Nitrogen fixation in microbial mats: complexity and dynamics of a small-scale ecosystem

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Chapter 6

NifH expression by five groups of phototrophs compared to nitrogenase activity in coastal microbial mats

Ina Severin\textsuperscript{1} and Lucas J. Stal\textsuperscript{1,2}

\textsuperscript{1}Department of Marine Microbiology, Netherlands Institute of Ecology, NIOO-KNAW;
\textsuperscript{2}Department of Aquatic Microbiology, Institute of Biodiversity and Ecosystem Dynamics, University of Amsterdam

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Abstract

Diazotrophic (nitrogen-fixing) Cyanobacteria are often structurally dominant in coastal microbial mats but diazotrophs from other bacterial lineages are also present and active. The expression of nifH by four non-heterocystous Cyanobacteria and one member of the Gammaproteobacteria was followed over a 24h cycle by using quantitative RT-PCR. Daily nifH expression patterns were compared to the actual nitrogenase activity of the entire mat community. Lyngbya sp. was identified as the dominant cyanobacterium but, although recognized as a diazotroph, its cell-specific and abundance-related nifH expression was low. Unexpectedly, the other three cyanobacterial phylotypes dominated community nifH expression at all stations. Also the gammaproteobacterium showed high levels of cell specific nifH expression but its nifH copy number was low. Its contribution to the whole community nifH expression was therefore low. These results demonstrate that there were varying levels of cell specific expression of nifH in the different mat types and more so, varying contributions to the overall nifH expression by the different diazotrophs. Furthermore, nitrogenase activity did not follow nifH expression patterns.
**NifH expression in microbial mats**

**Introduction**

Microbial mats are small-scale ecosystems harboring a highly diverse community of microorganisms, often assumed to be dominated by phototrophic bacteria (e.g., Krumbein et al., 1977; Jørgensen et al., 1983). They occur in a variety of habitats, including marine intertidal flats, hypersaline environments, hot springs and Antarctic ponds. Microbial mats are largely closed ecosystems and therefore possess a variety of metabolic pathways. This includes the fixation of atmospheric dinitrogen (N$_2$ fixation) which occurs in all microbial mats investigated for it (Severin & Stal, 2010). Especially in the marine environment, where a depletion of combined nitrogen often limits primary productivity (Paerl, 1990), diazotrophic (N$_2$ fixing) microorganisms play an important ecological role. It does not come as a surprise that the oxygenic phototrophic *Cyanobacteria* are the pioneer organisms and most conspicuous component of intertidal microbial mats. *Cyanobacteria* are well qualified for this task because of their low nutritional demands, their capability of photosynthesis, N$_2$ fixation, fermentative pathways and EPS-production (Stal, 2001).

However, nitrogenase, the enzyme complex catalyzing the reduction of atmospheric N$_2$ to ammonia, and hence the capability of N$_2$ fixation is widespread among the Domains *Bacteria* and *Archaea* but not in the *Eukarya*, except in symbiotic associations with *Bacteria* (Zehr et al., 2003). The highly conserved complex of two enzymes consists of dinitrogenase reductase, encoded by *nifH*, and dinitrogenase, encoded by *nifDK* (Postgate, 1982). *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).

With the development of general primers for *nifH* (Zehr & McReynolds, 1989), deeper insight into the diazotrophic community has been gained. The analysis of *nifH* genotypes in several cyanobacterial mats revealed a dominance of heterotrophic diazotrophs (e.g., Ben-Porath & Zehr, 1994; Olson et al., 1999). However, the presence of *nifH* does not indicate nitrogenase activity. Discrepancies between the presence of certain diazotrophs and the recorded pattern of nitrogenase activity (NA) gave rise to the question whether or not and under which circumstances these diazotrophs contribute to whole community N$_2$ fixation. Therefore, *nifH* transcripts were used in order to trace the active diazotrophs (e.g., Church et al., 2005b). Nevertheless, *nifH* expression does not necessarily translate into actual enzyme activity. In a number of diazotrophs nitrogenase is regulated post-transcriptionally (e.g., Ludden & Roberts, 1989; Ohki et al., 1991; Du & Gallon, 1993; Zehr et al., 1993). Steunou et al. (2008) observed that *Synechococcus nifH* expression was high in the evening and decreased overnight while NA peaked in the morning. In another study, *nifH* expression levels were generally correlated with cell-specific N$_2$ fixation rates (Zehr et al., 2007). Hence, gene expression, i.e., the synthesis of the enzyme, does not necessarily result in activity of that enzyme. On the other hand, enzyme activity can be measured even if no expression of the gene is detected (e.g., Steunou et al., 2008). In that case enzyme activity does not depend on *de novo* synthesis. Despite all these limitations, gene expression is often taken as an indication for the metabolic activity of an organism but must be interpreted with regard to the possible regulatory mechanisms.
In this study we aimed at the identification of key players in microbial mat N\textsubscript{2} fixation by following the daily pattern of \textit{nifH} expression for a selected number of diazotrophs. We developed primers that target several specific groups of closely related sequences accounting on average for more than 80\% of the sequences present in \textit{nifH} transcripts libraries. The \textit{nifH} expression patterns were compared to each other and to the NA patterns previously recorded for these mats (Severin & Stal, 2008). This approach yielded detailed information on the dynamics of \textit{nifH} expression in three different mat types and for five key groups of diazotrophs.

