

Use of different phosphorus sources by the bloom-forming cyanobacteria *Aphanizomenon flos-aquae* and *Nodularia spumigena*

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ABSTRACT: A laboratory experiment was conducted to study differences in the use of varying phosphorus sources by the bloom-forming filamentous cyanobacteria *Aphanizomenon flos-aquae* and *Nodularia spumigena*. Axenic strains were grown in typical bloom-time light regimes under phosphate-replete, added organic phosphorus phosphate-depleted and completely phosphorus-depleted conditions. Responses to the treatments differed clearly. *A. flos-aquae* growth was dependent on an ample supply of inorganic dissolved phosphorus, whereas *N. spumigena* grew equally well in all treatments. Cellular N:P ratios showed high plasticity for both species and phosphorus deficiency did not seem to affect nitrogen fixation of either species. Regarding bloom formation, the results call attention to the importance of the omnipresence, eurythermal growth and deeper vertical positioning of *A. flos-aquae* in comparison to *N. spumigena*. *N. spumigena* is able to form and sustain bloom biomasses relying on cellular phosphorus storage and effective remineralization of organic phosphorus compounds.

KEY WORDS: Filamentous cyanobacteria · *Aphanizomenon flos-aquae* · *Nodularia spumigena* · Phosphorus source · Phosphorus limitation · Organic phosphorus · Baltic Sea

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INTRODUCTION

In the Baltic Sea, filamentous diazotrophic cyanobacteria form extensive harmful blooms during the summer months when surface waters are depleted in both nitrogen and phosphorus (Kahru et al. 1994, 2000). Conventionally, it has been postulated that as a consequence of excess phosphorus, the nitrogen-fixing cyanobacteria gain competitive advantage. Human-induced eutrophication of the Baltic Sea has been ongoing since the beginning of the last century (Struck et al. 2000, Voss et al. 2000). The historically long eutrophication process and the characteristic haline stratification have led to widespread anoxic bottoms and subsequent internal loading of phosphorus from the sediments (e.g. Conley et al. 2002). In the Gulf of Finland, it has been observed that, as a result of the increasing water-column phosphorus reserves, phyto-

plankton biomass maxima have begun to show a bias towards late summer (Raateoja et al. 2005). This shift may have been caused by the increase in diazotrophic cyanobacteria. Among the most abundant species in conspicuous blooms formed by cyanobacteria are the nitrogen-fixing non-toxic *Aphanizomenon flos-aquae* (L.) Ralfs and the hepatotoxic *Nodularia spumigena* Mertens. However, their growth and nitrogen fixation are considered to be phosphorus or trace element limited in the Baltic Sea (Stal et al. 1999, Rydin et al. 2002, Moisander et al. 2003). They vary in their autecological traits (e.g. Huber & Hamel 1985, Huber 1986, Wallström et al. 1992, Kononen et al. 1996, Lehtimäki et al. 1997, Laamanen & Kuosa 2005).

Late winter surface-layer phosphate conditions have, to some degree, a determinant effect on the intensity of cyanobacteria blooms (Jansen et al. 2004, Laanemets et al. 2006). Before the onset of thermal

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stratification and the spring bloom in the Baltic Sea, waters are homogeneously mixed down to the permanent halocline, at about 60 to 80 m depth. The winter-time surface water has a lower than average dissolved inorganic nitrogen (DIN) to dissolved inorganic phosphorus (DIP) ratio compared to the average spring bloom phytoplankton demand of 16:1 (Suikkanen et al. 2007). After the establishment of thermal stratification, the spring bloom is terminated by nitrogen limitation and the biomass is sedimented from the surface layer. This creates a situation where an excess of phosphorus is formed in the upper mixed layer.

The excess phosphorus is channelled to nitrogen-fixing cyanobacteria through various pathways, e.g. through phosphate uptake during early summer with subsequent growth on intracellular stores (Larsson et al. 2001, Nausch et al. 2004) and through recycling of organic compounds in the planktonic food web (Tamminen 1989, Grönlund et al. 1996, Kangro et al. 2006). Deep-water excess phosphorus is channelled to the cyanobacteria by frontal hydrodynamic processes (Kononen et al. 1996), upwelling (Vahtera et al. 2005) and turbulent mixing (Kononen & Nömmann 1992) during the growth period. Thus, the excess phosphorus, present in different forms and available through pathways described above, can support bloom formation throughout the growth season. In contrast, it also forms a species selection mechanism through nutrient competition with the prevailing phosphorus sources and phosphorus supply mechanisms and autecological traits of the species as drivers.

Water-column stratification is a critical factor determining the intensity of cyanobacteria blooms at the species level (Kanoshina et al. 2003). In the Gulf of Finland, *Aphanizomenon flos-aquae* has been observed to thrive in areas of hydrodynamic activity with weaker stratification, while *Nodularia spumigena* is more dominant in inert, strongly stratified areas (Kononen et al. 1996, Vahtera et al. 2005). Water column stratification affects both the temperature and light climate experienced by the species, but also phosphorus availability and quality at different depths, with DIP concentrations rapidly increasing below the thermocline (Laanemets et al. 2004). The biomass maxima of the 2 bloom-forming species are usually vertically separated, *A. flos-aquae* having a deeper biomass maximum than *N. spumigena* (Niemistö et al. 1989, Kononen et al. 1998, Vahtera et al. 2005). Thus, in addition to the effects of the physical environment, the variation in prevalent phosphorus source with depth and sub-basin may affect the species composition of the cyanobacteria blooms.

To further understand phosphorus-related species selection and the main phosphorus sources for blooms of nitrogen-fixing filamentous cyanobacteria in the

Baltic Sea, a laboratory experiment was conducted. Growth, intracellular phosphorus storage capacity and the utilization of a synthetic phosphomonoester as phosphate source between *Aphanizomenon flos-aquae* and *Nodularia spumigena* were compared.

MATERIALS AND METHODS

Experimental set-up. The experiment was carried out with a 2 × 3 factorial design (2 different species, each of which were grown under 3 phosphorus conditions) to reveal differences in growth and phosphorus utilization of the 2 main bloom-forming cyanobacteria species. The 2 × 3 factorial experiment design was chosen so that the effect of different phosphorus sources on both main bloom-forming species could be examined. The experiment was carried out using the typical light regimes experienced by the species during bloom periods.

Prior to the experiment, axenic strains of *Aphanizomenon flos-aquae* (TR183) and *Nodularia spumigena* (AV1) isolated from blooms in the Baltic Sea (strains courtesy of Academy Professor Kaarina Sivonen) were grown in liquid modified Z8 growth medium without nitrogen and with salt (Z8XS) (Sivonen et al. 1989). The stock cultures were grown at 18°C in a climate-controlled room in low light (20 μmol q m⁻² s⁻¹) produced by white-light fluorescent tubes (Osram L18 W/22 Lumilux White); the same light source was used during the experiment. Aliquots of each strain were transferred to duplicate acid-washed 1000 ml Erlenmeyer glass experimental units in a laminar flow cabinet.

