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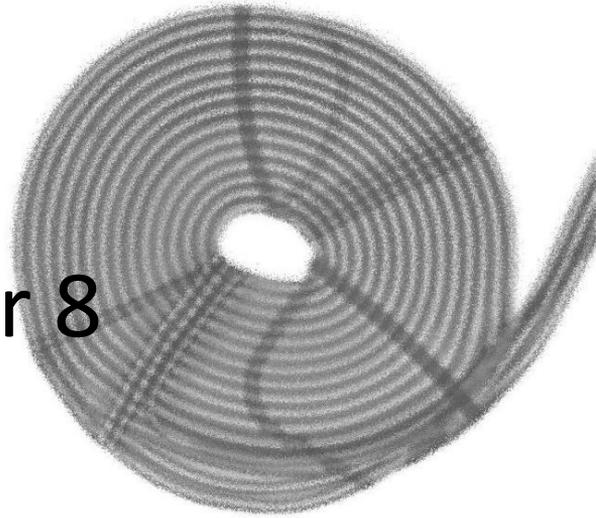
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# Chapter 8



*The effect of salinity on nitrogenase activity and on the composition of the active diazotrophic community in intertidal microbial mats*

Ina Severin<sup>1</sup>, Veronique Confurius-Guns<sup>1</sup> and Lucas J. Stal<sup>1,2</sup>

<sup>1</sup>*Department of Marine Microbiology, Netherlands Institute of Ecology, NIOO-KNAW;*

<sup>2</sup>*Department of Aquatic Microbiology, Institute of Biodiversity and Ecosystem Dynamics, University of Amsterdam*

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**Abstract**

Microbial mats are often found in intertidal areas experiencing a large range of salinities. This study investigated the effect of changing salinities on nitrogenase activity (acetylene reduction assay) and on the composition of the active diazotrophic community (*nifH* transcripts libraries) of three types of microbial mats situated along a littoral gradient. All three mat types exhibited highest nitrogenase activity at salinities close to ambient seawater salinities or lower. The degree of response to lowered or elevated salinities was highest for the mats higher up in the littoral zone. Changes in nitrogenase activity as the result of exposure to different salinities were accompanied by changes in the active diazotrophic community. The active diazotrophic community at ambient salinity differed between the stations. The two stations higher up in the littoral zone showed *nifH* expression by *Cyanobacteria* and *Proteobacteria*. *Oscillatoriales* and *Chroococcales* as well as *Gamma*- and *Deltaproteobacteria* prevailed. At both stations an increase in the contribution of *Gamma*- and *Deltaproteobacteria* was observed at increasing salinity which coincided with a decrease in nitrogenase activity. The station at the low water mark showed low cyanobacterial contribution to *nifH* transcript libraries at all salinities but an increase in *deltaproteobacterial nifH* transcripts under hypersaline conditions. In conclusion, increased salinities caused decreased nitrogenase activity which was accompanied by a lower proportion of cyanobacterial *nifH* transcripts.

## Introduction

Coastal ecosystems, including salt marshes, mangroves, wetlands, estuaries and bays, are often characterized by highly fluctuating environmental conditions, mainly due to the tidal cycle. One important factor in intertidal areas is salinity. Salinities range from almost freshwater, e.g., through runoff or upwelling groundwater, to hypersaline conditions due to evaporation, e.g., when seawater is confined to shallow areas with a negative water balance. These intertidal areas are colonized by a variety of microorganisms. Salt stress may limit the diversity of any group of organisms, including microorganisms, probably for reasons of impaired bioenergetics (Oren et al., 1999). Studies on the diversity of *Bacteria* and *Archaea* as well as their distribution along a salinity gradient in a coastal solar saltern showed a diverse community consisting of *Proteobacteria*, *Cyanobacteria*, high GC-Gram-positive bacteria and members of the *Cytophaga-Flavobacterium-Bacteroides* group (CFB) (Benlloch et al., 2002). The diversity of different clusters decreased with increasing salinity but a considerable degree of microdiversity in these remaining clusters was observed. Similar observations were made for a salinity gradient in a soda lake where the 16S RNA gene sequences of members of a variety of different bacterial groups, e.g., *Cyanobacteria*, *Proteobacteria*, *Actinobacteria* and *Clostridia* were retrieved (Foti et al., 2008). Again, the lowest bacterial diversity was found at the highest salinities. The change of bacterial communities along a salinity gradient has also been studied in other environments. Free-living bacterioplankton in two Chesapeake Bay estuaries showed a dominance of *Alphaproteobacteria* in the saltwater regions, members of the *Cytophaga-Flavobacterium* cluster in the turbidity maximum and *Betaproteobacteria* in the freshwater regions while *Gammaproteobacteria*-abundances exhibited only sporadic peaks (Bouvier & del Giorgio, 2002). Possible factors for these compositional changes were partly associated with but not exclusively driven by salinity. On a global scale salinity has been identified as the most important environmental determinant of microbial community composition (Lozupone & Knight, 2007). The effect of increased salinities on microbial activities and community composition has been studied for freshwater sediments (Edmonds et al., 2009). Despite changes in activity, the microbial community composition remained largely unaltered. It was therefore argued that changes in metabolic activity were driven by shifts in gene expression and regulation rather than by changes in the composition of the microbial community (Edmonds et al., 2009).

