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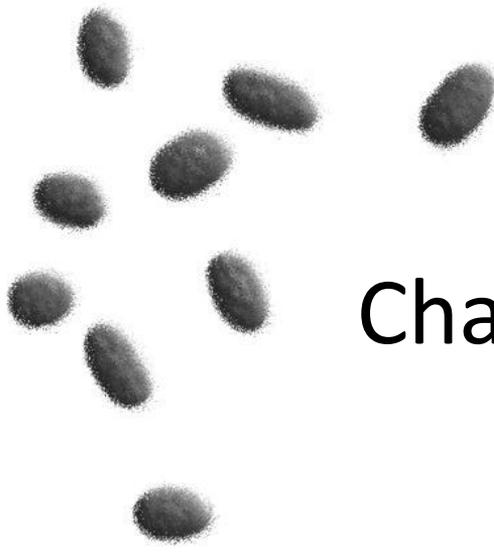
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Chapter 9

Spatial and temporal variability in nitrogenase activity and diazotrophic community composition in coastal microbial mats

Ina Severin¹ and Lucas J. Stal^{1,2}

¹*Department of Marine Microbiology, Netherlands Institute of Ecology, NIOO-KNAW;*

²*Department of Aquatic Microbiology, Institute of Biodiversity and Ecosystem Dynamics, University of Amsterdam*

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Abstract

Coastal microbial mats are highly diverse microscale ecosystems in an environment characterized by strongly fluctuating conditions. Nitrogen fixation is an important process in these often nitrogen-limited habitats. We investigated the characteristics of nitrogen fixation and the composition of the nitrogen-fixing (diazotrophic) community in three microbial mats situated along an intertidal gradient by using the acetylene reduction assay and by analyzing *nifH* clone libraries. Nitrogenase activity in the three mat types differed with respect to the daily pattern and daily integrated rates. The diazotrophic community composition in the three mat types was also dissimilar. The *nifH* clone libraries of the station that was most influenced by freshwater contained mostly cyanobacterial and alphaproteobacterial sequences whereas the other two stations were dominated by gamma- and deltaproteobacterial diazotrophs. These differences were attributed to the specific environmental conditions at each station resulting from their position along the intertidal gradient and likely caused the typical patterns of nitrogen fixation. Moreover, in each of the mat types, a dramatic shift in the diazotrophic community was observed over a period of two to three years. This shift varied greatly from station to station. The characteristics of nitrogenase activity at the same station in different years also varied considerably. Nitrogen fixation in the investigated microbial mats was spatially and temporally variable which was attributed to the shifts in diazotrophic community composition along the littoral gradient and during the three years of investigation.

Introduction

Microbial mats are versatile benthic communities of microorganisms, usually dominated by phototrophic bacteria (e.g., Krumbein et al., 1977; Jørgensen et al., 1983). They develop as vertically stratified populations of functionally different groups of microorganisms along physicochemical gradients. This stratification has been attributed to the prevailing gradients of oxygen, sulfide and light, which are generated and maintained by the metabolic activities of the community members (Revsbech et al., 1983; van Gemerden, 1993). Microbial mats are distributed globally and can be found in a wide variety of environments, such as cold Polar Regions, dry and hot deserts, hypersaline environments, hot springs and coastal environments. The latter are often characterized by strongly fluctuating environmental conditions, such as large variations in water availability and desiccation as well as salinity, temperature, oxygen, and sulfide gradients. They are also often depleted of combined nitrogen which then limits primary productivity (Paerl, 1990).

In most cases *Cyanobacteria* form the main structural element of microbial mats. As the primary colonizers on bare substrate they are the prerequisite for the development of this microbial ecosystem. The ability to fix atmospheric N₂ (dinitrogen) represents a distinctive advantage that allows *Cyanobacteria* to colonize the often nutrient-poor and particularly nitrogen-depleted environments in which microbial mats thrive. However, the ability to fix N₂ is widespread among other *Bacteria* and *Archaea*. Therefore, investigating the entire bacterial community of potential N₂ fixing organisms (diazotrophs) is essential to explain the characteristics of N₂ fixation in microbial mats. This can be done by detecting and characterizing *nifH*, the gene coding for the Fe-protein of nitrogenase, the enzyme complex which catalyzes the reduction of atmospheric N₂ to ammonia. *NifH* has been shown to be sufficiently variable to distinguish between *Cyanobacteria* and other *Bacteria* and *Archaea* as well as between heterocystous and non-heterocystous *Cyanobacteria* (Ben-Porath & Zehr, 1994). The analyses of *nifH* from the environment demonstrated that microbial mats contain a diverse diazotrophic community (e.g., Ben-Porath & Zehr, 1994; Olson et al., 1999).

For some microbial mats it has been shown that the community structure varies depending on the location of the mats along an intertidal gradient (Rothrock & Garcia-Pichel, 2005; Dijkman et al., 2010). In these studies the clear difference between the communities of the different microbial mats was attributed to a difference in desiccation frequency. However, several other factors including temperature, sedimentation patterns, oxygen concentration and oxygen penetration depth could also be responsible for changes in the community composition. Studies on the shift of the diazotrophic fraction of the microbial community along environmental gradients are rare. However, two different mat types in the intertidal area of Tomales Bay, California have been investigated with regard to the nitrogen cycle (Joye & Paerl, 1994). N₂ fixation in these mat types did not exhibit differences with regard to rates and daily cycle but seasonal changes were detected instead. Seasonal variations in community composition and the daily N₂ fixation pattern have also been observed for other microbial mats. Coastal microbial mats on North Carolina's Outer Banks barrier islands showed differences in daily N₂ fixation pattern and

nifH gene pool in summer and winter (Zehr et al., 1995; Paerl et al., 1996). This was attributed to changing environmental conditions supporting growth and activity of different types of diazotrophs in different seasons. In a changing environment, it may also be possible to find differences in the daily N₂ fixation pattern between different years, when the diazotrophic community has changed due to altered environmental conditions. The aim of this study was to investigate the differences in diazotrophic community composition and N₂ fixation in three microbial mats situated along an intertidal gradient. The results demonstrate that the community composition as well as daily patterns and integrated rates of N₂ fixation in the three mat types differed from each other. We also followed shifts in the diazotrophic community composition as well as differences in the daily N₂ fixation pattern over a period of three years. In each of the mat types, shifts in the diazotrophic community were observed. These shifts varied greatly between the different stations. The patterns of nitrogenase activity were also different and were attributed to morphological changes of the area during the period of this study.

