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ORIGINAL ARTICLE

# $N_2$-fixation, ammonium release and N-transfer to the microbial and classical food web within a plankton community

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**We investigated the role of $N_2$-fixation by the colony-forming cyanobacterium, *Aphanizomenon* spp., for the plankton community and N-budget of the N-limited Baltic Sea during summer by using stable isotope tracers combined with novel secondary ion mass spectrometry, conventional mass spectrometry and nutrient analysis. When incubated with $^{15}N_2$, *Aphanizomenon* spp. showed a strong $^{15}N$-enrichment implying substantial $^{15}N_2$-fixation. Intriguingly, *Aphanizomenon* did not assimilate tracers of $^{15}NH_4^+$ from the surrounding water. These findings are in line with model calculations that confirmed a negligible N-source by diffusion-limited $NH_4^+$ fluxes to *Aphanizomenon* colonies at low bulk concentrations ($<250$ nM) as compared with $N_2$-fixation within colonies. No $N_2$-fixation was detected in autotrophic microorganisms $<5$ μm, which relied on $NH_4^+$ uptake from the surrounding water. *Aphanizomenon* released about 50% of its newly fixed $N_2$ as $NH_4^+$. However, $NH_4^+$ did not accumulate in the water but was transferred to heterotrophic and autotrophic microorganisms as well as to diatoms (*Chaetoceros* sp.) and copepods with a turnover time of ~5 h. We provide direct quantitative evidence that colony-forming *Aphanizomenon* releases about half of its recently fixed $N_2$ as $NH_4^+$, which is transferred to the prokaryotic and eukaryotic plankton forming the basis of the food web in the plankton community. Transfer of newly fixed nitrogen to diatoms and copepods furthermore implies a fast export to shallow sediments via fast-sinking fecal pellets and aggregates. Hence, $N_2$-fixing colony-forming cyanobacteria can have profound impact on ecosystem productivity and biogeochemical processes at shorter time scales (hours to days) than previously thought.**
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## Introduction

Vast regions in the tropical ocean as well as brackish waters (for example, the Baltic Sea) and lakes are (periodically) characterized by low concentrations of dissolved inorganic nitrogen. $N_2$-fixation by cyanobacteria is a major source of new nitrogen and can have an important role sequestrating carbon in these regions and habitats. Extensive blooms of *Trichodesmium* occur in the tropical ocean, whereas the genera *Nodularia* and *Aphanizomenon* bloom in brackish waters, and *Aphanizomenon* in lakes (Capone, *et al.*, 1997; Ibelings and Maberly, 1998; Larsson *et al.*, 2001). These $N_2$-fixing cyanobacteria form colonies and leak a substantial fraction of newly fixed $N_2$ to the surrounding water (Mulholland and Capone 2001; Ploug *et al.*, 2010, 2011). However, quantitatively little is known about the fate of this newly fixed nitrogen, its role for the microbial and classical food webs and for large-scale biogeochemical fluxes (Mulholland, 2007).

In the Baltic Sea Proper, $N_2$-fixation contributes a yearly N input close to that of the entire riverine load (Wasmund *et al.*, 2001). Here the large $N_2$-fixing cyanobacteria are dominated by *Aphanizomenon* sp. (sometimes referred to as *A. flos-aquae*), *Nodularia spumigena* and, to a lesser extent, *Dolichospermum* spp. (formerly *Anabaena* spp.), (Wasmund 1997; Larsson *et al.*, 2001; Wasmund *et al.*, 2001). These filamentous, $N_2$-fixing cyanobacteria make up approximately 20–40% of the C biomass of phytoplankton during summer, while the remaining

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phytoplankton biomass is represented by a variety of prokaryotic and eukaryotic plankton organisms, for example, diatoms, dinoflagellates and picoplankton (Stal *et al.*, 2003; Hajdu *et al.*, 2007). Experimental studies as well as nitrogen budgets have suggested that $N_2$-fixing cyanobacteria leak a substantial fraction of their fixed nitrogen that is subsequently assimilated by picoplankton in the Baltic Sea (Ohlendieck *et al.*, 2000; Larsson *et al.*, 2001; Stal *et al.*, 2003). Various routes by which diazotrophic nitrogen may enter the pelagic food web have been investigated indirectly through experimental studies and by using the natural abundance ($\delta^{15}N$) signature of $N_2$-fixation in field studies (Wannicke *et al.,* 2013; Woodland *et al.*, 2013; Lesutiene *et al.,* 2014; Karlson *et al.,* 2015). Direct measurements of the routes and overall significance of N release by $N_2$-fixing cyanobacteria and subsequent N uptake within the phytoplankton community are missing, largely owing to technical and methodological limitations. Hence, the role of large, colony-forming $N_2$-fixng cyanobacteria in nutrient cycling, food webs and biogeochemical fluxes has remained unresolved.

