curves for 16S rRNA gene and *nifH* sequences were made in order to compare richness generated from the sequences retrieved from the Stations I and II (Figure 2A and 2B). 16S rRNA gene sequences the rarefaction curves 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.

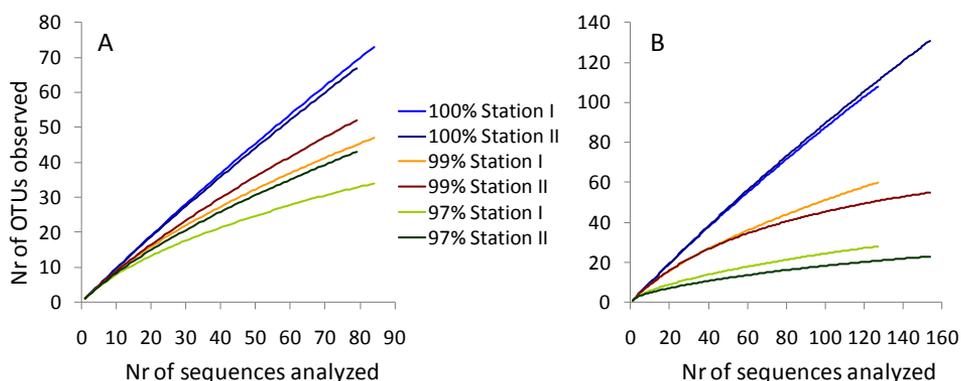


Figure 2. Rarefaction curves at 100%, 99% and 97% similarity cut-offs generated from 16S rRNA gene (A) and *nifH* (B) sequences from Station I and Station II in 2006

Clustering analyses using the 16S rRNA gene and *nifH* sequences were performed in order to compare the general structure of the abundant bacterial and diazotrophic taxa in both stations (Figure 3A and 3B). We found considerable 16S rRNA gene microdiversity at Station II with a higher proportion clusters within the 99% similarity cut-off than at Station I where the OTUs collapsed more gradually (Figure 3A).

The degree of microdiversity was even higher within the *nifH* gene clusters. Here, 45% and 58% of the sequences of Station I and II, respectively, were clustered at the 99% similarity level corresponding to 74% and 87% of total OTUs estimated by Chao1 (Table 1, Figure 3B).

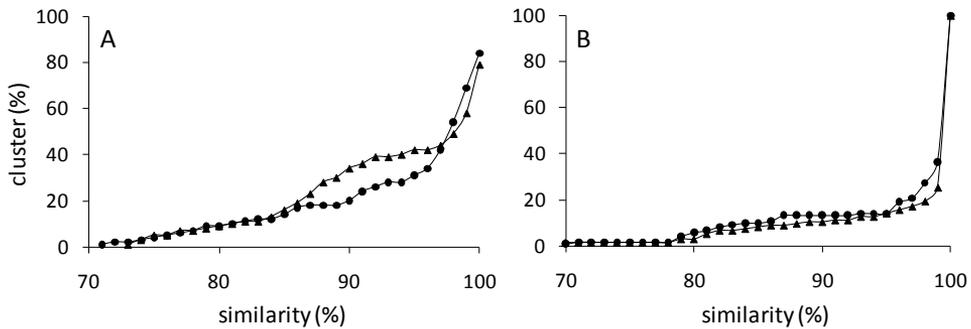


Figure 3. Clustering analyses of clone libraries based on the 16S rRNA gene (A) and *nifH* (B) at Station I (●) and Station II (▲) in 2006

Community composition

We used non-parametric richness diversity estimators to estimate similarity between both microbial mats (Station I and II) based on OTU membership. Using two genetic markers for total bacterial (16S rRNA gene) and diazotrophic taxa (*nifH*), we compared at three distance levels corresponding to 100%, 99% and 97% similarity: (i) the fraction of shared OTUs between two stations (Table 2), (ii) the fraction of sequences belonging to shared OTUs (Table 2), (iii) the abundance-based Jaccard and Sørensen similarity indices (Table 3), and (iv) the estimation of community structure similarity (theta) between the two microbial mats (Table 3).

