Ammonium ions inhibit nitrogen fixation but do not affect heterocyst frequency in the bloom-forming cyanobacterium _Nodularia spumigena_ strain AV1

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In the presence of ammonium ion, _Nodularia spumigena_ strain AV1, a filamentous, heterocystous cyanobacterium isolated from the Baltic Sea, lost aerobic nitrogen-fixation activity while maintaining heterocyst frequency along the filaments. Real-time RT-PCR showed that the expression of _nifH_ (encoding the dinitrogenase reductase component of the nitrogenase enzyme) was suppressed and the levels of _NifH_ protein decreased dramatically in response to treatment with ammonium. On the other hand, _ntcA_ (encoding the global nitrogen regulator in cyanobacteria) and _hetR_ (the key regulatory gene in heterocyst differentiation) were expressed and their expression patterns were not affected by the treatment with ammonium. These data demonstrate that _N. spumigena_ strain AV1 maintains heterocyst frequency along the filaments in the presence of ammonium and in the absence of detectable _N_2-fixation activity.

INTRODUCTION

_Nodularia spumigena_ is a planktonic, filamentous cyanobacterium capable of forming massive blooms when environmental conditions are suitable. Blooms of _N. spumigena_ are an annual phenomenon in the Baltic Sea, causing ecological and economic problems. The blooms are believed to have increased in recent years due to increased eutrophication (Bonsdorff _et al._, 2002; Kahru _et al._, 1994; Lundberg _et al._, 2005; Raateoja _et al._, 2005; Stalnacke _et al._, 1999; Voss _et al._, 2000; Wulff _et al._, 1990). _N. spumigena_ produces nodularin, a hepatotoxin that is a protein phosphatase inhibitor and tumour initiator and promoter (Fujiki & Suganuma, 1999; Ohta _et al._, 1999).

_N. spumigena_ fixes atmospheric nitrogen (N_2_) in heterocysts, specialized terminally differentiated cells that are the site of _N_2_ fixation in several filamentous _N_2_-fixing cyanobacteria. Nitrogenase, the enzyme complex responsible for fixing _N_2_, is irreversibly inactivated by oxygen. Heterocysts protect the nitrogenase complex under oxic conditions by the formation of a thick cell envelope, by loss of photosystem II activity, and by an increased rate of respiration (Fay, 1992). The formation and maintenance of a pattern of heterocysts along the filaments of heterocystous cyanobacteria are complex processes, not yet fully understood, which involve many genes in a developmental programme (Wolk, 2000). Among these genes, a few key regulatory genes and markers have been identified and extensively studied.

The protein NtcA is a key transcriptional factor required for the activation of many genes involved in nitrogen and carbon metabolism (Frias _et al._, 1994; Wei _et al._, 1994). NtcA belongs to the Crp family of bacterial regulators (Vega-Palas _et al._, 1992) and its activity is required for the development and function of mature heterocysts.

HetR is a serine-type protease with DNA-binding activity and is the master regulator of heterocyst differentiation (Buikema & Haselkorn, 1991; Dong _et al._, 2000; Huang _et al._, 2004; Zhou _et al._, 1998). Both NtcA and HetR are autoregulated and mutually dependent (Black _et al._, 1993; Muro-Pastor _et al._, 2002). _nifH_ encodes the dinitrogenase reductase component of the nitrogenase enzyme complex, and is traditionally used as a marker for the _N_2_ fixation process.

Present knowledge about _N_2_ fixation and heterocyst differentiation has been gained in large part from only a few cyanobacterial species, ‘model’ cyanobacteria, such as _Anabaena_ sp. PCC 7120 and _Nostoc punctiforme_. Extrapolation of this knowledge to understanding these processes in other cyanobacteria might not be accurate. Therefore, examining other cyanobacteria, especially the ecologically important species, is crucial to better understand their behaviour in nature.

In spite of the ecological importance of _N. spumigena_, few studies have addressed the _N_2_ fixation behaviour and heterocyst formation in this cyanobacterium (Lehtimäki _et al._, 1997; Sanz-Alférez and del Campo, 1994). Most
studies on *N. spumigena* so far have focused on conditions regulating nodularin production. The present investigation analysed the early response of *N. spumigena* to nitrogen supplementation, with a focus on N₂-fixation behaviour, expression patterns of key marker genes (*ntcA, hetR* and *nifH*), and heterocyst frequency.