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 at 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.

In 2006 two sampling 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 upwelling groundwater). This area is only irregularly inundated by the sea, usually at spring tide and with northern winds. The mats found at Station I revealed high cyanobacterial species diversity containing both heterocystous and non-heterocystous filamentous *Cyanobacteria* as well as unicellular types. Station II was situated near the low water mark. Due to tidal inundation, seawater is far more important than the occasional rain showers at this station 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 I was sampled again in 2007. Additionally, a third station (Station III) was chosen and sampled in 2007. 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 salt marsh 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. All three stations were sampled in 2008.

For each 24h measurement of nitrogenase activity (NA), samples of the mats were collected using a corer with a diameter of 50 mm made of a PVC tube. The upper 2 – 3 mm of the mat was dissected using a knife. After finishing the nitrogenase activity measurements, the sample was frozen in liquid nitrogen and stored at -80°C for later chlorophyll determination. Samples for molecular analyses 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 sectioned in four equal parts using a scalpel. Each part was transferred into a separate cryovial (Simpport Plastics, Beloeil, Qc J3G 4S5, Canada) and immediately frozen in liquid nitrogen.

Nitrogenase activity

Nitrogenase activity was measured using the acetylene reduction assay (ARA) (Hardy et al., 1968). The upper 2 – 3 mm of the mat was placed in a custom-made measuring cell (Staal et al., 2001) with a diameter of 46 mm and moisturized with filtered (Whatman GF/F glass microfiber filter) North Sea water. All mat samples were incubated simultaneously under exactly the same temperature (thermostat circulating water bath) and ambient light conditions by using a set-up with 6 measuring cells. Measurements of nitrogenase activity were carried out at regular intervals (every 4h). At each of the time points, 2 ml acetylene (Messer B.V., 4782 PM Moerdijk, The Netherlands) were injected. Incubations lasted 1h after which 10 ml of the headspace gas was transferred into evacuated and sealed 10 ml crimp top vials (Chrompack-Varian, Inc., 4338 PL Middelburg, The Netherlands). Between the incubations, air was flushed through the measuring cells using an air pump. 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 was used as carrier gas at a flow rate of 10 ml min^{-1} . The supply rates of H_2 and clinical air for the flame ionization detector were 30 ml min^{-1} and 300 ml min^{-1} , respectively. Gasses were purchased from Westfalen Gassen Nederland BV, 7418 EW Deventer, The Netherlands. 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). Based on the light response curves recorded for the mats at all three stations in 2006 and 2007 (Severin & Stal, 2008; Severin & Stal, unpublished), potential nitrogenase activities for the 2008-mats were calculated using the light intensities recorded during the incubations in 2008. This was done to elucidate whether the characteristics of nitrogenase activity, as derived from the corresponding light response curves, were similar for the different years. Nitrogenase activity rates were integrated for a period of 24h to compare daily rates between the three stations of one year and for each station in subsequent years.

Monitoring nitrogenase activity

To evaluate the influence of the environmental parameters light, temperature and tidal cycle, samples for nitrogenase activity measurements were taken twice a day at 10:00 am and 10:00 pm. Samples 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. 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 filtered seawater from the sampling location were added. Samples of all three mat types were incubated in duplicate. 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). Ethylene concentrations were measured by gas chromatography. Natural photon flux density (PFD, $\mu\text{mol m}^{-2} \text{s}^{-1}$) was recorded continuously using a PAR (photosynthetic active radiation) light sensor (LI-190 Quantum Sensor, Li-COR Biosciences, Lincoln, NE 68504-0425, USA) connected to a data logger (Licor 1000). The PFD was measured at intervals of 1 sec and averages were stored every minute. Temperature was recorded at the surface of the mat at the time of sampling. The tidal cycle was reconstructed based on publicly available information provided by the Dutch ministry of traffic and water state (Ministerie van Verkeer en Waterstaat, www.getij.nl).

Statistical analyses

The influence of the variation in the variables tide, light and temperature on the variation in nitrogenase activity was tested with one-way ANOVA (univariate analysis of variance) by using the SPSS software program version 17.0 (SPSS Inc., Chicago, IL 60606-6307, United States).

Nucleic acid extraction

DNA from all stations was extracted in triplicates with the MO BIO UltraClean Soil DNA Isolation-kit (MO BIO Laboratories, Inc., Carlsbad, CA 92010, USA according to the manufacturer's protocol. Quality and quantity of extracted DNA was checked on a 1% agarose gel and with the NanoDrop ND 1000 (NanoDrop Technologies, Inc., Wilmington, DE 19810, USA). The extracts of one station were combined and immediately used for amplification.

PCR, cloning and sequencing

For the amplification of *nifH*, 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 at all stations was estimated by the DOTUR and SONS software programs (Schloss & Handelsman, 2005; 2006) based on the alignment files for the *nifH* clone libraries. Clustering analysis for all stations in the three years was performed based on a similarity matrix including presence and absence of OTUs as well the amount of sequences contained in these OTUs. We used unweighed pair-group averages and the Euclidean distance measure (STATISTICA software package version 9, StatSoft, Inc., Tulsa, OK 74104, USA). All sequences are deposited at the NCBI GenBank database under the accession numbers GU193157 to GU193975.

Results

Spatial variability in nitrogenase activity

The daily nitrogenase activity (NA) dynamics for the three stations sampled in 2008 were measured using the acetylene reduction assay (ARA) and depicted in Figure 1 together with the daily light records.