Nanoscale secondary ion mass spectrometry (nanoSIMS) is a novel high spatial resolution (50 nm) technique that combines the qualities of a microscope with those of a mass spectrometer. Thus its use in isotope tracer experiments allows simultaneous determination of cell identity and activity in field samples of mixed microbial populations (Kuypers and Jørgensen, 2007; Wagner, 2009; Musat *et al.*, 2012). Using this technique, we recently demonstrated that the volume-specific $N_2$-fixation and $NH_4^+$ release were similar in *Aphanizomenon* sp. and *Nodularia spumigena* in the Baltic Sea. Furthermore, $N_2$-fixation by both cyanobacteria covered their cellular N-demands, and both species released ~35% of their newly fixed nitrogen to the surrounding water (Ploug *et al.*, 2010, 2011). *Aphanizomenon* does not form the conspicuous surface accumulations typical of *Nodularia*, but it often comprises >50% of the biomass of the $N_2$-fixing cyanobacteria during the summer season (Wasmund 1997, Larsson *et al.*, 2001,Walve and Larsson, 2007). In the present study, we combined nutrient measurements, stable isotope tracers analyzed with conventional mass-spectrometry and single-cell uptake-rate measurements by SIMS and nanoSIMS to directly identify and quantify the transfer of newly fixed $N_2$ by *Aphanizomenon* within the N-limited plankton community during early summer in the Baltic Sea.

## Materials and methods

*Sampling*
*Aphanizomenon* colonies were sampled in the upper 10 m of the water column using a plankton net (Hydrobios, 0.5 mø, 90-µm mesh) at station B1 (N 58° 48' 28, E 17° 37' 60) in the southern Stockholm archipelago of the NW Baltic Proper during the early phase of the *Aphanizomenon* bloom in June 2010, 2011, 2012 and 2013. Additional water from 5-m depth was collected using a 2-l water sampler (NM Tech AB, Stockholm, Sweden). The salinity was ~6 and the temperature ~10 °C. The samples were brought to the laboratory within 30 min and poured into a light trap in a thermostated room at *in situ* temperature to separate zooplankton from the cyanobacterial colonies. The light trap consisted of a funnel, which was covered by black foil except at its bottom where it was illuminated to attract zooplankton, while cyanobacterial colonies floated to the surface.

*Microscopy*
*Aphanizomenon* colonies disaggregate in 1% Lugol's solution. Lugol-fixed samples (each 1 ml) for the various incubations were transferred to a gridded Sedgewick Rafter counting chamber (Wildlife supply Company, New York, NY, USA). Numbers and dimensions of dispersed trichomes and vegetative cells were measured under an inverted microscope (Leica DMIRB, Wetzlar, Germany) at ×100 or ×400 magnification. Heterocyst frequency was measured as the number of heterocysts (trichome length)$^{-1}$ and in percentage of vegetative cells. Total cumulative trichome length per field was measured until its mean value was stable and the s.e. <2% of the mean. For each incubation which included *Aphanizomenon*, we counted and measured six replicates each with 32.5 mm trichomes and enriched the community with *Aphanizomenon* cells to the same final concentration of $5.0 \times 10^7$ ($\pm 0.3 \times 10^7$ cells l$^{-1}$; s.e.) by adding net-harvested colonies to bulk water samples.