The experiment consisted of 3 treatments: (1) a control treatment with a replete external dissolved orthophosphate (PO₄³⁻) supply (200 μM) in the growth medium (P-replete treatment); (2) a treatment with a dissolved orthophosphate-deficient growth medium with an added dissolved organic phosphorus (DOP) source (25 μM glycerol phosphate disodium salt hydrate [C₃H₇Na₂O₆P] added to Z8XS medium prepared without phosphorus) (DOP-enriched treatment) and (3) a treatment grown on Z8XS medium with phosphorus completely omitted (0 μM) (P-depleted treatment). Upon transfer to the experimental units the strains were washed on a 20 μM nylon mesh with treatment-specific medium. The initial biomasses (as chlorophyll *a* [chl *a*]) after the transfer to the experimental units were in the range of 18 to 22 μg l⁻¹ for *Aphanizomenon flos-aquae* and 25 to 28 μg l⁻¹ for *Nodularia spumigena* (see Table 1).

The experiment was run for 22 d at a temperature of 18°C in a climate-controlled room with a light:dark cycle of 12:12 h. The units were sampled 3 times a week for concentrations of chl *a*, phosphate phospho-

rus (PO_4^{3-} -phosphorus), nitrite and nitrate nitrogen ($\text{NO}_2^- + \text{NO}_3^-$ -nitrogen), particulate nitrogen (PN), particulate phosphorus (PP) and alkaline phosphatase activity (APA). On Day 14 of the experiment, 300 ml of treatment-specific growth medium was added to the experimental units to dilute the cultures. Transmission electron microscope (TEM) images and energy dispersive X-ray analyses were made at the end of the experiment from all the treatments.

The vertical separation of biomass maxima during blooms of the 2 species leads to the species experiencing different light environments. Thus, different light levels (*Aphanizomenon flos-aquae*: $40 \mu\text{mol q m}^{-2} \text{s}^{-1}$, *Nodularia spumigena*: $120 \mu\text{mol q m}^{-2} \text{s}^{-1}$) were used in the experiment for the 2 species to establish any differences in phosphorus utilization occurring during these specific conditions. The light levels were derived from species-specific optimal light conditions determined by laboratory experiments (Lehtimäki et al. 1997) and light profiles sampled from the northern Baltic Sea during July (data not shown).

Analytical methods. Duplicate 5 ml samples were taken for chl *a* analyses. The samples were filtered on GF/F (Whatman) filters that were left to dry in the dark for 15 min, after which the sample was extracted in 10 ml of 96% ethanol for 24 h. Chl *a* concentrations were measured after filtration through a GF/F filter to remove any interfering particles with a Jasco 750 spectrofluorometer. The spectrofluorometer was calibrated with pure chl *a* (Sigma Chemical).

Particulate nutrients were analyzed from 10 ml samples filtered on GF/F filters. Analyses for dissolved inorganic nutrients (analyzed according to Koroleff 1983) were performed from the filtrate of the same samples. The particulate nutrients were analyzed according to the same protocol after boiling the filter in 50 ml of persulfate solution.

APA was measured using the kinetic assay according to Petterson (1980), with modifications as in Kononen et al. (1993). Triplicate 4 ml samples were incubated at $+20^\circ\text{C}$ in the dark in acid-washed test tubes. 4-methylumbelliferone-phosphate (MUF-P) was used as fluorogenic substrate analogue at a final concentration of $0.0125 \mu\text{M}$ after determination of the saturation level. The assays were run for 60 min with fluorescence measurements every 15 min. Fluorescence readings were made with a Jasco 750 spectrofluorometer at an excitation wavelength of 365 nm and an emission wavelength of 460 nm. Fluorescence units were calibrated with MUF-P standard solutions over the range of 0.01 to $1 \mu\text{M}$ and the fluorescence intensity increase in the samples was used to cal-

culate the APA given as $\text{nmol MUF hydrolyzed h}^{-1}$. Soluble APA was measured after filtration through a $0.2 \mu\text{m}$ pore-size membrane filter.

Sub-samples for the ultra-structural analyses of the cultured cyanobacteria were preserved with glutaraldehyde (2% final concentration). Cells were concentrated using centrifugation (7 min 3000 rpm [$998 \times g$]) and dehydrated in a series of alcohol up to 96%, after which they were embedded in Spurr resin and processed according to Jensen & Moestrup (1999). A Leica ultracut UCT ultramicrotome was used for thin sectioning. Samples were examined with a JEOL JEM-1200EX transmission electron microscope with 60 kV tension and an FEI Tecnai F12 scanning electron microscope equipped with a Gatan 622 camera and an EDAX X-ray microanalyser operated with 120 kV tension.

Statistical analyses. Mixed model repeated measures analyses (PROC MIXED) were used to test for mean differences in chl *a* concentration, PN:PP ratio, chl *a* specific APA (SAPA), PP:chl *a* ratio and PN:chl *a* ratio between *Aphanizomenon flos-aquae* and *Nodularia spumigena* in the 3 treatments. We tested for the main effects of species and treatment and for the interactions of species-treatment, species-day and treatment-day on the measured parameters. All analyses were conducted with the Statistical Analysis System (SAS V8.02, SAS Institute). Appropriate transformations were applied where necessary to ensure normal distribution of model residuals.

RESULTS

Chlorophyll *a*

For *Aphanizomenon flos-aquae*, the P-replete treatment had the highest growth rate, whereas the 2 other treatments showed clearly slower growth rates (Table 1). There was less difference in the growth rates between the *Nodularia spumigena* treatments, with the DOP-enriched treatment having the highest growth rate. Of the 2 species, *N. spumigena* had a higher growth rate than *A. flos-aquae* in all cases except in the P-replete treatment.

Table 1. *Aphanizomenon flos-aquae* and *Nodularia spumigena*. Means \pm SD of initial biomasses and growth rates in the experimental units

Treatment	Chl <i>a</i> ($\mu\text{g l}^{-1}$)		Growth rate (μ)	
	<i>A. flos-aquae</i>	<i>N. spumigena</i>	<i>A. flos-aquae</i>	<i>N. spumigena</i>
P-replete	22.0 ± 0.0	25.0 ± 4.2	0.040 ± 0.001	0.039 ± 0.002
DOP-enriched	21.0 ± 1.4	26.0 ± 2.8	0.018 ± 0.007	0.045 ± 0.005
P-depleted	18.0 ± 2.8	28.0 ± 0.0	0.024 ± 0.009	0.035 ± 0.009

Chl *a* concentrations showed highly significant differences between species, with *Nodularia spumigena* having, on average, higher chl *a* concentrations than *Aphanizomenon flos-aquae* (Table 2, Fig. 1a,b). Treatments showed nearly significant differences and the species did not have a significant effect on the effect of treatment, i.e. the chl *a* concentrations of both species reacted in a similar way to the treatments. Both the effect of species and treatment were, however, affected by the elapsed time shown by the significant interaction terms for species and day of experiment and treatment and day of experiment. The significant interaction terms mirror the slower growth of *A. flos-aquae* in the DOP-enriched and P-depleted treatments.

PN:PP ratio

The P-depleted treatments of both species showed the most notable increases in the PN:PP ratios

(Fig. 1c,d). *Nodularia spumigena* had a clearer increase from PN:PP 12:1 at the beginning of the experiment to PN:PP 72:1 at the end of the experiment. The concurrent increase of the *Aphanizomenon flos-aquae* PN:PP ratio was from 11:1 to 65:1. The PN:PP-ratio seemed to increase more prominently for *A. flos-aquae* in the DOP-enriched (increase from 13:1 to 26:1) and in the P-replete (increase from 6:1 to 17:1) treatments than for *N. spumigena* in the DOP-enriched (increase from 13:1 to 22:1) and P-replete (increase from 7:1 to 11:1) treatments.