For Station I chlorophyll *a*-normalized ethylene production ranged from 0.3 – 2 $\mu\text{mol mg}^{-1} \text{h}^{-1}$ (Figure 1A). The trend was slightly different for the two days. At day 1 NA decreased towards the evening, showed slightly increased NA at sunset and minimum NA at night. At sunrise ($\sim 6:00$) NA started to increase and peaked at $\sim 10:00$. At day 2 NA was highest in the afternoon ($\sim 14:00 - 16:00$) and decreased afterwards, reaching a minimum just before midnight. Some increase of NA was observed in the early morning hours before sunrise ($\sim 4:00$). Daily integrated chlorophyll *a*-normalized NA at Station I was 19.5 and 17.7 $\mu\text{mol C}_2\text{H}_4 \text{mg}^{-1}$ at day 1 and 2, respectively. The daily cycle of nitrogenase activity at Station II differed from that of Station I. Nitrogenase activity at Station II showed two clear peaks at about sunrise ($\sim 8:00 - 9:00$) and sunset ($\sim 22:00 - 23:00$) at both days (Figure 1B). Activity during the sunrise peaks was higher than during at sunset. Total nitrogenase activity ranged from virtually zero at night to 11 $\mu\text{mol mg}^{-1} \text{h}^{-1}$ during the sunrise peak at day 1. Integrated for an entire day, chlorophyll *a*-normalized NA reached 106.9 and 86.5 $\mu\text{mol C}_2\text{H}_4 \text{mg}^{-1} \text{day}$ 1 and 2, respectively. NA at Station III also showed enhanced nitrogenase activity at sunrise ($\sim 7:00 - 9:00$) (Figure 1C). No increased activity was observed around sunset. The sunrise-maximum reached activities of 0.5 $\mu\text{mol mg}^{-1} \text{h}^{-1}$ and were therefore one order of magnitude lower than at the other stations. Daily integrated chlorophyll *a*-normalized NA was 3.7 $\mu\text{mol C}_2\text{H}_4 \text{mg}^{-1}$ at both days.

Factors influencing nitrogenase activity

We tested to what extent variations in the tidal amplitude, light and temperature conditions explained variations in NA (Table 1).

Based on One-Way-ANOVA, 56% of the variation in NA at Station I could be explained by recorded variations in light (27%, $p = 0.006$) and temperature (29%, $p = 0.007$). Variations in the tidal amplitude, i.e. the coverage with seawater, did not play a role. At Station II only light conditions seemed to influence NA (12% of the variation in AN explained by variation in light) but the result was not significant ($p = 0.055$). None of the investigated factors could explain any of the NA-variations at station III.

Microbial mat diazotrophic community composition

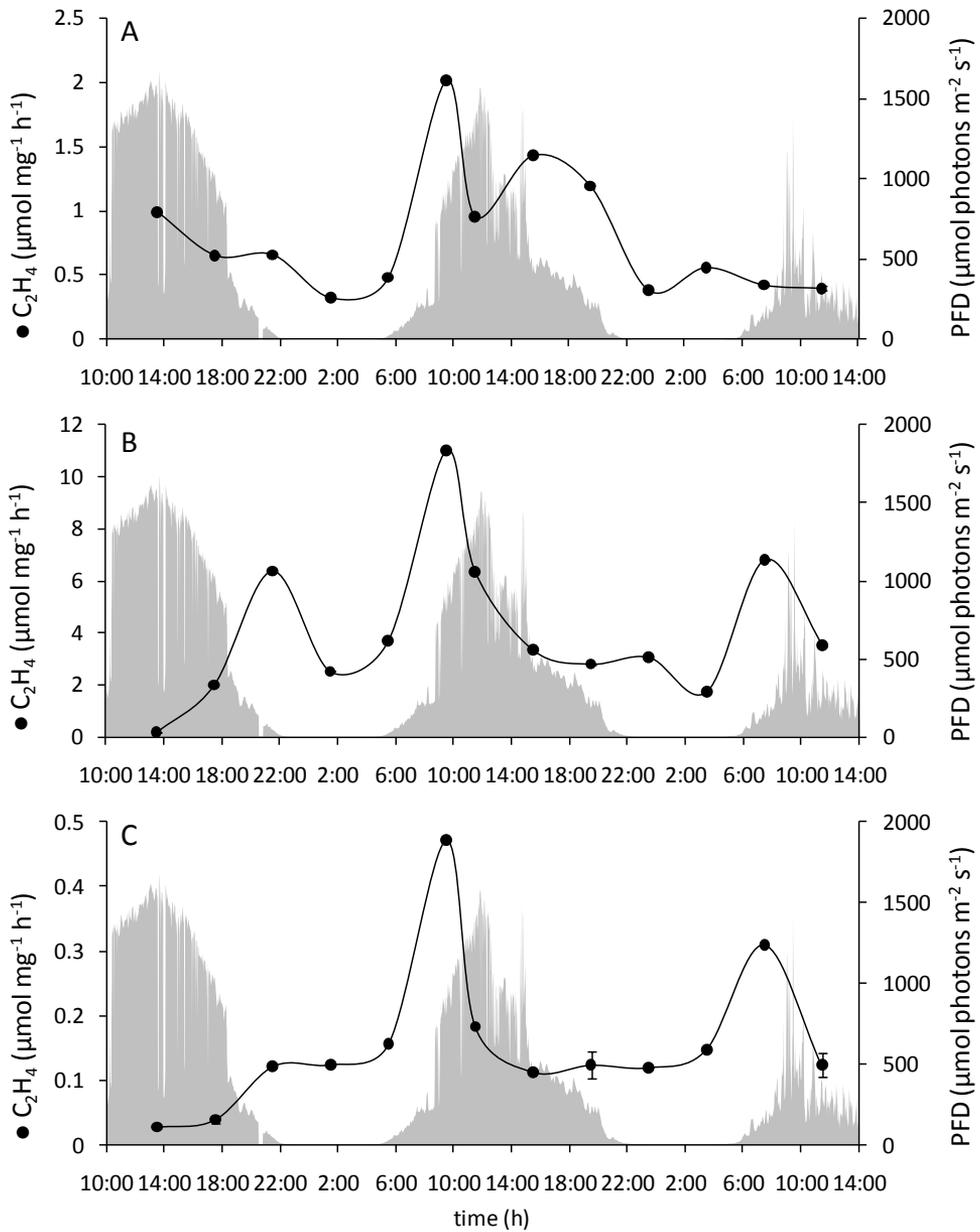


Figure 1. 24h cycle of chlorophyll *a*-normalized (mg^{-1}) nitrogenase activity as measured by acetylene reduction assay (ARA) (●). A: Station I 2008; B: Station II 2008. C: Station III 2008. Note different scales of the primary Y-axis

