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Stimulation of nitrogen-fixing cyanobacteria in a Baltic Sea plankton community by land-derived organic matter or iron addition

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ABSTRACT: In the Baltic Sea, floating blooms of nitrogen-fixing cyanobacteria occur yearly during late summer. These blooms can sometimes be limited by iron. Due to extensive foresting around the Baltic Sea, iron is entering the Baltic Sea partly bound to dissolved organic material (DOM) via rivers. An experiment was performed in 300 l laboratory mesocosms to test the hypothesis that riverine high-molecular weight dissolved organic matter (HMWDOM), extracted by tangential flow filtration >1000 Da, stimulates the biomass of nitrogen-fixing cyanobacteria, by increasing the availability of iron. The addition of iron/EDTA and of DOM resulted in 5 to 10 times higher biomass of nitrogen-fixing cyanobacteria. Accordingly, higher primary production and particulate nitrogen concentration at the end of the experiment were observed in those treatments compared to the control. The removal of mesozooplankton grazers did not have a significant effect on the microphytoplankton biomass and species composition. *Nodularia spumigena* biomass was highest in the treatments receiving DOM, but addition of iron alone had no significant effect on this. *N. spumigena* was less positively affected by iron addition than *Anabaena* cf. *inaequalis*, suggesting that *N. spumigena* is a better competitor for iron. Separate microcosms comparing additions of iron, manganese and cobalt showed that iron was limiting for cyanobacterial biomass development. The results strongly suggest that iron bound to DOM can contribute to the iron demands of nitrogen-fixing cyanobacteria in the Baltic Sea.

KEY WORDS: Cyanobacteria · Nitrogen fixation · Iron · High-molecular weight dissolved organic matter · HMWDOM · Humic acid · Mesocosm experiment

INTRODUCTION

Extensive cyanobacterial blooms are recurring phenomena in the Baltic Sea during late summer. The blooms consist of a number of diazotrophic (nitrogen-fixing) species. Most notably, the hepatotoxic species *Nodularia spumigena* occurs from mid- to late summer, forming massive floating layers over large areas (Sivonen et al. 1989). In lakes, diazotrophic cyanobacteria often become dominant in plankton communities at low N:P supply ratios (Smith 1983). However, energy and trace metal (especially iron) demands are high for diazotrophic growth (Raven 1988) compared to growth on nitrate or ammonium, which might restrict the occurrence of these species to areas with relatively high irradiance and trace metal availability.

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Dissolved inorganic N:P atomic ratios in open Baltic waters are low (7 to 10) before the onset of the spring bloom (Wulff & Stigebrandt 1989) and usually even lower during summer (Granéli et al. 1990). The growth of diazotrophic cyanobacteria in the Baltic Sea is further facilitated by a well-developed thermocline, availability of phosphorus and the relatively low salinity in the photic zone (Sivonen et al. 1989).

Iron has been identified as a limiting factor for nitrogen fixation and growth of cyanobacteria in the Baltic Sea (Stal et al. 1999). High-molecular weight dissolved organic material (HMWDOM, hereafter called DOM) from terrestrial sources has been suggested to influence the trace metal availability, such as iron (Thomas 1997 and references therein) and cobalt (Granéli & Haraldsson 1993), which is especially important for cyanobacterial blooms due to their relatively high demands for trace metals (Paerl et al. 2001). Furthermore, the concentrations of humic substances in freshwater in this region has been increasing (Andersson et al. 1991), probably resulting in higher loads of DOM in the Baltic Sea. It is therefore important to understand the effect of this DOM on plankton communities. We hypothesise that the addition of riverine DOM increases the iron availability, thereby stimulating the growth of diazotrophic cyanobacteria.

Nodularia spumigena produces a potent hepatotoxin, nodularin, and is at times avoided as a food source by some copepods (Engström et al. 2000). Avoidance by grazers could be an evolutionary advantageous strategy for this species at high grazing pressure. The extra supply of DOM from rivers could provide nutrition to the microbial food web and thereby stimulate micro- and mesozooplankton grazers. In this case, selection of phytoplankton species will be more strongly top-down controlled than in a system without DOM inflow. Therefore, our second hypothesis is that the high input of DOM leads to a stronger selection towards toxic phytoplankton species because of increased grazing pressure. A large fraction of riverine DOM is relatively refractory and not directly available for bacteria. The typical time scale of a mesocosm experiment (1 to 2 wk) might not be sufficient to observe enhanced bacterial production from addition of riverine DOM. Therefore, the hypothesis that enhanced grazing pressure would stimulate the development of toxic cyanobacterial species was tested directly by adding a natural density of mesozooplankton grazers, while mesozooplankton grazers were removed from the control. Because the exclusion of grazers was done by size-selection only (net filtration), the effect of microzooplankton could not be accounted for.

MATERIALS AND METHODS

DOM extraction. Riverine DOM (>1000 Da) was isolated from the river Ljungbyån in southern Sweden by tangential flow ultra-filtration using a Millipore Pelican™ system (Stolte et al. 2002b). River water was collected at the end of April to early May 2000. The water was submitted to ultra-filtration following filtration through a Water Technics filter cartridge (retention 1 µm) as quickly as allowed by the system. Three batches (250 l each) were processed, from which DOM >1000 Da was concentrated to a final volume of 8 l each. The whole process was performed at 10°C and was completed within 1 wk. The DOM extract was stored at 0 to 1°C until the experiment.

Mesocosm experiment. The experiment was carried out at the marine laboratory of Kalmar University, Sweden, during June 2000. One day before the start of the experiment, 5 m^3 of sea water was collected from the Baltic proper, 10 km off the east coast of Öland (17° 01′ 82″ E, 56° 55′ 85″ N). Water was pumped from 10 m depth and filtered through a 100 µm mesh size net (to remove mesozooplankton) to 1 m^3 polyethylene carboys. Within 5 h after collection, the water was siphoned into 18 mesocosm cylinders (volume 300 l each), changing cylinders every 30 s in order to avoid differences between the different cylinders due to phytoplankton patchiness.

The mesocosms consisted of 300 l polyethylene cylinders with a polyethylene lock. Treatments were done as in Table 1. Iron and EDTA were added together in equimolar amounts from one stock solution with EDTA-chelated $FeCl_2$, except in the EDTA treatment, where only EDTA was added. DOM or nitrate was added in equal amounts with respect to nitrogen.

