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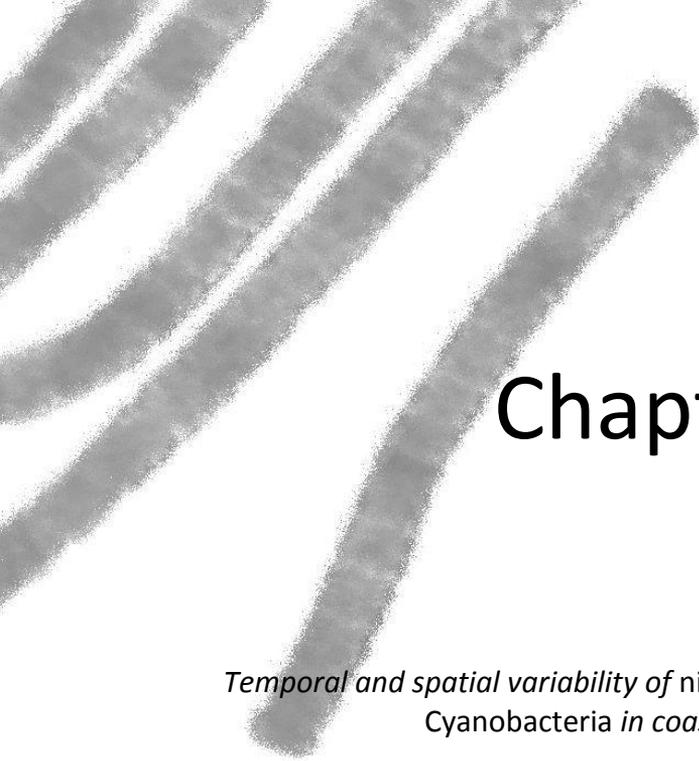
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Chapter 5

*Temporal and spatial variability of nifH expression in three filamentous
Cyanobacteria in coastal microbial mats*

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Abstract

Cyanobacteria are often the most conspicuous structural part of microbial mats. They are also the only oxygenic phototrophs capable of N₂ fixation (diazotrophy). This represents an important advantage for the persistence in the often N-depleted marine intertidal microbial mats. In this study the daily pattern of expression of the structural gene for dinitrogenase reductase, *nifH*, and of the 16S rRNA gene of three benthic filamentous diazotrophic *Cyanobacteria* (*Lyngbya* sp., *Nodularia* sp. and *Anabaena* sp.) was measured by using quantitative RT PCR. The gene expression patterns were compared and related to the daily pattern of nitrogenase activity. Microscopic observations revealed that the non-heterocystous *Lyngbya* sp. was the major cyanobacterial morphotype in all the microbial mats studied. When normalized to copy number, *nifH* as well as 16S rRNA gene expression by *Lyngbya* sp. was higher than by the heterocystous *Cyanobacteria* in all but one mat type. Related to the total amount of extracted RNA, *Lyngbya* sp. dominated 16S rRNA gene expression as well but was outcompeted by the heterocystous *Cyanobacteria* with respect to total *nifH* expression. This was due to the low DNA copy number of *Lyngbya* sp. *nifH*. The results revealed different cell-specific expression levels and varying contribution to the overall *nifH* and 16S rRNA gene expression by the three *Cyanobacteria* over the course of a daily cycle as well as in the different mat types. With respect to the overall *nifH* expression, the structurally dominant diazotroph was not the most active. Moreover, *nifH* expression pattern did not follow nitrogenase activity.

Introduction

The marine environment is often depleted of combined nitrogen and this nutrient might then limit primary productivity (Paerl, 1990). Such conditions provide diazotrophic (N_2 -fixing) microorganisms with an important ecological advantage.

The ability to fix atmospheric dinitrogen (N_2) is widespread among *Bacteria* and *Archaea*. *Cyanobacteria* received special attention because they are the only oxygenic phototrophs capable of N_2 fixation and are therefore carbon and nitrogen autotrophs. This property made *Cyanobacteria* the most conspicuous structural part of microbial mats because it enables them to meet the high energy- and electron demand of N_2 fixation (16 ATP and 8 low-potential electrons for the reduction of one molecule N_2). However, their aerobic and oxygenic phototrophic mode of life seems paradoxical considering the extremely oxygen-sensitive nitrogenase (Fay, 1992). *Cyanobacteria* have evolved a variety of strategies to comply with this incompatibility (for reviews see, e.g., Bergman et al., 1997; Berman-Frank et al., 2003; Gallon, 1992). Among the most ingenious adaptations is the differentiation of a special cell type, the heterocyst, which is devoted to the fixation of N_2 . Heterocysts have lost photosystem II and hence the capacity of oxygenic photosynthesis (Adams, 2000). Moreover, the cell envelope of heterocysts represents a gas diffusion barrier and any O_2 that enters the cell is scavenged by an efficient and high affinity respiratory system (Walsby, 2007). Hence, heterocysts provide an anaerobic environment for nitrogenase and are a means to spatially separate oxygen-evolving photosynthesis from oxygen-sensitive N_2 fixation. Most non-heterocystous diazotrophic *Cyanobacteria* fix N_2 exclusively under anaerobic conditions (avoidance). However, a few types have evolved strategies that enable them to carry out aerobic N_2 fixation (Bergman et al., 1997). Analogous to the heterocystous *Cyanobacteria*, some species separate N_2 fixation temporally from photosynthesis by confining the former to the night. All these strategies are reflected by the daily pattern of nitrogenase activity (NA) in an organism in laboratory culture but equally well in complex natural communities such as microbial mats.

