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ORIGINAL ARTICLE

Carbon, nitrogen and O_2 fluxes associated with the cyanobacterium *Nodularia spumigena* in the Baltic Sea

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Photosynthesis, respiration, N_2 fixation and ammonium release were studied directly in *Nodularia spumigena* during a bloom in the Baltic Sea using a combination of microsensors, stable isotope tracer experiments combined with nanoscale secondary ion mass spectrometry (nanoSIMS) and fluorometry. Cell-specific net C- and N_2-fixation rates by *N. spumigena* were 81.6 ± 6.7 and 11.4 ± 0.9 fmol N per cell per h, respectively. During light, the net C:N fixation ratio was 8.0 ± 0.8. During darkness, carbon fixation was not detectable, but N_2 fixation was 5.4 ± 0.4 fmol N per cell per h. Net photosynthesis varied between 0.34 and 250 nmol O_2 h^{-1} in colonies with diameters ranging between 0.13 and 5.0 mm, and it reached the theoretical upper limit set by diffusion of dissolved inorganic carbon to colonies (>1 mm). Dark respiration of the same colonies varied between 0.038 and 87 nmol O_2 h^{-1}, and it reached the limit set by O_2 diffusion from the surrounding water to colonies (>1 mm). N_2 fixation associated with *N. spumigena* colonies (>1 mm) comprised on average 18% of the total N_2 fixation in the bulk water. Net NH_4^+ release in colonies equaled 8–33% of the estimated gross N_2 fixation during photosynthesis. NH_4^+ concentrations within light-exposed colonies, modeled from measured net NH_4^+ release rates, were 60-fold higher than that of the bulk. Hence, *N. spumigena* colonies comprise highly productive microenvironments and an attractive NH_4^+ microenvironment to be utilized by other (micro)organisms in the Baltic Sea where dissolved inorganic nitrogen is limiting growth.

Subject Category: microbial ecosystem impacts
Keywords: stable isotopes; microsensors; N_2 fixation; photosynthesis; respiration; ammonium release
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Introduction

Summer blooms by filamentous, N_2-fixing cyanobacteria are dominated by *Nodularia spumigena*, *Aphanizomenon* sp. and *Anabeana* sp. in the Baltic Sea. These large cyanobacteria form colonies and contain gas vesicles providing buoyancy, whereby they can accumulate at optimal depth to sustain energy and nutrient demands for cellular growth and limit sedimentation losses (Walsby *et al.*, 1997). *N. spumigena* accumulate close to the sea surface, whereas *Aphanizomenon* sp. often occurs deeper in the water column (Hajdu *et al.*, 2007). *Nodularia* sp. is highly tolerant to ultraviolet radiation through the production of mycosporine-like amino acids and carotenoids (Wulff *et al.*, 2007; Mohlin and Wulff, 2009), and mm-thin *Nodularia* layers accumulating at the sea surface have recently been shown to be highly productive microenvironments with steep O_2 and pH gradients during light as well as during darkness (Ploug, 2008).

The large, filamentous cyanobacteria are thought to be the main N_2-fixing organisms in the Baltic Sea (Boström *et al.*, 2007; Degerholm *et al.*, 2008; Ploug *et al.*, 2010). Budget calculations of combined nitrogen as well as experimental studies have shown that these cyanobacteria must fix excess N_2 relative to their own nitrogen demand and channel the surplus nitrogen through the microbial food web and to picocyanobacteria (Ohlendieck *et al.*, 2000; Larsson *et al.*, 2001; Stal *et al.*, 2003). Using ^{15}N tracers combined with nanoscale secondary ion mass spectrometry (nanoSIMS) and elemental analysis isotope ratio monitoring mass spectrometry, it was recently shown that *Aphanizomenon* sp. releases a significant fraction (35%) of its newly fixed N_2 as NH_4^+ to the surrounding waters in the

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Baltic Sea (Ploug *et al.*, 2010). Hence, these large cyanobacteria can supply other algae and (micro)-organisms with nitrogen when bulk concentrations of dissolved inorganic nitrogen are low. The significance of N_2 fixation and NH_4^+ release by *N. spumigena* in the Baltic Sea is still unknown.

In this study, we used a combination of microsensors and isotope tracer experiments combined with nanoSIMS, and fluorometry to measure O_2 fluxes, C assimilation, N_2 fixation and NH_4^+ release in a field population of *N. spumigena* in the Baltic Sea. Distributions of photosynthesis and respiration across the size spectrum of colonies were analyzed in relation to diffusion limitation of photosynthesis and respiration by inorganic carbon and O_2, respectively. Functional similarities between *N. spumigena* and *Aphanizomenon* sp. and their impact on the Baltic Sea ecosystem are discussed.

Materials and methods

Sampling
Bulk water samples including *N. spumigena* colonies were directly collected at the sea surface using 1 l Duran bottles or a bucket in the upper meter of the sea at station B1 (N 58°48′28, E 17°37′60) in the Stockholm archipelago in August 2009. The salinity was 6 and the temperature was 19°C. Samples were immediately brought to the laboratory where they were incubated with stable isotopes or analyzed by the use of microsensors and fluorometry. All experiments were carried out at *in situ* temperature.

N. spumigena cell sizes, abundance and chlorophyll (Chl.a) colony structure
Cell sizes and abundance were studied using an inverted microscope (Zeiss, Bern, Switzerland) equipped with a digital camera (Axio Cam, Zeiss) connected to an image analysis program (AxioVision, Zeiss). Cell abundance and cumulative trichome length ($m\,l^{-1}$) were measured in Lugol-fixed samples in a gridded Sedgewick Rafter counting chamber (Wildlife Supply Company, Buffalo, NY, USA). Triplicate analysis of Chl. *a* in 0.5 l bulk water samples filtered onto GF/F filters (extracted in 15 ml Falcon vials containing 90% methanol) or in single *N. spumigena* colonies (extracted in 1.5 ml Eppendorf vials containing 90% methanol), and blanks were performed according to Meeks and Castenholz (1971).

