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Inorganic phosphorus enrichments in Baltic Sea water have large effects on growth, carbon fixation, and N_2 fixation by *Nodularia spumigena*

Malin Olofsson[1,*], Jenny Egardt[2], Arvind Singh[3], Helle Ploug[1]

[1]Department of Marine Sciences, University of Gothenburg, Box 460, 405 30 Göteborg, Sweden
[2]Department of Biological and Environmental Sciences, University of Gothenburg, Box 461, 405 30 Göteborg, Sweden
[3]Geosciences Division, Physical Research Laboratory, Navrangpura 380 009, Ahmedabad, India

ABSTRACT: Two strains of the filamentous N_2-fixing cyanobacterium *Nodularia spumigena* were inoculated separately in Baltic Sea water, and their growth, carbon (C)-fixation, and N_2-fixation rates were monitored during a 21 d laboratory experiment. Low amounts of P (1 µM final concentration) were added to otherwise un-amended Baltic Sea water (<0.5 µM P). Exponential growth was stimulated under P-enriched conditions, indicating that the natural Baltic Sea water contained all residual nutrients essential for growth, and that *N. spumigena* was limited by P. The molar ratio of C to N_2 fixation was >15 when ammonium was present at concentrations >5 µM on Day 0. This ratio was significantly different between the 2 strains, which indicated variable affinity for ammonium. It decreased in both strains as the ammonium concentration and C assimilation decreased and N_2 fixation increased during the experiment. After 7 d, C- and N_2-fixation rates covaried with a relatively stable C:N fixation ratio close to or below the C:N ratio of cells (range: 4.7 to 8.6). The C:N cellular ratio and the specific N_2 fixation varied significantly between strains, emphasizing the variability of eco-physiology between strains within the same species. The average growth rate during the experiment, the accumulated biomass, and the total N_2-fixation rate were significantly higher under P-enriched compared to P-limited conditions in both strains. Hence, summers with high influx of P may stimulate growth, CO_2 sequestration, and N_2 fixation by *N. spumigena* in the Baltic Sea.

KEY WORDS: Heterocystous cyanobacteria · Baltic Sea · Nutrient limitation · Carbon sequestration · N_2 fixation · Stable isotopes

INTRODUCTION

Atmospheric dinitrogen (N_2) fixation by aquatic cyanobacteria provides 'new' nitrogen (N) to N-limited ocean regions, where it drives the carbon sequestration. New N can be derived from upwelling events, river runoff, atmospheric deposition, and N_2 fixation. One of the world's largest estuaries is the Baltic Sea, where N_2 fixation contributes with a yearly import of N up to the size of the entire riverine load (480 Gg N yr^{-1}), and twice the atmospheric deposition (about 200 Gg N yr^{-1}) (Larsson et al. 2001, Wasmund et al. 2001, Moisander et al. 2007). Using stable isotope tracers and nanoscale secondary ion mass spectrometry (nanoSIMS), it has been demonstrated that diazotrophic cyanobacteria in the Baltic Sea can grow solely with N_2 fixation as the N source (Ploug et al. 2010, 2011, Zakrisson et al. 2014). Phosphorus (P) is the most significant growth-limiting nutrient for N_2-fixing cyanobacteria in the Baltic Sea (Stal et al. 1999, Moisander et al. 2003, 2007, Rahm & Danielsson 2007). P is required for e.g. cellular synthesis of nucleic acid, membrane phospholipids, ATP, and NADPH, and orthophosphate is the most biologically

*Corresponding author: malin.olofsson@marine.gu.se

available dissolved form (Shan et al. 1994). Anthropogenic P inputs to the Baltic Sea have increased during the past century and have been suggested to positively affect the cellular growth of diazotrophic cyanobacteria (Finni et al. 2001, Kahru & Elmgren 2014).

The Baltic Sea is stratified by a halocline. The orthophosphate concentration in the euphotic zone of the Baltic Proper varies annually between 0.1 and 1.0 µM, and occasionally decreases to 0.01 µM (Nausch et al. 2004, data from the Swedish Meteorological and Hydrological Institute [SMHI]). The supply of dissolved P in the euphotic zone originates from land sources, re-mineralization of organic matter, or from water below the halocline through e.g. eddy diffusion, internal waves, and episodic upwelling events in the Baltic Sea (Larsson et al. 2001). Orthophosphate is also bound to iron oxides in the sediment, and is released to the water column during anoxia in the sediments (Vahtera et al. 2007a, Conley et al. 2009).

Prior to the summer bloom of diazotrophic cyanobacteria, the yearly spring bloom of diatoms removes most dissolved inorganic nitrogen (DIN) and orthophosphate from the surface water (Larsson et al. 2001). Low DIN concentrations provide a niche for the summer blooms of N_2-fixing filamentous cyanobacteria, dominated by *Nodularia spumigena*, *Dolichospermum* sp. (formerly *Anabaena* sp.; Wacklin et al. 2009), and *Aphanizomenon* sp. (Lehtimäki et al. 1997, Bianchi et al. 2000, Hajdu et al. 2007). These N_2-fixing cyanobacteria have an advantage over non-N_2-fixing phytoplankton during the N-limited summertime in the Baltic Proper (Granéli et al. 1990). *N. spumigena* is potentially toxic (Sellner 1997), and can appear in high abundances, forming accumulations at the surface of the Baltic Sea (Pitkänen & Tamminen 1995, Degerholm et al. 2006, Ploug 2008, Mohlin & Wulff 2009).

N_2 fixation by a Baltic Sea cyanobacterial community (*Aphanizomenon* sp. and *N. spumigena*) has been shown to be highly sensitive to P availability; a single pulse of P enhanced gross N_2 fixation measured by acetylene reduction assay (ARA) up to 3-fold within 4.5 d (Moisander et al. 2007). The net C:N fixation ratio has been shown to be close to the Redfield ratio (C:N = 6.6), and ammonium (NH_4^+) was released to the ambient water in field samples of *N. spumigena* and *Aphanizomenon* sp. during late summer when orthophosphate concentrations are low in the Baltic Sea (Ploug et al. 2010, 2011). Hence, C and N_2 fixation and NH_4^+ release may be closely coupled to P limitation in *N. spumigena.*

Stable isotope tracer incubations combined with SIMS or nanoSIMS analysis have demonstrated a considerable variation of C- and N_2-fixation rates among single cyanobacterial cells within the same field population and in cultures (Finzi-Hart et al. 2009, Mohr et al. 2010, Ploug et al. 2010, 2011, Foster et al. 2013, Svedén et al. 2015). Furthermore, significant intraspecific variations of growth and pigment composition have been demonstrated in a culture study of *N. spumigena* (Wulff et al. 2007). Most culture studies have focused on only one strain and were performed with high nutrient concentrations (orthophosphate: ca. 2–200 µM) in f/2 or Z8 medium, which poorly reflect field conditions (Degerholm et al. 2006, Moisander et al. 2007, Vahtera et al. 2007b, Vintila & El-Shehawy 2007, Mohlin & Wulff 2009). We therefore lack insight into how different strains behave at low nutrient concentrations, similar to those occurring in the field.

