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Phosphorus limitation and diel control of nitrogen-fixing cyanobacteria in the Baltic Sea

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ABSTRACT: Up to half of the annual new nitrogen inputs into the Baltic Sea originate from blooms of N_2-fixing cyanobacteria. Estimates of the magnitude of this new nitrogen vary, partially because relatively few studies have investigated short-term changes in N_2-fixation rates in response to environmental changes *in situ*, including phosphorus availability, one of the major factors limiting N_2 fixation in the system. We examined cyanobacterial N_2 fixation in response to phosphorus amendments over the diel cycle during 2002 and 2003 in the Baltic Sea, when both *Nodularia spumigena* and *Aphanizomenon* sp. formed blooms. Phosphorus stimulated N_2 fixation in the open-sea areas in the Northern Baltic Proper and Gulf of Finland during both years. In microcosm experiments, both chlorophyll *a* concentration and N_2 fixation were positively related to time ($R^2 = 0.79$ and 0.54, respectively) for at least 4.5 d after the P amendment. N_2 fixation was enhanced up to 3-fold within 4.5 d by a single P pulse. N_2 fixation continued in the dark at 16 to 61% of maximum rates during the day, and there were no consistent changes in nitrogenase enzyme abundance in response to darkness. Immunoblotting showed that N_2 fixation is not regulated in response to darkness by size modifications of the Fe and MoFe proteins in *N. spumigena* or of the Fe protein of *Aphanizomenon* sp. Capability to fix N_2 at high rates at night allows these cyanobacteria to maximize their utilization of periodic P pulses for subsequent growth.

KEY WORDS: Diel cycle · Nitrogenase regulation · Baltic Sea · Phosphorus limitation · *Nodularia spumigena*

INTRODUCTION

Only a few coastal embayments and estuaries, including the Baltic Sea, experience annual summertime N_2-fixing cyanobacterial blooms (Niemi 1979, Paerl 1990). In places where these blooms occur, N_2 fixation can be an ecologically significant process. Summertime N_2-fixing cyanobacterial blooms provide as much as 10 to 55% of the annual N inputs into the Baltic Sea (Leppänen et al. 1988, Larsson et al. 2001, Wasmund et al. 2001). Three heterocystous cyanobacterial genera (*Aphanizomenon* sp., *Nodularia* spp., and *Anabaena* spp.) form the Baltic Sea cyanobacterial blooms, with *Nodularia spumigena* and *Aphanizomenon* sp. dominating the cell abundances. *Nodularia spumigena* is the most visible of these cyanobacteria: its surface accumulations are extensive enough to be mapped by remote sensing (Kahru et al. 1994). In spite of numerous studies on Baltic Sea cyanobacterial blooms, taxa-specific differences in environmental and diurnal controls of their N_2 fixation are not well understood (Lehtimäki et al. 1997, Evans et al. 2000).

Over large spatial and temporal scales (years to decades, hundreds of km), phosphorus (P) availability and cyanobacterial bloom intensities in the Baltic Sea

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are closely linked with bottom-water redox conditions (Kiirikki et al. 2001). Over long periods of stagnation, oxygen in waters beneath the Baltic Sea halocline is consumed and anoxia develops (Nehring 1981). Under anoxic conditions, phosphate and ammonia are released, while in hypoxic waters and sediments, denitrification is promoted (Kuparinen & Tuominen 2001). Both of these processes promote reductions in the N:P ratio in dissolved nutrients in the bottom waters. Baltic Sea bottom-water exchange is dependent on frequency of storms that force salty oceanic water over the sills at the Danish Sounds to the Baltic Sea from the North Sea. As this salty water oxygenates the Baltic Sea bottom, some of the lower salinity, low N:P ratio, P-enriched anoxic bottom waters are transported to the surface (Nehring 1981) and, as a result, intense cyanobacterial blooms develop over the next several years (Kahru et al. 2000, Kiirikki et al. 2001).

Superimposed on this large-scale forcing cycle, which has significant interannual variability in the Baltic Sea, P effects on growth and N_2 fixation of cyanobacteria are observed regionally and locally, at short time scales. Certain Baltic Sea regions are hydrodynamically more active than others, and susceptible to vertical water exchange such as upwelling and frontal transport that act as 'P pumps' into the surface waters (Kononen et al. 1996, Pavelson et al. 1997, Vahtera et al. 2005). These locations are often sites of intensive cyanobacterial growth. Cyanobacterial populations in the euphotic surface waters are likely to experience P depletion repeatedly over the growing season, depending on hydrodynamic conditions and how far they have advected from these P 'hotspots'. P regeneration within *Nodularia spumigena* aggregates may be an important mechanism by which blooms are maintained (Hagström et al. 2001). There is a general agreement that P regulates cyanobacterial bloom intensities in the Baltic Sea over interannual, basin-wide scales (Kahru et al. 2000, Kiirikki et al. 2001). However, estimates on the regulation and limitation of cyanobacterial growth and N_2 fixation in the Baltic Sea vary over time scales of hours to days (Wallström et al. 1992, Stal et al. 1999, Rydin et al. 2002, Moisander et al. 2003).

The goal of this study was to investigate short-term effects of P on the growth and N_2 fixation of Baltic Sea cyanobacteria, to better understand regional differences in bloom intensities and mechanisms for bloom maintenance. In order to study regulation of the nitrogenase proteins over the diel cycle, we additionally investigated potential changes in the quantity and size of the 2 cofactors of the nitrogenase enzyme (Fe and MoFe proteins) in response to darkness. This information is essential for predicting and modeling cyanobacterial bloom formation and N_2 fixation based on P availability in the Baltic Sea.