Microbial mats are examples of versatile benthic communities of microorganisms, usually dominated by phototrophic bacteria (e.g., Krumbein et al., 1977; Jørgensen et al., 1983). The barren intertidal sand flats are often colonized by cyanobacterial mats. *Cyanobacteria* are predestined for the task of colonization because of their low nutritional requirements and the capability of photosynthesis, N_2 fixation, anaerobic metabolisms and the production of extracellular polymeric substances (EPS) (Stal, 2001). Once the sediment is enriched with organic matter and fixed nitrogen and the matrix of EPS has stabilized and consolidated the sediment, higher organisms such as plants may settle and a marsh develops.

Although it is attractive to assign the diazotrophy of microbial mats to *Cyanobacteria* it has become clear that several other groups of *Bacteria* (anoxygenic phototrophic and chemotrophic bacteria) in the mat are also capable of fixing N_2 (e.g., Zehr et al., 1995; Olson et al., 1999). And, hence, a dispute started as to the extent *Bacteria* other than *Cyanobacteria* are responsible for the observed N_2 fixation in microbial mats. In this model, N_2 fixation is the result of the joint activities of *Cyanobacteria* and chemotrophic bacteria in which the former provide substrate and growth factors to the latter. In return

the chemotrophic bacteria provide the *Cyanobacteria* with fixed nitrogen and CO₂ (Steppe et al., 1996). No consensus on that matter has been reached yet.

Nitrogenase, the enzyme complex catalyzing the reduction of atmospheric N₂ to ammonia, is found in the Domains *Bacteria* and *Archaea* but not in the *Eukarya*, except in symbiotic associations with *Bacteria* (Zehr et al., 2003). The complex of highly conserved proteins consists of a Fe-protein (dinitrogenase reductase), encoded by *nifH*, and a FeMo-protein (dinitrogenase), encoded by *nifDK*, a structure which is evolutionary conserved in diazotrophs (Postgate, 1982). With the development of *nifH* primers (Zehr & McReynolds, 1989), this gene 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). This allowed for the detection and characterization of *nifH* genes from the environment and yielded a deeper insight into the diazotrophic members of complex microbial communities (e.g., Kirshtein et al., 1991; Church et al., 2005a; Yannarell et al., 2006).

Despite this progress, the link between diazotrophic genotypes detected in a sample and the actual diazotrophic activity remained unclear. Discrepancies between the presence of certain diazotrophs and the recorded pattern of NA gave rise to the question whether or not and under which circumstances these diazotrophs contribute to whole community N₂ fixation. Because detection of *nifH* alone is not necessarily indicative for diazotrophic activity, the transcripts were used to (quantitatively) trace presumed diazotrophic activity patterns. Church et al. (2005b) followed the temporal pattern of *nifH* expression in the subtropical North Atlantic Ocean by reverse-transcription quantitative PCR. Five of the six phylotypes that were considered in this investigation clustered with the *Cyanobacteria* and revealed a pronounced daily periodicity whereas the one phylotype that clustered with the *Gammaproteobacteria* did not exhibit a clear pattern of *nifH* expression. However, it remained unresolved whether gene expression translates into actual activity. It is known for a number of diazotrophs that nitrogenase is post-transcriptionally regulated (e.g., Ludden & Roberts, 1989; Ohki et al., 1991; Du & Gallon, 1993; Zehr et al., 1993). When expression patterns were compared to actual NA measurements, Zehr et al. (2007) found *nifH* expression levels to be generally correlated with cell-specific N₂ fixation rates whereas in another study, *Synechococcus nifH* expression was high in the evening and decreased overnight while NA peaked in the morning (Steunou et al., 2008).

The aim of this study was to follow the 16S rRNA gene and *nifH* expression patterns of one non-heterocystous and two heterocystous *Cyanobacteria* in three types of microbial mats *in situ*. The *Cyanobacteria* were previously isolated from coastal microbial mats exhibiting distinctly different daily NA patterns (Severin & Stal, 2008). Based on the *nifH* and 16S rRNA gene sequences of these cultures, a multiplex quantitative RT PCR assay using TaqMan chemistry was developed. The *nifH* expression patterns were then compared to actual NA.

Materials and Methods

Sampling

The study site is 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 (Table 1; for a comparison see also Dijkman et al., 2010).

Table 1. Description of the microbial mat types sampled for this study

	Location	Description	Dominant cyanobacterial species
I	high intertidal (close to the dunes)	medium coherent mat structure, clear stratification of the mat, green and purple layer	<i>Nostoc</i> sp., <i>Calothrix</i> sp., <i>Anabaena</i> sp., <i>Nodularia</i> sp., <i>Lyngbya</i> sp.
II	low intertidal (low water mark)	no coherent mat structure, fine sand, no clear stratification, only green coloring visible	<i>Lyngbya</i> sp.
III	intermediate	coherent mat structure, mostly organic, little sediment, clear stratification of the mat, green and sometimes purple layer	<i>Microcoleus chthonoplastes</i> , <i>Lyngbya</i> sp.