Our aim with this study was to test the impact of P limitation on C and N_2 fixation and cellular growth in 2 strains of *N. spumigena* under low nutrient conditions similar to those occurring in the Baltic Sea during summer. We hypothesized that even small pulses of P would increase cellular growth and C and N_2 fixation in *N. spumigena*, but not necessarily to the same extent in different strains.

MATERIALS AND METHODS

Cultures, experimental setup, and nutrient treatment

Two strains of the cyanobacterium *Nodularia spumigena* (KAC12 and KAC64), isolated from the Baltic Sea, were obtained from the Kalmar Algal Collection (KAC), Kalmar University, Sweden. Prior to the experiment, the 2 strains were acclimated to N-limited conditions, by growing them for 2 to 3 wk in f/2 medium without addition of combined nitrogen (–N) and with an initial P concentration of 50 µM. The f/2 medium (–N) was prepared from pre-filtered (0.7 µm pore size GF/F filter) and autoclaved seawater with a salinity of 6, collected at station B1 (Swedish Monitoring Program, SMP) in the Baltic Sea (58° 49′ N, 17° 38′ E). The Baltic Sea surface water was sampled during September 2012 when nutrients were low (nitrate+nitrite: 0.05 µM, ammonium: 0.13 µM, phosphate: 0.43 µM; SMP, SMHI) and kept in darkness at 4°C for 6 wk prior to the experiment, and then filtered (0.2 µm). In order to induce N_2 fixation and grow up sufficient biomass for the experiment, the strains were grown individually in f/2 (–N) in a temperature-controlled room at 18°C with a 16:8 h

light:dark cycle (light 07:00 to 23:00 h) (200 µmol photons m^{-2} s^{-1}, photosynthetically active radiation [PAR], measured in air with a LiCOR scalar irradiance sensor). The experiment was subsequently performed in un-amended Baltic Sea water.

For the experiment, 3 l of filtered (0.7 µm pore size GF/F filter) Baltic Sea water was added into each 4.5 l Plexiglas aquarium (Mohlin & Wulff 2009). Eight aquaria were inoculated with strain KAC12 and another 8 aquaria were inoculated with strain KAC64, all at a biovolume concentration in the aquaria of 1.24 mm^3 l^{-1} (14.56 m l^{-1} or 2.95×10^6 cells l^{-1}, see biovolume below) on Day 0, whereafter the aquaria were closed by a glass cover. On Day 0, the orthophosphate concentration was first measured and then enriched up to 1 µM in all aquaria, aiming at conditions above the average coastal summer concentration in the Baltic Sea of 0.5 µM (SMHI). Thereafter, orthophosphate was added every 2 to 4 d, aiming at a concentration above 1 µM (see 'Nutrient measurements' below) under P-enriched conditions. No orthophosphate was added after Day 0 under P-limited conditions. No other nutrients were added throughout the experiment, simulating nutrient-depletion during late summer in the Baltic Sea. The light intensity was 200 µmol photons m^{-2} s^{-1} (PAR) with a light:dark cycle of 16:8 h throughout the experiment. Each strain and treatment was replicated 4 times, i.e. the experiment was performed with 16 incubations in total in a thermo-constant room at 18°C. The aquaria were lightly bubbled with atmospheric air, to avoid C limitation. The dissolved inorganic carbon (DIC) measured with Trace gas-IRMS (UC Davis) did not decrease during the experiment (see 'Nitrogen- and carbon-fixation rates' below).

Biovolume and heterocyst frequency

Before each sampling, the water was lightly stirred with the sampling tube (or pipette tip, new for each treatment), to accomplish homogeneity. On Days 0, 2, 5, 7, 9, 11, 14, 16, 19, and 21, 4 ml were sampled from each aquarium, preserved with alkaline Lugol's solution, and stored in the dark until microscopic analysis. One ml of well-mixed sample was added to a Sedgewick-Rafter chamber to analyze biovolume (mm^3 l^{-1}). Trichome length and width were measured in 60 squares (1 square in the counting chamber = 1 µl), aiming at a minimum of 30 observed filaments, and a mean width together with the total trichome length were used to calculate the biovolume (mm^3 l^{-1}), assuming a cylindrical trichome shape (filament length × π × radius^2). In addition, heterocyst frequency (heterocysts $cell^{-1}$) was calculated based on counting of heterocysts in the same 60 squares.

Nutrient measurements

Orthophosphate (Strickland & Parsons 1972) and ammonium (Holmes et al. 1999) concentration was measured on Days 0, 2, 5, 7, 9, 14, 16, 19, and 21 with a Turner Trilogy fluorometer. One ml of orthophosphate (3 mM) was added to all aquaria (containing 3 l of sea water) on Day 0, and thereafter the P-enriched aquaria received 1 ml (3 mM) of orthophosphate every 2 to 4 d during the experiment, aiming at a concentration >1 µM. On sampling days when the orthophosphate concentration was already above 1 µM, no additional orthophosphate was added. No orthophosphate was added to the P-limited aquaria after Day 0.

Nitrogen- and carbon-fixation rates

C- and N_2-fixation rates were measured by incubation with ^{13}C and $^{15}N_2$ tracers, respectively (Montoya et al. 1996, Klawonn et al. 2015). On Days 0, 7, 14, and 21, 250 ml from each aquarium were incubated with spikes of ^{13}C (98 atom% ^{13}C; Sigma) resulting in 12.5% final labeling and $^{15}N_2$ (Sigma) (2% final labeling) in gas-tight serum bottles. The labeling % of $^{15}N_2$ to $^{14}N_2$ was measured by membrane inlet mass spectrometry (MIMS) at MPI (Bremen, Germany), while the labeling % of ^{13}C to ^{12}C-bicarbonate as well as total DIC were measured by trace gas-IRMS at the UC Davis Stable Isotope Laboratory (Davis, California, USA). The serum bottles with samples were incubated on a horizontal shaker for 6 h (18°C, 200 µmol photons m^{-2} s^{-1}), from 09:00 to 15:00 h. The incubation was terminated by filtering samples onto pre-combusted (450°C) GF/F filters (Montoya et al. 1996). The filters were dehydrated at 60°C for 8 h and de-calcified by HCl smoke in a desiccator overnight, and thereafter packed into tin cups. The samples were analyzed for incorporation of $^{15}N_2$ and ^{13}C by elemental analysis isotope ratio mass spectrometry (EA-IRMS) at the UC Davis Stable Isotope Laboratory. The standards were glutamic acid, peach leaves, and nylon, and the precision of measurements was 0.015%. The total net C assimilation (nmol C l^{-1} h^{-1}) to total net N_2 fixation (nmol N l^{-1} h^{-1}) ratio was calculated from the isotope enrichments and the particulate organic carbon (POC) and particulate organic nitrogen (PON), measured by EA-IRMS

(Montoya et al. 1996). The N-specific growth rates (h^{-1}) through N_2 fixation and C-specific growth rates (h^{-1}) through the C-assimilation rate, hereafter referred to as specific N_2-fixation rate (h^{-1}) and specific C-assimilation rate (h^{-1}), were calculated to compare the rates independent of variable biomass in the different incubations.