**METHODS**

**Growth conditions and nitrogen supplementation.** Axenic batch cultures of *Nodularia spumigena* strain AV1 (a Baltic Sea isolate) were grown in Z8XN₀ (nitrogen-free) medium (Sivonen *et al.*, 1989) on a shaker in a growth chamber with 16 h of white fluorescent light at 45 µmol m⁻² s⁻¹, at 20 °C.

For each experiment, 500 ml Z8XN₀ medium in 2 l flasks was inoculated with 10% of actively growing bacterial culture.

The medium was supplemented with ammonium (NH₄Cl) or nitrate (NaNO₃) to the final concentrations of 0 mM, 0.25 mM, 0.5 mM and 1 mM in the case of ammonium, and 0 mM, 30 mM, 60 mM and 100 mM in the case of nitrate.

In the reversion experiment, cells were grown for 6 days in the different ammonium concentrations. Cells were then filtered (nylon net filters, 11 µm; Millipore), rinsed with Z8XN₀ and resuspended to the same volume (500 ml) in Z8XN₀.

Chemostat cultures were grown in PC flasks (VWR) containing 1 l Z8XN₀. The medium was continuously supplied at a flow rate of 0.3 ml min⁻¹. The cultures were bubbled with filtered air provided by an aquarium pump. When the cultures reached a steady state of cell growth, as indicated by chlorophyll *a* (Chl *a*) measurements (Meeks & Castenholz, 1971) (Chl *a* = 1.88 ± 0.18 µg ml⁻¹), cultures were transferred to Z8XN containing either 0.25 mM or 0.5 mM ammonium (NH₄Cl). The pH of the medium did not change during any of the experiments. All experiments were independently repeated at least three times.

**RNA isolation.** Samples were collected in duplicate. Cells were harvested by filtration (PC filters 8.0 µm; Whatman), treated with RNAlater RNA stabilization buffer (Qiagen) and stored in 500 µl RLT buffer (RNaseasy mini kit; Qiagen) at -80 °C.

Cells were lysed using the FastPrep FP120 instrument (Qbiogene; Thermo Electron Corporation) in tubes containing acid-washed glass beads (212–300 µm; Sigma) at speed 6 for 8 × 20 s, with 1 min intervals of cooling on ice. Cell lysis was verified by light microscopy. The glass beads were removed by centrifugation and the supernatants transferred to clean microcentrifuge tubes. RNA was isolated using the RNeasy mini kit (Qiagen). The samples were treated with RNase-free DNase I during the isolation procedure. Before cDNA synthesis, the samples were analysed for DNA contamination using real-time PCR.

**Primer design.** Primers were designed using the Primer 3 software (http://workbench.sdsc.edu/). To verify primer specificity, PCR products were cloned using the TOPO-TA cloning kit (Invitrogen) and sequenced (DNA-technology, Aarhus, Denmark). The resulting sequences were analysed using BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/) and deposited in GenBank under accession numbers EF087988–EF087991.

**Real-time RT-PCR.** RNA was quantified using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). Five hundred nanograms of RNA from each sample was used for cDNA synthesis using the iScript cDNA Synthesis Kit (Bio-Rad).

Real-time PCR was performed in duplicate in an iCycler Real-time PCR machine (Bio-Rad) using the iQ SYBR Green Supermix and QuantiTect SYBR Green PCR Kit (Bio-Rad and Qiagen, respectively).

Standard curves were constructed using a 10-fold dilution series of genomic DNA from *N. spumigena* strain AV1 extracted as previously described (Wilson, 1998). After each run, a standard curve was automatically generated by the iCycler software version 3.0a (Bio-Rad). The efficiencies of all real-time PCR reactions were 95 ± 4%.

The real-time PCR programme was run as follows: 3 min (when using the iQ SYBR Green Supermix) or 15 min (when using the QuantiTect SYBR Green PCR kit) at 95 °C, 40 cycles of 30 s at 94 °C, 30 s at 54 °C, 55 °C or 56 °C (see Table 1), 30 s at 72 °C.

An end-point melt-curve analysis was performed after each run and analysed to ensure the absence of unspecific PCR products. The cDNA quantities of *nifH*, *ntcA* and *hetR* (and hence mRNA) were determined using the standard curves, and normalized by the relative cDNA quantities of 16S rRNA.