Mesozooplankton grazers were collected by vertical hauls from 20 m depth to the surface with a 100 µm mesh size plankton net at the same time as the water collection. Mesozooplankton grazers in field samples were quantified according to the Baltic Marine Biologists' recommendations (Hernroth 1985). After filling the mesocosms, mesozooplankton from the concentrated sample were added in selected treatments (labelled 'G' in text and tables) equal to the *in situ* mesozooplankton density. At the start of the experiment, *Nodularia spumigena* in the collected water were below detection. A low concentration (ca. 20 cells ml^{-1}) of *N. spumigena* KAC 66 (Kalmar Algal Collection) culture was therefore added to all treatments. This strain was previously isolated from the Baltic Sea. Genetic analysis has shown that KAC 66 is a common genotype of *N. spumigena* in the Baltic Sea (Janson & Granéli 2002). The *N. spumigena* culture was pregrown on F/20 medium without combined nitrogen based on aged Baltic Sea water (NO_3 < 0.1 µM, PO_4 < 0.02 µM).

Table 1. Set-up and additions in the different treatments (each in triplicate) during the mesocosm experiment. All treatments received NaH_2PO_4. Treatments that did not receive high-molecular weight dissolved organic matter (HMWDOM) received inorganic nitrogen instead, so that the nitrogen load was equal in all treatments. + = addition; – = no addition; NO_3 = nitrate added; DOM = HMWDOM added; Fe = dissolved iron/EDTA added; G = mesozooplankton grazers added; EDTA = EDTA added

Coding	Vol (l)	HMWDOM (0.1 µM N d^{-1})	$FeCl_2$/EDTA (0.1 µM d^{-1})	Mesozooplankton grazers (natural density)
NO_3	300	–	–	–
NO_3Fe	300	–	+	–
NO_3G	300	–	–	+
DOM	300	+	–	–
DOMFe	300	+	+	–
DOMG	300	+	–	+
NO_3EDTA[a]	10	–	–	–

[a]+0.1 µM d^{-1} EDTA: lower volume of EDTA was needed due to less intensive sampling programme in this treatment

Fe/EDTA concentration in this medium was 0.1 µM. Prior to addition to the mesocosms, the cells were collected on an 11 µm mesh size nylon net and washed with aged Baltic Sea water to remove excess nutrients.

Irradiance was provided by Osram powerstar HQI-E 250 W/D lamps at an incident photon flux density (16 h light/8 h dark) of 250 µmol photons m^{-2} s^{-1} (resulting in a 24 h average of 167 µmol m^{-2} s^{-1}) at the surface of the mesocosms, which were placed in a room with constant temperature of 18°C. Controls (in triplicate) to check the addition of EDTA alone were done in 10 l polycarbonate carboys under otherwise identical conditions as the 300 l cylinders. The volume was reduced, because only chlorophyll *a* and microphytoplankton species composition were monitored.

Samples were always taken 4 h after the onset of light, after careful mixing with a plastic Secchi disc-like device. All sampling equipment was made of plastic ware, and was rinsed before and between samplings with deionised water to reduce metal contamination.

Microcosm experiment. In addition to mesocosm experiments, a microcosm experiment was performed to test the effects of addition of Fe^{3+}, Mn^{2+} and Co^{2+} on the development and biochemical composition of the natural phytoplankton communities enriched either with NO_3^- or with the DOM extract. A 2^3 factorial experimental design combined the 3 trace metal additions for nitrate- and DOM-enriched phytoplankton communities (Table 2).

On Day 1 of the mesocosm experiment, 2 sets of eight 1 l acid-washed polycarbonate bottles were filled with mixed phytoplankton communities sampled in the 3 NO_3^- or DOM cylinders (⅓ l $bottle^{-1}$). Daily for 9 d, each set of 8 bottles was supplied with PO_4^{3-}, at the same concentration as in the mesocosms. Samples were taken for analysis of chlorophyll *a* (chl *a*) on Days 5, 6, 8 and 9, and on Days 6 and 9 for microphytoplankton counts.

Chemical and biological analyses. Chlorophyll *a* (chl *a*) was determined fluorometrically. Depending on the phytoplankton concentration, 25 to 100 ml was filtered in duplicate through 25 mm glass-fibre filters (Whatman GF/F) after which the filters were extracted for 8 h in the dark in 96% ethanol. Chl *a* concentration in the extract was measured using a Turner designs 10 absorption units fluorometer calibrated with a standard chl *a* solution (Sigma).

Analysis of total iron concentration in the DOM extract was performed by a Perkin Elmer 4100 atomic absorption spectrometer (AAS) with a conventional 10 cm slit burner head for the air acetylene flame at standard instrumental conditions. Total nitrogen and phosphorus concentrations in the DOM extract were analysed photometrically (Beckman DU 640) using standard methods (Valderrama 1995).

Particulate carbon and nitrogen in the mesocosms were measured after filtration of 100 to 200 ml of the samples on precombusted Whatman GF/F filters, using a Fisons NA 1500 CN analyser. A certified (OAS & IAS) analytical standard acetanilide (71.09% C and 10.36% N) (EMAL technology) was used for calibration. Dissolved nitrate + nitrite, ammonium and inorganic phosphate were analysed photometrically (Beckman DU 640) by standard methods (Valderrama 1995). During the last 3 d of the experiment, samples

Table 2. Microcosm experimental design. A 2^3 factorial experimental design was applied to 2 sets of 8 bottles. Set 1 = addition of NO_3^-: daily addition of 0.1 µM NO_3^- and 0.2 µM PO_4^{3-}; Set 2 = addition of >1000 Da riverine DOM: daily addition of DOM (concentration corresponding to 0.1 µM N) and 0.2 µM PO_4^{3-}. + = addition; – = no addition; + [$FeCl_3$] = addition of 3 nM d^{-1}; + [$MnCl_2$] = addition of 1 nM d^{-1}; + [$Co(NO_3)_2$] = addition of 0.1 nM d^{-1}

Treatment no.	$FeCl_3$	$MnCl_2$	$Co(NO_3)_2$
1	–	–	–
2	–	–	+
3	–	+	–
4	–	+	+
5	+	–	–
6	+	–	+
7	+	+	–
8	+	+	+

for nutrient analyses were filtered through Whatman GF/F in order to avoid interference of particulate material with the absorbance measurements. During this period, increased ammonium concentrations were observed in all cylinders, possibly caused by leakage from phytoplankton or protozoa during filtration.

Microphytoplankton samples (250 ml) were fixed with acid Lugol's solution and placed to settle in 10 to 25 ml sedimentation chambers. Cells were identified and quantified with inverted microscopes (Leica DM IL and Nikon Diaphot) at 100×, 200× and 400× magnification (Utermöhl 1958). The biomass (wet weight) of each microphytoplankton taxon was calculated from cell biovolumes according to the Baltic Marine Biologists' recommendations (Edler 1979). At least 20 cells were measured to estimate the cell volume. Simpson's species diversity index (D) was calculated according to (Washington 1984):

$$D = \sum_{i=1}^{S} \frac{n_i (n_i - 1)}{n(n-1)}$$

where S is the number of species, n the total number of individuals, and n_i the number of individuals in Species i of the population.