MATERIALS AND METHODS

Baltic Sea field studies. Field studies were carried out onboard RV 'Aranda' (Finnish Institute of Marine Research) in 2002 and 2003. The study area was located in the Gulf of Finland and the Northern Baltic Proper (Table 1). Diel cycles in the field were studied through incubation experiments on deck using Baltic seawater (Expts 02A, 02B, 03A, 03B), and by sampling directly from the sea (Expt An03), both types of samples defined as 'field samples' (Table 1). In the incubation experiments, surface water was collected from ~1 m depth using a pump (in 2002), or a 30 l water sampler (2003). All of the water collected was mixed in a 100 l container on deck, and then 14 l (2002) or 17 l (2003) were poured into each of six 20 l pre-cleaned (0.01 N HCl and deionized water) Cubitainers (Hedwin). Cubitainers are containers made of polyethylene and are approximately 85% transparent to incident photosynthetically active irradiation (PAR). The

Table 1. Diel experiments with natural communities in the Baltic Sea. Temperature, salinity, and nutrients are from 1.5 to 2 m depth

Expt	Date	Type	Station	Location	Treatment or depth	Temp. (°C)	Salinity	DIN (µM)	DIP (µM)
02A	Sampled 15 July 2002 Expt 16-17 July 2002	Cubitainer	H02_A1_4	59.2946° N, 23.0215° E	Control, P	18.3	5.6	0.12[a]	0.2[a]
02B	Sampled 18 July 2002 Expt 22-23 July 2002	Cubitainer	H02_EA	59.2210° N, 22.4309° E	Control, P	20.0	5.6	0.14	0.16
03A	Sampled 16 July 2003 Expt 19-20 July 2003	Cubitainer	LL3A	60.0403° N, 26.2079° E	Control, P	16.7	6.2		
03B	Sampled 14 July 2003 Expt 15-16 July 2003	Cubitainer	JML	59.3506° N, 23.3766° E	Control, P	19.3	5.2		
An03	24-25 July 2003	Rosette	JML	59.3506° N, 23.3766° E	0 m, 8 m	20.2–21.1	5.4–5.5		

[a]Determined at 0 m depth

Cubitainers were inoculated with a concentrated phytoplankton sample, consisting mostly of the filamentous cyanobacteria collected by vertical tows from the 0 to 10 m surface layer using a 100 µm zooplankton net (2.6 l in 2002 and 1 l in 2003). Three Cubitainers received a phosphorus addition (as K_2HPO_4; 2 µM final concentration), while the other 3 were controls. The Cubitainers were placed in tubs on the ship deck under natural irradiance with flowing surface seawater for temperature control. They were shaded with screening to alleviate photoinhibition by reducing PAR by 30%. Measurements were started within 16 to 88 h of the P addition. In each experiment, the diel measurements were initiated at 06:00 h. A sub-sample from the Cubitainers was collected every 3 h over the diel cycle in order to determine the rates of N_2 fixation using the acetylene reduction technique (AR). For immunoblotting of the nitrogenase enzyme, a sub-sample was collected every 3 h during the day and every 1 to 1.5 h at night. Sub-samples were also preserved in Lugol's solution for microscopic observations of community structure.

In addition to the incubation experiments, diurnal patterns of N_2 fixation were assessed in samples collected directly from the Baltic Sea at each time point of the diel study. A 24 h anchor station experiment was conducted on 24-25 July 2003 (Table 1, Expt An03), during which samples for N_2-fixation measurements were collected from 1 m and 8 m depths every 3 h with Niskin bottles. Samples for immunoblotting during this experiment were collected every 3 h during the day and every 1 h at night for 24 h. Additionally, CTD profiles and vertical profiles of PAR were obtained on each sampling occasion.

Culture studies. In order to study diel patterns of N_2 fixation in *Nodularia spumigena* in more detail, a diel experiment was carried out in the laboratory with *N. spumigena* strain FL2f isolated from the Baltic Sea (Moisander et al. 2002). The cultures were kept on a 15:9 h light:dark cycle. The experimental cultures were grown in a 1 l volume in duplicate 2 l flasks with constant aeration provided through a 0.2 µm filter and kept in an incubator at 27 to 30°C and 200 µmol m^{-2} s^{-1} PAR. The strains were grown in Z8 media (Rippka 1988), with combined nitrogen omitted and salts added at 0.15 M NaCl and 0.015 M $MgSO_4$ (final concentrations) for optimal growth (Moisander et al. 2002). Samples for the following assays were collected every 2 h during the diel experiments: CO_2 fixation ($NaH^{14}CO_3$ incorporation), N_2 fixation (acetylene reduction assay), abundance of the Fe and MoFe proteins in the nitrogenase enzyme (SDS-PAGE and immunoblotting with Fe and MoFe protein antibodies), chlorophyll *a* (chl *a*) concentration, and dissolved inorganic carbon (DIC) concentration.

Analytical methods. ***Acetylene reduction assay (AR):*** N_2-fixation activity was measured every 2 h in cultures and every 3 h in field samples. In the field studies, 1 AR measurement was taken from each Cubitainer (triplicate Cubitainers per treatment) or in triplicate from the Niskin bottle (Expt An03), whereby a 90 ml sample was incubated in 117 ml serum vials capped with red rubber serum stoppers. In culture experiments, duplicate AR measurements were made from 2 independent culture flasks each, whereby 8 ml of culture was transferred into 13 ml Exetainer tubes (Labco) and tubes were capped with serum screw caps. Acetylene was generated from calcium carbide, and 9 or 2 ml acetylene was injected with a syringe into each vial through the liquid phase in field and culture experiments, respectively. Next, the incubation vials were shaken for ~10 s. AR vials in the field were incubated for 3 h in the flowing surface water bath on the deck, while the AR vials in culture experiments were incubated for 2 h in the culture incubator. At the end of the incubation, tubes and flasks were shaken for ~20 s, and sub-samples of the headspace were collected into evacuated Vacutainers (Becton-Dickinson). To avoid potential trace ethylene contamination, Vacutainers were opened, aerated overnight, recapped, and manually re-evacuated using a 60 ml syringe prior to use. Ethylene content was measured using a Shimadzu GC-9A gas chromatograph equipped with a flame ionization detector and a Poropak T stainless steel column at 80°C, using ultrapure N_2 as a carrier gas. The gas chromatograph was calibrated daily using a standard curve constructed from ethylene (National Welders). Rates in blanks with Milli-Q water were subtracted from the rates in samples.