Table 1. Tidal amplitude (NAP: Normal Amsterdam Level), light intensity (PPFD: Photon Flux Density) and temperature at the mat surface at the time of nitrogenase activity measurements (NA, standard deviation given in brackets) as measured by acetylene reduction assay (ARA). n.m.: no measurement. Below, the statistical parameters of the one-way ANOVA (univariate analysis of variance) are given for Station I, II and III

Date	Time	Tide (NAP) [cm]	PPFD [$\mu\text{mol m}^{-2} \text{s}^{-1}$]	Temperature [°C]			NA (SD) [nmol C ₂ H ₄ mg chl a ⁻¹ h ⁻¹]		
				Station I	Station II	Station III	Station I	Station II	Station III
07.06.08	23:30	20	0.5	n.m.	n.m.	n.m.	59.3 (±40.0)	1033.7 (±1440.9)	1140.9 (±327.5)
08.06.08	11:30	40	1307.8	n.m.	n.m.	n.m.	5136.1 (±886.1)	367.1 (±118.8)	305.6 (±41.6)
08.06.08	23:30	-26	0.4	17.1	16.8	17.7	25.8 (±3.6)	496.4 (±423.9)	239.4 (±47.9)
09.06.08	11:30	-13	1344.5	21.5	23.1	21.5	3824.5 (±2611.8)	371.4 (±376.6)	692.6 (±164.0)
09.06.08	23:30	-81	0.2	19.3	17.6	18.8	31.4	58.3 (±3.1)	675.6 (±42.1)
10.06.08	11:30	-75	1035.3	19.4	18.6	18.4	1381.5 (±956.9)	565.9 (±100.5)	226.6 (±193.6)
10.06.08	23:30	-126	0.6	16.5	16.1	15.8	289.5 (±221.5)	30.6 (±18.2)	165.1 (±36.9)
11.06.08	11:30	-116	1079.5	19.6	17.5	17.8	1775.8 (±1121.8)	26.2 (±33.1)	342.2 (±61.8)
11.06.08	23:30	-137	0.7	14.4	14.0	13.5	n.m.	43.5 (±11.6)	392.2 (±5.8)
12.06.08	11:30	-117	623.5	15.8	15.0	15.4	336.2 (±262.2)	566.5 (±618.3)	1775.0 (±147.9)
12.06.08	23:30	-108	0.5	13.6	13.6	13.5	128.0 (±92.8)	25.9 (±16.4)	788.0 (±21.0)
13.06.08	11:30	-82	1069.5	14.9	15.0	14.4	536.5 (±452.5)	936.4 (±564.3)	113.6 (±104.9)
13.06.08	23:30	-53	0.6	13.0	12.5	12.8	36.8 (±30.6)	11.1 (±4.4)	742.7 (±332.0)
14.06.08	11:30	-27	847.6	16.0	5.0	4.8	434.9 (±66.2)	162.7 (±104.3)	428.2 (±11.6)
14.06.08	23:30	2	0.6	13.0	13.3	12.7	638.6 (±518.9)	27.1 (±5.7)	955.2 (±179.3)
15.06.08	11:30	24	1363.7	14.8	14.8	14.8	548.2 (±55.5)	463.3 (±50.5)	1135.5 (±347.9)
15.06.08	23:30	43	0.5	14.5	14.6	14.5	79.8 (±60.3)	11.8 (±2.0)	311.5 (±93.4)
16.06.08	11:30	61	1265.7	14.5	14.5	14.5	322.8	443.2 (±186.3)	357.2 (±152.5)
16.06.08	23:30	70	0.1	13.1	13.3	13.2	114.9 (±150.2)	10.4 (±4.7)	93.9 (±65.7)
17.06.08	11:30	86	1267.2	16.0	16.0	16.0	235.7 (±159.5)	309.1 (±7.4)	318.0 (±66.9)
17.06.08	23:30	84	0.8	16.0	16.4	16.2	11.1 (±13.4)	20.1 (±3.5)	746.3 (±176.4)
18.06.08	11:30	104	1173.1	18.5	18.0	16.8	259.3 (±6.3)	265.9 (±69.6)	88.8 (±33.8)
18.06.08	23:30	89	0.4	16.7	16.3	15.9	46.4 (±40.7)	38.8 (±3.7)	663.6 (±9.8)
19.06.08	11:30	112	295.0	20.1	18.9	18.7	186.0 (±53.7)	370.1 (±231.4)	106.4 (±3.2)

One Way-ANOVA		explained variation (p)
Tide	4.5% (0.805)	4.5% (0.935)
PPFD	27.2% (0.006)	11.9% (0.055)
Temperature	28.5% (0.007)	0.4% (0.312)
		0.3% (0.345)
		2.9% (0.562)
		4.4% (0.743)