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 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 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, *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 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. The samples were immediately taken to the lab and nitrogenase activity measurements started within 30 min after sampling. Each light response curve lasted for less than 1h and a fresh mat sample was used for each time point. After finishing the NA measurements, each sample was immediately frozen in liquid nitrogen and subsequently stored at -80°C for later chlorophyll determination. Samples for molecular analyses were collected 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 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 on-line 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Severin & Stal, 2008). Nitrogenase activity was normalized to the phototrophic biomass (mg chlorophyll *a*). Light response curves were fitted using the rectangular hyperbola model (Staal et al., 2002). Fitting the measured light-response curve to the rectangular hyperbola model was done with the SOLVER function of Microsoft Excel by nonlinear least-squares fitting.

Natural photon flux density 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 was calculated from the fitted parameters obtained from the hourly measured light response curves and the recorded natural PFD according to equation (1).

$$N = N_m \left(\frac{\alpha I}{N_m + \alpha I} \right) + N_d \quad (1)$$

where N_m is the nitrogenase activity at saturating irradiances minus N_d , N_d the nitrogenase activity measured in the dark, α the light affinity coefficient for nitrogenase activity and I the natural irradiance (PFD).

Nucleic acid extraction

DNA and RNA were extracted from the Stations I and II in 2006 and the 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 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 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 a DNase treatment (Deoxyribonuclease I, Invitrogen Corporation, Carlsbad, CA 92008, USA) as stated in the manufacturer'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 protocol. The resulting cDNA was immediately used for amplification or stored at -20°C .

Quantitative PCR

Based on the *nifH* and 16S rRNA gene sequence information of the 3 filamentous diazotrophic *Cyanobacteria* previously isolated from the site, primers and TaqMan probes were designed by TIB MOLBIOL (TIB MOLBIOL GmbH, 12103 Berlin, Germany). The isolates have previously been identified as *Lyngbya* sp. (CCY 0005), *Nodularia* sp. (CCY 0014) and *Anabaena* sp. (CCY 0015), the latter two being representatives of heterocystous *Cyanobacteria*. Primers and probes are listed in Table 2. The fluorescent reporter for the *nifH* TaqMan probes was 5'-labelled with FAM (6-carboxy-fluorescein) and 16S rRNA gene probes were 5'-labelled with Quasar 670 (indocarbocyanine). For both genes the probes were 3'-labelled with the BHQ-quenching dye. All probes were synthesized by Biosearch Technologies (Biosearch Technologies, Inc., Novato, CA 94949-5750, USA). They were checked against the NCBI database (Basic Local Alignment Search Tool, National Center for Biotechnology Information, Bethesda, MD 20894, USA) and tested for possible cross-amplification. All 16S rRNA gene assays as well as the *Lyngbya*- and *Nodularia-nifH* assays were specific at various target and non-target-concentrations and under the final cycling conditions. Due to the high similarity of the *nifH* sequences of the two heterocystous *Cyanobacteria*, the *Anabaena*-assay also appeared to amplify *Nodularia-nifH* sequences to a small extent. At non-target concentrations at least 10 times higher than the target concentration, up to 9.5 % of the detected fluorescence was caused by amplification of the non-target *nifH*. If such a high ratio of non-target (*Nodularia*) *nifH* to target (*Anabaena*) *nifH* was encountered in the samples, the results were corrected accordingly. Furthermore, amplification products from environmental samples using all the *nifH* and

16S rRNA gene primer combinations were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen Corporation, Carlsbad, CA 92008, USA) following the manufacturer's instructions. At least 48 clones per assay were sequenced using the BigDye Terminator chemistry (BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, Inc., Foster City, CA 94404, US) and confirmed the specificity of the assays for the samples used in this study. Primer concentrations were optimized for each dual assay separately and ranged from 300 to 900 nM to ensure highest possible efficiencies for both reactions (Table 2).

All quantitative PCR (qPCR) reactions were run on a Corbett Rotor-Gene 6000™ (Corbett Life Science, Sydney, NSW 2137, Australia). Environmental DNA as well as the cDNA reverse-transcribed from environmental RNA was used as a template. Cycling conditions were as follows: one cycle at 95°C for 15 min, 40 cycles 95°C for 10 sec and 55°C for 15 sec, and a final extension at 72°C for 15 sec. Environmental samples were run in triplicate and each run included two independently diluted series of linearized plasmids (containing the target *nifH* and 16S rRNA gene insert) as standard curves as well as non-template and non-target controls (linearized plasmids containing non-target *nifH* or 16S rRNA gene inserts). All 15 µl reactions contained 7.5 µl of the reaction mix (containing the reaction buffer as well as the DNA Polymerase and dNTPs, Absolute™ QPCR Mix, Thermo Fisher Scientific, Rockford, IL 61101, USA), forward and reverse primers (300, 333 or 900 nM) and 333 nM of the fluorogenic probe as well as 1 µl of template. *NifH* and 16S rRNA gene copy numbers for the plasmid standard curves ranged from 2.2 – 2.2×10^9 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 standard curves used in this study had an r^2 of at least 0.97. Reaction efficiencies for single reactions were between 95 and 100% but in the dual assay carried out for the final analyses, efficiencies dropped to ~ 80%. We therefore conclude that the reactions were slightly inhibited but stress that all the efficiencies were similar for all reactions and are therefore valid to for the comparison of gene expression levels. Expression levels for the individual strains were calculated as number of transcripts per DNA copy number (thereafter referred to as 'cell-specific' gene expression). In the case of multiple (but non-identical) *nifH* copies per cell, the designed TaqMan assay is specific and discriminates against any other *nifH* copy. In case of multiple (identical) genomes per cell, the term refers to the genome specific *nifH* expression. To also account for differences in the abundance (copy number) of the genes within a given sample, we calculated *nifH* expression per ng of extracted RNA (thereafter referred to as 'abundance-normalized' gene expression). In this way, the contribution of each of the three *Cyanobacteria* to gene expression within a sample was compared.