SDS-PAGE and western blotting: Cyanobacteria were filtered on 47 mm diameter, 5 µm pore-size Durapore filters (Millipore), snap frozen in liquid nitrogen, and stored at –80°C. The samples were extracted in Laemmli's buffer (Laemmli 1970), sonicated twice for 1 min in ice with a tip sonicator (with a minimum 10 min cooling period in ice between sonications), and heated to between 90 and 95°C for 5 min. Cell debris was removed by centrifugation, and the supernatant was transferred into a new tube and stored at –80°C. Total protein concentrations were determined using the Bio-Rad RCDC kit (Bio-Rad). For each experiment, a similar amount of protein for each time point was loaded on Bio-Rad polyacrylamide Tris-HCl ready-gels (10% resolving, 4% stacking gel). The amount of protein loaded varied from 7.5 to 20 µg, but was the same within each experiment. Wide range, colored molecular weight markers (6500 to 205 000 kDa) were used (Sigma). Purified Fe and MoFe proteins from *Azotobacter vinelandii* (Av2 and Av1, respectively), donated by G. Roberts (University of Wisconsin, Madi-

son), were used as controls. Av1 and Av2 served as positive controls when blotted with a specific antibody, and as negative controls when blotted with a non-specific antibody. Proteins were transferred onto 0.45 µm nitrocellulose membranes using the Bio-Rad minicell transfer unit with cooling. Membranes were frozen (–20°C) until blotting. For immunoblotting, membranes were first incubated for 1 h with TTBS (0.5 M NaCl, 20 mM Tris base, 0.1% Tween 20, pH 9.0), then blocked for 2 h in 2% bovine serum albumin in TTBS (pH 7.5) and washed twice for 5 min with TTBS (pH 7.5). Next, the membranes were incubated for 1 h with one of the primary antibodies in TTBS and immediately washed twice for 5 min with TTBS (pH 7.5). The primary antibodies were donated by P. Ludden (University of Wisconsin, Madison) and were generated against dinitrogenase (MoFe protein) from *Rhodospirillum rubrum* and dinitrogenase reductase (Fe protein) from *R. rubrum* and *A. vinelandii*. The membranes were then incubated with an anti-rabbit IgG secondary antibody with alkaline phosphatase conjugate (Sigma) for 1 h and washed twice for 5 min with TTBS (pH 7.5), and finally twice for 5 min with TBS (TTBS without Tween 20, pH 7.5). The bound antibodies were visualized using a Sigma BCIP/NBT liquid substrate system alkaline phosphatase detection kit according to the manufacturer's instructions. The blots were recorded using a Bio-Rad image analysis system and stored at –20°C.

Chl a, cyanobacterial abundance: For chl *a* analysis, samples were filtered onto Whatman GF/F filters, blotted dry on a paper towel, wrapped in aluminum foil, and frozen at –20°C. For determination of the chl *a* concentration in culture samples, the filters were extracted using a 90% acetone, 10% water mixture. Each sample was sonicated twice for 1 min in ice and kept at –20°C in the dark overnight. The extract was cleared by filtering through Whatman GF/F filters and fluorescence was detected using a Turner TD-700 fluorometer (Welschmeyer 1994). For chl *a* determination in field populations (deck incubations and direct sampling from the sea), the filters were extracted with 96% ethanol for >18 h at room temperature in the dark (Baltic Marine Environment Protection Commission 1988) and measured using a Perkin-Elmer LS-2 spectrofluorometer (Wellesley) at 662 nm. For the determination of cyanobacterial abundances in the field samples, 50 to 100 ml sample water was preserved with Lugol's solution and stored in the dark. The lengths of at least 50 filaments of each cyanobacterial taxon were measured and counted on a Sedgewick-Rafter counting cell using a Zeiss Axioplan 2 (Thornwood) microscope at 200× magnification.

CO_2-fixation, dissolved inorganic carbon (DIC): Photosynthetic activity in culture experiments was determined using the ^{14}C incorporation method (Steemann Nielsen 1952). From each culture, 5 ml was transferred into 20 ml liquid scintillation vials and 50 µl $NaH^{14}CO_3$ was added to give a final activity of approximately 0.02 µCi ml^{-1}. At each time point, the actual activity was determined parallel to the samples from vials containing 50 µl $NaH^{14}CO_3$ and 50 µl phenethylamine. The CO_2 fixation measurements were carried out in duplicate for each of the 2 culture flasks per strain. One additional vial was incubated in the dark at each time point, and the dark ^{14}C incorporation was subtracted from light values at that time point. The vials were incubated for 2 h beside the culture flasks in the incubator. After incubation, the contents of the vials were filtered on Whatman GF/F glass-fiber filters, and unincorporated ^{14}C was removed from the filters by fuming them in a concentrated HCl atmosphere for at least 1 h in the dark. The filters were air-dried overnight and placed in 7 ml liquid scintillation vials with 5 ml of liquid scintillation cocktail (Ecolume). Radioactivity was measured using a Beckman LS5000TD liquid scintillation counter (Fullerton). Counts per min were converted to disintegrations per min using a quench curve constructed by a ^{14}C toluene standard. The DIC concentrations used in calculations of CO_2 fixation were determined at each time point. Samples for DIC were collected in 7 ml tubes sealed without headspace and stored at 4°C until measurement with a Shimadzu TOC-5000A analyzer.

Physical measurements in the field: Vertical profiles of PAR in the water column were detected using an upward facing LI-COR (Lincoln) quantum sensor. Incident irradiance in air was measured using duplicate quantum sensors located on each side of the ship. Salinity and temperature profiles were obtained with a Seabird 911plus profiler (Bellevue). Nutrients onboard RV 'Aranda' were measured using an autoanalyzer following the guidelines of the Baltic Sea Monitoring Programme (Baltic Marine Environment Protection Commission 1983).