Dissolved inorganic carbon
Samples were injected into an acid stream (30 mM HCl) to convert dissolved inorganic carbon (DIC) into CO_2. Afterwards, samples were analyzed in a flow injection system coupled to a conductivity cell (Hall and Aller, 1992). Final concentrations of ^{13}C-bicarbonate in isotope incubation experiments (see below) were measured on a gas chromatography-isotope ratio monitoring mass spectrometry according to Assayag *et al.* (2006).

Incubations with stable isotopes
Bulk water samples were incubated in 1 l bottles. The bottles were closed with rubber stoppers through which ^{13}C-bicarbonate (9% final labeling) and $^{15}N_2$ (Sigma) (10% final labeling; Mohr *et al.*, 2010) was injected to the sample, except for three control bottles. The syringe was flushed with Argon gas between injections with $^{15}N_2$. Three replicates were incubated for 0, 3 or 6 h at 270 µmol photons $m^{-2}\,s^{-1}$ as measured using an LiCOR irradiance sensor, or in dark at *in situ* temperature (19 °C) in a thermostated room. The light sources were neon lamps. The incubations were stopped by filtration of samples onto pre-combusted GF/F filters (Montoya *et al.*, 1996). The filtrates were filled into 12 ml gas-tight exetainers to which 100 µl saturated $HgCl_2$ solution was added. Subsamples for nanoSIMS analysis were fixed with 2% paraformaldehyde and washed after filtration onto gold-palladium-coated GTTP filters (pore size 0.22 µm; diameter 25 mm; Millipore, Schwalbach, Germany).

Elemental analyzer-isotope ratio monitoring mass spectrometry analysis
The GF/F filters were analyzed through mass spectrometry, using N_2 and CO_2 released by flash combustion in excess oxygen at 1050 °C to determine the amount of ^{15}N gas and ^{13}C-bicarbonate incorporated into biomass. An automated elemental analyzer coupled to a Delta Plus Advantage mass spectrometer was used (Warembourg, 1993). Before analysis, GF/F filters were freeze-dried and dehydrated by HCl smoke in a desiccator overnight. Round punch-outs (Ø1 cm) of the GF/F filters were packed into tin cups, tightly pressed into a pill-shaped form and loaded onto an autosampler flushed with helium. As a standard, caffeine was used for calibration.

NanoSIMS analysis
For nanoSIMS analysis of single *N. spumigena* cells, the GTTP filters containing chemically fixed water samples were cut with a round stencil (Ø5 mm) and mounted onto a sample holder. The analysis was performed using a nanoSIMS 50 l manufactured by Cameca (Gennevilliers, France). For each individual cell, we recorded simultaneously secondary ion images of ^{12}C, ^{13}C, $^{12}C^{14}N$ and $^{12}C^{15}N$ using four electron multipliers. The measurements and the image and data processing were performed as described previously (Musat *et al.*, 2008; Ploug *et al.*, 2010).

Photosynthesis and respiration measurements in colonies using microsensors
Freshly sampled colonies, which appeared relatively homogeneous in density, were transferred to a Petri dish coated by a 3-mm-thick agar layer

(1% w:w) at the bottom and covered by water from the sampling site. The diffusion of gases, ions and solutes in 1% agar is close to that in sea water (Libicki *et al.*, 1988; Revsbech, 1989). Artifacts from a solid boundary, which limits solute exchange between colonies and the surrounding water, was hereby minimized (Ploug and Grossart, 1999). The Petri dish was placed in a thermostated container at *in situ* temperature. Oxygen concentration gradients were measured using a Clark-type oxygen microsensor (Revsbech, 1989) attached to a micromanipulator. The current was measured by a picoamperemeter (Unisense, PA2000) connected to a strip-chart recorder (Kipp en Zonen). The electrode was calibrated at anoxic conditions and at air saturation. Its 90% response time was <1 s and the stirring sensitivity $<0.3\%$. Its tip was 2 μm wide and its position was observed through a dissection microscope. Three replicates of the O_2 concentration gradient at the colony–water interface were measured at 50 μm step increments at steady-state in light and in dark, respectively. The light source was a Halogen Schott lamp (KL 1500 LCD) equipped with an infrared cutoff filter and calibrated using an LiCOR irradiance sensor. The O_2 fluxes and the exact position of colony surface relative to the center of the colony were calculated from the measured concentration gradients using the analytical solutions for diffusion in aggregates (Ploug *et al.*, 1997). The molecular diffusion coefficient for O_2 of $2.02 \times 10^{-5}\,cm^2\,s^{-1}$ at a salinity of 6 and at 19 °C was used in the calculations (Broecker and Peng, 1974). O_2 fluxes were converted into carbon assimilation rates assuming a photosynthetic quotient of 1.2 (Masotti *et al.*, 2007). The colony dimensions were measured under a dissection microscope with a calibrated ocular micrometer. Surface area and volume was calculated as for ellipsoids (Mass, 1994).

The theoretical limits of DIC and O_2 fluxes to colonies were calculated as a function of colony size as described in Ploug *et al.* (1997). For calculations of DIC fluxes, a bulk concentration of 1.35 mM (as measured) and a diffusion coefficient of $9.4 \times 10^{-6}\,cm^2\,s^{-1}$ (for HCO_3^-, the dominant species at pH of the bulk water) were used (Li and Gregory, 1974). For calculations of O_2 fluxes, a bulk concentration of 300 μM O_2 and a diffusion coefficient of $2.02 \times 10^{-5}\,cm^2\,s^{-1}$ were used. An apparent diffusivity of gases and solutes within colonies being 0.95 times the molecular diffusion coefficient in sea water was assumed (Ploug *et al.*, 2008). Both fluxes were calculated for a Sherwood number of 1, that is, no effective difference between the motion of the colony and that of the surrounding water.