For the analysis of nodularin, 200 ml samples were filtered through a 25 mm diameter Whatman GF/F filter, after which the filter was kept frozen at –18°C until analyses. Filters were freeze-dried prior to analysis and extracted in 1 ml 70% methanol by ultrasonic probe treatment (1 min). The extracts were centrifuged ($13\,000 \times g$, 5 min) and analysed by reversed phase high performance liquid chromatography with UV photodiode array detection according to Lawton et al. (1994) with minor modifications. Nodularin in extracts was quantified against standard nodularin (CalBiochem) dissolved in methanol.

Primary production was estimated during each of the first 3 d and later during every second day of the experiment, measured as ^{14}C-uptake using the method of Ærtebjerg-Nielsen & Bresta (1984). Two µCi of radioactive $NaH^{14}CO_3$ were added to 4 replicate 100 ml glass flasks containing sample water and incubated in the centre of each cylinder at middle depth for 2 h during midday every second day. One flask was kept dark using aluminium foil, and the dark uptake was subtracted from the values obtained from the light bottles. After the incubation, the water was filtered through 0.45 µm pore size membrane filters (Gelman GN-6) and the filters were placed in an acid atmosphere for 20 min to remove remaining non-assimilated $^{14}CO_2$. The filters were then placed in glass scintillation vials and 10 ml of scintillation cocktail (Packard, Ultima Gold) was added. Activities on the filters were measured using a Wallac scintillator and primary production was calculated as µg C l^{-1} h^{-1}.

Net oxygen production/consumption and community respiration was measured at Days 3, 5 and 7 after incubation in 125 ml glass-stopped flasks. Net oxygen production bottles (1 per mesocosm cylinder) were incubated for 24 h (t_{24}) at the same light regime as the mesocosms. Respiration was determined by incubating for 24 h in the dark. Oxygen production/consumption was calculated as the difference of concentrations between $t(24)$ (dark and light incubations) and $t(0)$ (initial concentration). Oxygen concentration was determined photometrically (Roland et al. 1999) using a Beckman DU640 spectrophotometer.

Bacterial cells were counted by flow-cytometry (Becton-Dickinson FACS-Calibur) after staining with SYTO13 (Molecular Probes) according to del Giorgio et al. (1996).

Bacterial production of heterotrophic bacteria was measured as uptake of ^{3}H-leucine (specific activity 150 Ci $mmol^{-1}$, Amersham), according to Smith & Azam (1992). The leucine was diluted with non-radioactive leucine and 5 µl was added to each subsample (1.7 ml) to give a final leucine concentration of 100 nM. These subsamples were incubated for 1 h in the dark in the same room as the mesocosms. All samples were incubated in duplicates and one blank was also included (killed by initial TCA addition). The incubations were terminated by adding TCA to the samples and the bacterial cells were rinsed with TCA (centrifuging at $13\,000 \times g$ for 10 min). Bacterial carbon production was calculated assuming an intracellular isotope dilution of 2 and a carbon-to-protein ratio of 0.86 (weight:weight) in bacterial protein (Simon & Azam 1989). After addition of liquid scintillation cocktail (1 ml Ultima Gold, Packard) samples were counted in a Wallac scintillator. Bacterial protein production (BPP) was calculated based on 7.3 mol% leucine in bacterial protein.

Statistical operations were performed using STATISTICA for Windows (StatSoft). After testing for normality, ANOVA was used to investigate differences in responses at different days, after which a post-hoc comparison (Tukey's HSD) was done to further identify significant differences between treatments. The multiple regression module of STATISTICA was used to investigate correlations between parameters.

RESULTS

DOM description

The concentrated DOM extract contained 600 µM total dissolved nitrogen. A minor part of the total nitrogen consisted of free dissolved inorganic nitrogen, namely 12 and 17 µM as nitrate and ammonium, re-

spectively. Total dissolved phosphorus concentration was 7.9 µM, of which 1.0 µM consisted of dissolved reactive phosphate. Total dissolved iron concentration in the extract was 6.7 mg l^{-1}. The final concentration added to the mesocosms was adjusted to 0.1 µM d^{-1} total nitrogen, resulting in addition of 20 nM d^{-1} total dissolved iron to the mesocosms that received DOM extract.

Mesocosm experiments – initial conditions

At the start of the experiment, microphytoplankton concentrations were low. The initial chl *a* concentration was 1.32 ± 0.06 µg l^{-1} and total microphytoplankton biomass as determined by microscope counts was 0.074 ± 0.007 µg wet wt (WW) ml^{-1}. The initial microphytoplankton community was dominated by the dinoflagellate *Dinophysis acuminata* (27 ± 4% of total wet weight), followed by *Aphanizomenon* sp. (25 ± 4%).

The zooplankton abundance in the mesocosm experiments is described in detail in an upcoming paper (Kozlowsky-Suzuki et al. in press). In the tanks with added grazers, the number of metazooplankton grazers (>100 µm) in all tanks at Day 0 was not significantly different from the *in situ* abundance (17 individuals l^{-1}). Cladocerans (mostly *Evadne nordmanni*) and copepods (mostly *Acartia bifilosa*) dominated initially.

Nitrate + nitrite, ammonium and dissolved inorganic phosphate concentrations were 0.11 ± 0.2, 0.1 ± 0.1 and 0.27 ± 0.01 µM, respectively, while the silicate concentration was 9.6 µM.

Mesocosms – inorganic nutrient concentrations

During the course of the mesocosm experiment, nitrate + nitrite levels were never detectable in any of the cylinders 24 h after daily addition of nitrate or DOM. Ammonium was below detection limit (<0.2 µM) after 2 d. No significant differences between treatments were observed at any time (ANOVA) (data not shown).

In all mesocosms, phosphate gradually accumulated with daily additions until Day 5. Then, phosphate quickly dropped in the NO_3Fe and all DOM treatments, and became depleted (<0.1 µM) from Day 8. In the NO_3 and NO_3G treatments, phosphate concentration decreased from Day 6 and levelled off around 0.5 µM from Day 8 (Fig. 1).

Silicate concentrations were lowest at Day 10 in all treatments (data not shown). In all NO_3 treatments silicate concentration had decreased to 4 to 5 µM, while concentrations were between 8 and 9 µM in the DOM treatments.

Biochemical data

In all treatments, chl *a* concentration did not vary dramatically during the first 4 d (Fig. 2). Then chl *a* concentration increased at different rates and for different periods of time depending on the treatment. From Days 8 to 10, chl *a* was significantly higher in the NO_3Fe treatment and all DOM treatments compared to the NO_3 treatment ($p < 0.05$, Tukey HSD). In the NO_3 and NO_3G treatments, the chl *a* concentrations were not significantly different from each other (Tukey HSD) and reached a maximum of 7 to 8 µg l^{-1} on Day 9, i.e. approximately 10 times lower than in the DOM treatments. In the NO_3EDTA control experiment, maximum chl *a* concentration was only 1.86 ± 0.06 µg l^{-1} on Day 6 (Fig. 2).