**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\textdegree 29' and E 6\textdegree 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 \textit{Cyanobacteria} as well as unicellular types. Station II is situated near the low water mark. Due to tidal inundation, seawater is far more important than the occasional rain showers and this distinguished it from Station I. The \textit{Cyanobacteria} at Station II are mostly non-heterocystous forms, predominantly \textit{Lyngbya aestuarii}. Occasionally, heterocystous \textit{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 higher plants were partly overgrowing the established mats. Based on microscopic observation, \textit{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 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 NA measurements, the sample was frozen in liquid nitrogen and stored at \textdegree 80°C for later chlorophyll determination. Samples for molecular analyses were collected using disposable 10 ml-syringes of which the lowest part had been removed to obtain a tube with a diameter of 1.5 cm. After
pushing the tube in the sediment and removing it again, the upper 2 – 3 mm of the mat was dissected and sectioned in four equal parts using a scalpel. Each part was transferred into a separate cryovial (Simport Plastics, Beloeil, Qc J3G 4S5, Canada) and immediately frozen in liquid nitrogen. Samples for NA measurements and nucleic acid extractions were taken over a 24h cycle in intervals of 4h (2006) or 2h (2007).

**Nitrogenase activity pattern**

NA was measured using the acetylene reduction assay (ARA) (Hardy et al. 1968). The online method of Staal et al. (2001) was used as described in Severin & Stal (2008). Light response curves of NA were calculated from ethylene production rates at photon flux densities (PFD) ranging from 0 – 300 µmol m⁻² s⁻¹ (Severin & Stal, 2008). Natural photon flux density (PFD, µmol m⁻² s⁻¹) was recorded 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 (LI-1000). The PFD was measured at intervals of 1 sec and averages were stored every minute. The NA rate at any time of the day and for any day was calculated from the fitted parameters obtained from the hourly measured light response curves and the recorded natural PFD (Severin & Stal, 2008).

**Nucleic acid extraction**

DNA and RNA were extracted from Stations I and II in 2006 and Stations I and III in 2007 at all sampling times. DNA was extracted using the MO BIO UltraClean Soil DNA Isolation-kit (MO BIO Laboratories, Inc., Carlsbad, CA 92010, USA) according to the manufacture’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 or stored at –20 °C. RNA was extracted from all time points in triplicates using the Qiagen RNeasy Mini-kit (Qiagen GmbH, 40724 Hilden, Germany) following the manufacturer’s protocol but replacing the enzyme-digest by a bead-beating step (glass-beads, 2 min). Immediately after checking quality and quantity of the extracted RNA, the triplicates per time point were combined and used for DNase treatment (Deoxyribonuclease I, Invitrogen Corporation, Carlsbad, CA 92008, USA) as stated in the manufacture’s descriptions. RNA was checked on a 1% agarose gel and the DNA-free RNA was instantly used for the RT reaction using Invitrogen chemicals (Superscript II Reverse Transcriptase and Random Primers, Invitrogen Corporation, Carlsbad, CA 92008, USA) and following the corresponding protocol. The resulting cDNA was immediately used for amplification or stored at –20 °C.
Quantitative PCR

Based on the sequence information retrieved from clone libraries of nifH and its transcripts constructed using general nifH primers (Zehr & McReynolds, 1989; Zani et al., 2000) and samples from Station I and II in 2006 (Supplement Table 1), primers were selected manually. The PCR products were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen Corporation, Carlsbad, CA 92008, USA) following the manufacturer’s instructions. PCR products of the clones were then 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). 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).