Commercial $^{15}N_2$ gas occasionally can be contaminated by ^{15}N-labeled ammonium and nitrate (Dabundo et al. 2014). We used one gas bottle for the whole experiment. The $^{15}N_2$-labeling % was low (2 %, equal to ca. 10 µM), and contaminated $^{15}NH_4^+$ may be 0.0006 to 0.030 µM if at all (Dabundo et al. 2014). This source is small relative to the total N demand by the high *N. spumigena* biomass during our 6 h incubations (<8.5 µM N; Fig. 5). We therefore do not consider a potential impurity of the $^{15}N_2$ gas to significantly bias our measured N_2-fixation rates.

Statistical analyses

Data were analyzed using 2-way ANOVA in SPSS software (PASW Statistics ver. 20, IBM) for Day 21, testing for differences between treatments (P-limited and P-enriched) and strains (KAC12 and KAC64), and also for possible interaction effects. The variables tested were growth rate (h^{-1}) from Day 0 to Day 21, biovolume, heterocyst frequency, specific N_2-fixation rate (h^{-1}), specific C-assimilation rate (h^{-1}), total N_2-fixation rate (nmol N l^{-1} h^{-1}), total C-assimilation rate (nmol C l^{-1} h^{-1}), cellular C:N ratio, and C:N fixation ratio. In addition, 2-way ANOVA was also run for specific C-assimilation rates (h^{-1}) and specific N_2-fixation rates (h^{-1}) on Days 7 and 14 for early differences in fixation rates, with respect to treatment and strain differences. It is challenging to culture organisms under naturally low nutrient conditions, and some inocula did not start to grow. Independent of strain and P treatment, the inoculum never started to grow in 4 cases out of 16 (2 P-enriched and 2 P-limited). These were excluded in all statistical tests. Significant differences were set to $p < 0.05$. Levene's test was used to check for homogeneity. In addition, Student's *t*-tests were also used (Microsoft Excel for Mac) to find specific differences between and within sampling days, and *F*-tests were used to check for homogeneity. When running the *t*-test for a particular parameter, strains were pooled when no significant difference was found, testing for treatment effects, and vice versa. Prism (GraphPad Software, ver. 6) was used to calculate growth rate (h^{-1}) by fitting exponential growth curves and extract the *k* value from the non-linear equation $N = N_0 \times \exp(kt)$, where N equals biovolume (mm^3 l^{-1}) at Time t, N_0 is the biovolume at Time 0, and k is the growth rate constant.

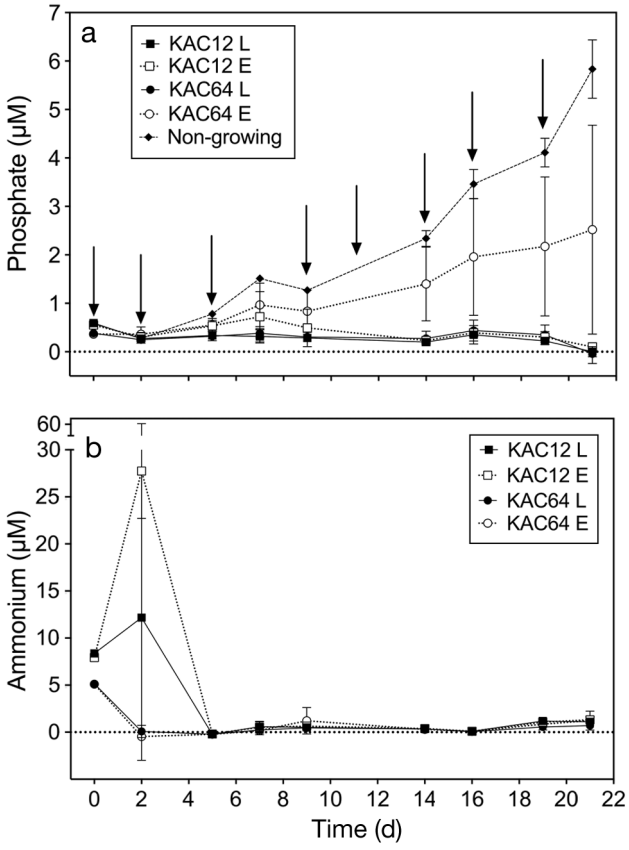

Fig. 1. (a) Phosphate and (b) ammonium concentrations (±SD) during the experiment with *Nodularia spumigena* strains KAC12 and KAC64 under P-limited (L) and P-enriched (E) conditions (n = 2–4). Panel (a) also shows the P concentration in the non-growing replicates. Arrows represent the time-points when P was added under P-enriched conditions

RESULTS

Phosphate and ammonium

The daily average P concentration ranged between 0.1 and 2.5 µM from Day 0 to Day 21 under P-enriched conditions (Fig. 1a). In incubations with KAC12, the P concentration did not increase above 1 µM despite the repeated P additions, and the orthophosphate therefore appeared to be immediately assimilated by KAC12. On Day 21, the average P concentration was 0.1 µM even under P-enriched conditions. In incubations with KAC64, the P concentration increased to an average value of 2.5 µM towards the end of the experiment under P-enriched

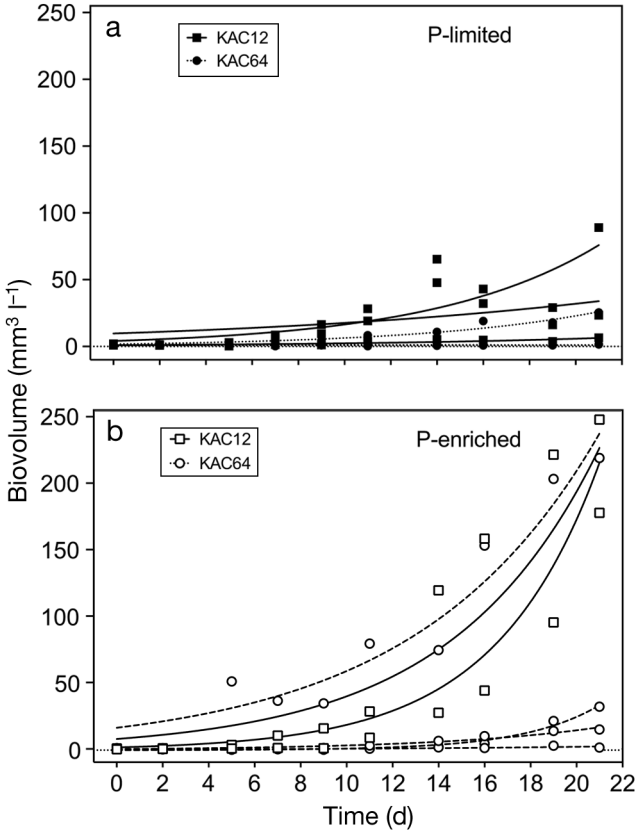

Fig. 2. Growth curves of *Nodularia spumigena* based on biovolume ($mm^3\ l^{-1}$) under (a) P-limited and (b) P-enriched conditions, and for the 2 strains (KAC12 and KAC64), n = 2–4, all replicates are shown

conditions. Under P-limited conditions, a low average concentration of P (<0.35 µM for KAC12 and <0.45 µM for KAC64) was measured from Day 2. At the end of the experiment, the average concentration of orthophosphate was below the detection limit (<0.04 µM) in P-limited conditions for both strains. The P concentrations measured in the 2 aquaria in which the inoculum never started to grow under P-enriched conditions are presented (Fig. 1a), showing the accumulation of P in the water with no cyanobacterial growth.