Temporal variability in nitrogenase activity

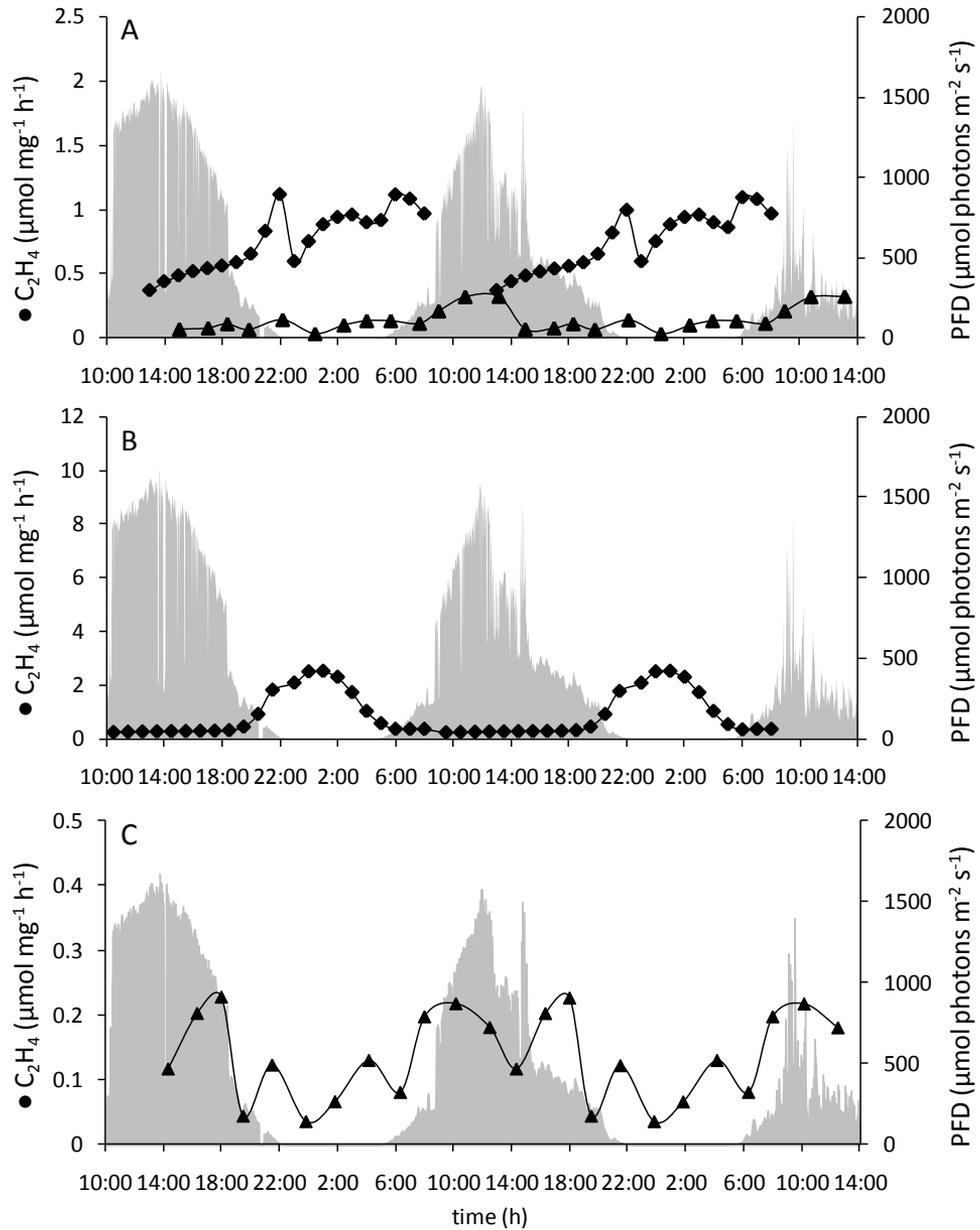


Figure 2. 24h cycle of chlorophyll *a*-normalized (mg^{-1}) nitrogenase activity as calculated on the basis of light response curves from 2006 (\blacklozenge) and 2007 (\blacktriangle) and the natural incident photon flux densities as recorded in 2008. A: Station I; B: Station II; C: Station III. Note different scales of the primary Y-axis; they are identical to those in Figure 1

The diel cycle of nitrogenase activity for all three mats was also calculated based on the light response curves generated in 2006 (Station I and II) and 2007 (Station I and III) and the light intensities measured in 2008. For all three stations calculated NA did not agree with the actual measurements in 2008 (Figure 2).

Using the light response curved from 2006 for the calculation of the daily NA cycle, enhanced NA at sunset and sunrise was predicted for Station I (Figure 2A). Activity was generally higher at night whereas the opposite was the case when NA was measured in 2008. The light response curves from 2007 predicted a NA pattern which was similar to the 2006-pattern at night but showed higher daytime activity. NA rates, however, were lower in 2007 compared to 2006 and 2008. Integrated over the entire day, chlorophyll *a*-normalized NA calculated from the 2006 NA pattern was similar to the one measured in 2008 (19.4 and 18.4 $\mu\text{mol C}_2\text{H}_4 \text{ mg}^{-1}$ per day, respectively) whereas the daily integrated NA calculated from the 2007 NA pattern was much lower than the one actually measured in 2008, reaching only $\sim 3 \mu\text{mol C}_2\text{H}_4 \text{ mg}^{-1}$ per day. For Station II only light response curves from 2006 were available for comparison. Based on these curves and the light intensities measured in 2008, the daily NA pattern was different from the one measured in 2008 (Figure 2B). In contrast to 2008, where highest NA occurred at sunset and sunrise, the 2006-pattern showed a confinement of NA to the night. Daily integrated chlorophyll *a*-normalized NA rates were lower when the light-response data of 2006 were used for the calculation than for the actual measurements in 2008, reaching $\sim 20 \mu\text{mol C}_2\text{H}_4 \text{ mg}^{-1}$ per day. The NA pattern of Station III as predicted from the light response curved obtained in 2007 was more variable than the one measured in 2008 (Figure 2C). Additionally to the peaks at sunset and sunrise, two more peaks occurred at daytime. Based on this calculation, daytime NA was only slightly higher than nighttime activity but daily integrated chlorophyll *a*-normalized rates were similar for the measured and the calculated NA cycle (3.2 and 3.7 $\mu\text{mol C}_2\text{H}_4 \text{ mg}^{-1}$ per day for 2007 and 2008, respectively).

Spatial variability in diazotrophic community composition

Based on the 2008 clone libraries of *nifH* the community of potential diazotrophs at the three stations was analyzed and compared (Figure 3).