Statistical methods: Linear regression analysis (SPSS for Windows) was performed for pooled N_2 fixation (acetylene reduction) rate and chl *a* data from the field study. The midnight datapoint in N_2 fixation was a large outlier in Expt 03B and was removed from the analysis.

RESULTS

The cyanobacterial community in the Baltic Sea was dominated by both *Nodularia spumigena* and *Aphanizomenon* sp. in 2002 and by *Aphanizomenon* sp. in 2003. Both cyanobacteria were present in the community during 2002 and 2003, with variable abundances among experiments (Fig. 1). *Anabaena* spp. were

present in the community in both years but at lower abundances than *N. spumigena* and *Aphanizomenon* sp. (Fig. 1). The ratio of DIN:DIP (in moles) was less than 1.3 in 2002, indicating that nitrogen limited phytoplankton growth (Table 1). Highest N_2 fixation rates were detected in incubations that started between 06:00 and 15:00 h (09:00 to 15:00 h in 2002, and 06:00 to 09:00 h or 12:00 to 15:00 h in 2003), while the lowest rates were observed in incubations that started between 21:00 and 03:00 h (Fig. 2). N_2 fixation rates in complete darkness (00:00 h time point) were 16 to 61% of the maximum daytime rates. In one experiment, there was a second peak in nitrogenase activity at midnight (Fig. 2D). In order to investigate the time scale in P stimulation during the 4 diel incubation experiments, the time from P addition to the beginning of the diel measurements was varied. In one experiment each year, the diel measurements were started 16 to 20 h after the experimental water was collected (Fig. 2A,C). In these experiments, P addition had only a minor visible effect on chl *a* concentration or N_2 fixation over the diel cycle. In a second experiment in 2002 and 2003, diel measurements were started after the water with added P had been incubated for 88 h (2002) or 64 h (2003) (Fig. 2B,D). In both of these experiments, N_2 fixation and chl *a* concentration were elevated throughout the diel experiment in response to P additions (Fig 2B,D). For a more detailed analysis, data from all incubation experiments were pooled and the difference between the P-amended and control Cubitainers was studied over time. This difference (increase in N_2 fixation or chl *a* compared with control

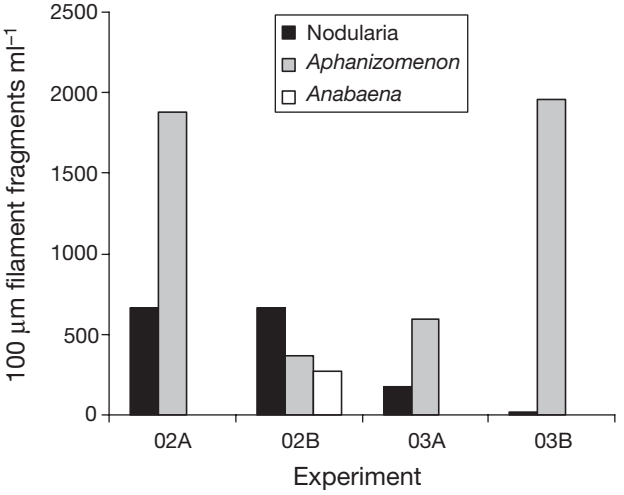

Fig. 1. *Nodularia spumigena*, *Aphanizomenon* sp. and *Anabaena* spp. Abundances of diazotrophic cyanobacteria in field experiments

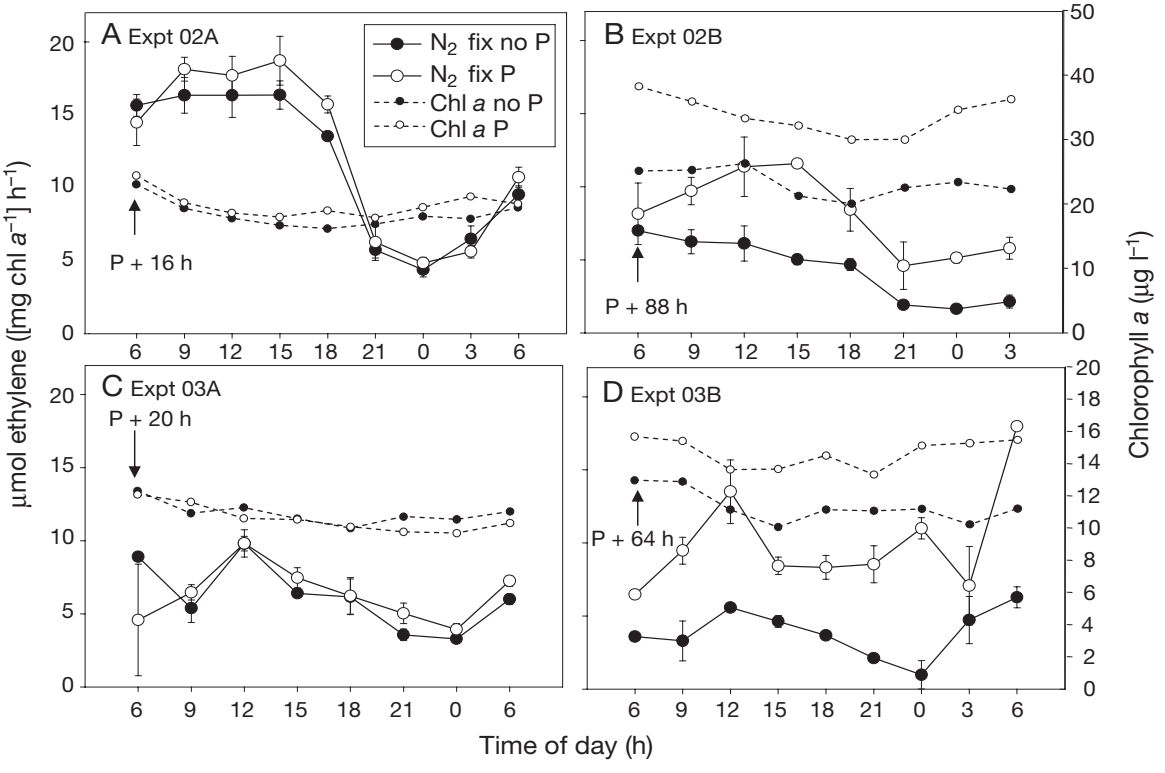

Fig. 2. N_2 fixation rates (acetylene reduction) and chl *a* concentrations in Baltic Sea diel incubation experiments with P additions. Time from addition of P to the first N_2 fixation measurement varied as indicated by arrows. (A) 16–17 July 2002 (Expt 02A); (B) 22–23 July 2002 (Expt 02B); (C) 15–16 July 2003 (Expt 03A); (D) 19–20 July 2003 (Expt 03B). Error bars: ±SD. For (C) period between final 2 measurements is 6 h