The average concentrations of ammonium (Fig. 1b) were 8 µM for KAC12 and 5 µM for KAC64 on Day 0, but after an increase on Day 2, the concentration decreased during the first week of the experiment. After 7 d, the average ammonium concentration was 0.3 µM under both P-enriched and P-limited conditions, and compared well to that occurring during summertime in the Baltic Sea (<0.3 µM, obtained from the SHARK database at the SMHI). The ammonium concentration increased from Day 7 to Day 21, to an average of 1 µM for both treatments.

Biovolume, growth rate, and heterocyst frequency

The average biovolume ($mm^3\ l^{-1}$) of *Nodularia spumigena* increased in both treatments and strains throughout the experiment, as shown by the exponential growth curves in Fig. 2a,b. However, a significantly higher average biovolume was measured on Day 21 under P-enriched compared to P-limited conditions ($p = 0.026$, ANOVA, n = 2–4, Table 1). At the end of the experiment, average cells l^{-1} had increased from 2.9×10^6 on Day 0 to 5.6×10^7 and 2.6×10^8 on Day 21, under P-limited and P-enriched conditions, respectively. The exponential growth rate (h^{-1}) was significantly higher under P-enriched as compared to P-limited conditions ($p = 0.041$, ANOVA, n = 2–4, Table 1). The average growth rate (h^{-1}) also showed significantly higher rates under P-enriched conditions compared to P-limited conditions when pooling the strains ($p = 0.029$, n = 6, Table 2), whereas no difference between strains was found when pooling the treatments. There was no significant difference between the 2 strains (KAC12 and KAC64) on any sampling day, when pooling the treatments in a *t*-test, or in the ANOVA when compared separately on Day 21.

The number of heterocysts $cell^{-1}$ (Fig. 3a) showed a significant difference between treatments on Day 21 ($p = 0.023$, ANOVA, n = 2–4, Table 1), and the highest heterocyst frequency was found under P-enriched conditions. However, no correlation was found between heterocyst frequency and specific N_2-fixation rate (h^{-1}) (Fig. 3b).

Nitrogen- and carbon-fixation rates

As the ammonium concentration decreased, the specific N_2-fixation rate increased and the specific C-assimilation rate (h^{-1}) decreased for both treatments during the experiment (Fig. 4a–d). The average specific C-assimilation rate (h^{-1}) during the experiment showed significantly higher values under P-enriched compared to P-limited conditions, when pooling the strains using a *t*-test ($p = 0.028$, n = 6, Table 2). However, no significant difference was found between treatments for average specific N_2 fixation during the experiment (Table 2), or on Days 7 and 21, for specific C assimilation and specific N_2 fixation in the ANOVA (Table 1). On Day 14, a significant difference was found between treatments for specific C-assimilation rate (h^{-1}), with significantly higher rates under P-enriched than under P-limited conditions ($p = 0.048$, ANOVA, n = 2–4, Table 1).

Table 1. Two-way ANOVA with significance values for treatment effects (P-limited and P-enriched conditions), differences between *Nodularia spumigena* strains (KAC12 and KAC64), and interaction effects. Asterisks indicate significant effects ($p < 0.05$); the asterisk in parentheses indicates a significant effect with a *t*-test for strain KAC12

Dependent parameter	Source	df	*F*	p
Biovolume ($mm^3\ l^{-1}$), Day 21	Treatment	1	7.4680	0.026*
n = 2–4	Strain	1	4.5030	0.067
	Treatment × Strain	1	1.8280	0.213
	Residual	8		
Heterocyst frequency (heterocysts $cell^{-1}$), Day 21	Treatment	1	7.8430	0.023*
n = 2–4	Strain	1	0.001	0.982
	Treatment × Strain	1	2.3960	0.160
	Residual	8		
Growth rate, Day 0 to Day 21	Treatment	1	5.9190	0.0410*
n = 2–4	Strain	1	0.8229	0.3909
	Treatment × Strain	1	0.1176	0.7405
	Residual	8		
Specific C-assimilation rate (h^{-1}), Day 7	Treatment	1	0.0989	0.7666
n = 2–3	Strain	1	4.0719	0.0996
	Treatment × Strain	1	0.3675	0.5708
	Residual	5		
Specific C-assimilation rate (h^{-1}), Day 14	Treatment	1	5.7539	0.0476*
n = 2–4	Strain	1	1.4401	0.2692
	Treatment × Strain	1	0.5567	0.4756
	Residual	7		
Specific C-assimilation rate (h^{-1}), Day 21	Treatment	1	3.9420	0.0823(*)
n = 2-4	Strain	1	0.0961	0.7646
	Treatment × Strain	1	0.0095	0.9251
	Residual	8		
Specific N_2-fixation rate (h^{-1}), Day 7	Treatment	1	0.1871	0.6805
n = 2–4	Strain	1	1.2934	0.0114*
	Treatment × Strain	1	0.0246	0.8806
	Residual	6		
Specific N_2-fixation rate (h^{-1}), Day 14	Treatment	1	2.0589	0.1945
n = 2–4	Strain	1	0.9367	0.3654
	Treatment × Strain	1	0.6056	0.4619
	Residual	7		
Specific N_2-fixation rate (h^{-1}), Day 21	Treatment	1	2.9140	0.1262
n = 2–4	Strain	1	0.3973	0.5461
	Treatment × Strain	1	0.0007	0.9794
	Residual	8		
Cellular C:N ratio, Day 21	Treatment	1	2.1999	0.176
n = 2–4	Strain	1	0.197	0.699
	Treatment × Strain	1	1.3240	0.283
	Residual	8		
C:N fixation ratio, Day 21	Treatment	1	0.32	0.5874
n = 2–4	Strain	1	5.8620	0.0418*
	Treatment × Strain	1	0.097	0.7636
	Residual	8		

The total C-assimilation to total N_2-fixation rate (nmol C l^{-1} h^{-1} and nmol N l^{-1} h^{-1}) was correlated from Day 7 to Day 21 independent of P limitation (Fig. 5). The slope of 4.1 derived from the linear equation ($y = 4.104x - 0.0$, $R^2 = 0.74$) represents the average ratio during Days 7 to 21, when the N demand by the cyanobacteria was covered by N_2 fixation. A significantly higher rate of total N_2 fixation (nmol N l^{-1} h^{-1}) was found on Day 21 under P-enriched conditions as compared to P-limited when