Ammonium analysis

The net production of $^{15}NH_4^+$ within the plankton community of cyanobacteria and other (micro) organisms was quantified in the filtrate after incubations with $^{15}N_2$ during 0, 3 and 6 h in light or in darkness using the method by Warembourg (1993). Samples were analyzed on a gas chromatography-isotope ratio monitoring mass spectrometry (VG Isogas Limited, Middlewitch, UK).

Colonies of different sizes, in which photosynthesis at 270 μmol photons $m^{-2}\,s^{-1}$ and dark respiration had been measured, were incubated afterwards for measurements of ammonium release during early evening and night. We used 15 ml acid-washed Falcon tubes rinsed with UV-treated MilliQ water. One single colony was added to each vial with 0.2-μm-filtered sea water from the sampling site, and closed without air bubbles. The vials were positioned on a roller table to avoid diffusion limitation during incubations. The colonies were first incubated at 270 μmol photons $m^{-2}\,s^{-1}$ in <9 h at *in situ* temperature in a thermostated room. Afterwards, the same colonies were harvested and re-incubated in darkness during 11 h in new vials containing 0.2-μm-filtered sea water from the sampling site. Three controls (nine vials) without colonies were incubated during 0, 9 and 20 h. Ammonium concentrations was measured on a fluorometer (Turner Designs, TD-700) using the method by Holmes *et al.* (1999). Ammonium concentration distributions within and around colonies were modeled from the measured NH_4^+ release rate and colony size using the analytical solutions for diffusion in aggregates (Ploug *et al.*, 1997). A molecular diffusion coefficient for NH_4^+ of $1.62 \times 10^{-5}\,cm^2\,s^{-1}$ at a salinity of 6 and 19 °C was used in the calculations (Li and Gregory, 1974).

Results

DIC, NH_4^+ and Chl.*a* concentrations in the bulk as well as total net C- and N_2-fixation rates, and net NH_4^+ release rates by the bulk plankton community are shown in Table 1. DIC decreased significantly within the beginning of the cyanobacterial bloom, which was composed of *Aphanizomenon* sp., *Anabaena* sp. and of *Nodularia* sp. occurring as free-living trichomes as well as in mm-size colonies. Dinoflagellates and picoplankton were also present at high abundance. Chl.*a* concentrations reached 9.2 μg l^{-1} during our study. Nitrate concentrations were <100 nM (Swedish monitoring program), and ammonium concentrations were <200 nM. Total net C- and N_2-fixation rates were 896 nmol C $l^{-1}\,h^{-1}$ and 41 nmol N $l^{-1}\,h^{-1}$, respectively, in light-exposed samples and 10-fold lower during darkness. The net production of NH_4^+ of the newly fixed N_2 (traced as $^{15}NH_4^+$ in the bulk after 3–6 h incubation with $^{15}N_2$) within the bulk plankton community was on average 1.0 and 1.3 nmol N $l^{-1}\,h^{-1}$ during light and dark, respectively.

An example of the isotopic compositions of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ in a *Nodularia* trichome after incubations with stable isotopes during 6 h is shown

Table 1 Bulk measurements of DIC, NH_4^+ and Chl. *a* during the course of time

Date	*31 July*	*2 August*	*4 August*	*8 August*
DIC (mM)	1.82	1.48 ± 0.09	1.38 ± 0.15	1.33 ± 0.10
NH_4^+ (nM)	ND	163 ± 12	142 ± 20	155 ± 38
Chl. *a* (μg l^{-1})	ND	ND	6.4 ± 2.0	9.24 ± 0.72
Total net C-fixation rate in light (nmol C l^{-1} h^{-1})	ND	ND	896 ± 30	ND
Total net C-fixation rate in darkness (nmol C l^{-1} h^{-1})	ND	ND	21 ± 4	ND
Total net N_2-fixation rate in light (nmol N l^{-1} h^{-1})	ND	ND	38 ± 6	ND
Total net N_2-fixation rate in darkness (nmol N l^{-1} h^{-1})	ND	ND	3.2 ± 0.3	ND
Total net NH_4^+ production rate in light (nmol N l^{-1} h^{-1})[a]	ND	ND	1.0 ± 0.4	ND
Total net NH_4^+ production rate in dark (nmol N l^{-1} h^{-1})[a]	ND	ND	1.3 ± 0.4	ND

Abbreviations: Chl., chlorophyll; DIC, dissolved inorganic carbon; ND, not determined.
Total C- and N_2-fixation rates measured using stable isotopes on the 4 August during light and darkness. Net NH_4^+ production rate within the total plankton community measured during light and darkness. All numbers represent average value with standard error of the mean value ($n = 3$).
[a]Traced by $^{15}NH_4^+$ release from newly fixed $^{15}N_2$.

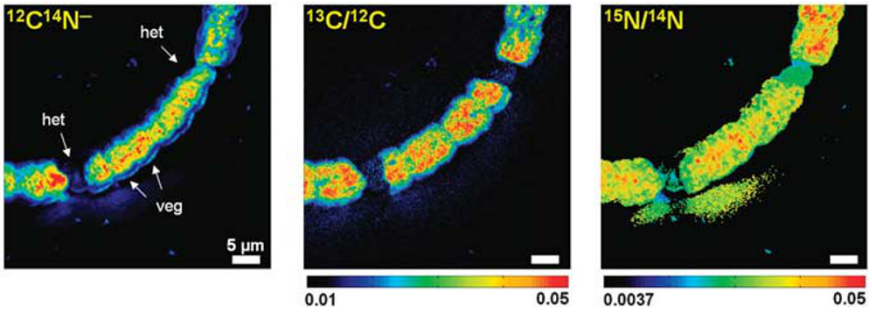

Figure 1 Distribution of $^{12}C^{14}N^-$, $^{13}C/^{12}C$ and $^{15}N/^{14}N$ in single *Nodularia* cells as measured by nanoSIMS after 6 h incubations with ^{13}C and $^{15}N_2$.