[% control]) represents the new activity or growth that occurred in response to P. There was a significant positive relationship (linear regression) between time and the 'new N_2 fixation' (R^2 = 0.54, p = 0.000) and 'new chl *a*' (R^2 = 0.79, p = 0.000) (Fig. 3). Cyanobacterial numbers of all taxa (*N. spumigena*, *Aphanizomenon* sp., *Anabaena* spp.) increased during the diel experiment that had a 64 h pre-incubation with P (Fig. 4).

Very calm, sunny conditions prevailed during the anchor station experiment in 2003 (An03) (Fig. 5). *Aphanizomenon* sp. was the only diazotroph detected in microscopic observations at this station. Nitrogen fixation continued at both 1 and 8 m depths throughout the diel cycle (Fig. 5A), and trailed the irradiance intensity in the water column and air (Fig. 5A,B). There was a deepening of the thermocline during the day and gradual shallowing again at night (Fig. 5C).

The Fe protein was detected throughout the diel cycle in both the Cubitainers and the anchor station experiment (Fig. 6). No changes over the diel cycle in molecular weight (MW) or consistent changes in the abundance of the nitrogenase Fe protein in immunoblots were observed either in the Cubitainer incubations or samples taken directly from the sea (Fig. 5). A second, higher MW band appeared sporadically in an experiment in which inhibitors were tested (data not shown). In the Baltic Sea field samples, the MoFe protein had a very weak antibody binding capacity and blots did not produce good results.

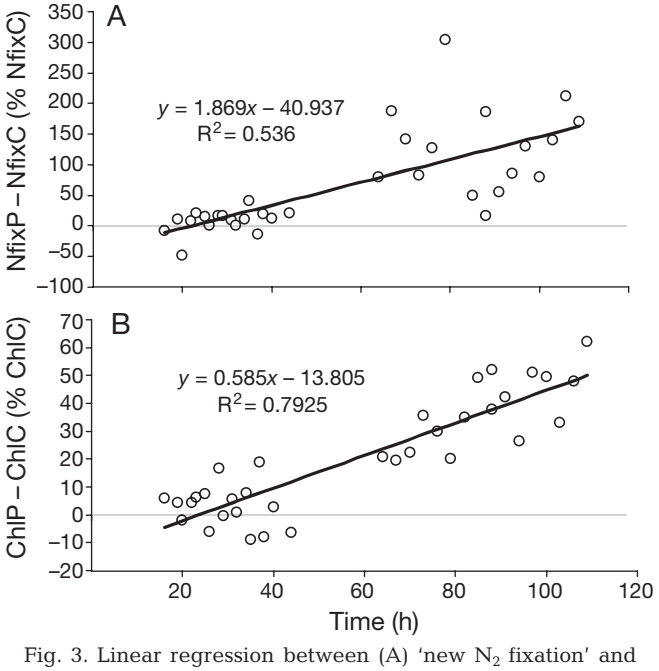

Fig. 3. Linear regression between (A) 'new N_2 fixation' and time; and (B) 'new chl *a*' and time, in response to P. Data from 4 enrichment experiments were pooled; *y*-axis unit is the difference between P treatment (suffix P) and control (suffix C) (% of control)

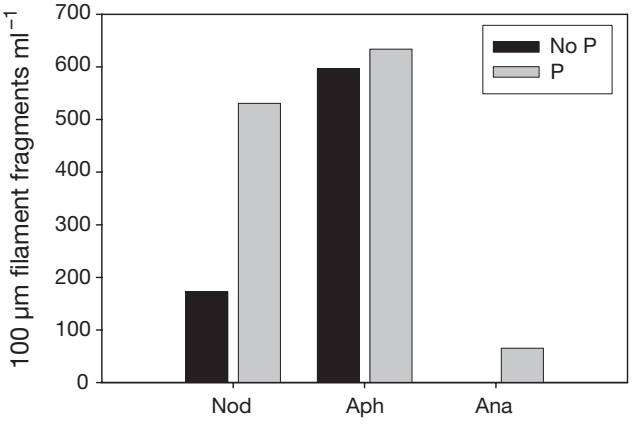

Fig. 4. *Nodularia spumigena* (Nod), *Aphanizomenon* sp. (Aph) and *Anabaena* spp. (Ana). Abundances of diazotrophic cyanobacteria at the end of diel Expt 03B. No P: control treatment; P: P added

A culture experiment was carried out with a *Nodularia spumigena* strain to investigate its diel cycle in N_2 and CO_2 fixation and abundances and motility of nitrogenase proteins, in order to compare these with the field data (Fig. 7). Carbon fixation was tightly coupled with light, while N_2 fixation continued throughout the diel cycle, albeit at reduced rates in the dark. The N_2 fixation rates in the dark were 21 to 45% of maximum rates in the light. Both Fe and MoFe proteins remained present throughout the diel cycle, with no detectable changes in abundance or MW. The MoFe protein from *N. spumigena* cultures had a weak binding capacity with the antibody and resolved as 3 bands, while the MoFe protein from *Anabaena aphanizomenoides* and *Anabaenopsis* sp. (both isolated from the Neuse River Estuary, North Carolina) resolved as 2 bands and had a much stronger binding capacity with the antibody (data not shown). DIC concentration gradually decreased during the day, but by the following morning had returned to the same level as the morning before.