At Station I *Cyanobacteria* and *Proteobacteria* dominated the clone libraries with a contribution of 45% and 40%, respectively. Most of the cyanobacterial sequences belonged to the *Oscillatoriales* (91%) but some heterocystous *Cyanobacteria* (*Nostocales*) (9%) were present as well. The proteobacterial fraction was dominated by *Alphaproteobacteria* that contributed 70% to the proteobacterial *nifH* sequences. *Deltaproteobacteria* were also abundant (27%) and *Gammaproteobacteria* (3%) were found as well. In contrast to Station I hardly any *Cyanobacteria*-related sequences were found in the *nifH* clone libraries of Station II. The few sequences that were found belonged to the *Oscillatoriales*. Proteobacterial sequences prevailed (66% of the sequences). *Gamma*- and *Deltaproteobacteria* dominated with 53% and 38% of the proteobacterial sequences, respectively. *Alpha*- and *Betaproteobacteria* were also found (7% and 2%, respectively). Another group of potential diazotrophs at Station II were the green sulfur

Microbial mat diazotrophic community composition

bacteria (*Chlorobi*) with an overall contribution of 14% to the clone libraries. At Station III *Proteobacteria* also dominated the *nifH* clone libraries with 80% of the sequences belonging to this group. *Gamma*- and *Deltaproteobacteria* (61% and 31% of the proteobacterial *nifH* sequences, respectively) prevailed but alphaproteobacterial sequences were also found. Green sulfur bacteria also contributed to the clone libraries (4%).

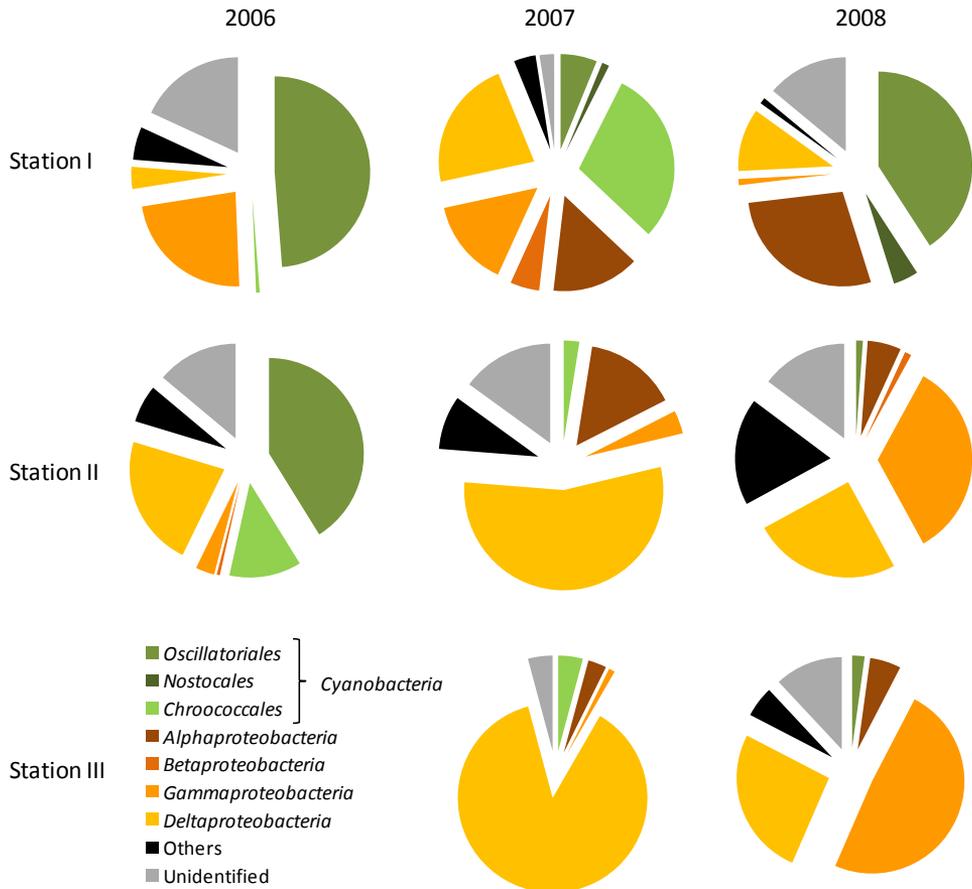


Figure 3. Relative contributions of major bacterial groups to the *nifH* clone libraries for Station I, II and III 2006, 2007 and 2008

Temporal variability in diazotrophic community composition

The *nifH* gene pool of the Stations I and II for the three years was compared (Figure 3) while for Station III only data for 2007 and 2008 were available. For the three stations we also compared the diversity based on the Chao1 diversity estimator (Table 2) and the overlap of OTUs found for the diazotrophic communities of each station in the successive years (Table 3) at a 99% similarity cut-off.

Table 2. Number of sequences per clone library, Chao1 diversity estimates and OTUs at 99% similarity based on *nifH* clone libraries

	Station I			Station II			Station III	
	2006	2007	2008	2006	2007	2008	2007	2008
Number of Sequences	127	92	93	154	80	88	96	92
Number of OTUs	60	45	37	55	58	64	34	62
Chao1 diversity (OTUs)	147	111	54	72	1581	277	150	177

Table 3. Summary of the diversity comparison of Station I, II and III in 2006, 2007 and 2008 based on shared OTUs at 99% similarity

	Station I			Station II			Station III
	06 vs. 07	06 vs. 08	07 vs. 08	06 vs. 07	06 vs. 08	07 vs. 08	07 vs. 08
Shared OTUs (#)	2	2	7	1	2	6	1
Shared OTUs (%)	3.3 / 4.4	3.3 / 5.4	15.6 / 18.9	1.8 / 3.7	3.6 / 3.1	10.3 / 9.4	2.9 / 1.6
Seq in shared OTUs (#)	2 / 18	2 / 6	11 / 37	9 / 1	3 / 2	9 / 12	1 / 1
Seq in shared OTUs (%)	1.6 / 9.6	1.6 / 6.5	12.0 / 39.8	5.8 / 1.3	1.9 / 2.3	11.3 / 13.6	1.0 / 1.1

In 2006 half of the *nifH* sequences retrieved at Station I belonged to *Cyanobacteria*, the majority was most closely related to the order of *Oscillatoriales*. As in 2008, *Deltaproteobacteria* represented a substantial fraction of the *nifH* gene pool whereas *Gammaproteobacteria* were the dominant proteobacterial diazotrophs in 2006 but not in 2008. In 2007 the cyanobacterial contribution to the *nifH* clone library was less than 40% and, in contrast to 2006 and 2008, dominated by *Cyanobacteria* most closely related to *Chroococcales*. Sequences belonging to *Alpha-*, *Beta-*, *Gamma-* and *Deltaproteobacteria* were found in 2007 and comprised more than half of the *nifH* clone libraries. Gammaproteobacterial *nifH* sequences were slightly more abundant than those clustering

with the other subgroups. The *nifH* diversity at Station I as predicted by the Chao1 diversity estimator was highest in 2006 and lowest in 2008. The similarity of the diazotrophic community composition between the years based on the number of shared OTUs was highest for the years 2007 and 2008. 12% of the sequences in 2007 were also found in 2008 while ~ 40% of the sequences retrieved in 2008 were already known from 2007.