Total particulate carbon and nitrogen concentration (TPC and TPN) showed similar trends as chl *a* (Fig. 2). The TPC and TPN concentrations in the treatments with iron and/or DOM were only significantly higher than in the NO_3 and NO_3G treatments at Day 10 (Tukey's HSD $p < 0.05$). Moreover, the NO_3 and the NO_3G treatments showed higher TPC/chl *a* ratios

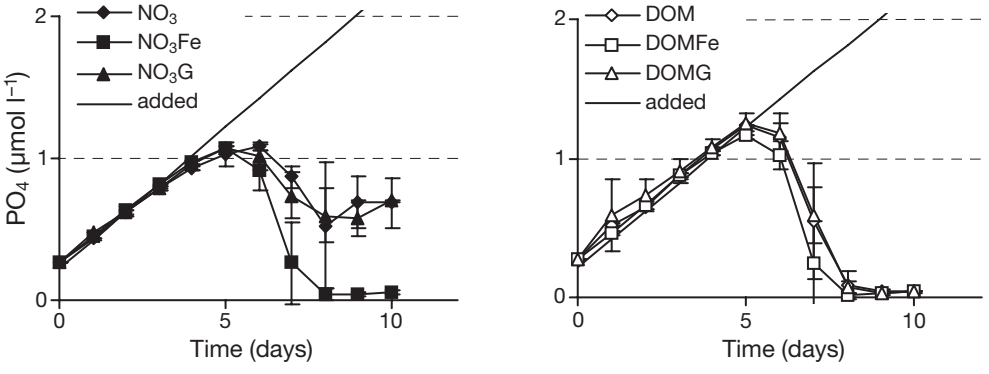

Fig. 1. Time course of dissolved reactive phosphate in the mesocosms. Treatments as in Table 1. Each data point represents average (± SD) of 3 replicate set-ups. Straight line represents the PO_4 concentration added to the mesocosms. No data were available for the NO_3EDTA treatment

compared to the treatments receiving iron and/or DOM from Day 8 (Fig. 3).

Primary production (PP) followed chl *a* concentration and consequently, PP: chl *a* ratios were not significantly different between treatments on any day (ANOVA).

Biological data

In all treatments but NO_3EDTA, the filamentous heterocystous cyanobacterium *Aphanizomenon* sp. was replaced by another heterocystous species, *Anabaena* cf. *inaequalis*. *Aphanizomenon* sp. biomass, initially 0.02 µg WW ml^{-1} contributing 25% of the total microphytoplankton community biomass, decreased to about 1% of the total microphytoplankton biomass on Day 5 (data not shown). The highest biomass of *A.* cf. *inaequalis* was reached at the last day in the 3 DOM treatments (7 to 9 µg WW l^{-1}), slightly higher than in the NO_3Fe treatment (5.4 µg WW l^{-1}) (Fig. 4). In the NO_3 and NO_3G treatments, *A.* cf. *inaequalis* biomass peaked at ca. 1.8 µg ww l^{-1} on Day 7. The average specific growth rate of *A.* cf. *inaequalis* based on cell counts between Days 0 to 7 was between 1.2 and 1.35 d^{-1}.

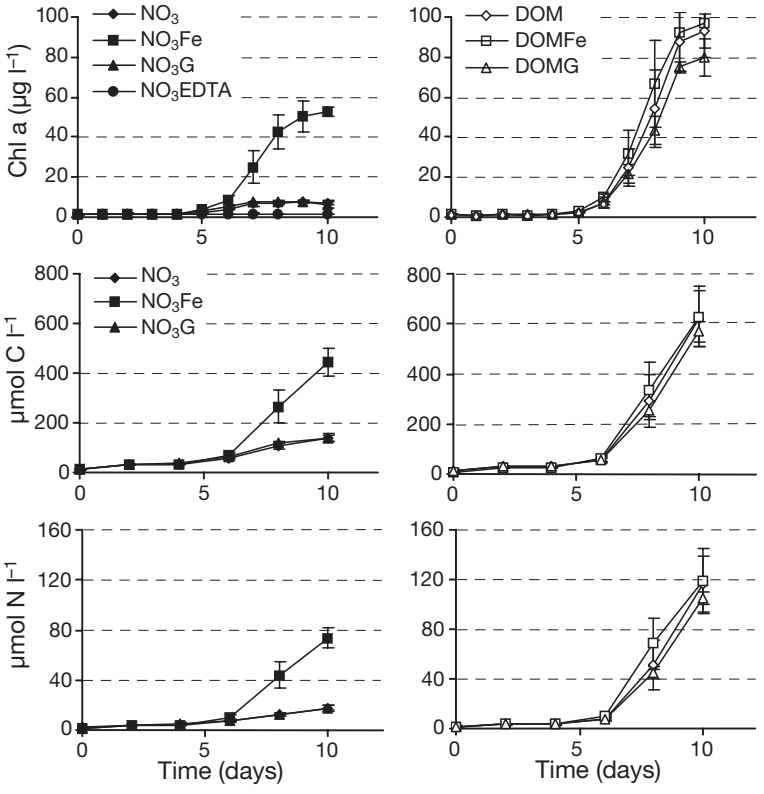

Fig. 2. Time course of chl *a*, total particulate carbon and total particulate nitrogen concentration in the different mesocosm treatments

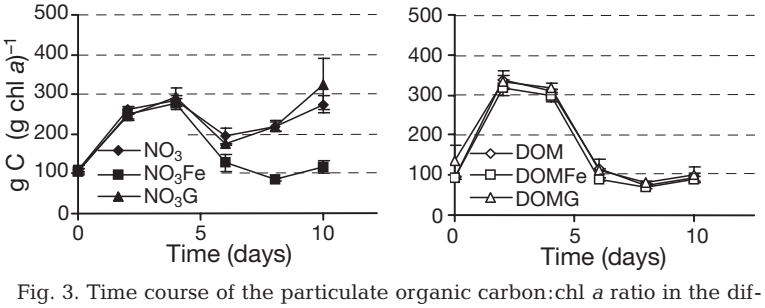

Fig. 3. Time course of the particulate organic carbon:chl *a* ratio in the different mesocosm treatments. No measurements available for NO_3EDTA treatment

Nodularia spumigena became the second dominant microphytoplankton species after *Anabaena* cf. *inaequalis* (Fig. 5) in all treatments but NO_3EDTA. *N. spumigena* average specific growth rate ranged from 0.4 to 0.6 d^{-1} in those treatments until Day 7. In the NO_3EDTA treatment, *N. spumigena* became the dominant microphytoplankton species, though growth rate was not above 0.2 d^{-1}. *A.* cf. *inaequalis* specific growth rate was only 0.17 d^{-1} between Days 0 and 7 in the NO_3EDTA treatment. At Day 10, *N. spumigena* biomass in the DOM treatment was significantly higher than in the NO_3 treatment ($p = 0.018$, Tukey HSD).