Table 2. TaqMan primer and probe sequences and final concentrations as used in the quantitative (RT) PCR

		final concentration (nM)			
		I 2006	II 2006	I 2007	III 2007
<i>Lynbgya</i> sp.					
16S forward	5'-CGG GAG CTT CGG CTC TAG T-3'	333	300	300	300
16S reverse	5'-GCT CAT CCG GGA TTA GCA GAA-3'	333	300	300	300
16S probe	5'-CTC TGT TGT CCC CGA CCT GAA GG-3'				
<i>nifH</i> forward	5'-AAG CTG ACT CTA CCC CTT TAA TCT T-3'	333	900	900	900
<i>nifH</i> reverse	5'-GCT TTC AAT ACT TCG TCT AGT TCT ACG-3'	333	900	900	900
<i>nifH</i> probe	5'-TAC TGT ACT TCA CGT TGC TGC TGA ACG C-3'				
<i>Nodularia</i> sp.					
		I 2006	II 2006	I 2007	III 2007
16S forward	5'-ACC CGA GCC GTA CCG TAG-3'	300	333	300	333
16S reverse	5'-GCA CTC TCT CCT TTC GGA AAG A-3'	300	333	300	333
16S probe	5'-TCA AGT CTT GGT AAG GTT CTT CGC GTT G-3'				
<i>nifH</i> forward	5'-GAT GCT ACA CAG TAA AGC TCA AAC CA-3'	900	333	900	333
<i>nifH</i> reverse	5'-CGG TCA GCA TTA CTT CTT CAA GTT-3'	900	333	900	333
<i>nifH</i> probe	5'-TCT ACA GCA CCA CGT TCA TCA GCC-3'				
<i>Anabaena</i> sp.					
		I 2006	II 2006	I 2007	III 2007
16S forward	5'-GAT GGA TAC TAG GCG TGG CTT-3'	333	900	300	333
16S reverse	5'-AAG GCA CTC TCT CCT TTC AAA GAG A-3'	333	900	300	333
16S probe	5'-AAC GCG TTA GCT CCG GCA CG-3'				
<i>nifH</i> forward	5'-GAT GCT ACA CAG TAA GGC TCA AAC AA-3'	333	300	900	333
<i>nifH</i> reverse	5'-CGG TCA GCA TTA CTT CTT CGA TTT-3'	333	300	900	333
<i>nifH</i> probe	5'-TCT ACT GCA CCA CGT TCA GCA GCC-3'				

Results

Nitrogenase activity pattern

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 $\mu\text{mol mg}^{-1} \text{h}^{-1}$ (Figure 1-3). The trend was increasing and revealed three slight maxima at sunset (~ 21:30), during the night (~ 2:00) and at sunrise (~ 5:00). The daily cycle of NA at Station II differed from that of Station I (Figure 1-3). 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 (~ 6:00). Total NA ranged from ~ 0.1 – 2.5 $\mu\text{mol mg}^{-1} \text{h}^{-1}$.

In 2007 Station I also showed several periods of enhanced NA (Figure 1-3). Again, increased activity was observed around sunset (~ 22:00) and in the early morning (~ 4:00). These nighttime-maxima reached activities of 0.14 $\mu\text{mol mg}^{-1} \text{h}^{-1}$ 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 9:00 and 14:00). The daytime maximum reached NA of up to 0.3 $\mu\text{mol mg}^{-1} \text{h}^{-1}$. Station III-2007 showed lower NA of up to 0.2 $\mu\text{mol mg}^{-1} \text{h}^{-1}$ (Figure 1-3). The daily pattern was similar to the one observed at Station I. Increased NA was observed from 8:00 – 12:00, around 18:00, 21:30 and 4:00. NA during the day was higher than NA at night.

Gene expression

Expression of *nifH* as well as the 16S rRNA gene within the same genome was monitored for three diazotrophic *Cyanobacteria* during the 24h cycles using the TaqMan qPCR approach (Figure 1-3).