At Station II most *nifH* sequences belonged to *Cyanobacteria* in 2006 whereas hardly any cyanobacterial *nifH* could be retrieved in 2007 and 2008. Most of the cyanobacterial sequences found in 2006 clustered with *Oscillatoriales*. In 2006 and 2008 approximately 25% of the proteobacterial community belonged to the *Deltaproteobacteria*. They dominated the *nifH* clone library in 2007 whereas *Gammaproteobacteria* were dominant in 2008. The diversity based on the Chao1 estimator was highest for 2008 and similar for 2006 and 2007. As for Station I, the highest overlap of OTUs was found for the diazotrophic communities in 2007 and 2008, corresponding to 11% and 14% of the sequences, respectively.

At Station III a clear prevalence of deltaproteobacterial *nifH* sequences was observed in 2007 whereas *Gammaproteobacteria* dominated in 2008. In 2007 only few sequences belonging to *Alpha*- and *Gammaproteobacteria* were present. Similar to 2008, only a minor fraction of the *nifH* sequences belonged to *Cyanobacteria*. In 2007, all of these cyanobacterial *nifH* sequences clustered within the order of *Chroococcales*. The diazotrophic diversity predicted by Chao1 was slightly higher in 2008. Compared to the other stations, the amount of shared OTUs in 2007 and 2008 was low, accounting for only ~1% of the sequences.

Clustering analyses of the diazotrophic communities of all samples based on the presence of OTUs and the numbers of sequences belonging to these OTUs demonstrated that the temporal variability of the diazotrophic community was largest from 2006 to 2007 (Figure 4).

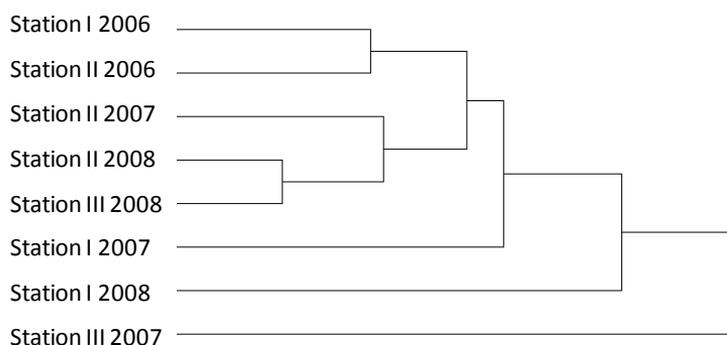


Figure 4. Clustering based on the presence of OTUs and the number of sequences belonging to these OTUs at a 99% similarity level for Station I, II and III in 2006, 2007 and 2008

Discussion

Spatial variability in nitrogenase activity and diazotrophic community composition

In 2008 a morning peak of nitrogenase activity (NA) was the only common feature in all three stations. Higher rates of NA in the morning might have been favored by an advantageous combination of increasing light intensities and low oxygen concentrations. If an evening peak of NA was detected as well, it was lower than the morning peak in all cases. The same pattern has also been observed in other cyanobacterial mats (Stal et al., 1984; Villbrandt et al. 1990). Apart from that, all three mat types showed differences in NA and their diazotrophic community compositions. The different patterns of NA in each of the mat types can most likely be attributed to the dissimilar diazotrophic communities at the three stations. As for the daily pattern of NA, the diazotrophic communities of Station II and III were more similar to each other than to Station I. This was confirmed by the comparison of common OTUs. 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 not overlook the microdiversity fraction observed in the majority of marine microbial communities (Acinas et al., 2004b; Klepac-Ceraj et al., 2004; Haverkamp et al. 2008). At this similarity level Station II and III shared more OTUs than either of the stations with Station I. The reason for this is not exactly known. Due to their location higher up in the intertidal, Station I and III were assumed to be more similar with regard to freshwater input and seawater coverage. Moreover, pore water salinity and desiccation time were proposed to be the main environmental variables determining community composition of the mats on Schiermonnikoog (Dijkman et al., 2010). However, environmental conditions other than salinity and desiccation time might have influenced the diazotrophic community. The different location of the mats within the intertidal area of the sandy North Sea beach also results in differences in water availability, temperature, sedimentation patterns, oxygen concentration and oxygen penetration depth. The reason for the almost complete lack of cyanobacterial *nifH* at Station II and III is not clear. The filamentous non-heterocystous *Cyanobacteria* observed at these two stations may not have been diazotrophic species. The dominant structural cyanobacterium *Lyngbya* sp., even if isolated strains have been shown to be diazotrophic, may not have been the main contributor to *nifH* clone libraries (see also, e.g., Omoregie et al., 2004a). However, it is known that discrepancies exist between clone libraries based on *nifH* and those based on their reverse transcripts (e.g., Hewson et al., 2007; Man-Aharonovich et al., 2007; Severin et al., unpublished). Therefore we cannot exclude that these *Cyanobacteria* may still have contributed to *nifH* expression and N₂ fixation.

Factors influencing nitrogenase activity

Another interesting point was the difference in the daily pattern and integrated rates of NA during the two subsequent days of our measurement. Because in phototrophic diazotrophs light is a direct or indirect source of energy supply for nitrogenase (e.g., Bebout et al., 1987) we supposed that the variation of light caused the difference in NA. Moreover, low light intensity may limit growth and metabolic activity of phototrophs as well as chemotrophs depending on products of photosynthesis, for instance to satisfy the demand of reducing equivalents for N₂ fixation (Bebout et al., 1993). However, although the total integrated daily light on the second day of the NA measurement was only 34% of that of the first day, integrated NA decreased only slightly, probably because the incident light intensities were still saturating for nitrogenase activity. The daily integrated NA might have been independent from the daily integrated incident photon flux. This has been shown for mats at Station I and II in 2006 (Severin & Stal, 2008). It is possible that during a day with a high daily photon flux more storage carbohydrate accumulates which can then be used during a successive day with less light so that overall NA is maintained. This does however not explain the difference in the pattern of NA between the two days.