Among other microphytoplankton species, diatoms were dominated by *Skeletonema costatum* which reached highest biomass concentration in the NO_3 treatment (Fig. 6). Maximum biomass of *S. costatum* in all DOM treatments was an order of magnitude lower than in the NO_3, NO_3EDTA and NO_3G treatments, but not significantly lower than in the NO_3Fe at Days 3 and 5 (Tukey HSD). *Dinophysis acuminata*, initially the dominant dinoflagellate, did not show any positive growth in any of the treatments and disappeared in all treatments (data not shown).

The species diversity of the microphytoplankton community increased in all treatments from Days 0 to 3, after which it was reduced in all treatments (Fig. 7). The microphytoplankton diversity was most reduced in all DOM and NO_3Fe treatments at Day 10. In contrast, microphytoplankton species diversity in NO_3, NO_3G and NO_3EDTA treatments increased again from Days 7 to 10. Combining measurements of all treatments and all sampling occasions, the number of detectable microphytoplankton species was negatively cor-

related with total microphytoplankton biomass (Fig. 8). After Day 5, the number of species decreased from 18 to around 14 at Day 10 in the NO_3, NO_3G and NO_3EDTA treatments, and to 10 in the NO_3Fe treatment. In contrast, in all DOM treatments the number of microphytoplankton species decreased continuously from on average 20 at the start of the experiment to 5 at the end of the experiment.

Total metazooplankton abundance ranged from 5 to 268 ind. l^{-1} throughout the experiment and was influenced by the manipulations after Day 4. At the end of the experiment, total zooplankton abundance and zooplankton diversity was lower in the DOMG treatment compared to the NO_3G treatment. In the prescreened tanks, some metazooplankton taxa (notably Synchaeta spp. and copepoda nauplii) had reached comparable numbers as in the tanks with added grazers after 5 d. At the end of the experiment, the abundance of most species in the NO_3Fe, DOM and DOMFe treatments were lower compared to the NO_3 treatment, and showed similar trends to the DOMG treatment (Kozlowsky-Suzuki et al. in press).

The particulate nodularin concentration was highest (between 3.3 ± 0.9 and 4.4 ± 1 µg l^{-1}) in the treatments where *Nodularia spumigena* biomass was highest (all DOM treatments); data not shown. Nodularin per *N. spumigena* biomass (Fig. 9) was rather constant between the treatments, but was only significantly elevated at Day 10 in the DOMFe treatment compared to the NO_3Fe treatment (Tukey HSD, $p < 0.05$).

Bacterial number developed equally in all treatments until Day 6. Thereafter, bacterial abundance was highest in the treatments with highest chl *a* (Fig. 10). At the end of the experiment (Day 10), bacterial production in the NO_3Fe treatment was highest and significantly higher than in the NO_3 and NO_3G treatments (Tukey HSD, $p < 0.05$); data not shown. The bacterial production correlated strongest with bacterial biomass ($p < 0.00001$, linear regression). Consequently, cell-specific bacterial production did not vary significantly between the treatments at any time.

In all 3 DOM treatments, net oxygen consumption occurred over a 24 h period and light/dark cycle at Day 3 indicating the

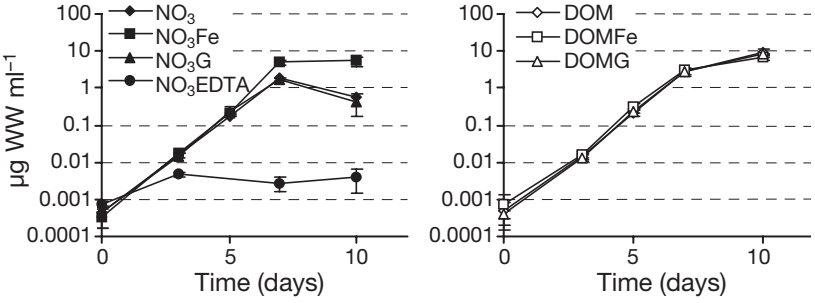

Fig. 4. *Anabaena* cf. *inaequalis*. Biomass development in the different mesocosm treatments

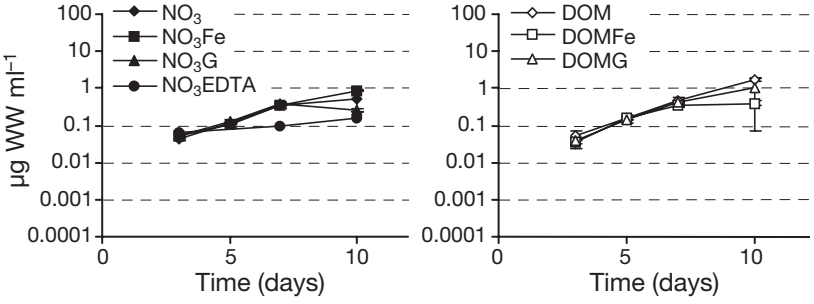

Fig. 5. *Nodularia spumigena*. Biomass development in the different mesocosm treatments. Biomass on Day 0 was calculated from concentration of cells in the culture that was added (final concentration in mesocosms = 20 cells ml^{-1} or 0.008 µg wet wt ml^{-1}

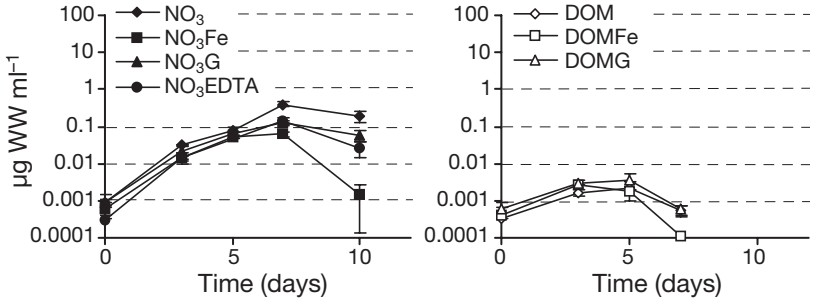

Fig. 6. *Skeletonema costatum*. Biomass development in the different mesocosm treatments