**Statistical analysis.** Two-way ANOVA on heterocyst frequency and Chl *a* concentrations was carried out using the R statistical software program, version 2.3.1 for Windows XP (http://www.r-project.org/).

**Ammonium and nitrate analysis.** Samples for ammonium analysis were filtered through 8.0 µm Whatman PC filters and the filtrate was analysed by the phenate method (Eaton *et al.*, 1995a). Absorbance was measured at 640 nm.

Samples for nitrate analysis were filtered through 8.0 µm Whatman PC filters and the filtrate was further filtered through sterile 0.20 µm filters (Sarstedt) to get rid of dissolved organic matter. The filtrate was analysed by the UV spectrophotometric screening method at 220 and 275 nm (Eaton *et al.*, 1995b).

**Rates of N₂ fixation.** N₂-fixation activity was measured using the acetylene reduction assay (Capone, 1993), with modifications as previously described (El-Shehawy *et al.*, 2003).

**Preparation of protein extracts and Western blotting.** Samples were collected by filtration (8.0 µm Whatman PC filters) after 6 days of growth in the presence of different concentrations of ammonium (0, 0.25, 0.5 and 1 mM) and resuspended in 500 µl Laemmli buffer (Laemmli, 1970) containing one tablet of protease inhibitor cocktail (Roche Diagnostics). Samples were stored at -20 °C until further analysis.

Table 1. Primer sequences used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
<th>Annealing temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S(Fw)</td>
<td>AAG CAT CGG CTA ACT CCG T</td>
<td>55</td>
</tr>
<tr>
<td>16S(Re)</td>
<td>TCT ACC CGG AAC GCA CTC TA</td>
<td>55</td>
</tr>
<tr>
<td>ntcA(Fw)</td>
<td>AGC GCC AAT TGA ACA GGT AG</td>
<td>55</td>
</tr>
<tr>
<td>ntcA(Re)</td>
<td>CTC ACC AAT CTC GAA CCC AT</td>
<td>55</td>
</tr>
<tr>
<td>hetR(Fw)</td>
<td>CCC AGT AAT CTG CCT GAT GC</td>
<td>54</td>
</tr>
<tr>
<td>hetR(Re)</td>
<td>TTC GCT TAA GGG CAT TTG AT</td>
<td>54</td>
</tr>
<tr>
<td>nifH(Fw)</td>
<td>CCT GAT CGT TGG TTG TGA CCC T</td>
<td>56</td>
</tr>
<tr>
<td>nifH(Re)</td>
<td>AAG AAG ATG ATG GCC GTG AT</td>
<td>56</td>
</tr>
</tbody>
</table>
Proteins were extracted by grinding the samples with a plastic pestle in tubes containing acid-washed glass beads (212–300 μm; Sigma) followed by heating the sample to 99 °C for 5 min. The samples were then centrifuged for 5 min at 15 000 g.

Western blotting was carried out as previously described (Braun-Howland et al., 1988). The membranes were incubated for 1 h with polyclonal anti-dinitrogenase reductase from *Rhodospirillum rubrum*, raised in rabbit at a 1 : 5000 dilution in PBS-Tween. Membranes were then incubated for 1 h with the secondary antibody (affinity-purified polyclonal pig anti-rabbit/HRP antibody; DAKO) at a 1 : 5000 dilution in PBS-Tween. Detection was performed using the ECL Plus system (Amerham/GE Healthcare) according to the manufacturer’s instructions. Visualization was performed on the Chemidoc system (Bio-Rad).

**Heterocyst counting.** Heterocyst frequency was determined by counting the number of heterocysts (late proheterocysts/early heterocysts were recognized by their thickened cell wall and pale appearance, and mature heterocysts were recognized by their poles) and vegetative cells that were present along filaments of *N. spumigena*. The total number of cells counted was approximately 1000 cells per sample.