(Figure 1). Vegetative cells were highly labeled by ^{13}C and ^{15}N after 6 h. In contrast, heterocysts were only faintly labeled by ^{13}C relative to that by ^{15}N. Total net C- and N_2-fixation rates were highly variable for individual cells (Table 2). The average net C-fixation rate was 81.6 ± 6.7 fmol C per cell per h, whereas the average net N_2-fixation rate was 11.4 ± 0.9 fmol N per cell per h. Paired measurements revealed an average net C:N fixation ratio of 8.0 ± 0.8 (s.e.; $n = 47$). Total net C assimilation by free-living *Nodularia* trichomes was 38 ± 3 nmol C l^{-1} h^{-1} and total net N assimilation was 5.4 ± 3.0 nmol N l^{-1} h^{-1}. Total average net carbon assimilation rate by colonies (>1 mm) was 50 ± 16 nmol C per colony per h and net N_2-fixation rate was 7.4 ± 0.6 nmol C per colony per h. Hence, 18% of the total net bulk N_2 fixation was on average associated with one single *Nodularia* colony per l, and total net N_2 fixation by *N. spumigena* (free-living trichomes and colonies) comprised 31% of total net N_2 fixation in the surface waters of the Baltic Sea in light. Dark N_2 fixation by *N. spumigena* was twofold lower than that of N_2 fixation in light, and it explained 78% of the bulk N_2 fixation during darkness.

An example of a *Nodularia* colony is shown (Figure 2). Oxygen concentrations within a 1.3 mm large colony during the early stage of the bloom (31 August) varied between 220 and 510 μM in dark and light at 270 μmol photons m^{-2} s^{-1}, respectively (Figure 3). Dark respiration rate calculated from the O_2 distribution was 5.5 nmol O_2 per colony per h. Net photosynthesis was 8.5 and 18 nmol O_2 per colony per h at 120 and 270 μmol photons m^{-2} s^{-1}, respectively. Photosynthesis was saturated in the entire colony at 270 μmol photons m^{-2} s^{-1} (data not shown). Volume-specific net photosynthesis was 8.5 and 18 nmol O_2 mm^{-3} h^{-1} at 120 and 270 μmol photons m^{-2} s^{-1}, respectively, because the colony volume was 1 mm^3. The colony appeared compact and its Chl.*a* content was 0.41 μg per colony. The Chl. *a*-specific photosynthesis in this colony was 0.44 mg C mg Chl. *a* per h at 270 μmol photons m^{-2} s^{-1}.

Dark respiration measured as a function of gross photosynthesis at 270 μmol photons m^{-2} s^{-1} in the same colonies is shown (Figure 4). Dark respiration was a significant linear function of gross photosynthesis during the early stage of the bloom. It was described by the equation: $R = 0.26 \times GP - 0.6$ ($R^2 = 1.00$), where dark respiration and gross photosynthesis is measured as nmol O_2 per colony per h. After few days, this relationship was described by: $R = 0.26 \times GP + 3.3$ ($R^2 = 0.83$), which indicates a higher background respiration by attached microbiota.

Table 2 C- and N-fixation rates in *Nodularia* sp. cells, trichomes and colonies measured by nanoSIMS and microsensors

Nodularia *sp. abundance*	*Specific net C-fixation rate*	*Specific net N_2-fixation rate*	*Total net C-fixation rate*	*Total net N_2-fixation rate*
4.7 ± 2.7 ($\times 10^5$ cells per l)	81.6 ± 6.7 (fmol C per cell per h) (ND)[a]	11.4 ± 0.9 (fmol N per cell per h) (5.4 ± 0.4)[a]	—	—
Free trichomes 1.5 ± 0.9 ($m\,l^{-1}$)	25.6 ± 1.6 (nmol $C\,m^{-1}\,h^{-1}$) (ND)[a]	3.6 ± 0.3 (nmol $N\,m^{-1}\,h^{-1}$) (1.7 ± 0.1)[a]	38 ± 3 (nmol $C\,l^{-1}\,h^{-1}$) (ND)[a]	5.4 ± 0.5 (nmol $N\,l^{-1}\,h^{-1}$) (2.5 ± 0.2)[a]
1 (colonies per l)	50 ± 16 (nmol C per colony per h)	7.4 ± 0.6 (nmol N per colony per h)	50 ± 16 (nmol $C\,l^{-1}\,h^{-1}$)	7.4 ± 0.6 (nmol $N\,l^{-1}\,h^{-1}$)

Abbreviations: nanoSIMS, nanoscale secondary ion mass spectrometry; ND, not detectable; s.e., standard error.
Nodularia sp. abundance measured as cell densities ($\times 10^5$ cells per l), cumulative length of free trichomes ($m\,l^{-1}$) and colonies (colonies per l) and their respective net C- and N_2-fixation rates. Total (all organisms) net C- and N_2-fixation rates in the bulk are also shown. All parameters are expressed as the average value (± s.e.) in samples analyzed by nanoSIMS ($n = 47$) and microsensors ($n = 14$).
[a]Numbers within parenthesis represent the rate in dark-exposed cells and trichomes.

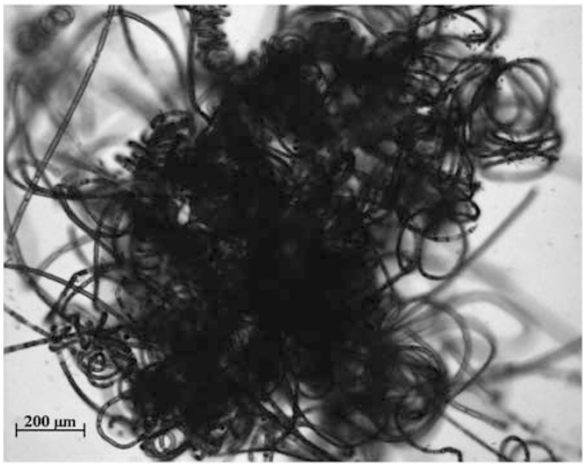

Figure 2 *N. spumigena* colony collected in the Baltic Sea.