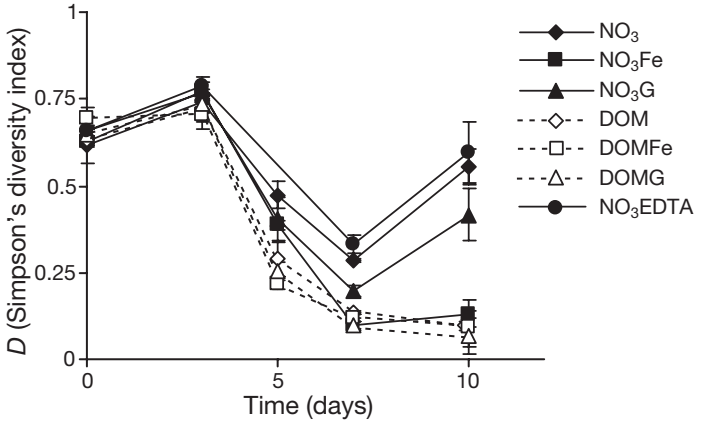

Fig. 7. Simpson's species diversity index of microphytoplankton species in the different treatments as a function of time. Each point represents average (± SD) of 3 replicate set-ups

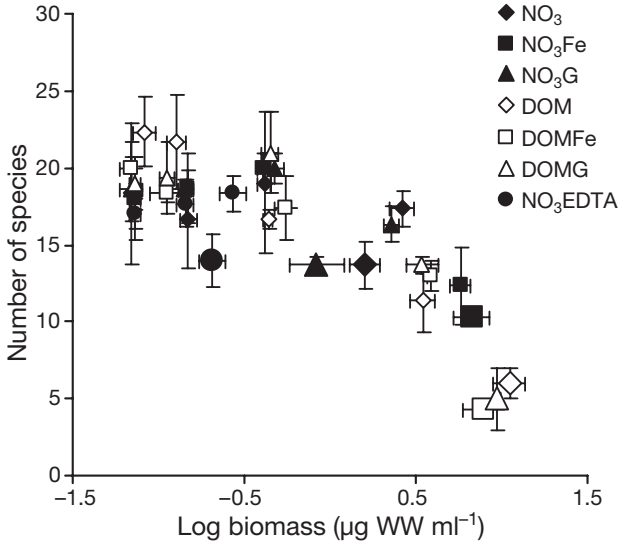

Fig. 8. Number of microphytoplankton species in the different treatments as a function of total phytoplankton biomass for all sampling days. Each data point represents average (± SD) of 3 replicate set-ups. Larger symbols refer to the last day of incubation (Day 10)

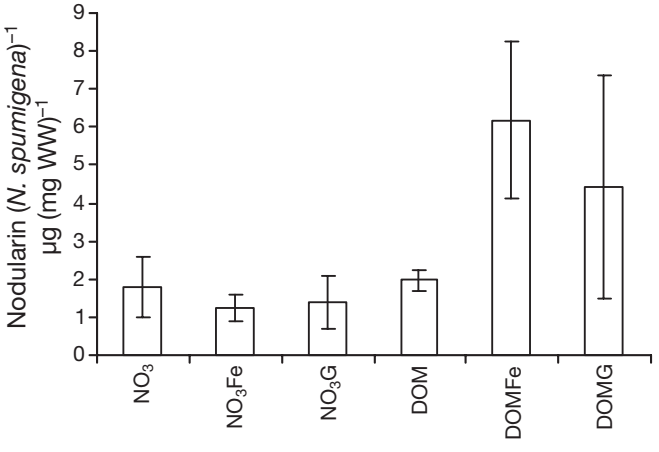

Fig. 9. Concentration of nodularin, normalized to *Nodularia spumigena* biomass on Day 10 in the different mesocosms. Before this day, no significant differences between the different treatments were observed

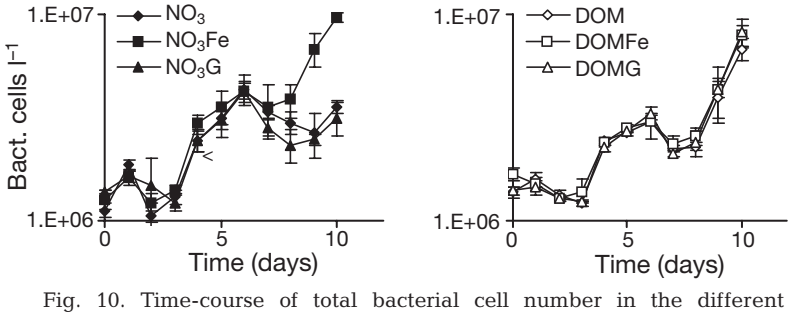

Fig. 10. Time-course of total bacterial cell number in the different mesocosm treatments

degradation of labile organic material. In those treatments, the net oxygen consumption during a 24 h period was in the range of 1.7 to 3.5 µM O_2 d^{-1}. At Days 5 and 7, net production of oxygen was observed in all treatments. The other treatments were net autotrophic throughout the experiment (Table 3).

Microcosm experiments

In the microcosms, chl *a* at Day 5 in all DOM treatments was about 2 to 4 times higher than in the NO_3 treatments (Fig. 11). The impact of Fe^{3+} addition in stimulating chl *a* concentration at Day 5 was significant (ANOVA, $p = 0.003$). In contrast, additions of Mn^{2+} and Co^{2+} did not have any significant effect (Fig. 11). Fe^{3+} additions caused a significant increase in the total cyanobacterial biomass on Day 6 (ANOVA, $p = 0.0018$) but not on Day 9 (data not shown). In the DOM treatments, the chl *a* concentration was significantly increased with the addition of Co^{2+} on Days 8 and 9 (ANOVA, $p < 0.05$; data not shown).

Anabaena cf. *inaequalis* biomass in the NO_3 microcosm treatments was significantly enhanced by iron on Day 6 (ANOVA, $p = 0.003$), but not on Day 9, while *Nodularia spumigena* biomass was enhanced by iron only on Day 9 (ANOVA, $p = 0.049$); data not shown. In the DOM treatments *A.* cf. *inaequalis* and *N. spumigena* biomass was significantly higher as a result of cobalt additions on Day 6 (ANOVA, $p = 0.018$ and $p = 0.006$, respectively; data not shown).