The primers developed on the basis of these nifH sequences (NCBI GenBank accession numbers GQ441193 to GQ442612) are listed in Table 1. The most prominent sequences represented four groups of diazotrophs characterized as (i) Lyngbya aestuarii, (ii) a unicellular cyanobacterium, (iii) organisms most closely related to Oscillatoria, and (iv) Thiorhodospira. Additionally, the primer pair designed and validated for Microcoleus was used (Bolhuis et al., 2010). The contribution of these sequences to the nifH transcript libraries constructed for different time points over a daily cycle varied (between 65 and 100%) but on average, these sequences comprised more than 80% of all nifH transcripts. Furthermore, 48 amplification products from environmental samples were cloned and sequenced for each primer set. All sequenced amplification products confirmed the specificity of the five primer pairs. Lyngbya aestuarii primers (hereafter termed L-nifH-F and L-nifH-R) as well as the Oscillatoria-related primer pair (O-nifH-F and O-nifH-R) and the primers targeting the unicellular cyanobacterium (U-nifH-F and U-nifH-R) showed no non-target hits at all, whereas amplification with the Thiorhodospira-related primer pair (T-nifH-F and T-nifH-R) resulted in 2% non-target hits. The specificity of the primers targeting nifH from Microcoleus chthonoplastes has been validated previously (Bolhuis et al., 2010). Additionally to these primers, general nifH primers (Poly et al., 2001) were used to approximate whole community nifH expression levels and gene copy numbers. These primers target a similar region as the ones proposed by Zehr and McReynolds (1989) but are less degenerated and do not require a nested PCR protocol. Cloning and sequencing of the amplification products analogous to the other primers used revealed a wide target range.
Table 1. Sequences of the primers used in the SYBR Green quantitative (RT) PCR

<table>
<thead>
<tr>
<th>nifH sequence type</th>
<th>primer</th>
<th>sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyngbya aestuarii</td>
<td>L-nifH-F</td>
<td>5'-TCA GGT TAC AGT TCT ATC GT-3'</td>
</tr>
<tr>
<td></td>
<td>L-nifH-R</td>
<td>5'-GGA GGT GAT AAT ACC ACG-3'</td>
</tr>
<tr>
<td>Oscillatoria-related</td>
<td>O-nifH-F</td>
<td>5'-TGC AGT GGA AGA CGT AGA ACT-3'</td>
</tr>
<tr>
<td></td>
<td>O-nifH-R</td>
<td>5'-CAC GTC CTG CAC AAC CTA CA-3'</td>
</tr>
<tr>
<td>unicellular cyanobacterium</td>
<td>U-nifH-F</td>
<td>5'-GAT TCT ACC CGC TTA ATG CTT-3'</td>
</tr>
<tr>
<td></td>
<td>U-nifH-R</td>
<td>5'-AAC GTC ACC CAA TAC GTC GTA-3'</td>
</tr>
<tr>
<td>Thiorhodospira-related</td>
<td>T-nifH-F</td>
<td>5'-AGG CTC AGA ATA CCA TTA TGC-3'</td>
</tr>
<tr>
<td></td>
<td>T-nifH-R</td>
<td>5'-TCG CCC AAC ACG TCA TAA AA-3'</td>
</tr>
<tr>
<td>Microcoleus sp.</td>
<td>Mc-nifH-f</td>
<td>5'-TTTACGGTAAAGGTGGAATCG-3'</td>
</tr>
<tr>
<td></td>
<td>Mc-nifH-QRT</td>
<td>5'-ATCTAACACACTCTTTGGTG-3'</td>
</tr>
<tr>
<td>general (Bacteria)</td>
<td>PolF</td>
<td>5'-TGC GAY CCS AAR GCB GAC TC-3'</td>
</tr>
<tr>
<td></td>
<td>PolR</td>
<td>5'-ATS GCC ATC ATY TCR CCG GA-3'</td>
</tr>
</tbody>
</table>