In addition to salinity changes, coastal areas are often characterized by low nutrient concentrations and different degrees of wave and wind energy. On some intertidal sand flats these harsh conditions largely exclude grazing organisms and thus allow for the development of microbial mats. These small-scale ecosystems are vertically stratified microbial communities, often built by *Cyanobacteria*. Especially coastal tidal flats with low slopes and fine sandy sediment serve as excellent habitats for mats (Stal, 2000). *Cyanobacteria* are oxygenic photoautotrophs with low nutritional requirements. Many of them are also capable of N<sub>2</sub> fixation, which provides them with a distinctive advantage that allows them to colonize nutrient-poor and nitrogen-depleted environments. The ability to fix N<sub>2</sub> is not limited to *Cyanobacteria* and occurs among a variety of other members of the *Bacteria* as well as in some *Archaea*. In addition to *Cyanobacteria*,

microbial mats may accommodate a variety of other functional groups of microorganisms, e.g., anoxygenic phototrophs, sulfate reducing bacteria and sulfur oxidizing bacteria. Especially members of the *Proteobacteria* were identified as potential diazotrophs based on the possession of *nifH*, the gene encoding for dinitrogenase reductase, one of the two components of nitrogenase, the enzyme catalyzing the reduction of  $N_2$  to  $NH_3$ . *Gamma*- and *Deltaproteobacteria* were most frequently encountered in different types of microbial mats (e.g., Zehr et al., 1995; Bauer et al., 2008) and within the *Deltaproteobacteria* especially the role of sulfate reducing bacteria in microbial mat  $N_2$  fixation was discussed (Steppe & Paerl, 2002). The high bacterial diversity of microbial mats has also been reported for mats in high salt environments and includes *Cyanobacteria*, *Chloroflexi*, *Bacteroidetes* and a variety of *Proteobacteria* (Fourçans et al., 2004; Ley et al., 2006). Hypersaline microbial mats are found in different regions of the world (Severin & Stal, 2010) and high salinities therefore do not seem to prevent the existence of microbial communities. Nevertheless, metabolic processes such as  $CO_2$  and  $N_2$  fixation can be severely inhibited by salt stress. In cultures of diazotrophic *Cyanobacteria* increased salinities greatly reduced nitrogenase activity (e.g., Fernandes et al., 1993; Fu & Bell, 2003). But also microbial mat community showed lower  $CO_2$  and  $N_2$  fixation rates at higher salinities (Pinckney et al., 1995a).

This study investigated the effect of different salinities on nitrogenase activity and the composition of the active diazotrophic community in coastal microbial mats. We show that, depending on the location along a littoral gradient, three microbial mats harbor different communities of active diazotrophic organisms that responded differently to changes in salinity with respect to nitrogenase activity and the composition of the active diazotrophic community.

## Materials and Methods

### Sampling

The study site was located on the Dutch barrier island *Schiermonnikoog*. The geographic coordinates of the study site were N 53°29' and E 6°08'. Microbial mats were found on the sandy beach covering the north bank of the island facing the North Sea. Areas of the beach are currently turning into a salt marsh, resulting in mats partly overgrown by higher plants. Due to this succession and the gradually changing influence of the North Sea, different mat types developed along the littoral gradient.