NifH expression levels for the non-heterocystous *Lyngbya* sp. were low at Station I-2006 and approximately four orders of magnitude higher at Station II-2006 (Figure 1) where maximum expression levels of ~ 560 *nifH* transcripts copy^{-1} were recorded at ~ 4:30. No clear pattern was seen at Station I. In 2007 expression levels reached maxima of 2.4 and 0.2 *nifH* transcripts copy^{-1} at Station I and III, respectively (Figure 1). At Station I-2007 higher expression levels were recorded during the day whereas higher values were seen at night at Station III-2007. 16S rRNA gene expression showed no clear pattern at either station in 2006 (not shown). As for *nifH*, a clear difference was seen for the levels of 16S rRNA expression; being six orders of magnitude higher at Station II-2006 than at Station I-2006. At Station I-2006 the highest expression was recorded in the afternoon and reached ~5.5 transcripts copy^{-1} whereas expression levels for Station II-2006 ranged between 2×10^6 and 20×10^6 transcripts copy^{-1} . In 2007 maximum 16S rRNA gene expression levels were similar for Station I and III, reaching ~1100 and 1200 transcripts copy^{-1} , respectively. As was the case for *nifH* expression, the highest values were recorded at daytime at Station I and at nighttime at Station III.

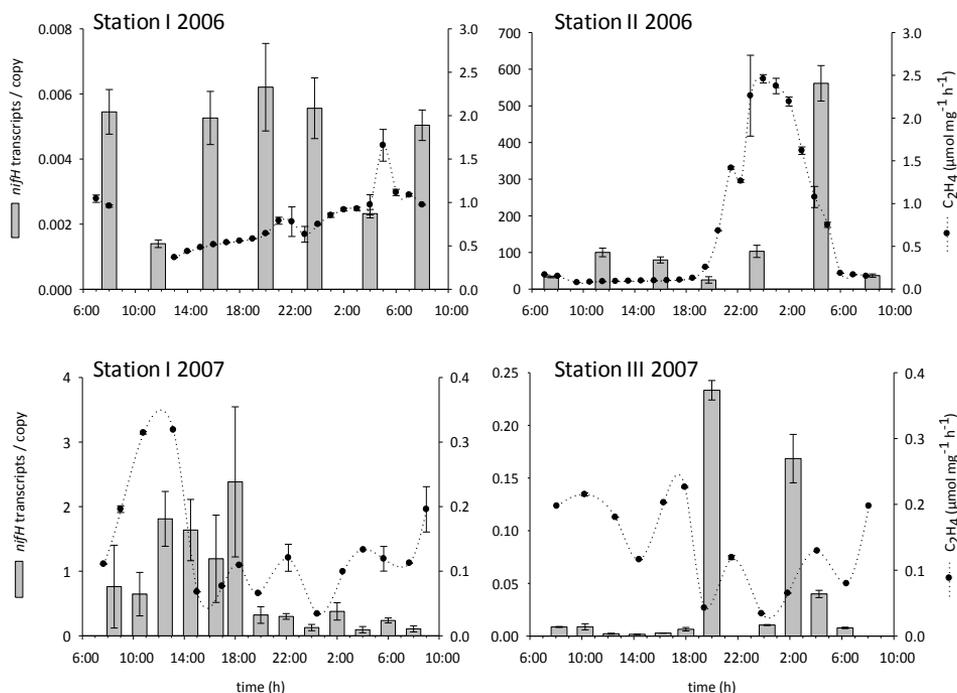


Figure 1. Cell-specific *nifH* expression determined by quantitative RT PCR using TaqMan primers and probe for *Lyngbya* sp. (Table 2) and nitrogenase activity measured by acetylene reduction assay and normalized to chlorophyll *a*; note differences in scales between the panels

For the heterocystous *Nodularia* sp., *nifH* expression levels were higher in 2006 than in 2007 (Figure 2). In both years *nifH* expression was highest at Station I. In 2006 maximum values at Station I were recorded during daytime and reached 2.8 transcripts copy⁻¹ (Figure 2). At Station II-2006 the highest expression level was recorded at 4:30 and reached 0.4 transcripts copy⁻¹. At Station I-2007 highest *nifH* expression was observed at daytime and reached ~0.2 transcripts copy⁻¹. There was no clear expression pattern observed for Station III-2007. Maximum expression levels reached 0.04 *nifH* transcripts copy⁻¹. 16S rRNA gene expression showed similar patterns as seen for *nifH* (not shown). 16S rRNA gene expression levels reached 935 and 42 transcripts copy⁻¹ at Station I and II-2006, respectively, and 32 and 105 transcripts copy⁻¹ at Station I and III-2007, respectively.