All quantitative PCRs (qPCRs) were run on a Corbett Rotor-Gene 6000™ (Corbett Life Science, NSW 2137 Sydney, Australia). Environmental DNA as well as the cDNA reverse-transcribed from environmental RNA were used as a template. Cycling conditions were the following: one cycle at 95 ºC for 15 min, 40 cycles at 95 ºC for 15 sec, 55 ºC for 20 sec and 72 ºC for 20 sec, followed by a melting curve analysis (72 – 95 ºC). Environmental samples were run in triplicate and each run included two independent dilution series of linearized plasmids (containing the target nifH insert) as standard curves as well as non-template and non-target controls (linearized plasmids containing non-target nifH inserts). All the 15 μl reactions contained 7.5 μl of the reaction mix (containing the reaction buffer as well as the DNA polymerase, dNTPs and SYBR Green I, Absolute™ QPCR SYBR® Mix, Thermo Fisher Scientific, Rockford, IL 61101, USA), 333 nM of the forward and reverse primer and 5.5 μl of MQ to adjust to a volume of 14 μl as well as 1 μl of template. NifH copy numbers for the plasmid standard curves ranged from approximately 2.2 x 10^2 to 2.2 x 10^7 copies per reaction. Least square linear regression analyses of ct values versus gene copies were used to quantify the original amount of target DNA or cDNA molecules in the sample. The correlation coefficient r^2 for the standard curves was ~ 0.96 and efficiencies for the dual assays were close to 95% for all reactions.

Expression levels were calculated as number of transcripts per DNA copy number for the individual organisms (thereafter referred to as ‘cell specific’ gene expression) and per ng of extracted RNA to account for differences in the abundance (copy number) of the genes within a given sample (thereafter referred to as ‘abundance-related’ gene expression).
Thereby the contribution of the three *Cyanobacteria* to nifH expression within that sample could be assessed.

## Results

### Nitrogenase activity pattern

For all stations the daily cycle of nitrogenase activity (NA) showed a consistent pattern almost independent from the differences in actual photon flux densities on several (4 – 12) consecutive days. Therefore, only the patterns for one day per year (May 31 – June 01, 2006 and June 26 – June 27, 2007) are shown (Figure 1 – 4).

NA dynamics for the two stations analyzed in 2006 have been described in detail elsewhere (Severin & Stal, 2008). For Station I-2006 chlorophyll a-normalized ethylene production ranged from 0.4 – 1.6 μmol mg⁻¹ h⁻¹. The trend was increasing and revealed three slight maxima at sunset (~21:30), during the night (~02:00) and at sunrise (~05:00). The daily cycle of NA at Station II differed from that of Station I. NA at Station II increased from 19:00 onwards, resulting in a peak at about midnight, subsequently decreasing again and reaching minimum values at sunrise (~06:00). Total NA ranged from ~0.1 – 2.5 μmol mg⁻¹ h⁻¹.

In 2007 Station I also showed several periods of enhanced NA. Again, increased activity was observed around sunset (~22:00) and in the early morning (~04:00). These nighttime maxima reached activities of 0.14 μmol mg⁻¹ h⁻¹ and were therefore one order of magnitude lower than those recorded in 2006 for the same station. In addition to these maxima, two more peaks were observed: a small peak in the early evening (~18:00) and a large peak during daytime (between 09:00 and 14:00). The daytime maximum reached NA of up to 0.3 μmol mg⁻¹ h⁻¹. Station III-2007 showed lower NA of up to 0.2 μmol mg⁻¹ h⁻¹.

The daily pattern was similar to the one observed at Station I. Increased NA was observed from 08:00 – 12:00, around 18:00, 21:30 and 04:00. NA during the day was higher than NA at night.

### NifH expression

Expression of nifH by five diazotrophs in four stations was monitored during a 24h cycle using the SYBR Green qPCR approach. Furthermore, whole community nifH expression was approximated by using a degenerate primer pair (Figure 1 – 4).

At Station I-2006 cell specific nifH expression levels by *Oscillatoria*-related organisms were approximately 200 times higher than those by the second and third most active groups, unicellular *Cyanobacteria* and *Thiorhodospira*-related organisms (Figure 1). For all three groups, expression was higher at day than at night. *Lyngbya aestuarii* and *Microcoleus* showed little to no cell specific nifH expression. Low cell specific expression levels were also detected with the degenerate primer pair.
At Station II-2006 *nifH* transcripts from the unicellular *Cyanobacteria* reached highest levels. *NifH* expression levels by *Thiorhodospira*-related organisms, *L. aestuarii* and *Microcoleus* were 30%, 22% and 18% of those recorded for the unicellular *Cyanobacteria*, respectively (Figure 2). *L. aestuarii* and *Microcoleus* cell specific *nifH* expression levels at Station II-2006 were approximately three times higher than at Station I-2006 whereas expression by *Thiorhodospira*-related organism was ~⅓ of that recorded for Station I-2006. There was very little to no cell specific *nifH* expression recorded for *Oscillatoria*-related diazotrophs. Again, very low expression levels were found when using the degenerate primer pair.