Three sites within this area were chosen based on microscopic observations of the cyanobacterial community composition as well as their situation along the littoral gradient. Station I was located near the dunes and influenced by both seawater and freshwater (rain and groundwater). This area was only irregularly inundated by the sea. The mats found at Station I revealed high cyanobacterial species diversity containing both heterocystous and non-heterocystous filamentous *Cyanobacteria* as well as unicellular species. Station II is situated near the low water mark. Due to tidal inundation, seawater was far more important than the occasional rain showers and this distinguished it from

Station I. The *Cyanobacteria* at Station II were mostly non-heterocystous forms, predominantly *Lyngbya aestuarii*. Occasionally, heterocystous *Cyanobacteria* have been observed, but these organisms did not seem to be a structural part of this community. Station III was located between Stations I and II and therefore represented an area influenced by seawater and fresh water depending on the tidal amplitude. At Station III higher plants were partly overgrowing the established mats. Based on microscopic observation, *Microcoleus chthonoplastes* was identified as the dominant cyanobacterial component. A variety of other non-heterocystous as well as heterocystous species were found as well.

For the incubations, mat samples of each location were collected by using disposable 10 ml-syringes of which the needle connector was cut off to obtain a corer with a diameter of 1.5 cm. The top 2 – 3 mm of the mat was sampled and transferred in the incubations vials. After finishing the nitrogenase activity measurements, sediment and biomass were retrieved by filtering the samples over a glass microfiber filter (Whatman GF/F, Whatman plc, Kent, United Kingdom). Each sample was divided in four parts and each part was transferred into a separate cryovial (Simport Plastics, Beloeil, Qc J3G 4S5, Canada) and directly frozen in liquid nitrogen.

#### *Incubations and nitrogenase activity measurements*

The mat samples were incubated in 30 ml serum flasks which were sealed with a butyl rubber stopper (Rubber B.V., 1211 JG Hilversum, The Netherlands). To each of the samples 2 ml of medium with the following salinities were added. Filtered North Sea water (Whatman GF/F glass microfiber filter) with a salinity of 33 PSU from the location served as the control ('natural salinity'). Tap water was used as the 0 PSU medium ('freshwater') and a 1:1 dilution of filtered seawater with tap water provided the 16.5 PSU medium ('half the natural salinity'). Salinities of 66 PSU and 165 PSU ('twice the natural salinity' and 'fivefold the natural salinity') were obtained by adding NaCl to the filtered seawater. Samples of all three mat types were incubated in duplicate. The entire incubation lasted 72h. Measurements of nitrogenase activity were carried out at the start of the experiment and after 12h, 24h, 60h and 72h. At each of the time points, the vials were closed and 5 ml acetylene (Messer B.V., 4782 PM Moerdijk, The Netherlands) were injected, the gas phase was mixed by pumping with the syringe and 5 ml of the headspace was removed to maintain normal pressure. Incubations lasted 3h after which 5 ml of the headspace gas were transferred into evacuated and sealed 5 ml crimp top vials (Chrompack-Varian, Inc., 4338 PL Middelburg, The Netherlands). Between the incubations, the vials were left open to permit gas exchange with the atmosphere. The temperature was kept constant by a thermostated water bath. Ethylene concentrations were measured using a gas chromatograph (Chrompack CP 9001) equipped with a flame ionization detector. The temperature of injector, detector and oven were 90°C, 120°C and 55°C, respectively. Helium served as a carrier gas (Westfalen Gassen Nederland BV, 7418 EW Deventer, The Netherlands) at a flow rate of 10 ml min<sup>-1</sup>. The supply rates of H<sub>2</sub> and clinical air (Westfalen Gassen Nederland BV, 7418 EW Deventer, The Netherlands) for the flame

ionization detector were 30 ml min<sup>-1</sup> and 300 ml min<sup>-1</sup>, respectively. The column was a 25 m long wide-bore silica fuse column (inner diameter of 0.53 mm) packed with Poropak U (Chrompack-Varian, Inc., 4338 PL Middelburg, The Netherlands).

#### *Determination of bulk chlorophyll a*

Chlorophyll *a* of the samples that were used for the nitrogenase measurements was extracted twice with 96% ethanol. The sediment was homogenized in the ethanol and left in the dark for 2 h at room temperature. The extract was centrifuged and the pellet was re-extracted overnight, while the first extract was stored in the dark at 4 °C. The two extracts were pooled and the absorption was measured at 665 nm in a spectrophotometer (Pharmacia Biotech – Novaspec II, Cambridge, England). The amount of chl *a* in each sample was calculated using an absorption coefficient of 72.3 ml mg<sup>-1</sup> cm<sup>-1</sup> and related to the sample surface. For stations I, II and III, the chl *a* contents were 101 µg chl *a*, 99 µg chl *a* and 73 µg chl *a* per mat sample, respectively.