Table 2. Summary of the diversity comparison between Station I and II in 2006 based on shared OTUs

target	Similarity cut-off	Shared OTUs	Shared OTUs (%)		Seqs in shared OTUs (# / %)	
			I	II	I	II
16S rRNA	100%	4	6	6	12 / 14.3	12 / 15.2
	99%	9	19	17	34 / 40.5	22 / 27.9
	97%	9	26	21	43 / 51.2	31 / 39.2
<i>nifH</i>	100%	9	8	7	15 / 11.8	13 / 8.4
	99%	19	32	35	61 / 48.0	67 / 43.5
	97%	8	29	35	93 / 73.2	103 / 66.9
			cDNA	DNA	cDNA	DNA
<i>nifH</i>	100%	21	4	19	303 / 34.9	71 / 55.9
cDNA-DNA	99%	23	10	38	576 / 66.4	110 / 86.6
	97%	21	16	75	725 / 83.6	118 / 92.9

Table 3. Abundance-based Jaccard and Sørensen similarity indices and the estimation of community structure similarity (theta) for Station I and II in 2006

target	Similarity cut-off	Jaccard	Sørensen	theta
16S rRNA	100%	0.10	0.18	0.17
	99%	0.26	0.41	0.29
	97%	0.38	0.55	0.51
<i>nifH</i>	100%	0.09	0.16	0.06
	99%	0.40	0.58	0.30
	97%	0.68	0.81	0.63
<i>nifH</i> cDNA-DNA	100%	0.36	0.53	0.11
	99%	0.81	0.90	0.09
	97%	0.93	0.97	0.09

Bacterial OTUs based on analysis of the 16S rRNA gene sequences shared between Station I and II increased significantly (3-fold) when decreasing the similarity cut-off from 100% to 99%. At 99% similarity nine OTUs (or 19% and 17% of the OTUs at Station I and II, respectively) were found at both stations. This corresponds to 34 and 22 sequences (or 41% and 28% of the sequences) at Station I and II, respectively. The increase of shared OTUs when decreasing the similarity cut-off from 99% to 97% was less pronounced (Table 2, Figure 4). This pattern was even more evident from the *nifH* analysis. For *nifH*, 19 OTUs (or 32% and 35% of the OTUs from Station I and II, respectively) were shared at 99% similarity, corresponding to 61 and 67 sequences (or 48% and 44% of the sequences) within the overlapping OTUs. A significant increase of shared OTUs was observed at 97%

similarity with 29% and 35% of the OTUs and 73% and 67% of the sequences being present at both stations (Table 2, Figure 4).

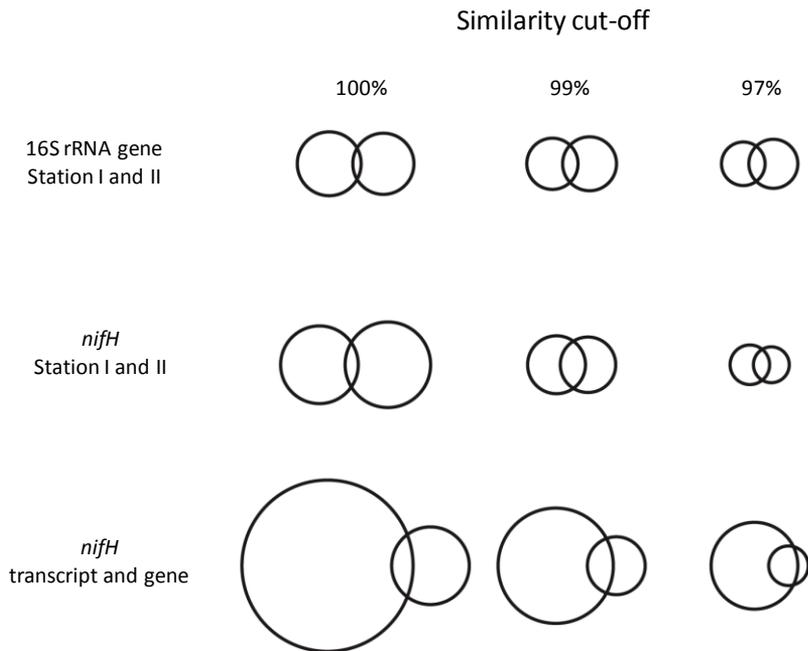


Figure 4. Venn diagrams representing the overlap of OTUs at 100%, 99% and 97% similarity cut-offs

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 2, Fig. 4). Despite the different sampling size for both fractions (867 vs. 127 sequences for cDNA and DNA, respectively) we observed a substantial proportion of identical sequences among the 21 shared OTUs (35% and 56% of the sequences for the cDNA and DNA library, respectively) at the 100% similarity cut-off. Only 4% 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 4% harbored 35% of the sequences within the shared OTUs. The comparison of overlapping OTUs at 99% and 97% similarity between the cDNA and DNA clone libraries assigned most of the sequences to shared OTUs. At 99% similarity 66% and 87% of the sequences within the cDNA and DNA clone libraries, respectively, belonged to shared OTUs. At the 97% similarity cut-off 84% and 93% of the cDNA and DNA sequences, respectively, were found at both stations. At the 99% and 97% 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 and highest for the present and active diazotrophic community at Station I (Table 3). Likewise, the structure of the diazotrophic community at both stations was more similar than the structure of the total bacterial community (Table 3).