In 2007 cell specific *nifH* expression levels were in a similar range for both stations. However, there were differences between the two stations with respect to the contribution of the different phylotypes. At Station I-2007 cell specific *nifH* expression by the unicellular *Cyanobacteria* was twice as high as by the gammaproteobacterium and four to almost seven times higher than those recorded for the other *Cyanobacteria* (Figure 3). Cell specific expression levels for the unicellular *Cyanobacteria* and *Microcoleus* were higher at day and therefore coincided with highest NA. For *L. aestuarii* highest levels were recorded at night, whereas *Oscillatoria*- and *Thiorhodospira*-related organisms showed enhanced expression at 08:30 and 18:00. Again, by using the degenerate primer pair, *nifH* expression levels were lower than the sum of expression levels of the investigated diazotrophs.

At Station III-2007 cell specific *nifH* expression by *Oscillatoria*-related organisms and those related to the unicellular *Cyanobacteria* were highest (Figure 4). *Oscillatoria*-related *nifH* expression reached maxima at sunset and shortly before sunrise. Expression levels by unicellular *Cyanobacteria* were also higher at night. All other organisms showed little cell specific *nifH* expression, reaching only 5% - 14% of the expression levels recorded for the unicellular *Cyanobacteria*. Very low *nifH* expression levels were also inferred from the use of the degenerate primers.

At Station III-2007 cell specific *nifH* expression by *Oscillatoria*-related organisms and those related to the unicellular *Cyanobacteria* were highest (Figure 4). *Oscillatoria*-related *nifH* expression reached maxima at sunset and shortly before sunrise. Expression levels by unicellular *Cyanobacteria* were also higher at night. All other organisms showed little cell specific *nifH* expression, reaching only 5% - 14% of the expression levels recorded for the unicellular *Cyanobacteria*. Very low *nifH* expression levels were also inferred from the use of the degenerate primers.
Figure 1. Cell specific nifH expression at Station I-2006 determined by quantitative RT PCR and nitrogenase activity measured by acetylene reduction assay; A: *Lyngbya aestuarii*; B: *Oscillatoria*-related filamentous cyanobacterium (note different scale for nifH expression); C: unicellular cyanobacterium; D: *Thiorhodospira*-related gammaproteobacterium; E: *Microcoleus chthonoplastes*; F: PolF/PolR primer pair.
Figure 2. Cell specific \textit{nifH} expression at Station II-2006 determined by quantitative RT PCR and nitrogenase activity measured by acetylene reduction assay; A: Lyngbya aestuarii; B: Oscillatoria-related filamentous cyanobacterium; C: unicellular cyanobacterium; D: Thiorhodospira-related gammaproteobacterium; E: Microcoleus chthonoplastes; F: PolF/PolR primer pair.
Figure 3. Cell specific \textit{nifH} expression at Station I-2007 determined by quantitative RT PCR and nitrogenase activity measured by acetylene reduction assay; A: \textit{Lyngbya aestuarii}; B: \textit{Oscillatoria}-related filamentous cyanobacterium; C: unicellular cyanobacterium; D: \textit{Thiorhodospira}-related gammaproteobacterium; E: \textit{Microcoleus chthonoplastes}; F: PolF/PolR primer pair
Figure 4. Cell specific nifH expression at Station III-2007 determined by quantitative RT PCR and nitrogenase activity measured by acetylene reduction assay; A: *Lyngbya aestuarii*; B: *Oscillatoria*-related filamentous cyanobacterium; C: unicellular cyanobacterium; D: *Thiorhodospira*-related gammaproteobacterium; E: *Microcoleus chthonoplastes*; F: PolF/PolR primer pair

Since the groups of closely related sequences chosen for the design of the nifH qPCR assays accounted for ~80% of all nifH transcripts, adding up the expression levels of all these groups was done to illustrate their relative contribution to approximated whole
community \textit{nifH} expression (Figure 5). For that purpose, transcript numbers were calculated per ng extracted RNA. The highest value of more than 30000 transcripts per ng extracted RNA was reached at night at Station III-2007 whereas lowest values (less than 8000 transcripts per ng RNA) were found for Station I-2007. At Station I-2006 cell specific \textit{nifH} expression by \textit{Oscillatoria}-related organisms was 200 times higher than \textit{nifH} expression by the other diazotrophs at all times. However, due to low numbers of \textit{nifH} copies, the overall \textit{nifH} expression pattern also revealed the contribution of the other diazotrophs. At Station II-2006 all investigated groups of diazotrophs contributed to overall \textit{nifH} expression. Due to high \textit{nifH} abundances, \textit{Microcoleus} \textit{nifH} expression levels were highest, followed by those found for unicellular \textit{Cyanobacteria}. In 2007 all groups were represented with respect to \textit{nifH} expression. At both stations \textit{nifH} transcripts of the unicellular \textit{Cyanobacteria} played a major role. At Station I-2007 \textit{Thiorhodospira}-related \textit{nifH} transcripts were also abundant whereas \textit{Microcoleus} \textit{nifH} expression appeared to be important at Station III-2007.