### RESULTS AND DISCUSSION

#### N₂ fixation

Cyanobacteria can utilize different sources of nitrogen, such as ammonia, nitrate and nitrogen gas. The preferred source of nitrogen is ammonium ion because it is readily incorporated into the carbon skeleton through the glutamine synthetase-glutamate synthase (GS-GOGAT) cycle (Flores & Herrero, 1994). Ammonium and nitrate generally repress N₂ fixation, with the latter being less effective and strain-dependent (Guerrero & Lara, 1987; Meeks et al., 1983; Ohmori & Hattori, 1972). Conversely, in *Anabaena CA* (ATCC 33047), nitrate is more efficient than ammonium in repressing N₂-fixation activity and heterocyst formation (Bottomley et al., 1979; Van Baalen, 1987).

In *N. spumigena*, N₂ fixation is modulated by ammonium (Fig. 1a). N₂-fixation activity dramatically dropped after incubation with NH₄Cl (0.25–0.5 mM) for 3 days. When ammonium was exhausted from the medium, the...
N₂-fixation activity was restored (Fig. 1a, Table 2). Two-way ANOVA on Chl a concentrations showed an enhanced growth in the presence of 0.25 mM in comparison to the controls (Fig. 1b). The enhancement of growth in low ammonium concentrations was previously reported by Lehtimäki et al. (1997). The statistical analysis also showed that the treatments did not negatively affect growth until day 6. From day 6 to day 9, growth slowed down in cultures treated with 0.5 and 1 mM ammonium, with the slowest growth being in the presence of 1 mM ammonium (Fig. 1c). In the presence of 1 mM ammonium, N₂-fixation activity was not detectable after 3 days incubation (Fig. 1a), and was not resumed because ammonium was not exhausted (Table 2).

Our data are in accordance with the results by Lehtimäki et al. (1997) and Sanz-Alférez and del Campo (1994), which showed that ammonium could limit both N₂ fixation and growth in Nodularia. Lehtimäki et al. (1997) also showed that prolonged incubation of N. spumigena strain BY1 (a Baltic Sea isolate) with high ammonium concentration resulted in reduced heterocyst frequency (and reduced N₂-fixation activity). It was not possible to maintain cultures of N. spumigena strain AV1 for a longer time period under non-fixing conditions. After day 9 of incubation in the presence of 1 mM NH₄Cl, growth was inhibited. To our knowledge and in support of our results, there is no report showing clearly that N. spumigena can grow for several generations with very low or no

Table 2. NH₄⁺ and NO₃⁻ concentrations in the medium during the experiments

<table>
<thead>
<tr>
<th>Incubation time (days)</th>
<th>[NH₄⁺] in the medium (mM)</th>
<th>[NO₃⁻] in the medium (mM)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.25</td>
</tr>
<tr>
<td>0</td>
<td>UD</td>
<td>0.24 ± 0.01</td>
</tr>
<tr>
<td>3</td>
<td>UD</td>
<td>UD</td>
</tr>
<tr>
<td>6</td>
<td>UD</td>
<td>UD</td>
</tr>
<tr>
<td>9</td>
<td>UD</td>
<td>UD</td>
</tr>
</tbody>
</table>

UD, Undetectable.

Fig. 2. (a) Frequency of heterocysts along filaments of N. spumigena strain AV1. (b, c) Light micrographs of N. spumigena strain AV1. Cultures were grown for 9 days in the presence of 0 mM NH₄Cl (b) or 1 mM NH₄Cl (c). Arrows indicate heterocysts. Error bars indicate SE. Scale bars, 10 μm.
N₂-fixation activity. Accordingly, *N. spumigena*, unlike 'model cyanobacteria', seems unable to efficiently utilize exogenous ammonium to support growth for a prolonged time under non-fixing conditions.

Nitrate supplemented as NaNO₃ up to 100 mM had no apparent effect on N₂-fixation activity or on growth (Fig. 1b, d), and nitrate was not taken up by the cells (Table 2). This finding is in accordance with the results of Lehtimäki et al. (1997), who showed that nitrate had no effect on growth and N₂ fixation in *N. spumigena* strain BY1, although the authors did not measure nitrate concentrations in the growth medium during the experiments. Furthermore, nitrate was also shown not to have any effect on heterocyst frequency in *Nodularia* sp. strains M1 and M2, even though cells exhibited nitrate-uptake activity (Sanz-Alférez & del Campo, 1994). Together, these results indicate that in *Nodularia*, nitrate uptake and/or assimilation might be inefficient.