The PN:PP ratio showed significant differences between species and treatments (Table 2), *Aphanizomenon flos-aquae* having on average a higher PN:PP ratio than *Nodularia spumigena*. The significant interaction terms for species and day and treatment and day showed that the effect of the species and treatments were influenced by the passing of time in the experiment. For both species the PN:PP ratios of the P-depleted treatments increased constantly and more rapidly than for the other treatments.

Table 2. Results for type III tests for fixed effects and the least square means \pm SE for the species and treatments in the mixed model repeated measures analysis for the dependent variables; chl *a*, particulate nitrogen to particulate phosphorus ratio (PN:PP), chl *a* specific alkaline phosphatase activity (SAPA), particulate phosphorus to chl *a* ratio (PP:chl *a*) and particulate nitrogen to chl *a* ratio (PN:chlorophyll *a*)

Effect	Num df	Den df	F-value	p > F	Least square means and standard errors		
Chl <i>a</i>							
Species	1	6	87.26	<0.0001	<i>A. flos-aquae</i>		<i>N. spumigena</i>
Treatment	2	6	4.89	0.0550	4.66 \pm 0.10		5.94 \pm 0.10
Species \times Treatment	2	6	4.11	0.0750	P-replete	DOP-enriched	P-depleted
Species \times Day	8	64	2.42	0.0236	5.60 \pm 0.12	5.17 \pm 0.12	5.12 \pm 0.12
Treatment \times Day	16	64	3.12	0.0006			
PN:PP							
Species	1	6	8.81	0.0250	<i>A. flos-aquae</i>		<i>N. spumigena</i>
Treatment	2	6	236.20	<0.0001	2.99 \pm 0.03		2.82 \pm 0.03
Species \times Treatment	2	6	3.52	0.0973	P-replete	DOP-enriched	P-depleted
Species \times Day	9	72	2.37	0.0210	2.31 \pm 0.04	2.91 \pm 0.04	3.55 \pm 0.04
Treatment \times Day	18	72	5.83	<0.0001			
SAPA							
Species	1	6	0.06	0.8174	<i>A. flos-aquae</i>		<i>N. spumigena</i>
Treatment	2	6	271.84	<0.0001	1.03 \pm 0.001		1.03 \pm 0.001
Species \times Treatment	2	6	8.00	0.0203	P-replete	DOP-enriched	P-depleted
Species \times Day	7	56	3.87	0.0017	1.01 \pm 0.001	1.03 \pm 0.001	1.05 \pm 0.001
Treatment \times Day	14	56	8.89	<0.0001			
PP:chl <i>a</i>							
Species	1	6	54.25	0.0003	<i>A. flos-aquae</i>		<i>N. spumigena</i>
Treatment	2	6	92.09	<0.0001	0.21 \pm 0.01		0.36 \pm 0.01
Species \times Treatment	2	6	12.27	0.0076	P-replete	DOP-enriched	P-depleted
Species \times Day	8	63	4.11	0.0005	0.47 \pm 0.02	0.26 \pm 0.02	0.17 \pm 0.02
Treatment \times Day	16	63	3.32	0.0003			
PN:chl <i>a</i>							
Species	1	6	62.93	0.0002	<i>A. flos-aquae</i>		<i>N. spumigena</i>
Treatment	2	6	0.98	0.4295	4.03 \pm 0.14		5.57 \pm 0.14
Species \times Treatment	2	6	2.43	0.1689	P-replete	DOP-enriched	P-depleted
Species \times Day	8	64	3.09	0.0053	4.62 \pm 0.17	4.84 \pm 0.17	4.95 \pm 0.17
Treatment \times Day	16	64	2.43	0.0062			

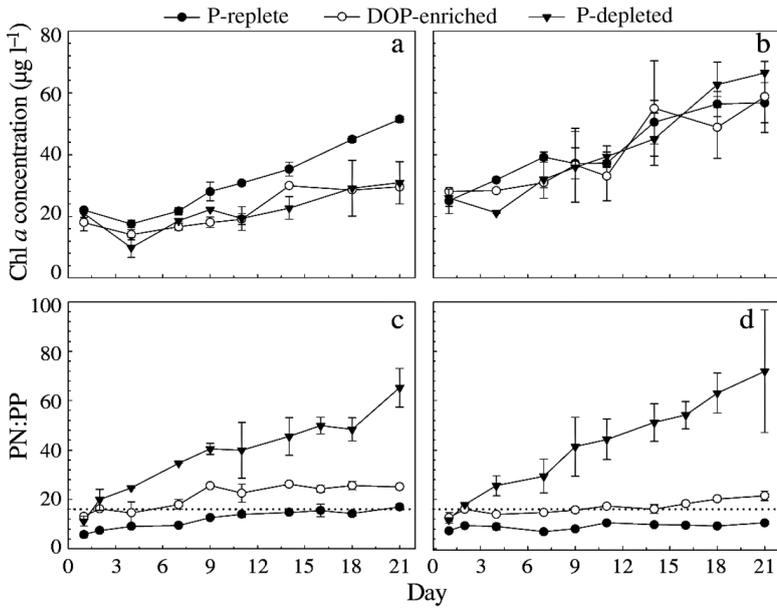


Fig. 1. (a,b) Chl *a* concentrations and (c,d) particulate nitrogen to particulate phosphorus ratios (PN:PP) during the experiment for (a,c) *Aphanizomenon flos-aquae* and (b,d) *Nodularia spumigena* in phosphorus-replete (P-replete), phosphorus-deficient with an organic phosphorus addition (DOP-enriched) and phosphorus-depleted (P-depleted) treatments. Error bars indicate \pm SD. (c,d) Dotted line shows the Redfield ratio of 16:1

SAPA and soluble APA

SAPA indicates culture-specific phosphorus stress. The species did not show a significant difference in SAPA (Table 2), which is probably partly due to the large variation in the *Nodularia spumigena* treatments (Fig. 2b). The treatments nevertheless evoked clearly statistically different responses, and all but the P-replete treatment showed increasing SAPA during the experiment (Fig. 2a,b) due to increasing phosphorus deficiency of the cultures. Both species reacted immediately to external phosphorus deficiency by increasing the enzyme activity (Figs. 2a,b & 3). *Aphanizomenon flos-aquae* showed a strong SAPA increase in the P-depleted treatment towards the end of the study after Day 14, which is mirrored by the significant species and day interaction term in the model (Table 2).