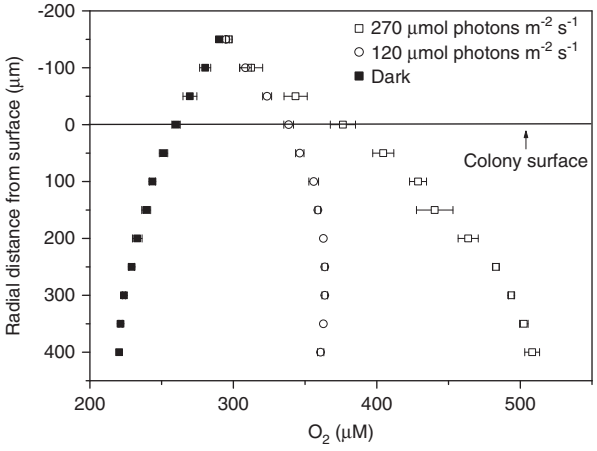

Figure 3 O_2 distributions measured in a *N. spumigena* colony at different light conditions. All data points represent the mean value of three series of measurements with the standard of the mean value shown as bars ($n = 3$).

Net NH_4^+ release from colonies to the 0.2-µm-filtered bulk water was measured during light and darkness in colonies of which size, photosynthesis and respiration had previously been measured. The

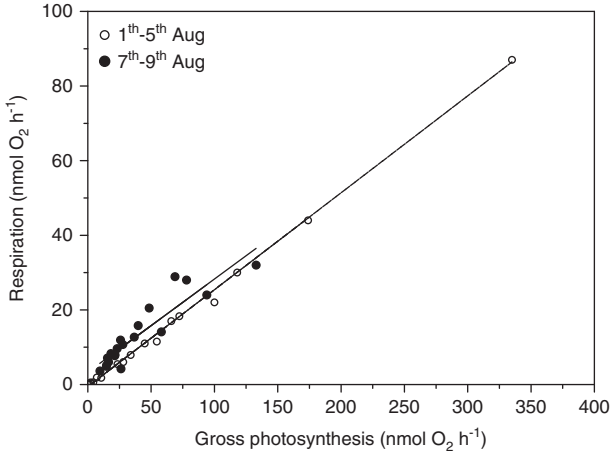

Figure 4 Dark respiration as a function of gross photosynthesis measured in differentially sized colonies during the bloom. All data points represent the average value of three to five series of measurements of O_2 gradients during light and dark, respectively (> 250 O_2 gradients).

average NH_4^+ release was sixfold higher in light as compared with that measured in darkness. The average net NH_4^+ release comprised 8–33% of the estimated gross N_2-fixation rate in light, when the ratio of dark respiration to gross photosynthesis was highest (Table 3). The NH_4^+ concentration gradients corresponding to the average value of measured net NH_4^+ release rates in light (0.77 nmol NH_4^+ per colony per h equal to the average value measured on 7 August; Table 3) and in dark-exposed 2 mm large colonies are shown (Figure 5). The maximum concentration varied up to 9 µM in the center of colonies during light when the bulk concentration was 150 nM, thus only *N. spumigena* colonies were on average < 60-fold enriched in NH_4^+ as compared with the bulk water during light. The net ammonium release and NH_4^+ gradient were substantially smaller in dark-exposed colonies.

We compared data of gross photosynthesis measured in *Aphanizomenon* sp. colonies during the same season and at the same temperature and station in the Baltic Sea, but exactly 1 year earlier

Table 3 Changes in volume, photosynthesis, respiration and ammonium release of *Nodularia* sp. colonies during time

Date	*Volume* (mm^3)	*Net phot.* (nmol $O_2\,h^{-1}$)	*Resp.* (nmol $O_2\,h^{-1}$)	*Dark resp: gross phot*	*Vol. gross phot.* (nmol $O_2\,mm^{-3}\,h^{-1}$)	NH_4^+ *release* (nmol $N\,h^{-1}$)	NH_4^+ *release in % of gross* N_2 *fix*
1 Aug (6)	0.20 ± 0.16	5.1 ± 2.4	1.3 ± 0.9	0.20 ± 0.03	32 ± 9.0	ND	ND
5 Aug (14)	9.3 ± 5.1	60 ± 19	20 ± 7	0.25 ± 0.01	8.6 ± 3.5	ND	ND
7 Aug (12)	8.3 ± 2.1	35 ± 8	15 ± 3	0.30 ± 0.03	6.0 ± 2.1	0.77 ± 0.57	33 ± 5
9 Aug (8)	7.1 ± 1.9	18 ± 5	11 ± 3	0.38 ± 0.01	4.0 ± 1.2	0.12 ± 0.03 (0.02 ± 0.01)[a]	8 ± 2

Abbreviations: ND, not determined; s.e., standard error.
Volume (mm^3), net photosynthesis (nmol $O_2\,h^{-1}$) at light saturation (270 μmol photons $m^{-2}\,s^{-1}$), respiration (nmol $O_2\,h^{-1}$), ratio of dark respiration to gross photosynthesis at saturating light intensity, volumetric gross photosynthesis (nmol $O_2\,mm^{-3}\,h^{-1}$), NH_4^+ release (nmol $N\,h^{-1}$) in light and NH_4^+ release in % of estimated gross N_2 fixation (net N_2 fixation plus NH_4^+ release) at measured in colonies sampled at different dates (number of colonies indicated within parenthesis). All parameters were measured in the same colony within the day of sampling and are expressed as the average value (± s.e.).
[a]Numbers within parenthesis represent the rate in dark-exposed colonies.