\textbf{Figure 5.} Abundance-related \textit{nifH} expression for \textit{Lyngbya aestuarii} (black); \textit{Oscillatoria}-related filamentous cyanobacterium (red); unicellular cyanobacterium (green); \textit{Thiorhodospira}-related gammaproteobacterium (yellow) and \textit{Microcoleus chthonoplastes} (blue); A: Station I-2006; B: Station II-2006; C: Station I-2007; D: Station III-2007
Discussion

The acetylene reduction assay (ARA) was used to investigate the 24h NA pattern at four stations sampled in 2006 and 2007. The NA pattern of the mats sampled in 2006 was assumed to reflect the major diazotrophs present in these mats (Severin & Stal, 2008). The variable daily pattern of NA in Station I was taken as an indication for the acclimation of the active organisms to the changing conditions, to shifts in the active community during a 24h day, or to a combination of both. Higher rates of NA in the morning as well as at sunset might have been favored by an advantageous combination of increasing light intensities and low oxygen concentrations. The same pattern has also been observed in other cyanobacterial mats (Stal et al., 1984; Villbrandt et al., 1990). The nighttime maximum of NA at Station II was typical for diazotrophic mats dominated by filamentous non-heterocystous Cyanobacteria in temperate intertidal areas (Villbrandt et al., 1990; Bebout et al., 1993; Joye & Paerl, 1994; Paerl et al., 1996), tropical habitats (Diaz et al., 1990; Steppe et al., 2001) and hypersaline mats (Omoregie et al., 2004a). In 2007 higher NA was observed during the day. This may hint towards a higher contribution of either heterocystous Cyanobacteria or diazotrophs other than Cyanobacteria or both. In Antarctic as well as tropical benthic microbial communities higher daytime NA was associated with a mixed community of cyanobacterial diazotrophs (Olson et al., 1998; Fernández-Valente et al., 2001; Charpy-Roubaud et al., 2001; Charpy-Roubaud & Larkum, 2005). In all these communities non-heterocystous Cyanobacteria were more abundant than heterocystous forms but non-cyanobacterial diazotrophs were also likely to contribute to NA. As was the case for Station I-2006, the highly dynamic NA patterns in 2007 might have been caused by the acclimation of active organisms to changing conditions, to shifts in the active community during a 24h day, or to a combination of both.

We also assessed nifH expression of five diazotrophs previously found to be present and active at the study site. In the early morning (4:00 and 8:00) the contribution of nifH transcripts of the phylotypes investigated in this study to the clone libraries was below the mentioned 80%. Therefore, other diazotrophs must have contributed to the overall nifH expression and possibly to community N₂ fixation. The results obtained by using quantitative PCR are considered as approximations due to theoretical and practical reasons (Smith & Osborn, 2008).