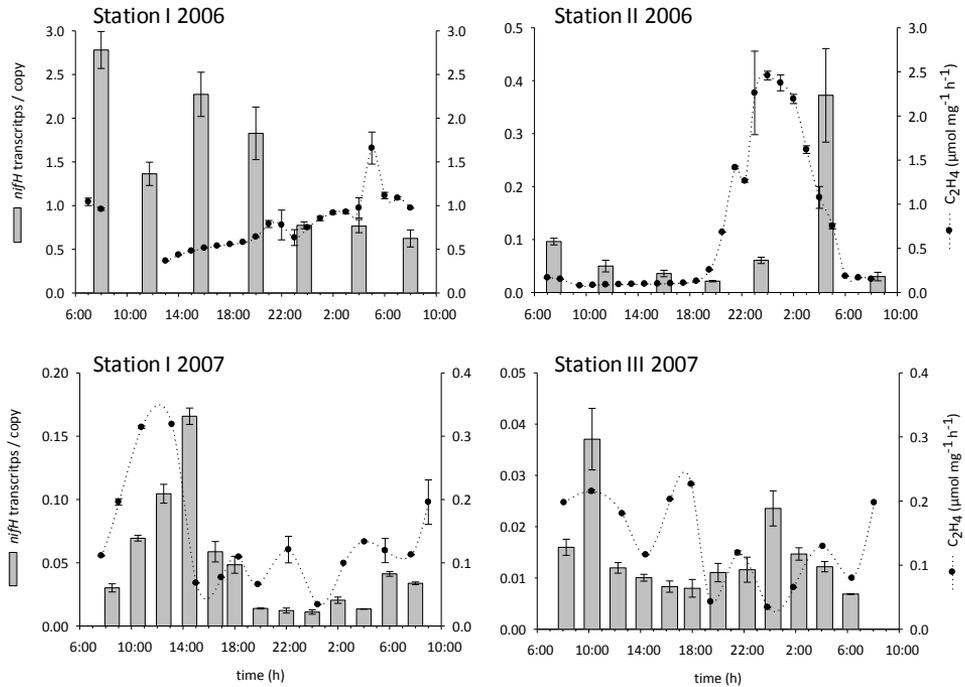


Figure 2. Cell-specific *nifH* expression determined by quantitative RT PCR using TaqMan primers and probe for *Nodularia* sp. (Table 2) and nitrogenase activity measured by acetylene reduction assay and normalized to chlorophyll *a*; note differences in scales between the panels

For the heterocystous *Anabaena* sp., *nifH* expression pattern and levels at Station I-2006 were similar to those recorded for *Nodularia* sp., whereas very little to no expression was detected at Station II-2006 (Figure 3). In 2007 expression levels were lower and ranged between 0.001 and 0.006 *nifH* transcripts copy⁻¹ and 0.004 and 0.03 *nifH* transcripts copy⁻¹ at Station I and III, respectively. No clear *nifH* expression pattern was observed in 2007. Expression levels of the *Anabaena* 16S rRNA gene varied between stations and years (not shown). In 2006 expression was higher at day- and nighttime for the Stations I and II, respectively. Furthermore, expression was three orders of magnitude higher at Station I than at Station II. In 2007 expression levels of the *Anabaena* 16S rRNA gene were in the same order of magnitude, reaching maxima of 90 and 45 transcripts copy⁻¹ at Station I and III, respectively. At Station I the maximum was reached at daytime whereas no such pattern was observed at Station III.

NifH expression in filamentous *Cyanobacteria*

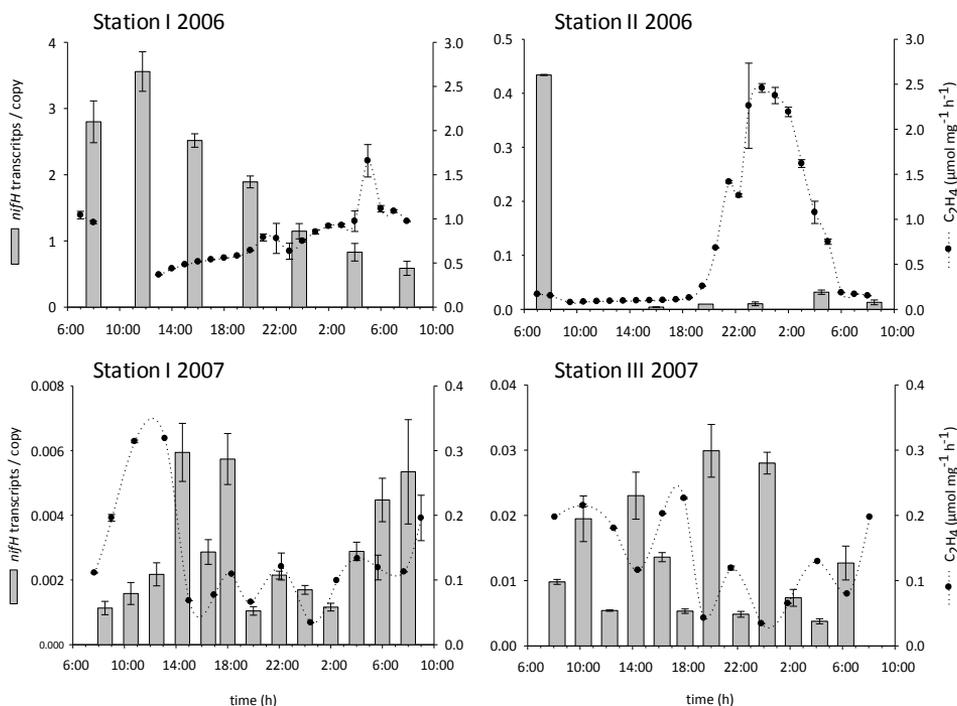


Figure 3. Cell-specific *nifH* expression determined by quantitative RT PCR using TaqMan primers and probe for *Anabaena* sp. (Table 2) and nitrogenase activity measured by acetylene reduction assay and normalized to chlorophyll *a*; note differences in scales between the panels

Summing up gene expression of the three diazotrophic *Cyanobacteria* showed their individual contributions (Figure 4). When *nifH* expression was normalized to the amount of extracted RNA, expression levels were highest at Station I in 2006 (Figure 4), one order of magnitude lower at Station II-2006 and at an intermediate level at Station I and III in 2007. 16S rRNA gene expression reached highest values at Station II-2006. *Nodularia* sp. contributed most to *nifH* and 16S rRNA gene expression at Station I-2006. Relative contributions of the other *Cyanobacteria* to 16S rRNA gene expression were higher than to *nifH* expression. At Station II-2006 *Nodularia* sp. and *Lyngbya* sp. dominated *nifH* expression while *Lyngbya* sp. was responsible for the majority of the 16S rRNA gene expression. At both stations in 2007 *nifH* expression by *Nodularia* sp. was dominant but some contribution was seen for *Anabaena* sp. as well. In contrast, 16S rRNA gene expression was dominated by *Anabaena* sp. and *Lyngbya* sp. at Station I and by *Lyngbya* sp. at Station III.