DISCUSSION

The phosphorus effect on N_2 fixation and chl *a* was striking and consistent in diel experiments that started 64 and 88 h after the P addition (Expts 03B and 02B, respectively). When all the data were pooled, it became apparent that P started to affect the population growth and N_2 fixation rates earlier than 64 h of P addition (Fig. 3). Chl *a* concentrations and cyanobacterial numbers paralleled N_2 fixation rates; therefore, it was not clear whether P stimulated growth, N_2 fixation, or both. Potentially, P could directly enhance nitrogenase gene mRNA copy numbers and consequently N_2 fixa-

tion rates per cell. At 109 h, 'new N_2 fixation' (N_2-fixation increase in response to P) was showing signs of reaching a plateau, but both 'new' N_2 fixation and chl *a* concentration continued to increase 4.5 d after the P amendment. It is likely that the continuously increasing chl *a* partially represents production from recycled nitrogen that became available to the non-diazotrophic portion of the community from cyanobacterial exudates. The time relationship of new N_2 fixation and growth in response to P is useful when estimating the potential for enhancement of N_2 fixation in the Baltic Sea after a P pulse, typical of upwelling or other mixing events. In 2002 there was on average a doubling (1.1-fold increase) in N_2 fixation in the period 88 to 109 h after P addition. In 2003, there was on average a 2.4-fold increase in N_2 fixation 64 to 88 h after P addition. At individual time points, differences between the P treatment and control were even higher. In contrast, the chl *a* concentrations had a lower slope value for increase under P amendment than did N_2 fixation rates, suggesting that the increase in chl *a* was less than the increase in N_2 fixation. Taken together, the data suggest there is a potential for up to a 300% (3-fold) increase in N_2 fixation and up to a 62% (0.6-fold) increase in chl *a* in Baltic Sea cyanobacterial blooms within 4.5 d in response to a single, saturating P pulse.

While only few studies have measured cyanobacterial N_2 fixation rates in response to P in the Baltic Sea, more data exist on the effects of P on their growth. In nutrient addition experiments and mesoscale field studies, P often stimulated growth of N_2-fixing cyanobacteria; however, other limitations were also apparent (Wallström et al. 1992, Kononen et al. 1996, Stal et al. 1999, Moisander et al. 2003, Vahtera et al. 2005). In a Baltic Sea mesocosm experiment, *Nodularia spumigena* growth rates were stimulated from 9.0 to 3.6 d^{-1} when P was added over a 14 d period (Wallström et al. 1992). In another mesocosm experiment with Northwest Baltic Proper N_2-fixing cyanobacteria, chl *a* increased 2- to 3-fold in response to P over a 25 d period (P amended every 4 d) with a linear regression similar to that obtained in this study (Rydin et al. 2002). Collectively, these studies suggest that P commonly limits both N_2 fixation and growth of cyanobacteria in the Baltic Sea. The magnitude of the *in situ* response to P shown in this study is of importance when estimating the impact of localized P pulses to existing cyanobacterial populations. In addition to P limitation, iron limitation and sulfate inhibition were previously proposed as controls of N_2 fixation in Baltic Sea cyanobacteria (Stal et al. 1999, Rydin et al. 2002).

In this study, the peak in nitrogenase activity in the Baltic Sea occurred between 09:00 and 15:00 h, which is similar to that observed in some previous studies (Vuorio et al. 1978, Evans et al. 2000) but at an earlier time of the