Diazotrophic and non-diazotrophic Cyanobacteria

In order to investigate the role of *Cyanobacteria* and other potential diazotrophs in microbial mat N₂ 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. A table containing a list of the most prominent sequences within the 16S rRNA gene and *nifH* clone libraries from 2006 is given as supplement information (Supporting Information Table 1).

At both stations clone libraries of the 16S rRNA gene were dominated by *Cyanobacteria*-related sequences (Figure 5A and 5B). *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 sequences (Figure 5C and 5D). *L. 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.

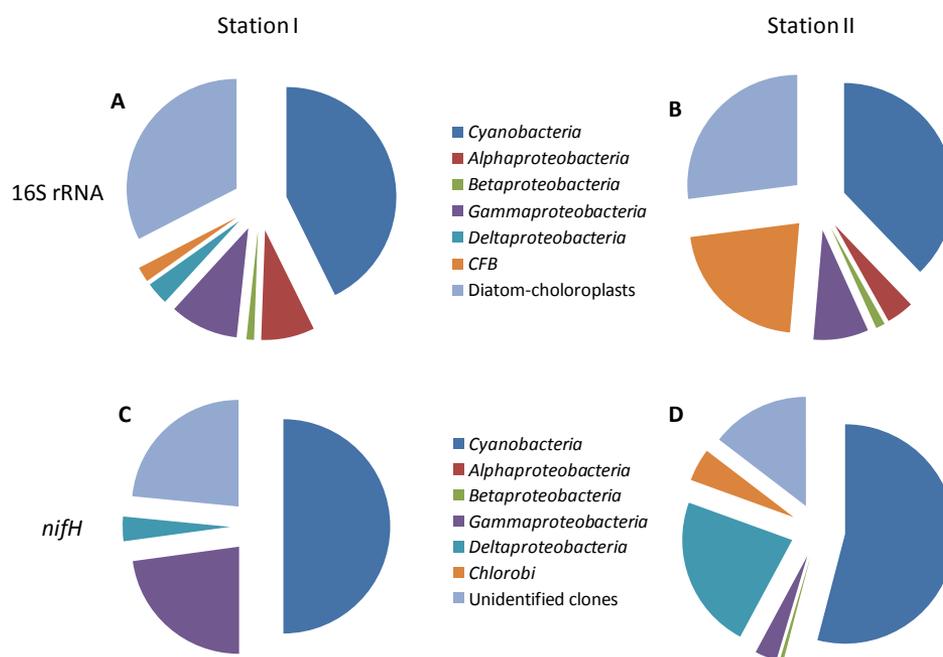


Figure 5. Relative contributions of major bacterial groups to the 16S rRNA gene and *nifH* clone libraries in 2006

Other diazotrophic taxa

The contribution of other bacterial groups to the 16S rRNA gene clone libraries varied between the stations (Figure 5A and 5B). At Station I *Proteobacteria*-related sequences were more important than at Station II whereas members of the Cytophaga-Flavobacter-Bacteroidetes (CFB)-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 more than 25% of the *nifH* sequences were closely related to members of the *Proteobacteria* which were the second most prominent group in the clone libraries (Figure 5C and 5D). *Gammaproteobacteria* were present at both stations but more prominent at Station I whereas *Deltaproteobacteria* prevailed at Station II. At Station II also *nifH* sequences belonging to the *Chlorobi* were 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* (Figure 6A and 6B). Furthermore, the cyanobacterial fraction of the diazotrophic community was analyzed (Figure 6C).