#### *Nucleic acid extraction*

RNA was extracted from triplicate samples at the end of the incubations 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. RNA extraction and therefore all subsequent steps failed for the Station III at freshwater conditions. The reasons for this failure are not known.

#### *PCR, cloning and sequencing*

For the amplification of *nifH* transcripts, 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, each 25 µl PCR reaction mix contained 25 pmol of each primer, 0.2 mM dNTPs, 1x reaction buffer and 0.625 U Qiagen HotStar Taq (Qiagen GmbH, 40724 Hilden, Germany) as well as 10-15 ng DNA. 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.

The fresh PCR product was cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen Corporation, Carlsbad, CA 92008, USA) following the manufacturer's instructions. Only white transformants were used for amplification with the 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. 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) and the M13 reverse primer.

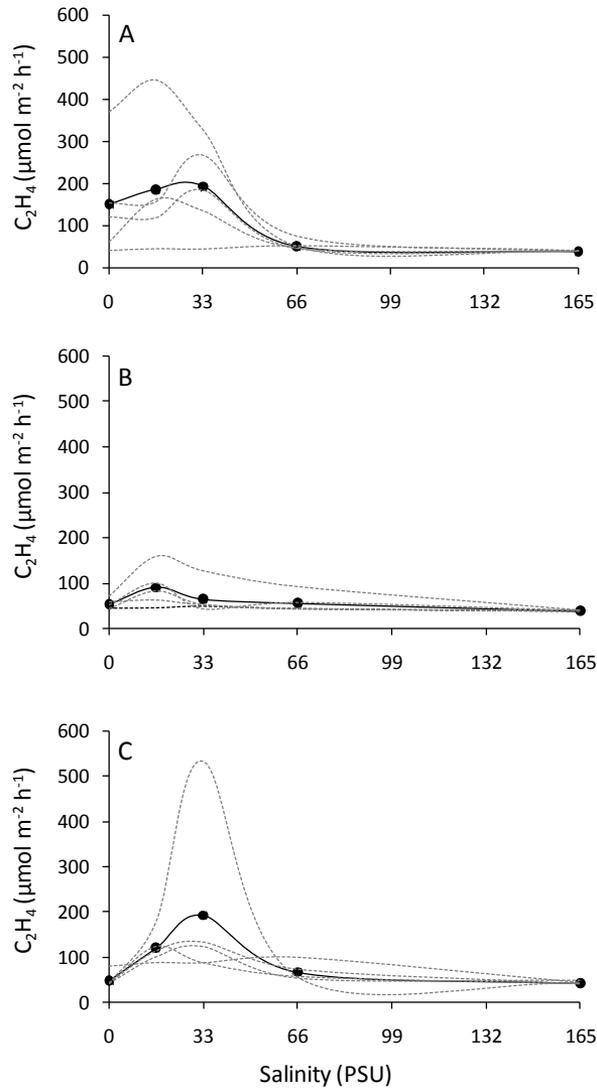
#### *Sequence analysis*

Sequences were aligned in BioEdit (Ibis Biosciences, Carlsbad, CA 92008, USA), corrected by manual inspection and analysed 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 active diazotrophic community at all three stations was estimated by the DOTUR and SONS software programs (Schloss & Handelsman, 2005, 2006) based on the alignment files of the *nifH* transcript libraries. All sequences are deposited at the NCBI GenBank database under the accession numbers GU192465 to GU193156.

## **Results**

#### *Nitrogenase activity*

Nitrogenase activity (NA) was measured at different time points during the incubation period of 72h and for five salinities ranging from 0 to 165 PSU. The results were averaged per salinity over the entire incubation period but the individual measurements per time point are also depicted to show variations due to the daily cycle of NA (Figure 1).