The impact of temperature, light intensity and tidal cycle on NA in 2008 was tested in order to elucidate whether these factors might control NA in the three mat types. When phototrophs were responsible for N₂ fixation, light would be an important controlling factor. In line with this, a significant impact of variations in light intensities on NA was found for the station that was shown to harbor many diazotrophic *Cyanobacteria*. Variations in light intensity influenced NA also in Station II although we hardly found any diazotrophic *Cyanobacteria* at this station. However, anoxygenic phototrophic diazotrophs might have been important at this station as indicated by the Gammaproteobacterial *nifH* in our clone libraries. On the other hand, diazotrophic *Gammaproteobacteria* were also found at Station III where light appeared to have no effect on NA. The reason for that is not clear. At both Station II and III most of these *Gammaproteobacteria* were phototrophs (purple sulfur bacteria). Nevertheless, we expect that also chemotrophic diazotrophs depend indirectly on light, because the photosynthesis by the *Cyanobacteria* drives the microbial mat. Obviously, other factors may also have played a role, among which temperature might have been important. It is known that the rate of metabolic processes vary with temperature according to the temperature quotient, Q₁₀, which is generally close to 2. This means that the rate of NA might double or halve with a rise or decrease, respectively of the temperature by 10°C. The difference in temperature recorded at the time of sampling on each of the mat surfaces was sufficiently large to cause such effect. However, the rate of NA will be controlled by a variety of factors and different organisms may have different temperature optima. The microbial mat is too complex to measure and interpret variations in metabolic rates such as NA. The tidal cycle and therefore the coverage with seawater might have been an important control of NA as well. The tidal position resulting in differences in seawater coverage and therefore desiccation frequency has been demonstrated to shape community structure, diversity and richness of microbial mats situated in an intertidal area (Rothrock & Garcia-Pichel, 2005) but we could exclude this as a factor controlling NA in the mats investigated in the present study. However, it

must be emphasized that only Station II became inundated at high tide during the sampling in 2008.

Temporal variability in nitrogenase activity and diazotrophic community composition

Besides the differences caused by the position of the microbial mats along the intertidal gradient, the change in beach morphology during the three years we carried out our investigation might have had an impact on the microbial mats. This has been demonstrated for the bacterial and diazotrophic community in 2006 and 2007 (Severin et al., unpublished). For Station II and III there was little overlap between the diazotrophic communities in 2007 and 2008 but comparing the diazotrophic communities of Station I and II showed that the largest shift happened from 2006 to 2007. The shift in community composition from one year to the next was most likely caused by morphological changes of the beach area, as visible in the increase of vegetation from 2006 to 2008, as well as by the difference in climatic conditions between these years, especially with respect to temperature, light and water availability. The spring preceding the sampling in 2006 was sunny but wet and allowed strong mat development. Both the spring in 2007 and 2008 were warm and dry and may have developed a diazotrophic community dissimilar from 2006 while similar climate conditions produced less variation between those two years. The extent to which the sampling area was covered by salt marsh vegetation was also more different between 2006 and 2007 than between 2007 and 2008.

We compared the characteristics of NA by calculating the daily cycle of NA based on the light response curves generated in 2006 and 2007 and the light intensities measured in 2008. If NA characteristics of one station were similar over the years, a similar daily NA pattern should have been observed. However, daily patterns of NA in the three years were different both with respect to total activity as well as to the daily patterns. This is in line with the shift in diazotrophic community composition. Different diazotrophs adapt differently to changing environmental conditions within a 24h cycle and different diazotrophic communities are therefore expected to exhibit different daily patterns of NA. The lower daily integrated chlorophyll *a*-normalized NA rates calculated for Station I in 2007 and Station II in 2008 as compared to the other years at these stations are also attributed to the shift in diazotrophic community composition. In both cases, a decrease in the contribution of *Oscillatoriales* to the diazotrophic community might have caused the lower NA rates. An increase of *nifH* sequences related to *Oscillatoriales* at Station I in 2008 coincided with an increase in daily integrated chlorophyll *a*-normalized NA rates and substantiates this hypothesis.

Studies to follow community composition and metabolic activity of microbial mats over the course of several years are rare. There are, however, microbial mats located in the intertidal zone of Bird Shoal within the Rachel Carson National Estuarine Research Reserve (RCNERR) which have been studied rather extensively with regard to the diazotrophic community and NA during the last decade (e.g., Zehr et al., 1995; Paerl et al., 1996; Steppe & Paerl, 2002; 2005). Most obvious was the seasonal change in NA pattern and rate, most

likely due to a shift in the diazotrophic community. Earlier studies conducted in 1992 and 1993 showed a shift from cyanobacterial diazotrophs in summer to chemotrophic diazotrophs in winter which was accompanied by high NA rates and nighttime activity during summer and low NA rates and daytime activity during winter (Zehr et al., 1995; Paerl et al., 1996). In the period between 1997 and 1999 these daily NA patterns in different seasons were shown again but were less obvious at times and also complemented by a third pattern of high sunrise NA in summer. The diazotrophic community included the same major groups in all seasons and years (Steppe & Paerl, 2002; 2005). In contrast to the mats investigated by us, the Bird Shoal mats did apparently not experience the same large changes in environmental conditions (light, temperature, tidal range) from year to year and therefore might have been more stable with respect to community composition and NA.

Summary

The spatial differences observed for the diazotrophic community in 2008 were attributed to different environmental conditions at each station due to their position along the intertidal gradient and were assumed to cause the differences in NA. The temporal variability in characteristics of nitrogenase activity was most likely caused by large shifts in diazotrophic community composition observed for each of the mat types from 2006 to 2008. From 2007 onwards the spatial variability in the diazotrophic communities due to the location of the stations along a littoral gradient was probably more important than the temporal changes between years.

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