The species affected the response to treatment statistically significantly, as

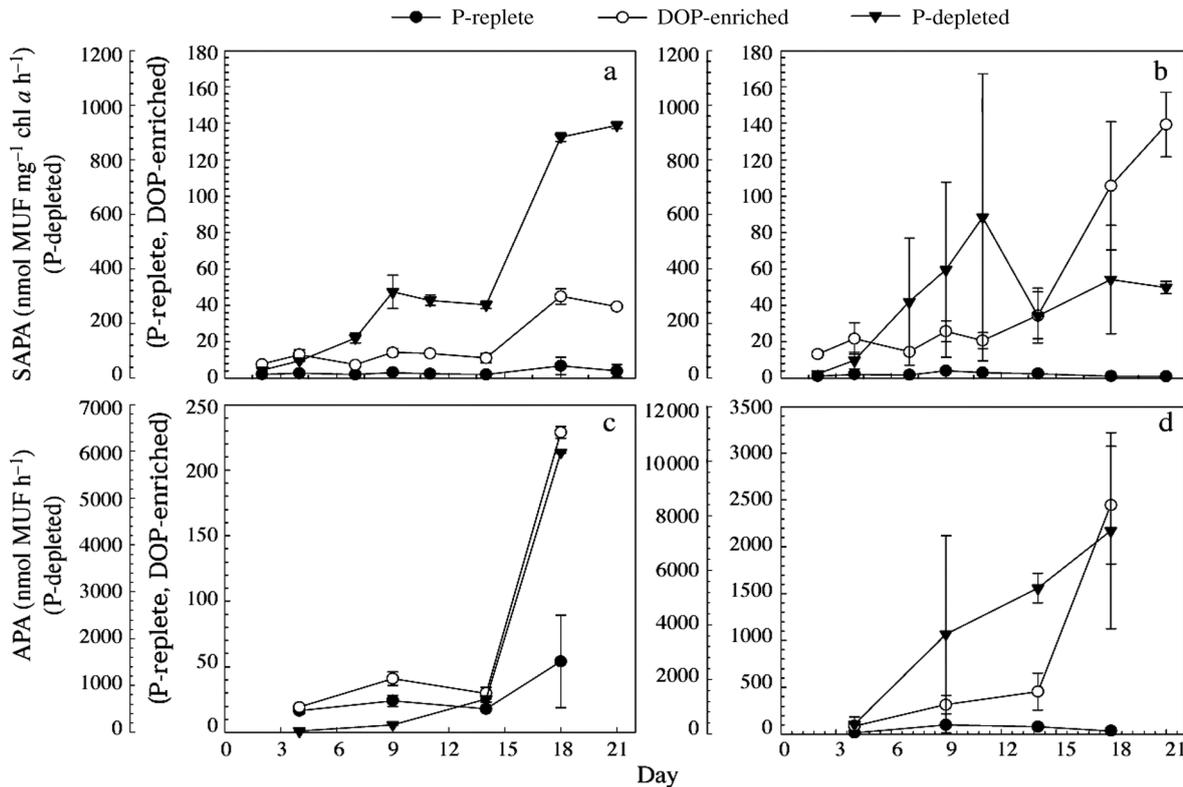


Fig. 2. (a,b) Chl *a* specific alkaline phosphatase activity (SAPA) and (c,d) soluble alkaline phosphatase activity (APA) during the experiment for (a,c) *Aphanizomenon flos-aquae* and (b,d) *Nodularia spumigena* in phosphorus-replete (P-replete), phosphorus-deficient with an organic phosphorus addition (DOP-enriched) and phosphorus-depleted (P-depleted) treatments. Error bars indicate \pm SD

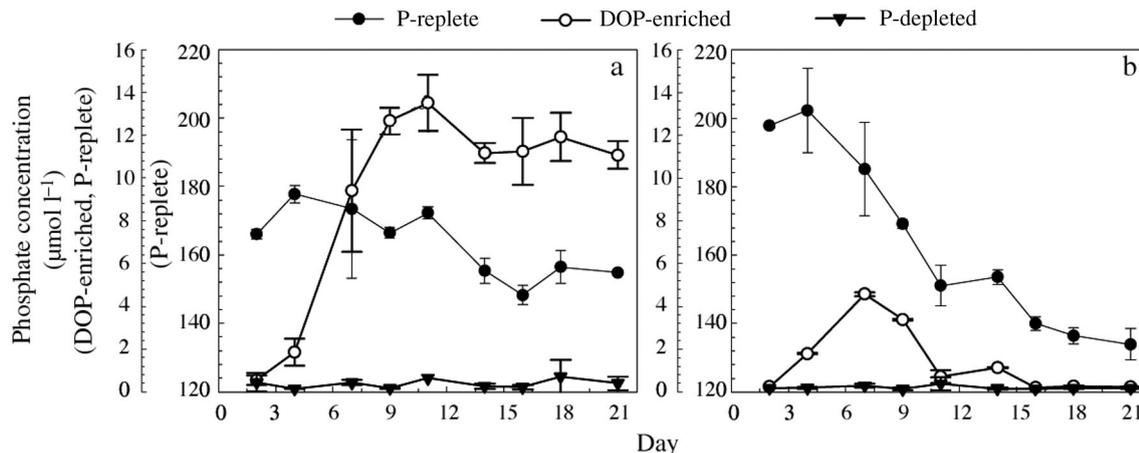


Fig. 3. Phosphate (PO_4) concentrations during the experiment for (a) *Aphanizomenon flos-aquae* and (b) *Nodularia spumigena* in phosphorus-replete (P-replete), phosphorus-deficient with an organic phosphorus addition (DOP-enriched) and phosphorus-depleted (P-depleted) treatments. Error bars indicate \pm SD

shown by the species and treatment interaction term. *Nodularia spumigena* SAPA in the DOP-enriched treatment showed a more pronounced increase than that of the *Aphanizomenon flos-aquae* DOP-enriched treatment, indicating stronger phosphorus stress. Generally, *A. flos-aquae* had on average a higher SAPA in the P-depleted treatment (*A. flos-aquae*: $364.35 \text{ nmol MUF } \mu\text{g}^{-1} \text{ chl a h}^{-1}$; *N. spumigena*: $269.48 \text{ nmol MUF } \mu\text{g}^{-1} \text{ chl a h}^{-1}$), while *N. spumigena* had on average a higher SAPA in the DOP-enriched treatment (*A. flos-aquae*: $18.71 \text{ nmol MUF } \mu\text{g}^{-1} \text{ chl a h}^{-1}$; *N. spumigena*: $46.69 \text{ nmol MUF } \mu\text{g}^{-1} \text{ chl a h}^{-1}$). Both species showed negligible SAPA in the P-replete treatment throughout the study, reflecting the good phosphorus nutritive status of the cells.

Soluble APA was highest in the P-depleted treatments, and lowest in the P-replete treatments (Fig. 2c,d), in accordance with general levels of SAPA. The *Nodularia spumigena* P-depleted and DOP-enriched treatments had consistently higher levels of soluble APA than the corresponding *Aphanizomenon flos-aquae* treatments, even though the P-depleted treatment showed large variation. The percentages of soluble to total APA varied between 3 to 35 and 19 to 67% for *A. flos-aquae* and *N. spumigena*, respectively. This indicates possibly a larger fraction of excess enzyme synthesis for *N. spumigena*.

Dissolved inorganic nutrients

Phosphate concentrations in the P-replete treatments declined more rapidly for *Nodularia spumigena* (Fig. 3). However, phosphate did not decrease to levels where it would have affected the nutritive status of the cells for either species shown by constantly low SAPA.

The P-depleted treatments of both species showed consistently low phosphate concentrations. Thus, growth conditions in the P-replete or P-depleted treatments regarding phosphorus supply did not markedly change during the experiment.