The mats under investigation differed in location, microscopically observed cyanobacterial community and NA pattern. We therefore investigated which of the diazotrophs represented in nifH transcript clone libraries were responsible for the observed NA pattern. We could not detect any direct relationship between the NA pattern and any of the nifH expression patterns. This is not surprising because expression of a certain gene does not necessarily translate directly into enzyme activity (e.g., Zehr et al., 1993). Due to post-transcriptional modification, expression can be detected without measurable activity. On the other hand, NA can be measured without detecting nifH expression. For instance, Synechococcus nifH expression in a hot spring microbial mat was high in the evening and decreased overnight while NA peaked in the morning (Steunou et al., 2008). In that case NA is not dependent on de novo synthesis of nitrogenase. In 2006 Station I was dominated
by the filamentous cyanobacterium *Lyngbya aestuarii* but there was only very little *nifH* expression measured for this diazotroph. In contrast, *nifH* expression by *Oscillatoria*-related diazotrophs was very high. *Oscillatoria* was considered to be the main pioneer organism in mats growing in the intertidal zones of the North Sea island Mellum (Stal et al., 1985). When grown under alternating light-dark cycles, two NA maxima were observed (Stal & Krumbein, 1987). The smaller of the peaks was detected just after the onset of the dark phase and the larger one as soon as there was light available. This is in agreement with the NA pattern found at Station I-2006. However, there is growing evidence that diazotrophs other than *Cyanobacteria* may be important in microbial mats (e.g., Omoregie et al., 2004b). Among them, proteobacterial *nifH* sequences were retrieved frequently (e.g., Zehr et al., 1995; Steppe et al., 2001). The non-cyanobacterial target of the primer pair used in this study was a gammaproteobacterial diazotroph most closely related to *Thiorhodospira*. This proteobacterium belongs to the family *Ectothiorhodospiraceae* which, together with the *Chromatiaceae*, constitute the purple sulfur bacteria. Many of these organisms possess *nifH* (Tourova et al., 2007). Being anoxygenic phototrophs, one would expect optimal growth conditions in anoxic but still illuminated parts in the mat. NA at daytime seems plausible under such conditions and would agree with the *nifH* expression pattern. However, when cell abundances were taken into account, *Thiorhodospira*-related organisms did not seem to be important in whole mat *nifH* expression. It is unknown whether similar expression levels result in similar activities or not. Owing to the fact that different organisms may be characterized by different post-transcriptional and post-translational regulatory mechanisms, their contribution to whole community NA may still be different from what we might expect based on gene expression levels. Nevertheless, oxygenic phototrophs seem to be the major contributors to NA at Station I-2006.

Station II-2006 was structurally dominated by *Lyngbya aestuarii*. Since the NA pattern recorded for this mat was in agreement with those known from *Lyngbya* cultures and from other *Lyngbya*-dominated cyanobacterial mats (Omoregie et al., 2004a), this organism was suspected to be responsible for the bulk of the observed NA. However, the *nifH* expression levels observed for this mat indicate that the dominant organism, even when it is a known diazotroph, is not necessarily the most active one. The conditions at Station II-2006 might not have supported diazotrophic growth of *Lyngbya*, or, alternatively, the majority of this cyanobacterium in this mat could have been not diazotrophic. Similar observations have been made for a *Lyngbya* dominated mat from Guerrero Negro, Mexico (Omoregie et al., 2004b). There, several *nifH* transcripts belonging to *nifH* cluster 1 (conventional nitrogenases from *Cyanobacteria*, *Proteobacteria* and *Firmicutes*) were retrieved but no *nifH* transcripts belonging to *Lyngbya* were found. However, since the number of sequences that were analyzed was low, less active diazotrophs might have been missed out.

Similar to 2006, Station I in 2007 was characterized by a rather mixed cyanobacterial composition. The NA pattern was highly dynamic but highest rates were, in contrast to 2006, recorded during daytime. It was therefore not surprising to find other organisms being most active with regard to *nifH* expression. These differences could originate from the slightly different location of the mat along the dune area but are more likely to be
caused by the morphological changes of the whole beach area over the years. Climatic differences between the years, especially with respect to temperature, light and water availability during the onset of the mat development but also at the time of the investigation, may have influenced the diazotrophic community. The spring preceding the sampling in 2006 was sunny but wet and allowed strong mat development. In 2007 spring was dry and might have had negative effects on biomass build-up as well as activity of the mat organisms.

At Station III-2007 most of the nifH expression associated with the unicellular *Cyanobacteria* was detected during the night. This was in contrast to the other stations but the reason for this exception is unclear. *M. chthonoplastes*, which has been characterized as the structurally most important filamentous cyanobacterium at Station III-2007, showed very low cell specific nifH expression levels. The repeated failure to detect NA in pure cultures of *M. chthonoplastes* led to the general assumption that this organism lacked the genes for dinitrogen fixation and, hence, lacked the ability to fix $N_2$ (de Wit et al., 2005; Steppe & Paerl, 2002; Zehr et al., 1995). However, Bolhuis et al. (2010) discovered that the genome of *M. chthonoplastes* PCC7420 possesses a complete nif-gene cluster and reported that several other strains of this species contained nifH, nifD and nifK. We therefore expected to find some nifH expression in this mat but previous analyses also showed low cell specific expression rates in the same mat type (Bolhuis et al., 2010). However, when taking the measured nifH copy number into account, *M. chthonoplastes* appeared to be the most important contributor to overall nifH expression and, hence, it might not only be the structurally dominant organisms but also one of the key diazotrophs in this mat type.