Table 2. Growth rate (h^{-1}) calculated from the exponential growth curves in Prism, from Day 0 to Day 21, of *Nodularia spumigena* strains KAC12 and KAC64 under P-enriched and P-limited conditions. Average specific C-assimilation rate (h^{-1}) from Day 0 to Day 21 and the growth rate (h^{-1}) showed a significant difference between P-enriched and P-limited conditions (marked with *, $p < 0.05$), when strains were pooled in a *t*-test. The average specific N_2-fixation rate (h^{-1}) from Day 0 to Day 21 is also shown; significant differences ($p < 0.05$) between the 2 strains were found when treatments were pooled in a *t*-test (marked with a +). Values are given ± SD

	Growth rate (h^{-1})	Average specific C-assimilation (h^{-1})	Average specific N_2-fixation (h^{-1})
KAC12 P-enriched	0.0079 ± 0.0019*	0.0090 ± 0.0001*	0.0128 ± 0.0018+
KAC12 P-limited	0.0040 ± 0.0017	0.0069 ± 0.0007	0.0089 ± 0.0019+
KAC64 P-enriched	0.0068 ± 0.0039*	0.0084 ± 0.0016*	0.0073 ± 0.0028
KAC64 P-limited	0.0017 ± 0.0032	0.0065 ± 0.0018	0.0050 ± 0.0026

pooling the strains in a *t*-test ($p = 0.037$, $n = 6$, Table 3), while no significant effect was found between treatments for total C-assimilation rate (nmol C l^{-1} h^{-1}) when pooling the strains on Day 21 ($p = 0.066$, $n = 6$).

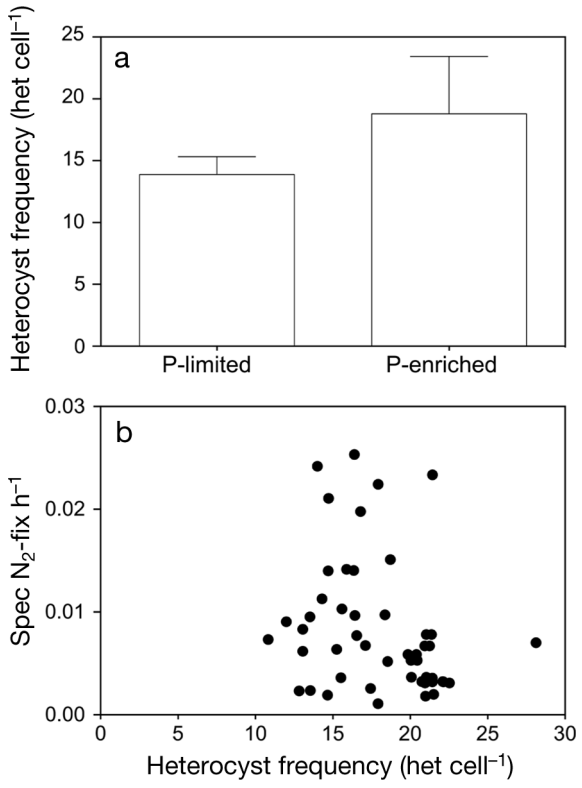

Fig. 3. (a) Heterocyst frequency (heterocysts $cell^{-1}$) in *Nodularia spumigena* on Day 21 under P-limited and P-enriched conditions, where a significant difference between treatments was found ($n = 6$). Error bars show standard deviation. (b) Specific N_2-fixation rate (h^{-1}) and heterocysts $cell^{-1}$ (no significant correlation was found)

Strain-specific C- and N_2-fixation rates

On Day 0, both the specific C-assimilation rate (h^{-1}) ($p = 0.052$, $n = 2$ for KAC12, 4 for KAC64; Table 3) and the specific N_2-fixation rate (h^{-1}) ($p = 0.014$, $n = 2$ for KAC12, 4 for KAC64), Table 3) showed a significant difference between strains in a *t*-test. In KAC12, the average specific C-assimilation rate (h^{-1}) decreased from Day 0 to Day 21 by a factor of 2.5 and 7.1 under P-enriched and P-limited conditions, respectively. In KAC64, these parameters decreased by a factor of 2.8 and 7.6 under P-enriched and P-limited conditions, respectively (Table 3). We found significantly higher specific C-assimilation rates (h^{-1}) under P-enriched conditions compared to P-limitation for KAC12 on Day 21 (*t*-test, $p = 0.035$, $n = 2–3$; Table 3). No significant difference between strains was found for specific C-assimilation rate (h^{-1}) on Days 7, 14, or 21 in the ANOVAs (Table 1). A significantly higher value of the average specific N_2-fixation rate (h^{-1}) during the experiment was found in KAC12 as compared to KAC64, when pooling the treatments using a *t*-test ($p = 0.027$, $n = 5–7$, Table 2). A significantly higher specific N_2-fixation rate was also found in KAC12 than in KAC64 on Day 7 in the ANOVA ($p = 0.011$, $n = 2–3$, Table 1), and when pooling treatments in a *t*-test ($p = 0.011$, $n = 2–3$, Table 3). However, no significant difference was found between the 2 strains for total N_2 fixation at any time (Table 3), partly because of large variations in total biomass when pooling treatments.

On Day 0, the C:N fixation ratio showed up to 5 times higher values compared to the cellular C:N ratio (Fig. 6a–d) when ammonium concentrations were high. The pattern was similar for both strains and treatments. There was no significant difference in biovolume between the 2 strains on Day 0. However, total C assimilation was significantly higher in KAC64 as compared to KAC12. Thus, the C:N fixation ratio by KAC64 (34) was significantly higher than that by KAC12 (15) on Day 0, since the N_2-fixation rate was lower for KAC64 compared to KAC12. From Day 0 until Day 21, the C:N fixation ratios decreased to 3.6 and 3.7 in KAC12 under P-limited and P-enriched conditions, respectively. In KAC64, it decreased to 2.6 and 2.9 under P-limited and P-enriched conditions, respectively. On Day 21, the C:N fixation ratio was significantly higher for KAC12