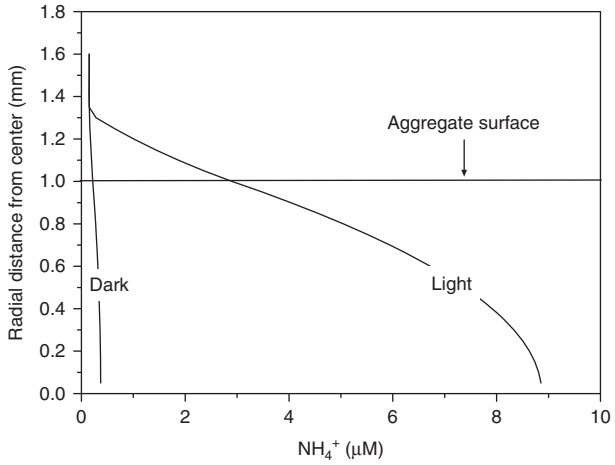

Figure 5 NH_4^+ distributions during dark and light at 270 μmol photons $m^{-2}\,s^{-1}$ modeled from measured net fluxes (Table 3).

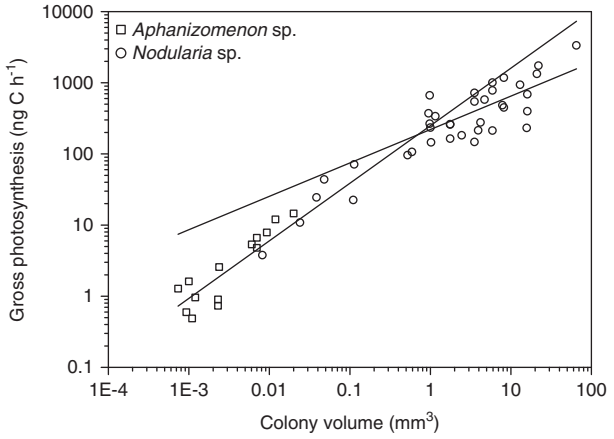

Figure 6 Gross photosynthesis as a function of colony size of *Aphanizomenon* sp. (squares) and *N. spumigena* (circles). All data points represent the average value of three series of measurements.

(Ploug *et al*., 2010) with those measured in *N. spumigena* colonies sampled during this study (Figure 6). Concentrations of orthophosphate and inorganic nitrogen were similarly low during the two studies (Swedish monitoring program). Photosynthesis was saturated during all measurements performed at 270–300 μmol photons $m^{-2}\,s^{-1}$ of both *Aphanizomenon* sp. and *N. spumigena* Gross photosynthesis varied almost four orders of magnitude in colonies, with volumes varying five orders of magnitude. It ranged between 0.5 ng C per colony per h and 3.5 μg C per colony per h, and was significant functions of colony volume. For small colonies (<1 mm) it could be described by: $GP = 250 \times (vol)^{0.81}$ ($R^2 = 0.91$, $P < 0.001$; $n = 20$), where gross production is measured as ng C per colony per h and volume is measured as mm^3. For large colonies (>1 mm), it was described by: $GP = 201 \times (vol)^{0.51}$ ($R^2 = 0.51$, $P < 0.001$; $n = 31$). Hence, the volumetric gross photosynthesis was lower in large colonies relative to that in small colonies.

Diffusion to a stagnant sphere increases proportionally to the sphere radius, whereas biological rates of photosynthesis and respiration may increase proportionally to r^3, that is, colony volume, as shown in *Aphanizomenon* sp. (Ploug *et al*., 2010). Larger colonies may, therefore, be subjected to increasing diffusion limitation of biological processes at a certain colony size. The volumetric net photosynthesis rates measured at saturating light intensities are shown as a function of colony volume (Figure 7a). It was high and invariable with size for small colonies (<1 mm^3 equivalent to ~1.25 mm diameter) of both *Aphanizomenon* sp. and *N. spumigena* and hence independent of the potential DIC flux. In larger colonies composed by *N. spumigena*, however, the volumetric net photosynthesis rates decreased likely as a result of DIC limitation. Prognostic modeling showed that C-fixation rates due to photosynthesis were close to the theoretical maximum fluxes of DIC from the surrounding water to colonies (>1 mm) in the Baltic Sea.

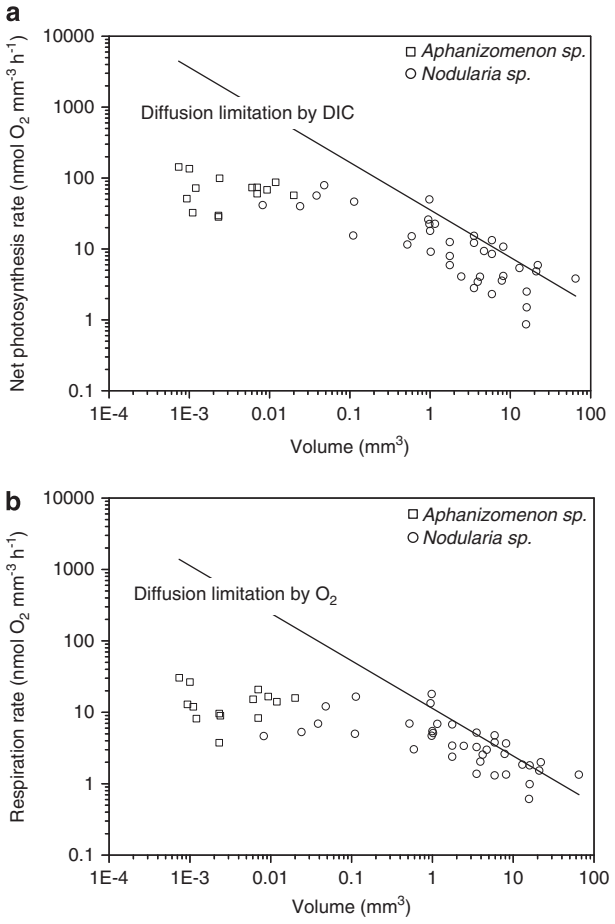

Figure 7 (**a**) Volumetric net photosynthesis rate as a function of colony size of *Aphanizomenon* sp. (squares) and *N. spumigena* (circles). The maximum diffusive flux of DIC to colonies is indicated (see text). (**b**) Volumetric respiration rate as a function of colony size of *Aphanizomenon* sp. (squares) and *N. spumigena* (circles). The maximum diffusive flux of O_2 to colonies is indicated (see text). All data points represent the average value of three series of measurements.