**Figure 1.** Nitrogenase activity averaged for the 72h incubation period (solid line) and for each of the time points separately (dotted lines) as measured by acetylene reduction assay (ARA, normalized to  $\text{m}^2$ ) at the different salinities. A: Station I; B: Station II. C: Station III

At Station I highest NA at all time points was recorded at half the natural salinity (16.5 PSU) or natural salinity (33 PSU) (Figure 1A). Averaged NA at half the natural salinity was only slightly lower (98%) than maximum NA at natural salinity (100%). At freshwater conditions NA was still 78% of the maximum NA. At double and fivefold the natural salinity

(66 PSU and 165 PSU, respectively) a strong decrease of NA was observed. NA only reached 27% and 20% of the maximum NA at double and fivefold the salinity, respectively. At Station II differences between the averaged NA at the five salinities were less pronounced (Figure 1B). Highest NA was recorded at half the natural salinity. At natural salinity NA reached 71% of the maximum NA. At freshwater conditions and under double the salinity 60% and 62% of the maximum NA were recorded. At the highest salinity, NA was lowest but still reached 44% of the maximum NA. Station III showed the most pronounced differences in NA at different salinities (Figure 1C). Highest NA was recorded at natural salinity, followed by those at half the natural salinity (63%) and at double the natural salinity (35%). Lowest NA was recorded at freshwater conditions (25%) and fivefold the natural salinity (22%).

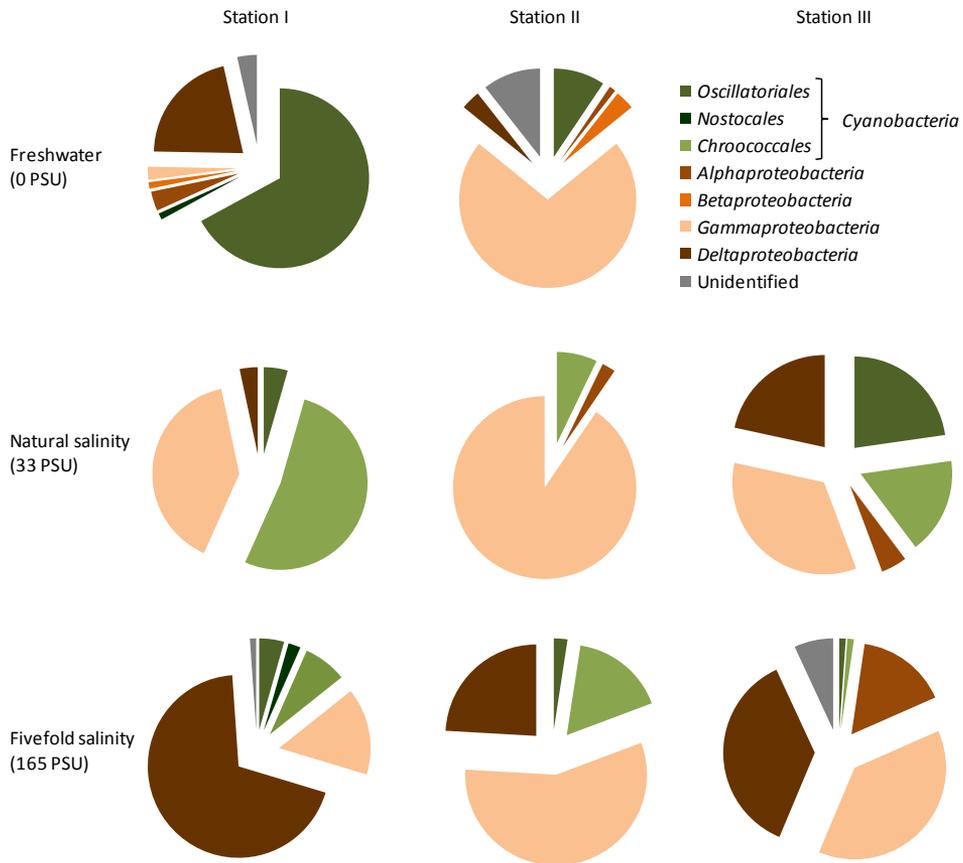
#### *Composition of the active diazotrophic community*

Diversity of *nifH* transcripts, based on the Chao1 richness estimator, varied between the stations and at different salinities (Table 1). We used a 99% similarity cut-off level to distinguish different OTUs (operational taxonomic units). Chao1 diversity richness estimator based on the *nifH* transcripts predicted highest diversity at natural salinities for station II and III. At Station I highest diversity was predicted for the highest salinity. Differences in the estimated diversity of the active diazotrophic community at different salinities were highest for Station II. The estimated diversity was similar for Station I and II at freshwater conditions and highest for Station III at natural and fivefold the natural salinity.