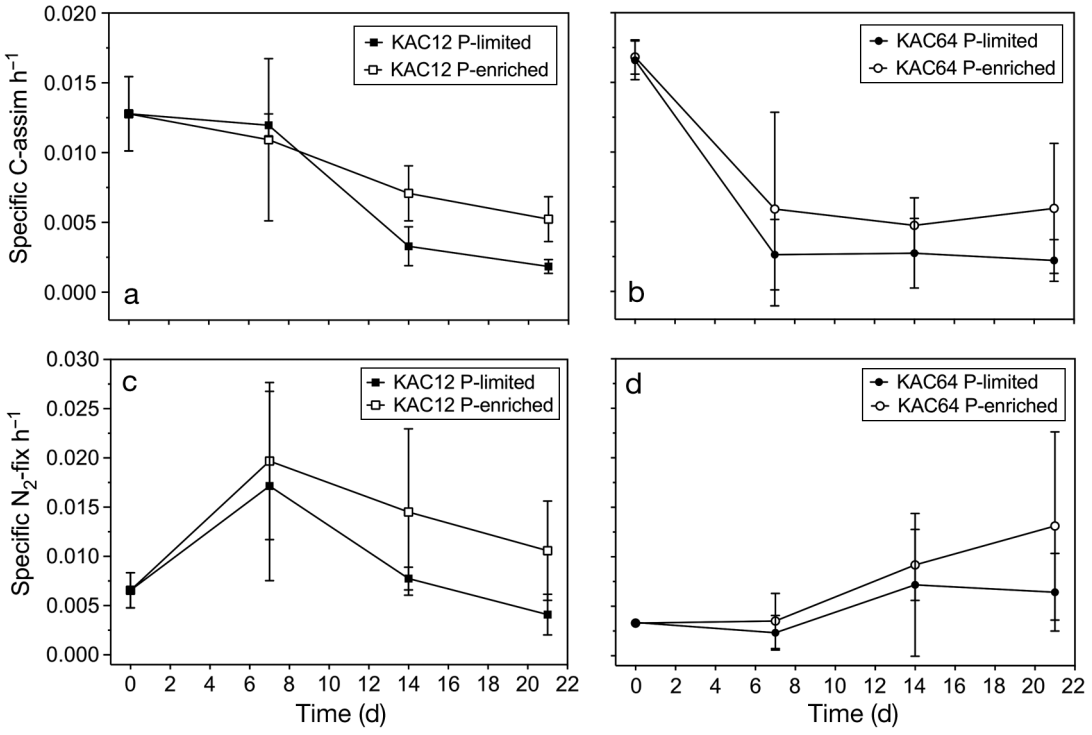

Fig. 4. (a,b) Specific C-assimilation rate (h^{-1}) and (c,d) specific N_2-fixation rate (h^{-1}) for *Nodularia spumigena* strains (a,c) KAC12 (n = 2–3) and (b,d) KAC64 (n = 2–4) under P-limited and P-enriched conditions. Error bars show standard deviation

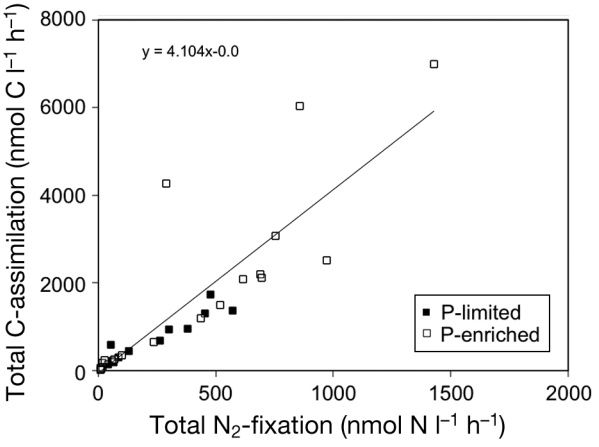

Fig. 5. Correlation between total C assimilation (nmol C l^{-1} h^{-1}) and total N_2 fixation (nmol N l^{-1} h^{-1}) from Day 7 to Day 21 for *Nodularia spumigena*. Closed (open) symbols show rates under P-limited (P-enriched) conditions. The equation of the line is $y = 4.104x$ ($R^2 = 0.74$), where the slope (4.1) equals the average ratio during Days 7 to 21

as compared to KAC64, independent of treatments (p = 0.042, n = 2–4, ANOVA, Table 1).

The cellular C:N (mol:mol) ratio (Fig. 6a,b) was 8.3 for KAC12 and 6.7 for KAC64 on Day 0, showing a significant difference between strains when using a *t*-test (p = 0.024, n = 4). From Day 0 to Day 7, the C:N ratio decreased in KAC12 while it increased in KAC64, ending up with a significantly higher C:N ratio in KAC64 under P-limited (8.7) compared to P-enriched (6.9) conditions on Day 21 (p = 0.020, n = 3–4), and compared to Day 0 (p = 0.002, n = 3–4). On Day 21, the C:N ratio in KAC12 was above the Redfield ratio (6.6) under both P-enriched (7.4) and P-limited (7.6) conditions. However, no significant difference was found for the cellular C:N ratio between strains or treatments on Day 21 in the ANOVA (Table 1).

DISCUSSION

Genome-transcriptome analysis has revealed complex systems for both inorganic and organic P uptake in *Nodularia spumigena* (strain:CCY9414) (Voß et al. 2013), and it has been experimentally demonstrated that *N. spumigena* can efficiently use both dissolved inorganic P (DIP) and dissolved organic P (DOP) as a P source (Vahtera et al. 2007b). The ambient DOP in the Baltic Sea water was not measured during our study, but was probably similar under P-limited and P-enriched conditions since we only added inorganic P in our treatments, and the community was only

Table 3. Specific C-assimilation rate (h^{-1}), specific N_2-fixation rate (h^{-1}), total C-assimilation rate (nmol C l^{-1} h^{-1}), and total N_2-fixation rate (nmol N l^{-1} h^{-1}) on Days 0, 7, 14, and 21. On Day 0, a significantly higher specific C-assimilation rate was found for *Nodularia spumigena* strain KAC64 as compared to KAC12 when pooling the treatments in a *t*-test. Specific N_2-fixation rates were significantly higher in KAC12 than in KAC64, when pooling the treatments, on Days 0 and 7. On Day 0, a significantly higher total C-assimilation rate was found for KAC64 as compared to KAC12 when pooling the treatments. On Day 21, a significantly higher rate for total N_2-fixation was found in P-enriched conditions compared to P-limited conditions when pooling the strains. Significant differences between treatments are marked with a * and between strains with a $^+$. Standard deviation is shown for all rates

	Day 0	Day 7	Day 14	Day 21
Specific C assimilation				
KAC12 P-enriched	0.0128 ± 0.0026	0.0109 ± 0.0058	0.0071 ± 0.0019	0.0052 ± 0.0016
KAC12 P-limited	0.0128 ± 0.0026	0.0119 ± 0.0008	0.0033 ± 0.0014	0.0018 ± 0.0005
KAC64 P-enriched	0.0168 ± 0.0012$^+$	0.0059 ± 0.0069	0.0047 ± 0.0019	0.0059 ± 0.0047
KAC64 P-limited	0.0168 ± 0.0012$^+$	0.0026 ± 0.0025	0.0027 ± 0.0025	0.0022 ± 0.0015
Specific N_2 fixation				
KAC12 P-enriched	0.0065 ± 0.0018$^+$	0.0197 ± 0.0079$^+$	0.0165 ± 0.0084	0.0105 ± 0.0050
KAC12 P-limited	0.0065 ± 0.0018$^+$	0.0171 ± 0.0096$^+$	0.0088 ± 0.0012	0.0041 ± 0.0021
KAC64 P-enriched	0.0033 ± 0.0002	0.0035 ± 0.0028	0.0105 ± 0.0036	0.0131 ± 0.0095
KAC64 P-limited	0.0033 ± 0.0002	0.0023 ± 0.0018	0.0082 ± 0.0072	0.0064 ± 0.0039
Total C assimilation				
KAC12 P-enriched	238.88 ± 57.57	1214.79 ± 1223.26	2799.47 ± 392.24	4247.19 ± 3885.19
KAC12 P-limited	238.88 ± 57.57	747.38 ± 668.87	1095.28 ± 809.16	323.84 ± 113.98
KAC64 P-enriched	433.28 ± 54.22$^+$	1558.32 ± 2351.22	1760.48 ± 2862.88	1386.72 ± 1011.36
KAC64 P-limited	433.28 ± 54.22$^+$	326.82 ± 373.64	497.65 ± 639.16	281.29 ± 349.05
Total N_2 fixation				
KAC12 P-enriched	15.56 ± 4.02	357.46 ± 365.38	863.14 ± 154.00	974.47 ± 643.22*
KAC12 P-limited	15.56 ± 4.02	263.18 ± 210.76	371.41 ± 268.88	91.42 ± 35.23
KAC64 P-enriched	12.78 ± 2.29	110.76 ± 153.54	294.65 ± 386.02	457.99 ± 321.37*
KAC64 P-limited	12.78 ± 2.29	31.09 ± 29.57	195.68 ± 261.57	104.08 ± 137.42