The volumetric respiration rates measured in colonies composed by *Aphanizomenon* sp. and *N. spumigena*, respectively, are shown as a function of colony volume in Figure 7b. As in the case for photosynthesis, the smallest colonies by *Aphanizomenon* sp. and *N. spumigena* show uniform volumetric respiration rates independent of colony size. In *Aphanizomenon* sp., these respiration rates were up to 100-fold lower than that needed to create anoxia in the center of a sphere (Figure 7b). Measured respiration rates in *Nodularia* colonies ($>1\,mm^3$) decreased in parallel to the theoretical respiration limit set by diffusion limitation by O_2 from the surrounding water to the colonies. Community respiration by *N. spumigena* with their associated (micro)organisms in these large colonies were, therefore, close to the theoretical concentration limit of respiring biomass under aerobic conditions in the Baltic Sea.

Discussion

Combining microsensor techniques with stable isotope enrichments and nanoSIMS, we have recently shown that *Aphanizomenon* sp. is a key player in biogeochemical C and N fluxes in the Baltic Sea (Ploug *et al.*, 2010). The present study was focused on the contribution by trichomes and colonies formed by *N. spumigena* to C and N fluxes in the Baltic Sea. NanoSIMS analysis showed a high enrichment of ^{15}N, which had been transferred from heterocysts to vegetative cells within hours. In contrast, heterocysts were only faintly labeled with ^{13}C as compared to vegetative cells within the same time course, presumable due to the fact that no oxygenic photosynthesis occurs within heterocysts. Similar patterns have been observed in *Aphanizomenon* sp. (Ploug *et al.*, 2010) as well as *Anabaena* sp. (Popa *et al.*, 2007). Both the cell-specific net C- and N_2-fixation rates by *N. spumigena* were ca. 2.4-fold higher than that of the respective rates reported for *Aphanizomenon* sp. in light (Ploug *et al.*, 2010). Cell-specific dark N_2 fixation by *Nodularia* sp. was 20-fold higher than that reported for *Aphanizomenon* sp. Dark C-fixation was not detectable, and respiration of stored carbon presumably supported this N_2 fixation. The volume of *N. spumigena* cells is approximately 2.5-fold larger than that of *Aphanizomenon* sp., and volume-specific net C- and N_2-fixation rates at light saturation of photosynthesis in *N. spumigena* were thus slightly lower than those reported for *Aphanizomenon* sp. The abundance of filamentous cyanobacteria is, at times, measured as meter filament per liter. The net C- and N_2-fixation rates associated with 1 m cumulative length of *N. spumigena* trichomes was almost sixfold higher than those reported for *Aphanizomenon* sp. (Ploug *et al.*, 2010). The abundance of *N. spumigena* and *Aphanizomenon* sp. varies between 1 and $50\,m\,l^{-1}$ in the Baltic Sea (Hajdu *et al.*, 2007; Rolff *et al.*, 2007). In this study, the abundance of free-living trichomes of *N. spumigena* was $1.5\,m\,l^{-1}$, only. *N. spumigena* colonies did not disintegrate in Lugol's solution and their cell content could, therefore, not be quantified as in *Aphanizomenon* sp. colonies (Ploug *et al.*, 2010). In that study, cell-specific net carbon assimilation rate in *Aphanizomenon* sp. colonies measured with microsensors was similar to that measured by nanoSIMS. Total net C- and N_2-fixation by *N. spumigena* of this study was dominated by colonies. Both photosynthesis and respiration, which provide the energy for N_2-fixation, were so high that they reached the physical limits set by diffusion limitation of bicarbonate and oxygen, respectively, in the Baltic Sea. Hence, despite their small volume and low abundance, *N. spumigena* colonies can contribute substantially to total N_2-fixation and NH_4^+ release during cyanobacterial blooms in surface waters of the Baltic Sea.

Shear and fluid motion as well as floatation to the air–water interface may enhance mass transfer of O_2 and CO_2 to colonies when photosynthesis and

respiration are limited by the diffusive supply of these gases from the surrounding water to colonies (Ibelings and Maberly, 1998; Ploug *et al.*, 1999; Ploug, 2008). Gross photosynthesis in 1–6 mm thin layers of primarily *N. spumigena* accumulating at the sea surface has previously been shown to vary between 1 and 7 nmol $O_2\,mm^{-3}\,h^{-1}$ (Ploug, 2008). The average gross photosynthesis in single colonies of this study varied between 4 and 32 nmol $O_2\,mm^{-3}\,h^{-1}$ depending on colony size and time during the bloom (Table 3). The average gross production rate by much smaller *Aphanizomenon* sp. colonies in the Baltic Sea was 78 nmol $O_2\,mm^{-3}\,h^{-1}$ (Figure 7). The measured carbon-specific photosynthesis of *Aphanizomenon* sp. collected in the Baltic Sea (Ploug *et al.*, 2010) as well as the Chl.*a*.-specific photosynthesis of *N. spumigena* colonies of this study were in the same range as those measured in cultures of the respective cyanobacteria isolated from the Baltic Sea (Moisander *et al.*, 2007; Degerholm *et al.*, 2008).

This study suggests that bicarbonate in addition to CO_2 must be an important carbon source in *N. spumigena* colonies, because the net photosynthesis rate can be as high as the potential diffusive fluxes of DIC to colonies. Net photosynthesis in the smaller *Aphanizomenon* sp. colonies was 10- to 100-fold lower than that of the potential DIC flux, although these colonies belong to the most productive microenvironments reported in aquatic systems (Ploug *et al.*, 2010). Bicarbonate uptake in cyanobacterial cells must be accompanied either by uptake of H^+ or by excretion of OH^- to keep balance of charges (Wolf-Gladrow *et al.*, 2007). High uptake rates of H^+ are problematic at alkaline pH as shown using a kinetic model of the marine carbonate system (Wolf-Gladrow and Riebesell, 1997; Wolf-Gladrow *et al.*, 1999). The uptake of HCO_3^- in combination with efflux of OH^- leads to an alkaline microenvironment in the colonies. The pH within surface accumulations of *N. spumigena* has previously been shown to vary between 7.4 during darkness and 9.0 in light (Ploug, 2008). The high photosynthesis rates associated with *N. spumigena* colonies suggests that this cyanobacterium is adapted not only to high pH in the interior of colonies, but may also benefit from CO_2 release through respiration by attached heterotrophic bacteria during photosynthesis.