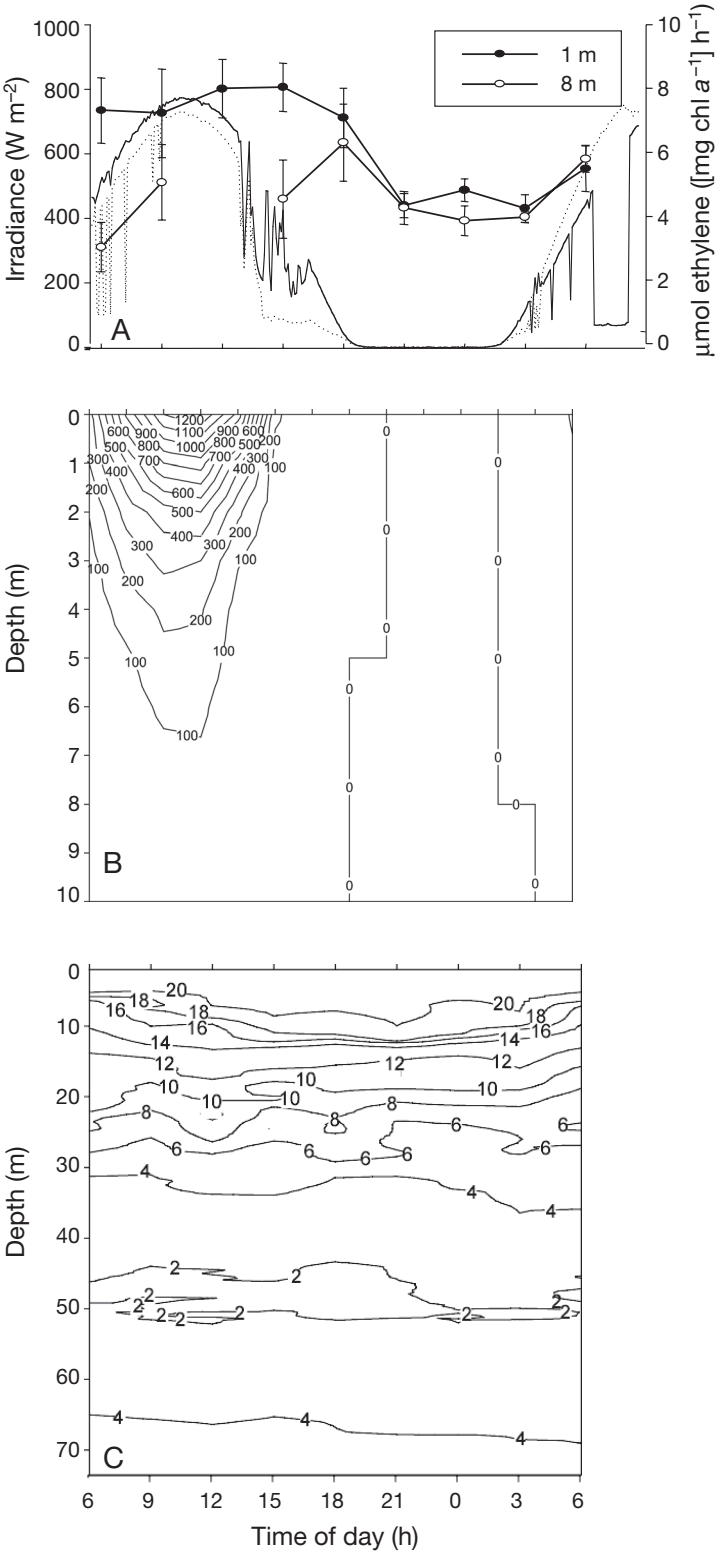

Fig. 5. Anchor station (An03) on 24–25 July 2003. (A) N_2 fixation (µmol ethylene [mg chl a^{-1}] h^{-1}) (circles) measured from water sampled from 1 and 8 m depths. Daily incident irradiance (W m^{-2}) measured on each side of the ship (solid and dotted lines with no symbols); (B) irradiance in the water column (µmol m^{-2} s^{-1}); and (C) temperature (°C) in the water column

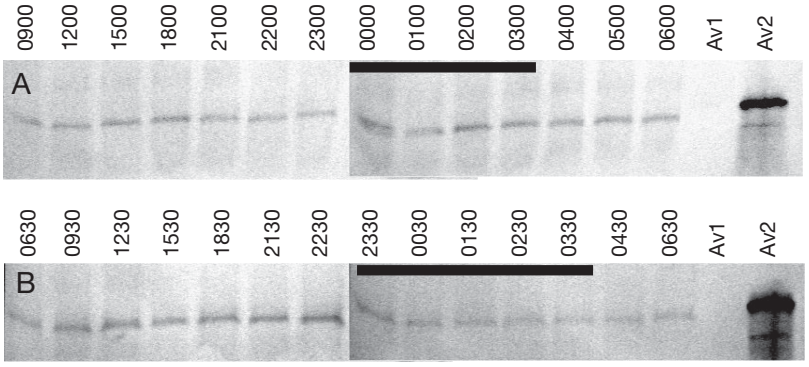

Fig. 6. Immunoblots from diel experiments in the Baltic Sea. (A) 15–16 July 2003, Cubitainer sampling, control treatment (water sampled 14 July 2003) (Expt 03A); (B) 24–25 July 2003, rosette sampling from 8 m depth (Expt An03). Av1: dinitrogenase (MoFe protein) from *Azotobacter vinelandii*; Av2: dinitrogenase reductase (Fe protein) from *A. vinelandii*. Sampling times (h) are shown at the top of each lane. Black bar indicates the dark period

day than reported by Gallon et al. (2002). These differences may partially reflect cloudiness during the days of measurement. N_2 fixation continued throughout the dark period, as reported in other studies of the Baltic Sea in which the dominating genus was mostly *Aphanizomenon* (Vuorio et al. 1978, Stal & Walsby 1998, Evans et al. 2000, Gallon et al. 2002). *Nodularia spumigena* and *Aphanizomenon* sp. fixed N_2 in the dark at 68 to 97% and 31 to 33% of maximum rates in light, respectively (Evans et al. 2000). In this study, the N_2 fixation rates for *N. spumigena* culture were slightly lower, 21 to 45% of maximum rates in the light. In field samples, dark fixation rates ranged from 26 to 61% of maximum rates in the light. These rates are higher than is typical of heterocystous cyanobacteria. In identical culture experiments, and under a 15:9 h light:dark cycle, N_2 fixation in the heterocystous cyanobacteria *Anabaena aphanizomenoides* and *Anabaenopsis* sp. isolated from the Neuse River Estuary was significantly more reduced in the dark (data not shown). In the *N. spumigena* cultures from this study, CO_2 and N_2 fixation continued at more or less constant rates during the light period, exhibiting no lag periods or exhaustion of resources for either process. *Nodularia spumigena* may be able to store energy in the form of carbohydrates more effectively than other heterocystous cyanobacteria, allowing it to continue N_2 fixation at high rates throughout the dark period (Evans et al. 2000). Alternatively, it may be able to utilize other mechanisms for energy generation in the dark.

Nitrogen fixation rates at the anchor station at 8 m depth were 45 to 105% of the rates in the surface water, and were detectable throughout the night. Interestingly, the diel pattern at 8 m depth was still present even though irradiance at this depth was only 2 to 4% of the surface irradiance, varying between 0.3 and 61 µmol m^{-2} s^{-1} during the day (06:00 to 23:00 h) (Fig. 5A). *Aphanizomenon* sp. was present at this station, whereas *Nodularia spumigena* was not detected in microscopic observations. The peak in nitrogenase activity lagged behind the peak in irradiance intensity at the anchor station, providing evidence for light dependence of N_2 fixation in *Aphanizomenon* sp. The anchor station experiment demonstrated the ability of natural populations of *Aphanizomenon* sp. to continue to fix N_2 at a constant rate long after irradiance levels are drastically reduced, indicating a reliance on carbohydrate stores generated during the day.