Phosphate concentrations increased in the DOP-enriched *Aphanizomenon flos-aquae* treatment for the first 8 d of the experiment, after which the concentration stabilized at around 11 to 12 μM. Phosphate concentrations in the DOP-enriched *Nodularia spumigena* treatment increased initially until Day 7 of the experiment, and subsequently decreased to levels comparable to the P-depleted treatment. The completely depleted phosphate concentrations and rapidly increasing SAPA for the *N. spumigena* DOP-enriched treatment imply substrate limitation of hydrolysis.

Nitrite and nitrate concentrations were consistently low ($<0.5 \mu\text{M}$) (data not shown) for all but the *Aphanizomenon flos-aquae* P-replete and P-depleted treatments, which showed a slight variation in DIN concentrations (0.5 to 2.0 μM).

PP:chl a ratio

Both species showed their lowest PP:chl a ratios in the P-depleted treatments. The DOP-enriched treatments had intermediate, and the P-replete treatments the highest PP:chl a ratios (Fig. 4a,b). The differences between species and treatments were significant (Table 2). *Aphanizomenon flos-aquae* had on average a lower PP:chl a ratio compared with *Nodularia spumigena*.

The species and treatment interaction term was also significant, implying differing responses of the species to the treatments. *Nodularia spumigena* had higher ratios

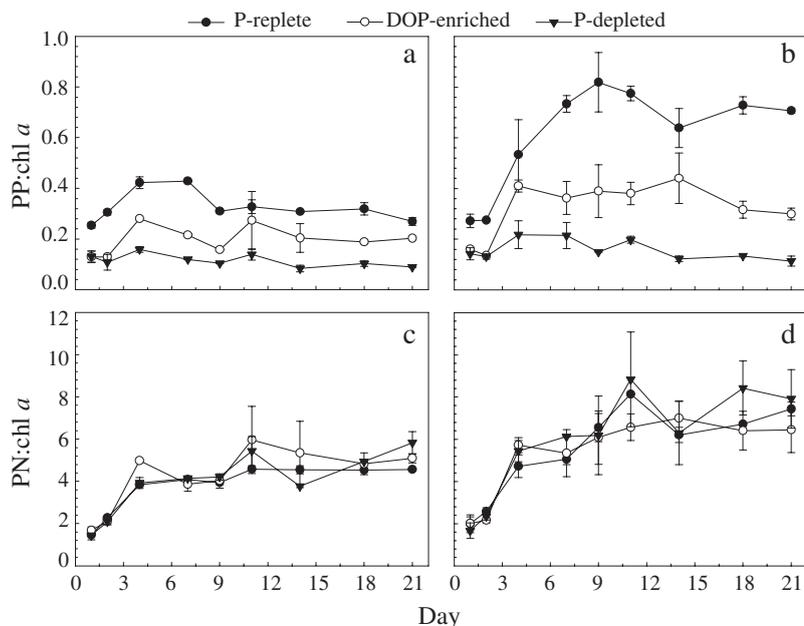


Fig. 4. (a,b) Particulate phosphorus to chl *a* ratio (PP:chl *a*) and (c,d) particulate nitrogen to chl *a* ratio (PN:chl *a*) during the experiment for (a,c) *Aphanizomenon flos-aquae* and (b,d) *Nodularia spumigena* in phosphorus-replete (P-replete), phosphorus-deficient with an organic phosphorus addition (DOP-enriched) and phosphorus-depleted (P-depleted) treatments. Error bars indicate \pm SD

in both the P-replete and DOP-enriched treatments. However, both species had similar PP:chl *a* ratios in the P-depleted treatment (*Aphanizomenon flos-aquae*: 0.12 ± 0.03 ; *N. spumigena*: 0.16 ± 0.04) (Fig. 4a,b).

PN:chl *a* ratio

Nodularia spumigena PN:chl *a* ratios were higher than the corresponding *Aphanizomenon flos-aquae* values (Fig. 4c,d) and the difference between species was significant, whereas no treatment effects were found (Table 2). The ratios increased for both species during the experiment, but dilution of the cultures on Day 14 of the experiment seemed to have a decreasing effect on the ratios in both species. The ratio stabilized more or less after Day 4 of the experiment for both species in all treatments. The average PN:chl *a* ratio for all *A. flos-aquae* and *N. spumigena* treatments was 4.7 ± 0.7 and 6.8 ± 1.0 , respectively.

TEM images and energy dispersive X-ray analyses

The polyphosphate granules were distinctly small and characteristically electron-dense, appearing as dark grains in the images (Fig. 5). The granules were on average only a few nm in diameter and were evenly

distributed in both vegetative cells and heterocytes. Energy-dispersive X-ray analyses from an area abundant in electron-dense granules (Fig. 6a) and from a gas vacuole (Fig. 6b) taken from a *Nodularia spumigena* P-replete unit shows a distinct phosphate peak that is overlapped by an α -osmium peak at approximately 2 KeV in the area of electron-dense granules. The spectrum from the gas vacuole devoid of electron-dense granules shows no phosphate.

The P-replete control treatments of both species had abundant polyphosphate granules distributed throughout the cells (Fig. 5a,d). The DOP-enriched and P-depleted treatments showed successively less polyphosphate granules than the P-replete treatments (Fig. 5b,c,e,f).

DISCUSSION

The present study illustrates the phosphorus utilization of the 2 main bloom-forming nitrogen-fixing filamentous cyanobacteria species from the Baltic Sea during bloom conditions. Our aim was to investigate the possible effects of phosphorus source on species-specific phosphorus utilization and the potential of the principal phosphorus source as a species selection mechanism during bloom formation and sustenance.

The growth of the 2 main bloom-forming species, *Nodularia spumigena* and *Aphanizomenon flos-aquae*, responded with distinct patterns to the phosphorus treatments in the simulated bloom-time light regimes. *A. flos-aquae* growth suffered from the lack of available abundant dissolved phosphate. The growth rates of both the P-depleted and DOP-enriched treatments were slower than that of the P-replete treatment, which was of the same magnitude as that in all the *N. spumigena* treatments.

The experiment was done in batch cultures; thus, the growth environment changes with time as phosphate is utilized and biomass accumulates. The growth environment, regarding phosphorus supply, in the P-replete and P-depleted treatments for both species were most likely not markedly affected by the density effect. Phosphate concentrations in the P-depleted treatments were constantly low (Fig. 3) and the particulate phosphorus concentrations for both species were stable (data not shown). The P-replete treatments showed marked drawdown of phosphate, especially the *Nodularia spumigena* treatment, but the absolute