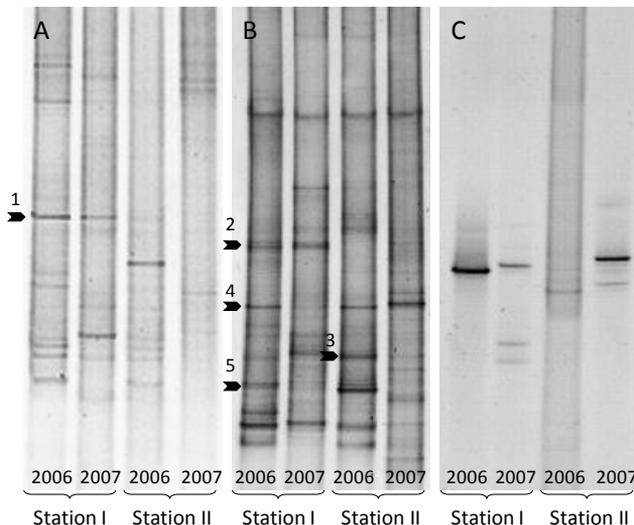


Figure 6. DGGE profiles of the bacterial 16S rRNA gene (A), bacterial *nifH* (B) and cyanobacterial *nifH* fragments (C) in 2006 and 2007

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 24h 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 of contribution of different bacterial groups over a 24h cycle (Fig. 7). 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 important in the afternoon (4 pm). Sequences related to unicellular *Cyanobacteria* were dominant at all time points although they were a minor component in the *nifH* clone libraries. After midnight the proportion of cyanobacterial *nifH* transcripts dropped to only 25% and *Gammaproteobacteria*-related sequences became much more important (Fig. 7). *Ectothiorhodospira*-related sequences were the dominant *Gammaproteobacteria* at all times.

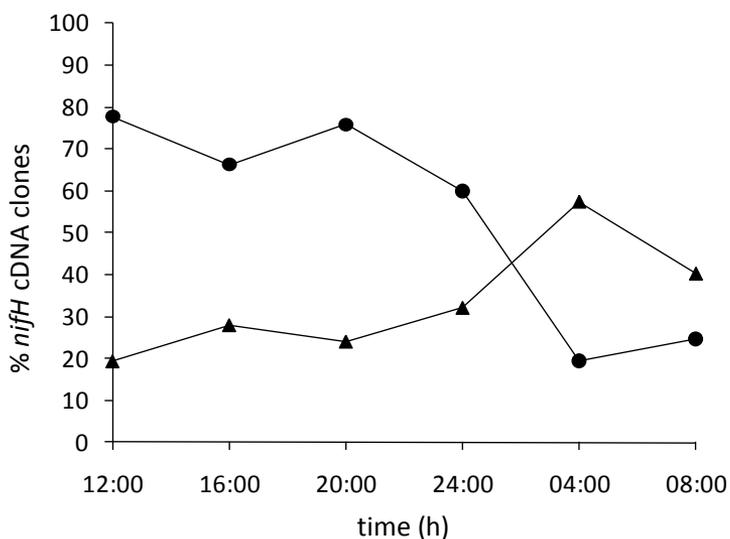


Figure 7. Relative contribution of *Cyanobacteria*-related (●) and *Gammaproteobacteria*-related (▲) sequences to the *nifH* cDNA clone libraries at 6 time points over a 24h cycle in 2006

Discussion

Species richness and community composition

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 (operational taxonomic units) 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., 2004a) and, most importantly, to not overlook the fraction of microdiversity observed in the majority of marine microbial communities (Acinas et al., 2004b; 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. However, the curves leveled off at the 85% similarity cut-off (data not shown), suggesting that the high-ranking phylogenetic groups were represented in our dataset. Most of the *nifH*-diversity was concentrated at the 99% similarity level. This hinted towards a predominance of microdiverse clusters (Acinas et al., 2004b) 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 which 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.

The analysis of OTUs shared between the present and 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 (at 99% similarity) 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 *Gloeotheca* 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 to other (active) diazotrophs and, hence, might be major contributors to N₂ fixation in this mat. There were OTUs in the *nifH* cDNA library which 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 presumably is 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 to 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.