In both the field samples (including the anchor station at which *Aphanizomenon* sp. was the only diazotroph detected) and *Nodularia spumigena* cultures, dinitrogenase reductase (Fe protein) and dinitrogenase (MoFe protein) (*N. spumigena* cultures only) remained present throughout the diel cycle, and no apparent or consistent changes in intensities or MW were observed. These results contrast those of another Baltic Sea study, in which a reduction in the Fe protein intensity was reported during the night (Gallon et al. 2002). Some diazotrophs, including some cyanobacteria, regulate nitrogenase activity in response to environmental factors such as NH_4^+ or darkness through structural modifications of the Fe protein (Ludden & Burris 1978, Zehr et al. 1993, Nordlund 2000). This modification often occurs through ADP-ribosylation and appears as a slower migrating, inactive form of the Fe protein in SDS-PAGE. The lack of change in motility of the nitrogenase proteins during reduced N_2-fixation activities in the dark suggests that Baltic Sea *N. spumigena* and *Aphanizomenon* sp. do not use size modification as a mechanism to regulate their nitrogenase activity in response to darkness, or that the modification is too labile to be detected. The absence of this mechanism in these heterocystous cyanobacteria is supported by a prior study with *Anabaena variabilis* in which the ribosylation pathway enzymes were not found to be functional and a secondary band was not present (Durner et al. 1994). Furthermore, ADP ribosylation has not been found in all non-cyanobacterial diazotrophs studied. For example, no size modification of the Fe protein was observed for *Rhodobacter sphaeroides* in response to either NH_4^+ or darkness (Yoch et al. 1988).

Most environmental nitrogenase enzyme studies have focused on the Fe protein, while the MoFe protein has received considerably less attention. In this study, we obtained novel information on the MoFe protein of *Nodularia spumigena*. Structure of the Fe protein is relatively conserved among diazotrophs as

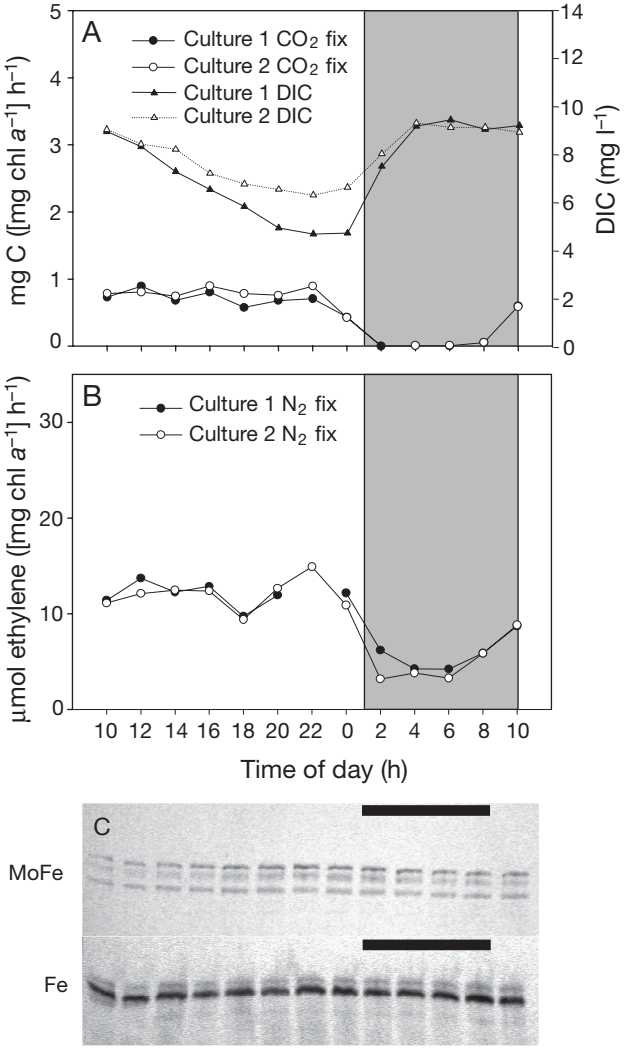

Fig. 7. *Nodularia spumigena*. (A) CO_2 fixation (^{14}C incorporation) and dissolved inorganic carbon concentration (DIC); (B) N_2 fixation (acetylene reduction); and (C) immunoblotting of the 2 nitrogenase proteins (Fe, dinitrogenase reductase; MoFe, dinitrogenase) from the 15:9 h light:dark diel experiment. The *N. spumigena* strain was cultivated in duplicate cultures (Cultures 1 and 2). Darkened areas (A,B) and bars (C) indicate the time period when cultures were in darkness. Incubations that started at midnight were kept in light in the first hour, and in darkness in the second hour

evidenced by the high efficiency by which the antibody against the *Azotobacter vinelandii* (γ-Proteobacterium) Fe protein binds the Fe protein from cyanobacteria. However, the observed differences in binding capacity and mobility of the MoFe protein between *N. spumigena* and 2 other heterocystous cyanobacteria (*Anabaena aphanizomenoides* and *Anabaenopsis* sp., data not shown) suggest that MoFe proteins in these cyanobacteria have important structural differences that require clarification. The data also revealed that the MoFe protein in *N. spumigena* was not structurally modified or subject to changes in abundance in response to darkness. Taken together, the data suggest that diel patterns of N_2 fixation in *N. spumigena* and *Aphanizomenon* sp. are either under transcriptional or posttranslational control over the diel cycle, by a mechanism that does not significantly affect the integrity of either Fe or MoFe (in *N. spumigena*) proteins. The ability to keep fixing N_2 at relatively high rates and maintain integrity of the nitrogenase proteins in the dark in Baltic Sea cyanobacteria may play a role in their ecological success in this environment. This ability allows these cyanobacteria to maximize their N_2 fixation and growth during periodic P pulses to the euphotic layer.

Cyanobacterial blooms have been occurring in the Baltic Sea for thousands of years (Bianchi et al. 2000), but recently the blooms appear to have intensified. Long-term data sets suggest that phosphate concentrations are increasing in some surface and deep waters, while they are decreasing or remaining constant in others (Rahm & Danielsson 2001). Yet there is a net input of approximately 32 000 t P yr^{-1} into the Baltic Sea (Wulff et al. 2001). The sediments and bottom waters in the Baltic Sea continue to serve as a rich source of P that, under conditions of bottom water anoxia enhanced by eutrophication, maintains the potential to support future cyanobacterial blooms.

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