DISCUSSION

The observed increase in particulate nitrogen, up to 120 µmol l^{-1} in the DOM treatments, could not be explained by the total amount of nitrogen added (1 µmol l^{-1} in 10 d) in all treatments. The possibility that heterotrophy contributed significantly to the observed growth of the cyanobacteria in the DOM treatments was unlikely, considering the relatively small amounts of HMWDOM added. The biomass production can therefore only be explained by the additional input of nitrogen by nitrogen-fixing cyanobacteria, which became dominant in all treatments. Iron addition (NO_3Fe treatment) led to about 5 times higher biomass of diazotrophic cyanobacteria compared to the NO_3 treatment. Iron was also identified as the limiting factor in the separate microcosms (see below). From these results, we conclude that iron was limiting for diazotrophic cyanobacteria at the beginning of the experiment, i.e. in the

Table 3. Net oxygen production (µM O_2 d^{-1}) in the dark and in the mesocosm light regime. Average of 3 replicate experiments (± SD). Negative values indicate net consumption over a 24 h period

Day	NO_3	NO_3Fe	NO_3G	DOM	DOMFe	DOMG
Dark						
3	−10.92 ± 0.42	−11.33 ± 0.29	−9.90 ± 0.69	−8.30 ± 0.55	−8.17 ± 0.82	−8.54 ± 1.40
5	−9.95 ± 0.58	−10.21 ± 0.85	−8.16 ± 0.42	−7.37 ± 0.44	−7.12 ± 0.82	−7.13 ± 0.52
7	−21.18 ± 8.77	−26.96 ± 10.76	−21.26 ± 9.79	−23.59 ± 8.90	−24.48 ± 9.15	−21.52 ± 4.01
Light						
3	0.30 ± 1.26	0.13 ± 0.62	0.40 ± 1.26	−2.03 ± 1.82	−3.45 ± 0.63	−1.69 ± 2.24
5	11.64 ± 8.28	28.63 ± 9.07	18.50 ± 3.06	24.60 ± 8.27	27.16 ± 6.23	17.11 ± 5.94
7	28.39 ± 6.45	152.75 ± 105.29	27.59 ± 2.01	194.61 ± 47.80	199.96 ± 26.62	179.75 ± 0.96

original Baltic Sea water, probably due to the high iron demand of N_2-fixing cyanobacteria (Raven 1988). The relatively high POC:chl *a* ratio in the treatments without any iron or DOM was consistent with the concept of iron limitation (Van Leeuwe & Stefels 1998). We furthermore conclude that the daily addition of iron and/or DOM relieved iron limitation in the NO_3Fe and the 3 DOM treatments, respectively. Extra iron added in addition to DOM did not further stimulate the cyanobacteria biomass development, indicating that another factor, presumably phosphate, had become the limiting factor for cyanobacterial growth in the DOM treatments. Consistent with the hypothesis of iron limitation of diazotrophic growth, biomass development was lowest in the NO_3EDTA treatment, presumably due to effective binding of free dissolved iron by EDTA.

In the microcosm experiments receiving DOM, extra addition of iron had no significant effect, while cobalt additions had a small, but significant additional positive effect on cyanobacterial biomass. Cobalt can be limiting for some phytoplankton species in Swedish coastal waters (Granéli & Haraldsson 1993), and the relatively high concentration of DOM in our experiment could have enhanced cobalt limitation. Humic acid material can form complexes with cobalt, lowering the free dissolved cobalt concentrations by orders of magnitude (Boyer & Brand 1998 and references therein). Moreover, in the DOM treatments there might have been a higher cobalt requirement of the phytoplankton community due to the abundant cyanobacteria. The combination of these factors might have induced cobalt deficiency in the DOM treatment.

The studied effects of humic materials on aquatic biota are numerous, but often contrasting. Besides providing nitrogen for phytoplankton growth (reviewed by Carlsson & Granéli 1998), stimulation of the growth of aquatic organisms by humic substances can be caused by specific growth promoters (hormone-like molecules), sequestering and subsequent release of phosphate or trace nutrient metals such as iron, dissolution of insoluble phosphate species, reduction of harmful UV-B radiation or reduction of harmful solutes by adsorption. Negative effects include reduction of photon irradiance, direct inhibition of enzymes, and release of oxygen radicals (reviewed by Thomas 1997). The effect of humic substances on the availability of trace metals for phytoplankton is not clear. *Microcystis aeruginosa* growth was inhibited by humic substances, supposedly by a chelating effect that reduced the dissolved free iron availability (Imai et al. 1999). In contrast, stimulation by humic material through enhanced trace metal availability has been suggested for iron (Arvola & Tulonen 1998) and selenium (Doblin et al. 1999).

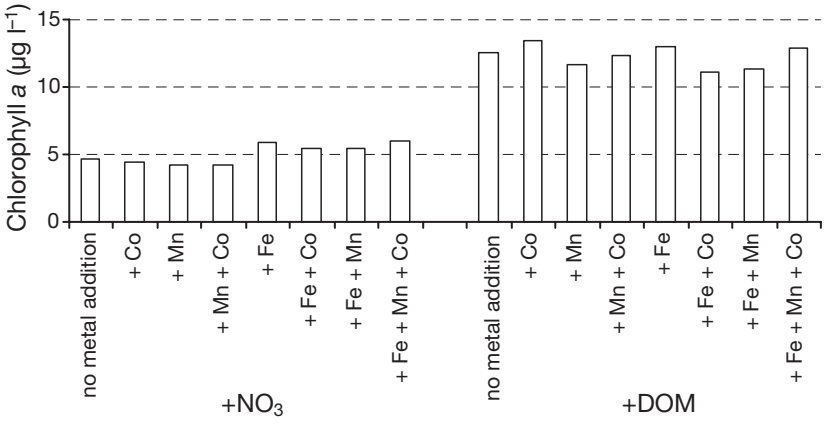

Fig. 11. Chlorophyll *a* concentration on Day 5 in the microcosms. In the NO_3 treatments, Fe addition had a significant positive effect on chl *a* concentration. In the DOM treatments, chl *a* was significantly higher than in the NO_3 treatments (ANOVA, $p < 0.05$)

In the Baltic Sea, iron limitation of cyanobacterial growth and nitrogen fixation has been documented (Stal et al. 1999). However, blooms of *Anabaena* cf. *inaequalis*, the dominant cyanobacterium in our experiment, are not common for open Baltic waters. The *hetR* gene sequences of repre-

sentative filaments of *A.* cf. *inaequalis* from our mesocosms have been deposited at GenBank as Strain M14-2 under Accession AF14178 and AF14179 (Carpenter & Janson 2001). The fact that this species grew well in our mesocosms could be explained by the relatively high irradiance levels and temperature, high phosphate concentrations, and elevated iron concentrations, possibly even in the control experiments compared to the open Baltic Sea. *Nodularia spumigena*, typically the more common cyanobacterium in the open Baltic Sea during late summer, became dominant in the NO_3EDTA treatment, and was the second most dominant species in all other treatments. This species was therefore less dependent on iron supply under the conditions of our experiments. We hypothesise that iron concentrations in open Baltic waters are high enough to allow blooms of nitrogen-fixing cyanobacteria to occur, although competition for iron between different cyanobacteria might determine the species composition of the diazotrophic phytoplankton. Possibly, bloom development of opportunistic species such as *A.* cf. *inaequalis* is rare in the Baltic Sea due to the limited availability of iron. In line with this conclusion, the high specific growth rate of *A.* cf. *inaequalis* in our experiments (max. 1.2 d^{-1}) indicates that this is a typical opportunistic species, benefiting from the relatively nutrient-rich conditions in our experiments. *N. spumigena* had a lower maximum specific growth rate (max. 0.6 d^{-1} during our experiments). Nevertheless, *N. spumigena* became dominant in the NO_3EDTA treatment where *A.* cf. *inaequalis* did not grow. Furthermore, the ratio of *N. spumigena*:*A.* cf. *inaequalis* was close to 1 and increasing in the NO_3 and NO_3G treatments (Fig. 12). Both observations suggest that *N. spumigena* is a better competitor for iron than *A.* cf. *inaequalis*.