Members of the total bacterial and diazotrophic community

Microbial mats all over the world have been shown to harbor diazotrophic organisms and exhibit nitrogenase activity (e.g., Falcón et al., 2007; Olson et al., 1999; Omoregie et al., 2004b; Steppe et al., 1996; 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 has questioned the role of *Cyanobacteria* in N₂ fixation in microbial mats and suggested that other groups might contribute as well (e.g., Steppe et al., 1996; Steppe & Paerl 2002; Zehr et al., 1995; Ley et al., 2006). Analyses of the bacterial community by the two molecular markers (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 the 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 location results in differences in environmental conditions such as water availability, salinity and temperature. It also affects 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 non-heterocystous *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. A possible explanation for this could be that an excess of *L. aestuarii* template might have inhibited amplification through re-association. 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 able 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 sub-divisions of the *Proteobacteria* to the *nifH* clone libraries was strikingly different between both stations. The difference in contribution of *Bacteria* to microbial mat communities may be caused by the presence of dissimilar habitats due to different prevailing environmental conditions, e.g., 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 sulfate reducing bacteria (SRB) that are known to be an important functional group in intertidal microbial mats (Steppe & Paerl 2002). *Proteobacteria* comprise a wide range of physiologically diverse *Bacteria*, many of them capable of N₂ fixation. However, the predominance of *Deltaproteobacteria* at Station II (23% of all clones) should be interpreted with caution because it has recently been shown that the *nif* gene-cluster 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. The result was nevertheless surprising because *Deltaproteobacteria* were not found in the corresponding 16S rRNA gene clone library. This may be due to the small number of clones analyzed. However, prominent members of the diazotrophic community may also have been a minor component of the total bacterial community. This discrepancy has been observed for a variety of microbial mats dominated by non-heterocystous *Cyanobacteria* (e.g., Fernández-Valiente et al., 2001; Omoregie et al., 2004b; 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. *Cyanobacteria* were the main contributors to the *nifH* pool in the mats studied here, but members of the *Proteobacteria* were also important. Some *Chlorobi* were found as well. These green sulfur bacteria are obligate anaerobic anoxygenic phototrophs or photoheterotrophs which are often found in hot spring microbial mats but also occasionally occur in coastal intertidal microbial mats (Pierson et al., 1987).

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 whole 24h 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 contribution to *nifH* DNA and cDNA clone libraries indicated that *Gammaproteobacteria* showed a higher expression of *nifH* compared to 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 different diazotrophic organisms expressed *nifH* at different times. The reason for this shift is not known. It cannot be explained in terms of the prevailing light and oxygen conditions. The observed nitrogenase activity peak at sunset and sunrise hinted to the activity of non-heterocystous *Cyanobacteria* (Severin & Stal 2008). This pattern has also been reported for a similar mat dominated by filamentous non-heterocystous *Cyanobacteria* (Villbrandt et al., 1990) although our Station I also exhibited nitrogenase activity at night (Severin & Stal 2008). The authors explained the daily variations of nitrogenase activity in terms of (i) an adaptation of the active diazotrophs to changing conditions, (ii) to shifts in the active community during the day-night cycle, or (iii) 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 24h 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).

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Supplementary Information

Table 1: Phylogenetic summary based on analyses of bacterial 16S rRNA gene (A) and *nifH* (B) clone libraries

Supplementary Table 1. Phylogenetic summary based on analyses of bacterial 16S rRNA gene (A) and nifH (B) clone libraries

A 16S rRNA gene	Station I	Station II
	no of clones closest relative in genbank (identity)	no of clones closest relative in genbank (identity)
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>Thermonomas brevis</i> (94 %)
	<i>Alkalilimnicola ehrlichei</i> (93 %)	<i>Halilea</i> sp. (95-96 %)
	<i>Lysobacter</i> sp. (92 %)	
presumed CFB	2 <i>Algoriphagus</i> sp. (98 %)	16* <i>Maribacter</i> sp. (91 %)
		<i>Lewinella cohaerens</i> (95-96 %)
		<i>Algoriphagus</i> sp. (96 %)
Chloroplasts	29	20
unidentified clones	5	11

* > 90 % similarity shown

Diversity of nitrogen fixing microbial mat

B nifH	Station I		Station II	
	no of clones	closest relative in genbank (identity)	no of clones	closest relative in genbank (identity)
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>Geothallobacter ferrihydriticus</i> (80-82 %)	42	<i>Geothallobacter 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 %) <i>Ectothiorhodospira mobilis</i> (86 %) <i>Pseudomonas stutzeri</i> (86-87 %) <i>Thiorhodospira sibirica</i> (81 %) <i>Methylomonas</i> sp. (79 %)	6	<i>Pseudomonas azotifigens</i> (86 %) <i>Methylomonas methanica</i> (79-82 %) <i>Ectothiorhodospira mobilis</i> (89 %) <i>Ectothiorhodospira shaposhnikovii</i> (84 %)
presumed Chlorobi	0		9	
Others*	38		27	

*none of the groups above or unidentified