Previous studies have shown that *N. spumigena* colonies are microbial consortial communities, and it has long been thought that the close associations of bacteria as well as protozoans and metazoans associated with these colonies are important in nutrient, O_2 and CO_2 exchange (Paerl and Kuparinen, 2002). Dark respiration and gross photosynthesis was tightly coupled in colonies with a ratio of 0.26. Dark respiration comprised 16% of gross photosynthesis in *Aphanizomenon* sp. colonies during the same season and at the same temperature in 2008 (Ploug *et al.*, 2010). This difference may be explained by higher dark respiration rates by *N. spumigena* cells during higher dark N_2 fixation as well as by higher densities of heterotrophic bacteria on the *N. spumigena* trichomes as compared with *Aphanizomenon* sp. *N. spumigena* may also harbor heterotrophic N_2-fixing bacteria, which have been shown to play a significant role in the Baltic Sea (Boström *et al.*, 2007). Cyanobacterial aggregates have earlier been shown to be anoxic in their interior during an ephemeral stage (12 h) of their decay (Ploug, 2008). During such relatively short periods with very high respiration rates, however, a substantial fraction of organic matter must be respired in aggregates (Ploug *et al.*, 1997). During a light–dark cycle new organic matter is synthesized during daytime. Anoxic conditions have long been suspected to occur within cyanobacterial colonies (Pearl and Bebout, 1988; Hietanen *et al.* 2002). This study shows that anoxic conditions potentially occur within colonies >1 mm in the Baltic Sea (Figure 7b). The colonies composed by *Aphanizomenon* sp. are too small and poorly colonized by bacteria for which respiration is too low to develop anoxia inside colonies when the bulk O_2 concentration is at air saturation. This was not the case for the larger *N. spumigena* colonies.

The net C:N fixation ratio varies between 1 and 40 in the bulk plankton community during a developing cyanobacterial bloom in the Baltic Sea, presumably partly due to a variable NH_4^+ release by the large cyanobacteria (Gallon *et al.*, 2002). In our study, the C:N fixation ratio in the bulk was 20, whereas that of *N. spumigena* was 8 during light. During a 16:8 h light dark cycle, the net C:N fixation ratio would be $(16\times 82-8\times 28):(16\times 11.4+8\times 5.4) = 4.8$ assuming dark respiration of the cyanobacteria to be 26% of gross photosynthesis. If 25% of the fixed N_2 is released as dissolved organic nitrogen and NH_4^+ to the surrounding water, the resulting C:N fixation ratio would be 6.4 integrated over the diel cycle. Release of dissolved organic nitrogen and NH_4^+ to the surrounding water, during the early and late light phase, has recently been shown in cultures of *N. spumigena* (Wannicke *et al.*, 2009), and $^{15}N_2$ fixed by large cyanobacteria (*Aphanizomenon* sp. and *N. spumigena*) in the Baltic Sea has been traced in organisms represented by the 2–5 μm size class, for example, picocyanobacteria (Ohlendieck *et al.*, 2000). *N. spumigena* appears to use NH_4^+ inefficiently, but N_2-fixation in *N. spumigena* cultures decreases when bulk NH_4^+ concentrations are very high (250 μM) (Vintila and El-Shehawy, 2007; Vintila *et al.*, 2010). In our study, the NH_4^+ release from light-exposed *N. spumigena* colonies to 0.2-μm-filtered bulk water comprised 8–33% of the estimated gross N_2-fixation rates. These are minimum estimates, because dissolved organic nitrogen may be an additional nitrogen source released by the cyanobacteria that attached biota presumably assimilate N for growth. The lowest NH_4^+ release was measured in colonies with the highest respiration

relative to gross photosynthesis (Table 3). *N. spumigena* is usually colonized by a diverse community of bacteria exhibiting high ecto-enzymatic activities (Hoppe, 1981; Stoecker *et al.*, 2005; Tuomainen *et al.*, 2006), and a significant fraction of the primary production, measured by use of $H^{14}CO_3$ in *N. spumigena* colonies from the Baltic Sea, was incorporated into the associated bacteria (Hoppe, 1981). Many pelagic bacteria are motile, show chemotaxis and their colonization rates of particles are in the order of minutes (Kiørboe *et al.*, 2002). The nitrogen sources leaking from *Nodularia* colonies may sustain growth of other biota when bulk concentrations of dissolved inorganic nitrogen are low. A similar substantial NH_4^+ release was shown to be associated with *Aphanizomenon* sp. in the Baltic Sea (Ploug *et al.*, 2010). The net production of $^{15}NH_4^+$ within the whole plankton community was 2% and 26% of net $^{15}N_2$ fixation during light and dark, respectively (Table 1). The low $^{15}NH_4^+$ traced in the bulk sample during 3–6 h incubation in light provides additional evidence that this nitrogen source is rapidly used by other organisms in the plankton community and channeled through the (microbial) food web during daytime when C- and N-fluxes within the plankton community are highest.

Aphanizomenon sp. is a key player in biogeochemical C- and N-fluxes owing to its high productivity and summer blooms lasting 2–3 months in the Baltic Sea (Larsson *et al.*, 2001; Rolff *et al.*, 2007; Ploug *et al.*, 2010). This study shows that *N. spumigena*, due to its large size and productivity, including high dark N_2 fixation, can contribute substantially to total C and N fluxes even at low cellular abundance in the Baltic Sea.

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