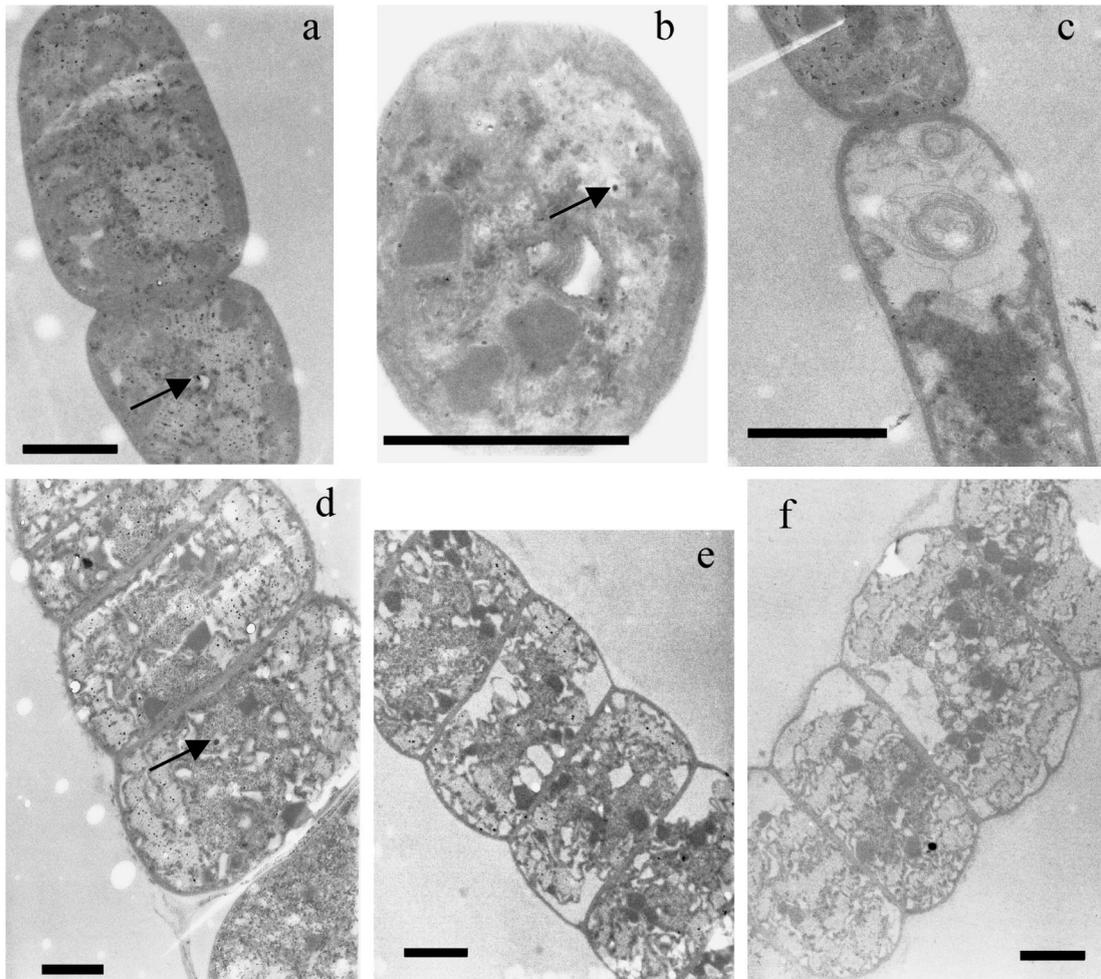


Fig. 5. *Aphanizomenon flos-aquae* and *Nodularia spumigena*. Transmission electron microscope images of cells from the experimental treatments. (a) *A. flos-aquae* phosphorus-replete control, (b) *A. flos-aquae* phosphorus-depleted treatment with added organic phosphorus, (c) *A. flos-aquae* phosphorus-depleted treatment, (d) *N. spumigena* phosphorus-replete control, (e) *N. spumigena* phosphorus-depleted treatment with added organic phosphorus and (f) *N. spumigena* phosphorus-depleted treatment. Polyphosphate bodies appear as black grains (black arrows). Scale bar = 1 μm

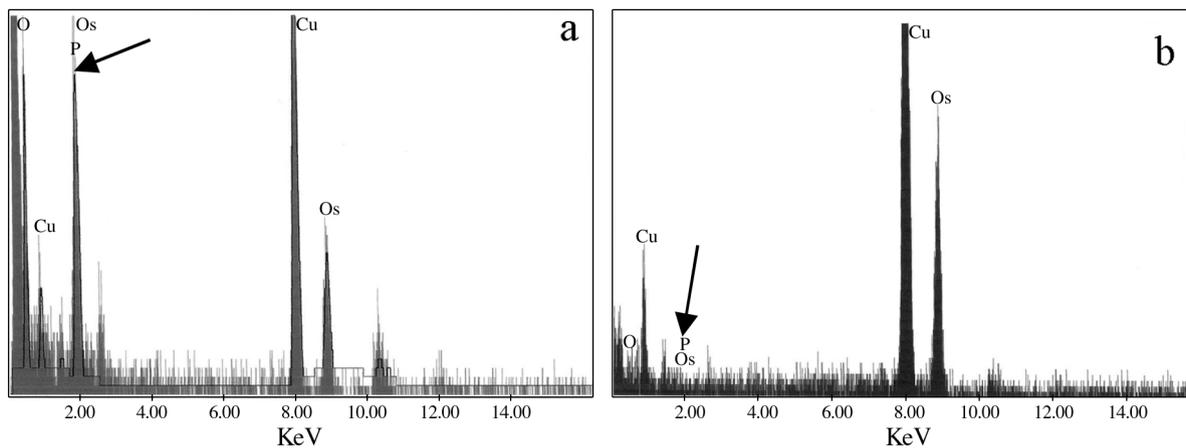


Fig. 6. Energy dispersive X-ray analysis elemental spectra from (a) area with electron-dense granules, and (b) gas vacuole of a *Nodularia spumigena* control treatment vegetative cell. The y-axis shows relative intensity. Diameter of the X-ray beam was 500 nm. Arrows show the position of the phosphorus peak

concentrations were still so high at the end of the experiment that they most likely did not affect phosphate availability of the experimental units, as also indicated by constantly low SAPA. The DOP-enriched units, especially that of *N. spumigena*, showed clear signs of substrate limitation after Day 14 of the experiment, with depleted phosphate concentrations and rapidly increasing SAPA. While, *A. flos-aquae* did not show as clear an increase in SAPA and phosphate concentrations were not depleted. *A. flos-aquae* SAPA and N:P ratios indicated a phosphorus deficiency, but an affinity constraint for phosphorus uptake, as shown by the steady phosphate concentrations, caused continuous phosphorus stress. *N. spumigena* was able to satisfy its phosphorus requirements as long as substrate was available for hydrolysis. The quicker drawdown of phosphate in the *N. spumigena* DOP-enriched treatment might be a density effect. However, the faster growth, when compared to *A. flos-aquae*, nevertheless indicates a more efficient use of the available phosphorus source.

The fact that *Aphanizomenon flos-aquae* showed less efficient growth in both phosphorus-deficient treatments places it in an inferior position in relation to *Nodularia spumigena* in the utilization of phosphate from hydrolysable organic compounds during bloom periods. Regarding species selection during bloom formation, our results point to the importance of the omnipresence and eurythermal growth, and thus larger inoculum for summer blooms of *A. flos-aquae*. Populations of *A. flos-aquae* are present throughout spring and early summer in free water (e.g. Laamanen & Kuosa 2005), constituting thus a larger bloom inoculum than that of *N. spumigena*, and allowing populations with less efficient growth to still form substantial bloom biomasses. *N. spumigena* biomass is temporally and spatially more patchy (Kononen & Nömmann 1992, Laamanen & Kuosa 2005); it is observed later in the year in free water than *A. flos-aquae* and its growth is more stenothermal, thus partly not being able to grow efficiently at deeper depths where dissolved phosphate is available (e.g. Lehtimäki et al. 1997).