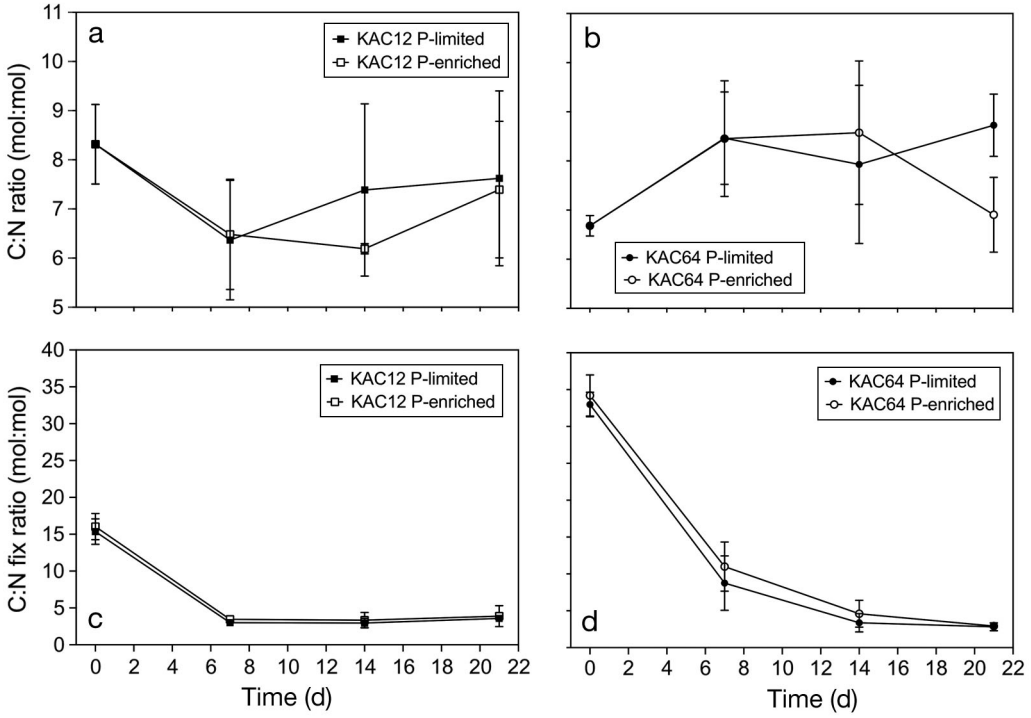

Fig. 6. (a,b) Cellular C:N ratio (mol:mol) and (c,d) the C:N fixation ratio (mol:mol) for *Nodularia spumigena* strains (a,c) KAC12 and (b,d) KAC64 under P-limited and P-enriched conditions (n = 2–4). Error bars show standard deviation

composed of *N. spumigena* and bacteria. Low inorganic P enrichments to Baltic Sea water stimulated exponential growth in *N. spumigena*, indicating that the natural Baltic Sea water contained all other residual nutrients essential for sustained growth during the 3 wk experiment.

The specific C- and N_2-fixation rates by *N. spumigena* were in a similar range as those measured by nanoSIMS in field samples of *N. spumigena* from the Baltic Sea and correspond to doubling times of 3 to 4 d (Ploug et al. 2011). At the end of our study, the maximum cell abundances were equal to those reported for the Baltic Sea in July (Hajdu et al. 2007, Klawonn 2015). This observation is presumably a result of a relatively large inoculum combined with a lack of competition from other organisms except bacteria during our 3 wk study. Photosynthesis by field populations of *N. spumigena* is saturated at a PAR intensity of 200 µmol photons m^{-2} s^{-1} (Ploug 2008). No indication of reduced growth rates due to self-shading occurred under P-enriched conditions because the cultures were very dilute compared to e.g. biomass accumulation in f/2 medium (<100-fold higher than that measured on Day 21 in our study). Culture studies of *N. spumigena* have previously demonstrated a significant reduction in cellular growth rate, a decreased number of heterocysts per vegetative cell, and reduced N_2-fixation rate measured by ARA in P-limited compared to P-enriched conditions (Degerholm et al. 2006, Mohlin & Wulff 2009). These studies, however, were performed with reference to high nutrient concentrations often used in cultures (f/2 or Z8 medium: up to 200 µM orthophosphate) and using ARA or heterocyst frequency as a proxy for N_2-fixation in one strain only (Degerholm et al. 2006, Moisander et al. 2007, Vahtera et al. 2007b, Mohlin & Wulff 2009). In our study, the sensitivity of growth and C- and N_2-fixation rates in 2 different *N. spumigena* strains were demonstrated at naturally low P concentrations (0.1 to 1.0 µM), which occur in the Baltic Sea during summer.

The heterocyst frequency in *Aphanizomenon* sp. is higher during early summer than in late summer, and it is negatively correlated to the C:P ratio of cells (Walve & Larsson 2007, Zakrisson & Larsson 2014). The high heterocyst frequency may compensate for temperature limitation of N_2-fixation by *Aphanizomenon* sp. during early summer when ambient inorganic P concentrations are still high (Svedén et al. 2015). Heterocyst frequency in Baltic Sea cyanobacteria has previously been used as a proxy for N_2 fixation (Mohlin & Wulff 2009). However, genomic-transcriptomic analysis of *N. spumigena* has indicated that heterocyst formation is poorly constrained by availability and usage of DIN (Voß et al. 2013). This is confirmed by experimental studies, which showed that heterocyst frequency was similar in *N. spumigena* grown under N-replete (nitrate) and N-limited (no combined N) conditions (Mohlin & Wulff 2009), and that heterocyst formation is poorly regulated by NH_4^+ usage (Vintila & El-Shehawy 2007). In the present study, the heterocyst frequency of *N. spumigena* was significantly lower under P limitation than under P-enriched conditions. The average heterocyst frequency decreased from 0.105 to 0.085 heterocysts $cell^{-1}$, and from 0.104 to 0.068 heterocysts $cell^{-1}$ under P-enriched and P-limited conditions, respectively, although N_2 fixation increased from Day 0 to Day 21. Hence, no direct correlation between heterocyst frequency and N_2 fixation was found, as also observed by Vintila & El-Shehawy (2007). Consequently, heterocysts can be present without NifH activity in *N. spumigena*, but under N-limited conditions, they may quickly up-regulate the NifH activity and the N_2-fixation rate as observed in this and other studies (Vintila & El-Shehawy 2007, Vintila et al. 2010).