**Heterocyst frequency**

The general consensus is that ammonia and nitrate suppress N₂ fixation and heterocyst formation in filamentous heterocystous cyanobacteria such as *Anabaena* and *Nostoc* (Adams & Duggan, 1999). However, several studies have demonstrated that heterocysts can differentiate in the presence of a nitrogen source. For example, in *Anabaena variabilis*, growth on glutamine as the sole nitrogen source gave rise to patterned heterocysts even though nitrogenase activity was lost (Thiel & Leone, 1986). Other examples are from mutation studies (Buikema & Haselkorn, 1991; Wolk, 1982; Yoon & Golden, 1998) such as overexpression of *hetR* or mutations in *patS*, which led to formation of heterocysts in the presence of nitrate (Buikema & Haselkorn, 1991; Yoon & Golden, 1998), although the heterocyst pattern was aberrant.

Ammonium supplementation to the growth medium had no statistically significant effect on heterocyst frequency along the filaments of *N. spumigena* strain AV1 until day 9 of incubation in batch cultures (Fig. 2). In chemostat cultures, ammonium supplied at a concentration of 0.25 mM NH₄Cl was consumed and had a slightly negative effect on N₂-fixation activity after 4 days of incubation (Fig. 3), which was relieved at day 12. The treatment had no statistically significant effect on heterocyst frequency or growth. Higher concentration of ammonium (0.5 mM) negatively affected the growth as in the batch cultures, which highlights that the continuous supply of this concentration of ammonium may be toxic to the cells.

**Expression of nifH, ntcA and hetR**

Our data demonstrated that *N. spumigena* strain AV1 maintains heterocyst frequency along the filaments in the presence of ammonium and in the absence of detectable N₂-fixation activity. This behaviour of *N. spumigena* differs from the known behaviour of ‘model’ cyanobacteria such as *Anabaena* sp. PCC 7120, *A. variabilis* and *Nostoc punctiforme*, in which ammonium or nitrate have a pronounced negative effect on heterocyst differentiation and pattern formation, leading to inhibition of the differentiation process, reduction in heterocyst numbers, or loss of heterocysts when the filaments are not fixing N₂ aerobically (Adams & Duggan, 1999; Bothe, 1982; Guerrero & Lara, 1987). The response of *N. spumigena* to ammonium resembles that of *Anabaena cylindrica*, which was reported to form heterocysts (and to fix N₂) in the presence of ammonium (Stewart & Rowell, 1975).

To investigate the genetic background of the observed behaviour, we analysed the expression patterns of *nifH*, *ntcA* and *hetR* using real-time RT-PCR.

In the presence of combined sources of nitrogen, the expression of *hetR* in *Anabaena* sp. PCC 7120 is at a low level (Black et al., 1993; Buikema & Haselkorn, 1991). Within 2 h of nitrogen step-down, the expression of *hetR* is induced and after 3.5 h, enhanced gene expression occurs in cells that will become heterocysts (Black et al., 1993). NtcA perceives the signal of nitrogen step-down and a molecular cascade operates between activation of NtcA and *hetR*-enhanced expression (Ehira & Ohmori, 2006; Muro-Pastor et al., 2002; Zhang et al., 2006). The enhanced
expression of hetR in turn has a positive feedback on ntcA expression (Muro-Pastor et al., 2002). In general, ammonium is known to repress heterocyst formation and expression of ntcA and hetR in cyanobacteria (Adams & Duggan, 1999; Herrero et al., 2001; Meeks & Elhai, 2002).

As expected, ammonium negatively affected the expression of nifH (Fig. 4a), which correlated well with the N₂-fixation activity of the cultures (Fig. 1a), and the expression was abolished at day 3 of incubation in the presence of 1 mM NH₄Cl. This result was further supported by Western blot analysis, which showed the disappearance of NifH protein bands in the presence of 1 mM NH₄Cl after 6 days incubation (data not shown).

On the other hand, N. spumigena expressed ntcA and hetR continuously in the presence of ammonium in all cultures, and the expression patterns of these genes were not affected by the treatment (Fig. 4b, c).

When the cultures were grown in different concentrations of ammonium for 6 days, washed and resuspended in nitrogen-free medium, the cells quickly recovered N₂-fixation activity and nifH expression, with no effect on ntcA and hetR expression patterns (Fig. 5).

Therefore, analysis by real-time RT-PCR demonstrated that N. spumigena expresses the key markers of heterocyst differentiation in the presence of ammonium while the expression of nifH drops to undetectable levels.