In the treatments with highest cyanobacterial biomass, the development of other microphytoplankton was dramatically reduced. Since the effect was strongest in the treatments receiving DOM, direct effects of DOM on microphytoplankton cannot be excluded. The decrease in non-cyanobacterial species might otherwise be explained by competition for limiting nitrogen. The nitrogen-fixing cyanobacteria could increase in biomass in the NO_3Fe and all DOM treatments, and deplete the already limiting dissolved inorganic nitrogen pool even more, leading to competitive exclusion of non-cyanobacterial species. However, it is possible that extracellular products from the cyanobacteria in those treatments had a negative impact on the growth of other microphytoplankton, because proto- and metazooplankton development was also impaired by the dominant cyanobacteria (Kozlowsky-Suzuki et al. in press). For instance, the dominant copepod *Acartia bifilosa* had much higher survival, by the end of the experiment, when kept in filtered seawater, than when incubated with plankton from the NO_3Fe and all DOM treatments. Since this copepod readily fed upon the dominant *Anabaena* cf. *inaequalis* during the first half of the experiment, with no effect on its survival, the negative effect was presumably due to extracellular compound(s) produced by cyanobacteria (Kozlowsky-Suzuki et al. in press). Negative allelopathic effects of cyanobacteria on other phytoplankton have been reported (e.g. Keating 1977) including those in the Baltic Sea (Suikkanen et al. 2004), and the strong correlation between the number of species and total microphytoplankton biomass, which consisted mainly of cyanobacteria, suggests that allelopathy also might have played a role in our experiments. The pronounced differences in the diversity index between the treatments can be explained by the decrease in number of species, parallel with the increasing dominance of cyanobacteria in the NO_3Fe and all DOM treatments.

The toxicity of *Nodularia spumigena* (indicated by the nodularin:*N. spumigena* biomass ratio) was rather constant, and only significantly elevated in the DOMFe treatment compared to the NO_3Fe treatment (Tukey post-hoc). Cell-specific toxicity of *N. spumigena* increases as a result of phosphorus limitation (Stolte et al. 2002a). The DOMFe treatment was the first to be phosphorus-depleted, possibly caused by precipitation of $Fe_3(PO_4)_2$ due to the extra addition of iron over DOM (Fig. 1). The slightly longer time of phosphorus limitation might explain the enhanced toxicity of *N. spumigena* in those treatments.

The mechanism that could have lead to higher iron availability in the DOM treated mesocosms remains unclear. In natural marine waters, only 0.1 to 1% of the total iron concentration is biologically available (van den Berg 1995). Small

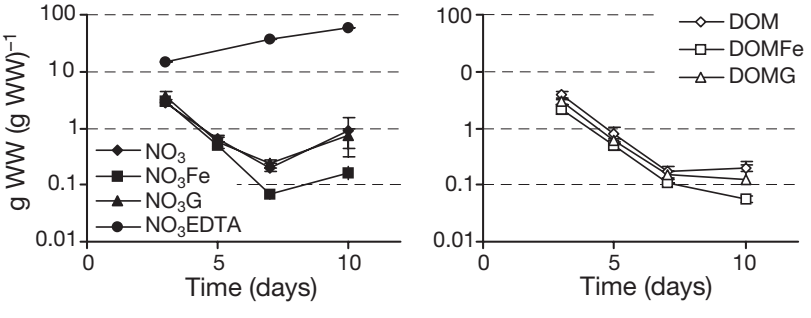

Fig. 12. *Nodularia spumigena*:*Anabaena* cf. *inaequalis*. Time-course of biomass ratio in the different mesocosm treatments (note the logarithmic scale)

changes in total iron and/or chelating substance concentrations might therefore result in large changes of the bio-available dissolved iron concentration. The concentration of total iron in the DOM that was daily added to the mesocosms was low (ca. 20 nM d^{-1}) compared to the amount of iron added in the NO_3Fe treatment (100 nM d^{-1}). However, since the speciation of iron in the mesocosms or the DOM extract was not measured, no conclusions can be drawn as to whether the stimulating effect in the DOM treatments was caused by the added iron or the chelating effect of the DOM material.

In our experiments, mesozooplankton removal or re-addition did not significantly affect chl *a*, microphytoplankton biomass, species composition, or toxin production. Therefore, the hypothesis that enhanced mesozooplankton grazing pressure leads to enhanced selection towards poorly edible or toxic species was not supported. Instead, toxic and/or otherwise poorly edible filamentous cyanobacteria developed in all mesocosms. The species succession was apparently mainly caused by bottom-up effects, while the presence or absence of mesozooplankton did not have an additional effect. Net oxygen consumption in the beginning of the experiment suggests that at least part of the added DOM material was consumed, but no effects of DOM could be detected in bacterial abundance and/or bacterial production. It cannot be excluded that net oxygen consumption was caused by enhanced phytoplankton respiration or (photo)chemical oxidation of the dissolved organic material. Since we did not manipulate the microzooplankton biomass in our experiments, the hypothesis should be further tested with respect to this group.

In summary, although it is not known which fraction of the iron is available to phytoplankton, the total iron concentrations in the open Baltic Sea surface waters are around 50 nM. The largest part of this is in particulate (5 nM) and colloidal (35 nM) form. Dissolved cationic forms of iron were below 9 nM throughout the water column (Brügmann et al. 1998). Compared to dissolved iron concentrations of 0.1 nM in the open ocean (e.g. Martin & Gordon 1988), iron is not scarce in the Baltic Sea. This might be one of the reasons explaining the success of iron-demanding diazotrophic cyanobacteria in this area. However, nitrogen fixation can become limited by iron availability during summer (Stal et al. 1999). In this study, iron and riverine high molecular weight DOM has been shown to release this possible iron limitation for cyanobacteria in a Baltic Sea community. Further studies are needed to reveal whether the ongoing increased leaching of humic substances from Swedish soils (Andersson et al. 1991) will lead to increased N fixation and noxious cyanobacterial blooms.

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