**Table 1.** Number of sequences and OTUs per clone library and number of OTUs predicted by Chao1 at 99% similarity on the basis of the *nifH* transcript libraries. n.a.: not analyzed

	Number of sequences			Number of OTUs (99%)			Chao1 (predicted OTUs at 99%)		
	I	II	III	I	II	III	I	II	III
Freshwater (0 PSU)	85	85	n.a.	36	36	n.a.	50	47	n.a.
Natural salinity (33 PSU)	90	84	88	41	49	51	59	111	117
Fivefold salinity (165 PSU)	91	83	87	42	29	48	80	38	95

The active diazotrophic community, as assessed by the *nifH* transcripts present at the end of the incubations at three of the salinities, showed differences between the salinities at one station as well as between the different stations. Most sequences, however, belonged to *Cyanobacteria* and *Proteobacteria* at all stations (Figure 2).



**Figure 2.** Relative contributions of major bacterial groups to the *nifH* transcript libraries for Station I, II and III at three different salinities

At Station I the contribution of *Cyanobacteria* to the *nifH* transcript libraries decreased with increasing salinity from 68% at freshwater conditions to 57% at natural and 14% at fivefold the natural salinity. At freshwater conditions *Oscillatoriales*-related sequences dominated whereas members of the *Chroococcales* prevailed at natural salinity. At the highest salinity both groups as well as some sequences belonging heterocystous *Cyanobacteria* (*Nostocales*) were found. The percentage of proteobacterial sequences in the *nifH* transcript libraries increased with increasing salinity from 28% at freshwater

conditions to 43% at natural salinity and 85% at fivefold the natural salinity. At the highest salinity *Deltaproteobacteria* dominated whereas at natural salinity *Gammaproteobacteria* prevailed. At Station II *Proteobacteria* dominated the *nifH* transcript libraries at all salinities with 80%, 93% and 81% at freshwater, natural and fivefold the natural salinity, respectively. As was the case for Station I, the contribution of *Deltaproteobacteria* increased with increasing salinity but unlike Station I *Gammaproteobacteria* were dominant at all salinities (90%, 97% and 70% of all proteobacterial sequences at freshwater, natural and fivefold the natural salinity, respectively). However, certain members of the *Gammaproteobacteria* were only found at one of the three salinities, indicating some shift within this group with increasing salinity. Among the few cyanobacterial *nifH* transcripts no heterocystous *Cyanobacteria* were found. *Chroococcales* dominated at higher salinities. At Station III no good-quality RNA could be extracted from the incubation at 0 PSU and therefore no analysis of the active diazotrophic community at freshwater conditions was possible. At natural salinity 40% of the *nifH* transcripts belonged to *Cyanobacteria* whereas almost no cyanobacterial *nifH* transcripts were found at fivefold the natural salinity. The *Cyanobacteria* at natural salinity belonged to *Oscillatoriales* and *Chroococcales* (57% and 43 %, respectively). With increasing salinity there was an increase of proteobacterial sequences from 60% to 91% and also an increase of the contribution of *Alphaproteobacteria* from 8% to 18% at natural and fivefold the natural salinity, respectively. *Gamma-* and *Deltaproteobacteria* dominated the *nifH* transcript libraries with 57% and 36% at natural salinity and 42% and 40% at fivefold the natural salinity, respectively.

We also used non-parametric richness diversity estimators to estimate the similarity between the three different salinities at each station based on OTU membership. We compared the fraction of shared OTUs between the salinities and the fraction of sequences belonging to shared OTUs (Table 2).

**Table 2.** Summary of the OTU overlap within the *nifH* transcript libraries at 99% similarity for the different salinities at Station I, II and III

	Shared OTUs (#)	Shared OTUs (%)		Sequences in shared OTUs (# / %)	
<b>Station I</b>					
0 PSU – 33 PSU	3	8.3	7.3	22 / 25.9	3 / 3.3
33 PSU – 165 PSU	10	24.4	23.8	30 / 33.3	16 / 17.6
<b>Station II</b>					
0 PSU – 33 PSU	1	2.8	2.0	1 / 1.2	1 / 1.2
33 PSU – 165 PSU	5	10.2	17.2	11 / 13.1	15 / 18.1
<b>Station III</b>					
33 PSU – 165 PSU	7	13.7	14.3	14 / 15.9	9 / 10.3

At Station I more OTUs were shared between the active diazotrophic communities at natural and fivefold natural salinity (24.4% and 23.8% of the OTUs and 33.3 and 17.6% of the sequences, respectively) than between freshwater conditions and natural salinity (8.3% and 7.3% of all OTUs and 25.9% and 3.3% of the sequences, respectively). The higher overlap between the active diazotrophic communities at natural and fivefold the natural salinity was also observed for Station II (10.2% and 17.2% of the OTUs and 13.1% and 18.1% of the sequences at natural and fivefold the natural salinity, respectively). Only one common OTU containing one sequence was found at freshwater and natural salinities. At Station III seven OTUs were found at natural and fivefold the natural salinity, corresponding to 13.7% and 14.3% of the OTUs and comprising 15.9% and 10.3% of the sequences, respectively.