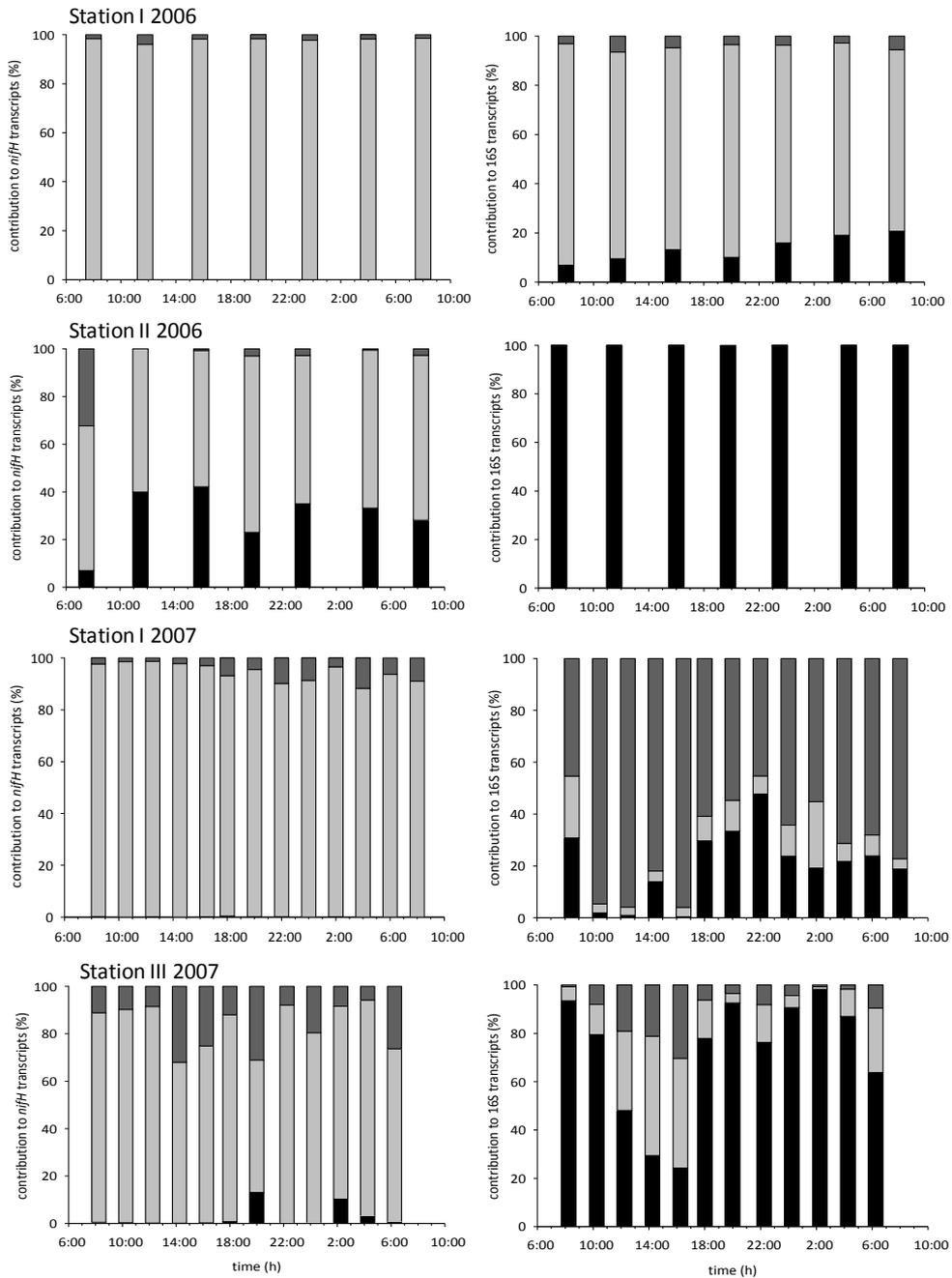


Figure 4. Percentage contribution to abundance-normalized *nifH* (left panel) and 16S rRNA gene expression (right panel) for *Lyngbya* sp. (black), *Nodularia* sp. (light grey) and *Anabaena* sp. (dark grey)

Discussion

The acetylene reduction assay (ARA) was used to investigate the 24h nitrogenase activity (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 adaptation of the active organisms either to the changing environmental conditions, or to shifts in the active community during a 24h day, or to a combination of both. The nighttime maximum of NA at Station II was typical for diazotrophic mats dominated by filamentous non-heterocystous *Cyanobacteria* (Villbrandt et al., 1990; Bebout et al., 1993; Omoregie et al., 2004b). The higher daytime NA that was observed in the samples of 2007 may hint towards a larger contribution of either heterocystous *Cyanobacteria* or diazotrophs other than *Cyanobacteria* or both. As was the case for Station I-2006, the highly dynamic NA pattern in 2007 might have been caused by the adaptation of active organisms to changing conditions, to shifts in the active community during a 24h day, or to a combination of both.