Variations between cells and strains within the same species are prerequisite for evolution at a long time scale. At a shorter time scale, it can be an advantage for the overall population to exploit temporal and spatial variability in the natural environment, e.g. in coastal regions.

Our study showed significant differences between 2 strains of *N. spumigena* with respect to C:N fixation ratio when ammonium was present in the ambient water, C:N cellular quota, specific N_2 fixation, and capacity of P uptake. Hence, it is important to study more than one strain in order to quantify eco-physiological stress responses within a species.

In incubations with KAC64, P concentration increased to an average value of 2.5 µM towards the end of the experiment under P-enriched conditions. In contrast, the ambient P concentration did not increase despite repeated P additions in incubations with KAC12, which suggests that this strain efficiently assimilated the added P. The capacity to assimilate and possibly store P therefore apparently varied between the 2 strains. The more efficient P uptake by KAC12 relative to KAC64 co-occurred with significantly higher specific N_2 fixation by KAC12 relative to KAC64 (Table 2). However, significant effects of P limitation on growth were observed in both strains within 3 wk, suggesting that stored P was not sufficient to cover P demands for growth during 3 wk. On Day 21, the P concentration had decreased below 0.04 µM in 4 out of 6 aquaria under

P limitation. The cyanobacteria grown under P-limited conditions were pale and orange, and apparently lost pigments or changed the pigment composition during the experiment. Loss of photosynthetic pigments may be common among phytoplankton limited by macronutrients (Latasa & Berdalet 1994). In a previous culture study by Mohlin & Wulff (2009), a decrease in the pigment concentration was also observed under P limitation. Furthermore, they also demonstrated 15% reduction in cellular growth rate and 10% reduction in heterocysts per vegetative cell under P limitation. However, nitrate was added to the medium according to f/2 (ca. 800 µM) at the beginning in that study. Nitrate concentrations are <300 nM during summer in the Baltic Sea (SMP, SMHI), and thus of minor significance for cellular growth by *N. spumigena* in the field. In our study, the nitrate concentration was <50 nM when we sampled the water in the field. The average reduction in growth rate for both strains was 75%, under P limitation relative to that measured under P-enriched conditions. A similar high reduction in C-specific growth was also found in a transition from P-enriched to P-limiting conditions in N_2-fixing cells by Degerholm et al. (2006). Hence, P limitation appeared to have a strong negative effect on growth rate in *N. spumigena,* when N_2 fixation was the sole N source, probably because N_2 fixation is a highly energy-demanding process (Flores & Herrero 2005). At the end of our experiment, the significantly reduced growth rate (h^{-1}) and accumulated biomass resulted in a lower total N_2 fixation under P limitation as compared to P-enriched conditions.

The specific C assimilation decreased during our experiment in both P-enriched and P-limited *N. spumigena*, which may indicate a co-limitation of growth by another nutrient. However, increasing N_2 fixation also leads to a decrease in net C assimilation in cyanobacteria because N_2 fixation is a highly energy-demanding process. Less fixed carbon is used for respiration and energy-generation during ammonium uptake compared to N_2 fixation (Kranz et al. 2011, Eichner et al. 2014). During our experiment, net C assimilation was high and N_2 fixation low when ammonium was present at high concentrations (<7 d; Fig. 4). Ammonium inhibits N_2 fixation in *N. spumigena* cultures (Vintila & El-Shehawy 2007). The higher C:N fixation ratio by KAC64 relative to KAC12 may therefore indicate a higher affinity for ammonium by KAC64 as compared to KAC12 during the first week of the experiment when ammonium concentrations were high. Specific N_2-fixation rate was significantly higher in KAC12 than in KAC64 on Day 7 (Table 3). Specific C assimilation decreased in both strains and treatments as N_2 fixation increased and finally covered N demands. Degerholm et al. (2006) found only a small decrease in gross N_2 fixation (net N_2 fixation plus ammonium release) by *N. spumigena* under P-limited compared to P-enriched conditions. Our observations during the beginning of the experiment may thus also indicate a high gross N_2 fixation but low net N_2 fixation and high ammonium release by the cyanobacteria. From Day 7 until Day 21, the ammonium concentration decreased, while the net N_2 fixation increased and appeared to be the sole N source for the cells because the C:N fixation ratio was lower than or similar to the cellular C:N ratio. The difference in average specific N_2-fixation rates (h^{-1}) between strains may indicate a difference in capacity of N_2 fixation between KAC12 and KAC64 (Table 2).

After 7 d, the average C:N fixation ratio was ca. 4 and was below the cellular C:N ratio in both strains. Hence, N_2 fixation covered cellular N demand, and cells presumably released ammonium to the surrounding water to be used by bacteria in the ambient water. A culture study showed that most ammonium was released by *N. spumigena* at the beginning and at the end of a light period (Wannicke et al. 2009). The net C:N fixation ratio may thus be adjusted to the cellular C:N ratio over 24 h. Field populations of *Aphanizomenon* sp. and *N. spumigena* both release ca. 35% of the newly fixed N_2 as ammonium to the surrounding water in the Baltic Sea (Ploug et al. 2010, 2011). Surrounding organisms that benefit from the release are non-heterocystous picocyanobacteria such as *Synechococcus* (Ohlendieck et al. 2000, Stal et al. 2003) heterotrophic bacteria, and microalgae (Larsson et al. 2001, Salomon et al. 2003, Ploug 2008, Ploug et al. 2011, Adam et al. 2016). In the Baltic Sea, the turnover time of ammonium is 5 to 6 h, and released ammonium derived from N_2 fixation is efficiently assimilated by co-occurring heterotrophic and autotrophic bacteria, and microalgae (Adam et al. 2016).

Phosphate-rich upwelling events have experimentally been shown to be important for the initiation of filamentous cyanobacteria blooms (Nausch et al. 2009, Wasmund et al. 2012). The availability of dissolved P has been suggested to determine the spatiotemporal distributions of diazotrophic cyanobacteria, due to species-specific P-uptake kinetics and P-storage capacities in the Baltic Sea (Grönlund et al. 1996, Walve & Larsson 2007, Mohlin 2010). In the Baltic Sea, *Aphanizomenon* sp. occurs from May until September, while *N. spumigena* mostly occurs

during July and August when temperature is higher and orthophosphate concentrations are low (Larsson et al. 2001, Walve & Larsson 2007). Our study demonstrated significant variations of eco-physiological parameters, e.g. P-storage capacities and affinity for ammonium, between strains of the same species of *N. spumigena.* However, the total biovolume and growth in both strains increased at small enrichments of inorganic P, which resulted in a significantly increased total N_2 fixation due to higher biomass when the Baltic Sea water was repeatedly enriched with pulses of inorganic P. By comparison, stored P apparently was a small source to support growth, C-, and N_2- fixation rates. Hence, *N. spumigena* may benefit from even small pulses of P from anoxic sediments in shallow, coastal environments during summers with strong stratification and low influx of P in the Baltic Sea.

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