### Discussion

We measured nitrogenase activity (NA) in three different microbial mat types over a salinity range from 0 PSU (freshwater) to 165 PSU (fivefold the natural salinity) and also analyzed the *nifH* transcripts to investigate the effect salinity changes have on the active diazotrophic community and its performance with respect to N<sub>2</sub> fixation. Studies of this kind are rare and have been limited to hypersaline microbial mats (Pinckney et al., 1995a; Yannarell et al., 2006).

The difference in NA rates per station and salinity observed for the different time points result from the fact that measurements were performed both at day and at night. The highly variable daily NA pattern which has been observed for these and other diazotrophic microbial mats (Omeregic et al., 2004 a, b; Steppe & Paerl 2005; Severin & Stal, 2008) results in different NA rates depending on the time of the measurement, even if environmental conditions, e.g., temperature, are kept constant in laboratory experiments (e.g., Severin & Stal, 2008). However, the general tendency, i.e., the response to changing salinities, was the same at all time points and is therefore discussed below.

Station I, the mat highest up in the littoral zone, is less influenced by seawater than the other two stations. Highest NA at salinities ranging from freshwater to ambient seawater salinity demonstrates the adaptation to conditions likely to occur at this station but also the intolerance of the present diazotrophic community with regard to elevated salinities. The shift within the active diazotrophic community seemed more pronounced for the transition from freshwater to natural salinity than from natural to fivefold the natural salinity. This might illustrate the plasticity of the diazotrophic community within the natural salinity range at this station. The diazotrophic fraction of the community best adapted to each of these salinities is most active and ensures high NA. No such plasticity is expected for conditions unlikely to occur and in agreement with decreasing NA at higher salinities. The active diazotrophic community which is presumably responsible for high NA under freshwater and seawater conditions and showed a remarkable change within the most common groups, *Cyanobacteria* and *Proteobacteria*. The reason why members of the structurally dominant *Oscillatoriales* seemed to be better adapted to lower salinities whereas members of the *Chroococcales* contributed the major part of cyanobacterial *nifH*

transcripts at ambient salinity is unknown. Hypersaline microbial mats in different environments contain filamentous as well as unicellular forms (e.g., Fourçans et al., 2004; Yannarell et al., 2006) but there is some evidence that unicellular *Cyanobacteria* become more important at elevated salinities (e.g., Garcia-Pichel et al., 1998 and references therein). In the mats investigated in this study gamma- and deltaproteobacterial *nifH* transcripts dominated the clone libraries at higher salinities. *Proteobacteria* are common member of intertidal microbial mat communities and have been shown to contribute substantially to the *nifH* gene pool (Olson et al., 1999; Bauer et al., 2008) and to the part of the diazotrophic community that is actually expressing *nifH* (Steppe & Paerl, 2002, 2005). Their prevalence in the *nifH* transcript libraries at hypersaline conditions is in line with their presence and activity in hypersaline microbial mats (Omeregíe et al., 2004a, b). A different picture has been drawn for a Bahamian hypersaline mat where *Cyanobacteria* were the dominant contributors to the *nifH* gene pool at high salinities whereas a lower salinity of the water during wet months caused a higher diversity of other diazotrophs (Yannarell et al., 2006). The environmental conditions for these mats were different from the ones affecting temperate intertidal mats and substantial differences in the diazotrophic community and its response to salinity changes are therefore not surprising. Nevertheless, it has been shown that higher salinities correlate with lower NA in these types of mats as well (Pinckney et al., 1995a). This is generally attributed to bioenergetic aspects (for a review see Oren, 1999). The synthesis and/or uptake of compatible solutes to balance the osmotic pressure of the environment as well as excreting salt ions from the cytoplasm are expensive restrict other energy-demanding metabolic processes, e.g., N<sub>2</sub> fixation. Early studies on cultured organisms showed the optimum of NA to be at salinities close to those in the natural environment of the diazotroph (e.g., Dicker & Smith, 1981). Wieland and Köhl (2006) demonstrated that microbial communities were well adapted to short-term fluctuation within the natural range of salinity but that an increase of salinity above a certain transition point limited microbial activity. It therefore seems plausible that energy constrains N<sub>2</sub> fixation and could be the main reason for a decrease in NA under increased salinities.