The attempt to approximate whole community nifH expression by using the degenerate nifH primers PolF/PolR (Poly et al., 2001) proved to be inappropriate for the samples investigated in this study. Since its publication, the PolF/PolR primer pair has been used in a number of soil studies, including SYBR Green based qPCR analyses (e.g., Park et al., 2007; Bowers et al., 2008; Coelho et al., 2008). In several studies covering various habitats the authors detected either low numbers of cyanobacterial nifH sequences (Bauer et al., 2008; Terakado-Tonooka et al., 2008) or no cyanobacterial nifH sequences at all (Coelho et al., 2009; Lau & Pointing, 2009). An evaluation of two widely used primer pairs for universal nifH targeting (the primers designed by Zehr & McReynolds, 1989 and those introduced by Poly et al., 2001) showed that the PolF/PolR primer pair skewed template-to-product ratios and was a selectivity for proteobacterial nifH sequences (Diallo et al., 2008). Our test on nucleic acids extracted from the study site demonstrated that it was possible to retrieve proteobacterial as well as cyanobacterial nifH sequences with this primer pair. However, nifH sequences belonging to the unicellular *Cyanobacteria* which have been found to be abundant at all our stations were not detected. Together with a presumed lower specificity for the other nifH sequences, this could explain why the nifH expression levels determined by using this primer pair were lower than the sum of the expression levels of the five groups of diazotrophs investigated in this study. It must therefore be concluded that the use of a degenerated primer pair to approximate whole community gene expression is not always possible and has to be validated carefully from case to case,
e.g., by comparison of the primer pair target range to clone libraries constructed with valid general primers.

In this study we used, to our knowledge for the first time, *nifH* qPCR assays based on groups of closely related sequences to follow *nifH* expression of a wider range of presumed key diazotrophs over a diel cycle. *NifH* expression patterns by these five groups of diazotrophs as well as their contribution to whole community *nifH* expression varied greatly between the different mat types and in the two years we investigated. Diel NA pattern also differed between the stations and years but did not show any relation to *nifH* expression patterns. We showed that the structurally dominant *Lyngbya aestuarii* was not the main contributors to *nifH* expression in these mats. However, based on the discrepancy between the daily patterns of *nifH* expression and NA, it is also possible that diazotrophs other than those identified as the dominant ones based on previously constructed clone libraries contributed significantly to whole community NA.

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**Supplementary Information**

*Table 1*: Contribution of bacterial phyla to clone libraries of *nifH* and its transcripts from Station I and II in 2006. Additionally, the phylotypes used for Q-PCR primer design are listed.
Supplementary Table 1. Contribution of bacterial phyla to clone libraries of \( nifH \) and its transcripts from Station I and II in 2006. Additionally, the phylotypes used for Q-PCR primer design are listed.

<table>
<thead>
<tr>
<th></th>
<th>Station I ( nifH )</th>
<th></th>
<th>Station II ( nifH )</th>
<th></th>
<th>Station I ( nifH ) transcript</th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>no of clones</td>
<td>sequence similarity</td>
<td>contribution (%)</td>
<td>no of clones</td>
<td>sequence similarity</td>
<td>contribution (%)</td>
</tr>
<tr>
<td>presumed <strong>Cyanobacteria</strong></td>
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<td>100</td>
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<td>Lyngbya aestuarii</td>
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<td>97-99%</td>
<td>45.1</td>
<td>74</td>
<td>97-99%</td>
<td>40.0</td>
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<tr>
<td>Oscillatoria sp.</td>
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<td>88%</td>
<td>3.1</td>
<td>1</td>
<td>96%</td>
<td>0.5</td>
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<tr>
<td>Gloeothece sp.</td>
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<td>83%</td>
<td>0.6</td>
<td>23</td>
<td>83-84%</td>
<td>12.4</td>
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<tr>
<td>presumed <strong>Proteobacteria</strong></td>
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<td>26.5</td>
<td></td>
<td>49</td>
<td>26.5</td>
<td></td>
</tr>
<tr>
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<td>0.0</td>
<td></td>
<td>0</td>
<td>0.0</td>
<td></td>
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<tr>
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<td></td>
<td>1</td>
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<tr>
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<tr>
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<td></td>
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<td>17.3</td>
<td>3</td>
<td>84-89%</td>
<td>1.6</td>
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<tr>
<td><strong>Others</strong></td>
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<td>23.5</td>
<td></td>
<td>36</td>
<td>19.5</td>
<td></td>
</tr>
</tbody>
</table>

*none of the groups above or unidentified