Cell-specific gene expression

One of the organisms investigated in this study, the non-heterocystous *Lyngbya* sp., is a structurally important cyanobacterium in a variety of different microbial mats (e.g., Omoregie et al., 2004a, b). Microscopic observations revealed the presence of *Lyngbya* sp. in all the investigated mats and *nifH* expression confirmed transcription of one of the structural genes coding for nitrogenase. Although this cyanobacterium was abundant and exhibited high cell-specific *nifH* expression levels at Station II-2006, the pattern of *Lyngbya* sp. *nifH* expression did not correspond to the recorded NA pattern. This may not be surprising since gene expression does not necessarily translate directly into an active enzyme (e.g., Ohki et al., 1991; Du & Gallon, 1993; Zehr et al., 1993). For instance, *Synechococcus nifH* gene 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 addition, nitrogenase may be post-translationally regulated (e.g., Ludden & Roberts, 1989; Durner et al., 1994; Colón-López et al., 1997). At Station I-2006 hardly any cell specific *nifH* expression was detected although *Lyngbya* sp. was present. The conditions at this station might not have supported diazotrophic growth of this cyanobacterium, or alternatively the majority of the *Lyngbya* sp. found in this mat might have been of a non-diazotrophic type. *NifH* expression of less than 1 transcript copy⁻¹ could have two reasons. On the one hand, all cells could be transcribing but only with a very low rate. On the other hand, transcription per cell could be high but only taking place in very few cells. The reason for higher expression of *nifH* in the light at Station III in 2007 is unclear. Maximum nitrogenase activity by *Lyngbya* sp. is usually observed in the dark (e.g., Paerl et al., 1991). Nevertheless, some degree of light-mediated N₂ fixation might be possible due to spatial separation (lateral partitioning) of photosynthesis and N₂ fixation during illumination, with N₂ fixation being confined to terminal regions (Paerl et al., 1991).

Heterocystous *Cyanobacteria* are not common in marine microbial mats but they were isolated from the study sites. At Station I-2006 the pattern of *nifH* expression by *Anabaena* sp. and *Nodularia* sp. corresponds well with earlier observations of NA patterns for these organisms (Evans et al., 2000, Stal et al., 2003). At Station II-2006 the cell specific *nifH* expression below one transcript per gene copy can be explained by the fact that in heterocystous *Cyanobacteria* only a fraction of the cells, namely those that differentiate to heterocysts, are transcribing *nifH*. Transcription of less than one transcript copy⁻¹ is therefore plausible. Additionally, transcription in terminally differentiated heterocysts could be low due to limited protein turnover, resulting in even lower measurements of cell-specific *nifH* expression.

Abundance-normalized gene expression

At Station II-2006 16S rRNA gene expression normalized to gene copy numbers was clearly dominated by *Lyngbya* sp.. This agrees with the assumption that this station was, at least structurally, dominated by this cyanobacterium. The low contribution of *Lyngbya* sp. to overall *nifH* expression compared to the heterocystous *Cyanobacteria* might be due to conditions that allowed growth and activity of *Lyngbya* sp. but might not have supported diazotrophic growth of this cyanobacterium. With respect to N₂ fixation, *Lyngbya* sp. might be outcompeted by heterocystous *Cyanobacteria* or other diazotrophs without being excluded from the mats. This indicates that structurally dominating diazotrophs might be active but not necessarily the key players in N₂ fixation. Furthermore, the observed *Lyngbya* sp. might have been of the non-diazotrophic type. The lower abundance-normalized 16S rRNA gene expression at Station II-2006 is in line with the location of this station close to the low water mark and the larger influence of North Sea water. Low abundances of heterocystous *Cyanobacteria* compared to non-heterocystous forms have been shown for intertidal microbial mats before (Stal et al., 1985).

Summary

The results of this study demonstrated the varying cell-specific as well as abundance-normalized 16S rRNA gene and *nifH* expression levels of three benthic filamentous diazotrophic *Cyanobacteria* (*Lyngbya* sp., *Nodularia* sp. and *Anabaena* sp.) which had been identified microscopically as structural elements of the investigated microbial mats and were isolated in pure culture. This allowed following their metabolic activity in general through the quantitative measurement of their 16S rRNA gene expression and specifically by following expression of their *nifH* in natural mat samples by the application of specific primers and probes for these two markers belonging to the same genome. 16S rRNA gene expression indicated varying metabolic activities throughout a daily cycle as well as between the samples and was in line with microscopical observations of the dominant cyanobacterial components of the microbial mats. The expression patterns of *nifH* showed that the relative contribution of these *Cyanobacteria* to overall *nifH* expression varied as

well. The structurally dominant *Lyngbya* sp. did not always appear as the key player in *nifH* expression. However, gene expression does not translate into enzyme activity and therefore, the contribution to actual N₂ fixation by these three filamentous *Cyanobacteria* remains unknown.

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