Station III showed the most pronounced response of NA to salinity changes. This indicates a less tolerant diazotrophic community with regard to salinity stress. We were unable to analyze the active diazotrophs at freshwater conditions but the community shift from natural salinity to hypersaline conditions demonstrated a relatively stable proteobacterial contribution to the *nifH* transcript libraries. *Cyanobacteria*, however, almost disappeared from the *nifH* transcript libraries at the higher salinity which could be another reason for the observed decrease in NA.

Station II was situated close to the low water mark and therefore frequently covered with seawater. It harbored the most stable active diazotrophic community under increasing salinities, except for the increase in contribution of *Deltaproteobacteria*. *Deltaproteobacteria* have been encountered in microbial mats experiencing salt stress (Omeregíe et al., 2004a, b) but were also found in marine microbial mats at normal seawater salinities (i.e. ~35 PSU) and have been proposed as possible components of the diazotrophic community (Steppe & Paerl, 2002, 2005). Other *Proteobacteria* that have frequently been demonstrated in microbial mats are *Gammaproteobacteria* (Olson et al.,

1999; Steppe & Paerl, 2005; Bauer et al., 2008). They also appear as active diazotrophs in hypersaline microbial mats (Omeregic et al., 2004b) and seem to harbor members well adapted to fix  $N_2$  at increased salinities. Along with the more stable active diazotrophic community at Station II we also observed smaller changes in NA at the different salinities. This could be caused by the selection for a halotolerant diazotrophic community resulting from the more frequent changes of salinity experienced at the lower intertidal region during the tidal cycle. The reason for the observation that highest NA occurred at half the natural salinity is unknown. NA in general was lower at Station II than at Station I and III, likely caused by the low contribution of *Cyanobacteria* to the *nifH* transcript libraries, i.e., the active diazotrophic community, at all salinities. In a study on the effect of salinity changes on cyanobacterial mats in the intertidal zone of the Arabian Gulf, mats in the lower intertidal experienced more severe inhibition of metabolic processes (photosynthesis and oxygen consumption) than those situated further away from the water line (Abed et al., 2007). This is in contrast to the findings of this study. The reason for this can be found in the different conditions prevailing at both intertidal zones. At the Arabian Gulf site larger variations in salinities were observed for the upper intertidal, possibly due to more severe desiccation during low tide. In the present study the mats higher up in the intertidal were usually not inundated by seawater and therefore experienced smaller changes in salinity than Station II at the low water mark where periods of emersion and immersion frequently alternated.

The difference in perception of the active diazotrophic community based on the *nifH* transcript libraries alone or on the analysis of OTU membership is likely to be caused by the approach. The latter analyzes the overlap of all the OTUs irrespective of their affiliation to the major groups assigned to the *nifH* sequences in order to display major shifts. This can cause a higher overlap of OTUs within minor groups of active diazotrophs present at different salinities while major groups without substantial OTU overlap change without altering the result of this type of analysis. Furthermore, a cut-off level of 99% sequence similarity was chosen to account for possible Taq errors but allow the consideration of microdiverse clusters observed in the majority of marine microbial communities (Acinas et al., 2004b; Klepac-Ceraj et al., 2004; Haverkamp et al., 2008). A general decrease of diversity but an increase of microdiversity with increasing salinity has also been observed for the bacterial community of a solar saltern (Benloch et al., 2002) and a soda lake (Foti et al., 2008). This phenomenon might also apply for the active diazotrophic community of microbial mats. However, the diversity of OTUs at 99% similarity predicted by the Chao1 diversity indicator showed higher diversity at hypersaline conditions only for Station I.

In conclusion, mats naturally exposed to smaller salinity changes showed a more variable active diazotrophic community and a stronger response to decreased or increased salinities with regard to NA than mats situated in a zone with a larger range in natural salinity. In general, the diazotrophic community responded better to salinities that likely occur in nature than to those beyond the normal salinity range. *NifH* expression accompanying low NA at higher salinities was dominated by members of the *Proteobacteria*, mainly *Gamma*- and *Deltaproteobacteria*. Their actual contribution to

whole mat NA, however, remains unresolved. In all three microbial mats salinity was an important factor shaping the active diazotrophic community and consequently also NA.

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