In our experiment *Nodularia spumigena* grew best, but at similar growth rates as in the other treatments, on organic phosphorus. *N. spumigena* seems to have developed an adaptation to efficient growth in conditions of phosphate deficiency in systems that nevertheless have abundant phosphorus available in other forms. The fraction of the DOP pool during summer in the surface layer of the Baltic Sea may represent 70 to 100% of the total phosphorus (Kononen & Nömmann 1992); Grönlund et al. (1996) observed that during short (40 min) incubations, more than half of the added phosphate ended up in the DOP pool. Nausch & Nausch (2004) estimated that 4 to 43% of DOP would

be bioavailable. Our results support the assumption that *N. spumigena*, located typically close to the surface in an environment where phosphorus occurs predominantly in particulate and dissolved organic forms during summer, presumably satisfies its phosphorus needs through remineralization pathways.

The TEM images showed a gradual decreasing trend of possibly identified polyphosphate granules from P-replete to DOP-enriched to P-depleted treatments (Fig. 5). However, the size and distribution of the polyphosphate granules were uncharacteristic, and they were rather small in comparison to an earlier report (Janson et al. 1994). For *Anabaena flos-aquae* (Lyngb.) Bréb, it has been observed that phosphorus is stored as sugar phosphorus under nitrogen-fixing conditions (Thompson et al. 1994), which might explain the peculiar form and distribution of the phosphorus-rich granules in our strains that also relied on nitrogen fixation as the sole nitrogen source.

The presence of phosphorus in areas dense with supposed polyphosphate granules was verified with energy-dispersive X-ray analysis. Phosphorus produces a peak in the spectrum at approximately 2 KeV (Fig. 6a). Osmium, which is used as a fixative in the sample preparation to increase contrast, produces an α -peak with nearly the same energy as phosphorus, but it also produces a β -peak at approximately 9 KeV (Fig. 6a,b). A spectrum from a gas vacuole (Fig. 6b), devoid of the supposed polyphosphate granules, still shows the osmium β -peak, indicating the presence of osmium. The osmium α -peak and the phosphorus peak are not preserved, indicating that the overlapping osmium and phosphorus peaks are indeed related to the presence of phosphorus and not of only osmium.

The PN:PP ratios in the experiment showed distinct patterns between treatments. The particulate phosphorus analyses in the experiment include both intracellular and surface-adsorbed phosphate. The P-depleted treatments showed the most distinct increase in PN:PP ratios (Fig. 1c,d). This was caused by the decrease in cellular phosphorus stores (cf. Fig. 5c,f), but possibly also due to the increase in some nitrogenous compound in the cells, reflected by the increasing PN:chl *a* ratios (Fig. 4c,d). In our experiment, the strains relied completely on nitrogen fixation, and the P-depleted treatments of both species accumulated nitrogen in relation to biomass in a similar manner to the other treatments (Fig. 4c,d), indicating high plasticity of nitrogen fixation in relation to phosphorus stress. Neither the source of phosphorus nor the intensity of phosphorus starvation significantly affected the nitrogen fixation ability of the cells. Previous field and experimental studies have noted that nitrogen fixation of filamentous heterocyte possessing cyanobacteria is stimulated by phosphorus addition and general growth

promoting factors (Huber 1986, Lehtimäki et al. 1997, Moisander et al. 2003). Based on our results it seems, nevertheless, as if nitrogenase synthesis is rather robust in relation to cellular phosphorus depletion. The increase in PN:PP ratios to >60:1 did not seem to affect nitrogen fixation to a large extent.

The P-replete treatments of both species showed constantly low PN:PP ratios, more so for *Nodularia spumigena*. *Aphanizomenon flos-aquae* showed a slight but constant increase in the PN:PP ratio. The increasing PN:PP ratio in the *A. flos-aquae* P-replete treatment might not be a sign of usage of cellular phosphorus stores but may indicate accumulation of nitrogen storage compounds and an equilibrium state in phosphorus storage.

The *Nodularia spumigena* P-replete treatment showed a very low PN:PP ratio throughout the experiment (<11:1). The particulate nutrient analysis used was not able to discriminate between surface-adsorbed and intracellular phosphorus. Sañudo-Wilhelmy et al. (2004) and Fu et al. (2005a) have found that true intracellular phosphorus concentrations are much smaller than anticipated from bulk particulate nutrient analyses due to ample surface adsorption of phosphate. Intracellular N:P ratios are up to 1.2 to 2 times higher than measured bulk ratios. This would yield an average N:P ratio of 13.2 to 22 for the *N. spumigena* P-replete treatment. Fu et al. (2005a) observed that the amount of surface-adsorbed phosphorus depends on the physiological state and phosphorus demand of the cell, with actively growing and phosphorus-depleted cells having less surface-adsorbed phosphate. We can assume that the P-replete treatments had an ample supply of phosphate (Fig. 3), and thus the amount of surface-adsorbed phosphate would be in the upper part of the range. This would mean that *N. spumigena* and *Aphanizomenon flos-aquae* have cellular N:P ratios exceeding the Redfield ratio under conditions when phosphate is not limiting. The P-depleted treatments reached N:P ratios of approximately 70:1; according to the range given by Fu et al. (2005a), these values would yield true intracellular N:P ratios of approximately 80 to 140:1, of which the lower values in the range are more probable, since nutrient-depleted cells utilize to some degree the surface-adsorbed phosphate. Nevertheless, the results show that the N:P ratio of *N. spumigena* and *A. flos-aquae* has high plasticity, and that efficient growth can occur at N:P ratios far exceeding the Redfield ratio of 16:1.

Nodularia spumigena showed a significantly higher PN:chl *a* ratio than *Aphanizomenon flos-aquae* (Table 2), indicating a more efficient nitrogen fixation under the experimental conditions. This may have been caused by the different light levels used in the

experiment. Nitrogen fixation has been observed to depend on photon flux density, and colonies adapted to lower light levels did not increase nitrogen fixation to levels equalling colonies adapted to high light conditions, even when transferred to higher light conditions (Evans et al. 2000). Our cultures were pre-acclimated at low photon flux densities ($20 \mu\text{mol q m}^{-2} \text{s}^{-1}$) for weeks; the rapid initial increase in the PN:chl *a* ratios indicates the high plasticity of the nitrogen fixation capacities of the strains used.

However, it is also possible that the higher PN:chl *a* ratios for *Nodularia spumigena* would have been caused by a lower C:chl *a* ratio due to the higher light levels used, thus increasing the PN:chl *a* ratio in excess in relation to the *Aphanizomenon flos-aquae* treatments. We can consider particulate phosphorus a rather stable parameter in the P-depleted treatments since the treatments received no other phosphorus source than the cellular phosphorus present at the start of the experiment. The *A. flos-aquae* PP:chl *a* ratio was only slightly higher than that of *N. spumigena*. At the beginning of the experiment upon transfer from the stock cultures *A. flos-aquae* was transferred to $40 \mu\text{mol q m}^{-2} \text{s}^{-1}$, whereas *N. spumigena* was transferred to $120 \mu\text{mol q m}^{-2} \text{s}^{-1}$. As a consequence, we could anticipate a more pronounced decrease in the relative chl *a* amount per cell for *N. spumigena*. The PP:chl *a* ratios for the P-depleted treatments nevertheless do not show any decrease, which would be a sign of decreased C:chl *a* ratio, and there is no marked difference between the species. Thus, this implies that the changes in the PP:chl *a* and PN:chl *a* ratios would mainly be because of changes in cellular nutrient reserves and not the change in relative cellular chl *a* content in relation to light intensity.