The possible mechanism, whether molecular or physiological, operating behind the observed ‘uncoupling’ between N₂ fixation and heterocyst formation processes during the early response of N. spumigena strain AV1 to ammonium treatment needs to be further investigated. The genome of N. spumigena strain 9414 has recently been released. BLASTP analysis has revealed that the NtcA protein of N. spumigena strain CCY 9414 shares 99% identity with its homologues from Anabaena sp. PCC 7120 and A. variabilis, while HetR shares 91% and 90% identity with its homologues from Anabaena sp. PCC 7120 and A. variabilis, respectively.

Alignment of NtcA protein sequences from Anabaena sp. PCC 7120, A. variabilis, Nostoc punctiforme and Nodularia

![Fig. 4. Expression pattern of (a) nifH, (b) ntcA and (c) hetR in N. spumigena strain AV1 measured using real-time RT-PCR. Cultures were treated with 0, 0.25, 0.5 and 1 mM NH₄Cl at the beginning of experiments. SQ, starting quantity. Error bars indicate SE.](http://mic.sgmjournals.org)
spumigena strain CCY 9414 has shown that amino acids L112 and K201 are substituted with F and E, respectively, in *N. spumigena* strain CCY 9414. Partial sequencing of *ntcA* from *N. spumigena* strain AV1 and BLASTX analysis revealed that these two substitutions are conserved in this strain. Alignment of HetR sequences from the same organisms revealed that amino acids V150, D169, I273 and K279 are substituted by T, V, V and R, respectively, in *N. spumigena* strain CCY 9414. However, these amino acids were not shown to be necessary for HetR function in *Anabaena* sp. PCC 7120 (Risser & Callahan, 2007). Partial sequencing of *hetR* from *N. spumigena* strain AV1 and BLASTX analysis revealed that the first two substitutions are conserved in this strain and also in strain KAC 17 available in the database.

Therefore, the insensitivity of the heterocyst formation process in *N. spumigena* strain AV1 to ammonium seems unlikely to be due to a genetic variation in the protein sequences of NtcA and HetR. Variations in their genetic regulation need to be investigated.

Further analysis of the genome of *N. spumigena* revealed that it possesses *amt*, *nir*, *nar* and *nrtP* (coding for ammonium permease, nitrite reductase, nitrate reductase and nitrate/nitrite permease, respectively) (Flores et al., 2005; Flores & Herrero, 2005). Therefore, the observed uncoupling behaviour does not seem to be due to a genome reduction.

Possibly, *N. spumigena* in the Baltic Sea does not experience high nitrogen concentrations (millimolar quantities) in open water, and has adapted to a nitrogen-poor environment.

Despite the ecological and economic importance of *N. spumigena*, few studies have addressed the physiology of...

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**Fig. 5.** (a) Nitrogenase activity measured by acetylene reduction assay after ammonium removal (see Methods). (b–d) Expression pattern of *nifH* (b), *ntcA* (c) and *hetR* (d) in *N. spumigena* strain AV1 measured using real-time RT-PCR, after ammonium removal. Cultures were grown in the presence of 0, 0.25, 0.5 and 1 mM NH₄Cl. SQ, starting quantity. Error bars indicate SE.
this cyanobacterium. The data presented here clarify some novel aspects of this ecologically important organism. First, we were able to demonstrate that ammonium had a pronounced inhibitory effect on the N₂-fixation activity of *N. spumigena*, while nitrate did not. Second, we demonstrated that ammonium had no effect on heterocyst frequency along the filaments. Third, *N. spumigena* continued to express the master genes *ntcA* and *hetR*; their expression was not affected by the treatment, while the expression of *nifH* ceased.

Our findings might explain (together with environmental factors) why the blooms of *N. spumigena* in the Baltic Sea continue to develop even while the sea continues to receive nitrogen from different sources (Bonsdorff *et al.*, 2002; Enell & Fejes, 1995; Kahru *et al.*, 1994; Lundberg *et al.*, 2005; Raateoja *et al.*, 2005; Stalnacke *et al.*, 1999; Voss *et al.*, 2000; Wulff *et al.*, 1990). Thus, this work shows the necessity for further study of the physiology of *N. spumigena* in response to any measure being proposed to manage the blooms in the Baltic Sea.

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