The P-replete *Nodularia spumigena* PP:chl *a* ratio shows a very strong initial accumulation of cellular phosphorus, after which a stabilization is seen at a rather high PP:chl *a* ratio (approximately 0.7). In the P-replete *Aphanizomenon flos-aquae* treatment the PP:chl *a* ratio stabilizes at approximately 0.3. Both species seem to be very efficient in cellular phosphorus storage, in accordance with earlier reports (Kromkamp 1987). Nevertheless, *N. spumigena* seems to have a relatively higher cellular phosphorus quota. A similar pattern as found with the other treatments is reflected in the DOP-enriched treatments. *A. flos-aquae* shows slightly higher and increasing PN:PP ratios and slightly lower PP:chl *a* ratios throughout the experiment. Even though the PP:chl *a* ratio is somewhat compromised by the possibility of deviant C:chl *a* ratios due to different light levels used, the higher growth rates and lower PN:PP ratios for *N. spumigena* in the DOP-enriched and P-depleted treatments emphasise the better capacity of *N. spumigena* to use the synthetic phosphomo-

noester and cellular phosphorus stores as phosphorus sources for growth under the experimental conditions.

Light intensity does not affect phosphorus uptake in cyanobacteria as such, but may stimulate it and lead to increased production of polyphosphate storage products (e.g. Fu et al. 2005b and references therein). This is, along with lower C:chl *a* ratios, a potential explanation for the higher cellular phosphorus quota (expressed as PP:chl *a* ratio) of *Nodularia spumigena* in the experiment. As the biomass maxima of the 2 species are vertically separated during blooms, this general observation is likely to apply in the field as well, with *N. spumigena* phosphorus uptake being stimulated by high light environments and the higher cellular phosphorus quota compensating for the relative scarcity of phosphate.

The P-depleted treatments clearly showed the highest SAPA, and for both species activities started to increase concurrently at the beginning of the experiment (Fig. 2a,b). SAPA for both species seemed to be repressed by the absence of dissolved phosphate, but also the cellular phosphorus content showed simultaneous decrease with increasing SAPA, especially in the *Nodularia spumigena* DOP-enriched treatment (Figs. 2a,b & 4a,b). *N. spumigena* alkaline phosphatase seemed to leak more readily into the growth medium than it did for *Aphanizomenon flos-aquae* (Fig. 2c,d). This leads in nature to a poorer ratio of acquired phosphate to produced enzyme amount, since most probably heterotrophic bacteria and smaller phototrophs will out-compete the filamentous cyanobacteria in phosphorus uptake when nitrogen and carbon are in sufficient supply.

During summer, DIN is depleted from the surface waters in the Baltic Sea, and phytoplankton production is generally nitrogen-limited (e.g. Granéli et al. 1990, Moisander et al. 2003); bacterial production is limited by the supply of carbon, nitrogen and phosphorus in the proper ratios (Heinänen & Kuparinen 1992, Kuparinen & Heinänen 1993). Thus, in summer, during periods when other phytoplankton are nitrogen-limited and bacteria are limited by the resource ratios, available phosphorus from inorganic and organic pools might be channelled to the diazotrophic cyanobacteria despite excess leakage of alkaline phosphatase. However, filamentous cyanobacteria have been usually reported to be responsible for only a minor fraction of phosphorus uptake during blooms (Grönlund et al. 1996, Nausch et al. 2004). Their phosphorus demand in any case seems to be small, and the N:P ratios display remarkable plasticity, as growth rates can withstand increases in cellular N:P ratios of at least up to 80:1 (Fig. 1c,d).

Nodularia spumigena SAPA varied greatly between replicates in the P-depleted treatment, which is thus somewhat ambiguous in relation to *Aphanizomenon flos-aquae*. *A. flos-aquae* had a more consistent re-

sponse, with a strong increase at the end of the experiment. On average, *A. flos-aquae* SAPA was higher in the P-depleted treatment (364.35 nmol MUF mg⁻¹ chl *a* h⁻¹) than the corresponding *N. spumigena* SAPA (269.48 nmol MUF mg⁻¹ chl *a* h⁻¹). The values observed are generally an order of magnitude higher than ambient SAPA activities measured in the field during a cyanobacteria bloom dominated by *A. flos-aquae*, and up to 2 times higher than the maximum values measured during the same bloom event (Grönlund et al. 1996).

Both species showed slightly elevated SAPA in the DOP-enriched treatments (Fig. 2a,b), along with increasing phosphate concentrations (Fig. 3). The increasing phosphate concentrations indicate efficient liberation of phosphate from the synthetic phosphomonoester. *Nodularia spumigena* was able to utilize the liberated phosphate efficiently, as seen by declining concentrations after Day 7 of the experiment, but *Aphanizomenon flos-aquae* had a clear affinity constraint at phosphate concentrations of approximately 11 to 12 mmol m⁻³. *N. spumigena* showed rapidly increasing SAPA after the depletion of phosphate from the medium, indicating efficient repression of enzyme synthesis by the absence of the product. Stoecker et al. (2005) found elevated APA activities during a cyanobacteria bloom dominated by *N. spumigena*, and they concluded that colonies and aggregates of cyanobacteria can be important sites of hydrolytic activity in the Baltic Sea, affecting nutrient regeneration. Panosso & Granéli (2000), on the other hand, noted that *N. spumigena* was unable to utilize dissolved organic matter derived by filtration from river water as a phosphorus source. The quality of the organic phosphorus source plays a large role in whether phosphorus requirements can be met through the enzymatic liberation of phosphorus from organic compounds.

CONCLUSIONS

Efficient *Aphanizomenon flos-aquae* growth during summer has to be supported by higher phosphate concentrations; the biomass maximum is thus frequently found just above the thermocline or in frontal regions where vertical advection occurs. *A. flos-aquae* has also been noted to grow more efficiently in low light than *Nodularia spumigena* during phosphorus-replete conditions, emphasizing the difference. The results thus strengthen the conception, based on field studies, that under bloom conditions *N. spumigena* is a superior competitor for phosphorus at low concentrations. It is more efficient in acquiring phosphate from organic sources and grows better on intracellular phosphorus stores.

There is a difference between *Aphanizomenon flos-aquae* and *Nodularia spumigena* phosphorus acquisition strategies. *A. flos-aquae* is dependent on a sufficient DIP supply. *A. flos-aquae* growth is eurythermal, and summer blooms already have relatively large inocula in surface waters during spring and early summer, allowing substantial biomasses to form. The internal loading of phosphorus that has been a recurrent phenomenon in the Gulf of Finland since the mid-1990s, and also in the Baltic Proper in recent years, will most likely especially promote the growth of *A. flos-aquae*. *N. spumigena* growth is more independent of a DIP supply, allowing it to thrive in more stratified and inert near-surface layers, with organic compounds and efficient phosphorus recycling being the main phosphorus supplies.

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