*Incubations with $^{13}C$-labeled bicarbonate and $^{15}N_2$*
During 2010, bulk samples enriched with *Aphanizomenon* were incubated in 280-ml serum bottles, whereas other bottles (1-L Duran, Main, Germany) only contained the fraction of cells <5 µm after gentle filtration of the bulk water community through a 5-µm polycarbonate filter. $^{13}C$-labeled bicarbonate and $^{15}N_2$ gas (ISOTECH, #CX0937; Sigma-Aldrich, Stockholm, Germany) was injected to a final concentration of 11% and 25% or 33% labeling, respectively, except for three control bottles. We started our experiments during the night at 0200 hours to prolong the equilibration time prior to $N_2$-fixation during day time, and incubations were stopped after 12 and 24 h when the underestimation of $N_2$-fixation rate relative to true $N_2$-fixation rates is <20% owing to disequilibrium between the injected $^{15}N_2$ gas bubble and the surrounding water (Mohr *et al.*, 2010). We injected a $^{15}N_2$ gas bubble instead of using a large aliquot of $^{15}N_2$-enriched water because a relatively high labeling—percentage of $^{15}N_2$ (>25%)—is required to track its fixation by cyanobacteria, $^{15}NH_4^+$ release and transfer to other phytoplankton, as well as grazing within 24 h. In 2012 and 2013, we repeated

the experiments using a small aliquot of $^{15}N_2$-enriched, 0.2-μm-filtered Baltic Sea water to a final labeling of 1% to ensure that measured $N_2$-fixation by Aphanizomenon was independent of labeling% and that lack of measured $N_2$-fixation by cells $<5$ μm when adding a $^{15}N_2$-bubble was not due to a long equilibration time relative to the incubation time and a long diffusion distance to small cells in the bottles (Mohr *et al.*, 2010).

During 2010, 2011 and 2012, all samples were incubated at 0.5-m depth in an outdoor mesocosm at ambient light with running sea water at *in situ* temperature (10 °C). Incident light intensity was measured using a LiCor irradiance sensor (Biospherical Instruments, San Diego, CA, USA) and varied from 0.2 μmol photons $m^{-2}\,s^{-1}$ (at night) to 1800 μmol photons $m^{-2}\,s^{-1}$ (no clouds at noon). In 2013, we repeated the experiment with cells $<5$ μm in a thermostated room at *in situ* temperature at 200 μmol photons $m^{-2}\,s^{-1}$ during 18 h followed by 6 h darkness, with samples taken at $T_0$, $T_6$, $T_{12}$ and $T_{24}$. In parallel to the incubation of cells $<5$ μm, field samples of *Aphanizomenon* sp., large diatoms and zooplankton were concentrated using a plankton net (90 μm) and incubated in 2-L Duran bottles. They contained $5.3\times10^7\pm0.3\times10^7$ *Aphanizomenon* cells $l^{-1}$ (± denotes s.e.). $^{13}$C-labeled bicarbonate and $^{15}N_2$ gas were injected to 11% and 25% final labeling, respectively, except for three control bottles, and incubated in the thermostated room at *in situ* temperature and 200 μmol photons $m^{-2}\,s^{-1}$ for 18 h followed by 6 h darkness. Afterwards, copepods were separated from the rest of the sample in the light trap and collected on precombusted GF/F filters (~120 copepods each) for elemental analysis isotope ratio mass spectrometry (EA-IRMS) analysis.

After all incubations during all years, subsamples of 50 ml for SIMS and nanoSIMS analysis were preserved with 2% paraformaldehyde for 24 h at 4 °C and then filtered onto gold–palladium-coated GTTP filters (pore size 0.22 μm; diameter 25 mm; Millipore, Eschborn, Germany), washed, dried and stored at −20 °C. The remaining water of each incubation was immediately filtered onto precombusted GF/F filters for determining bulk carbon and nitrogen assimilation rates of the total plankton community using EA-IRMS (please see below). The filtrate from each incubation was filled into 7.7 ml Exetainers (Labco, Lampeter, Wales, UK) and biological activity immediately stopped by adding 100 μl saturated $HgCl_2$ solution for $^{13}$C-labeled bicarbonate and $^{15}NH_4^+$ analysis. Our $^{15}N_2$ gas did not belong to the batches that have recently been documented to contain contaminations by $^{15}NH_4^+$ and $^{15}NO_3^-$ (Dabundo *et al.*, 2014). However, we examined leftover fixed filtrates from the start of our experiments and detected low contamination of $^{15}NH_4^+$ (<1 nM final concentration) and $^{15}NO_3^-$ (<16 nM final concentration) (Warembourg, 1993; Füssel *et al.*, 2012). These concentrations were insignificant relative to the measured $^{15}NH_4^+$ released from $^{15}N_2$-fixation during experiments (please see Discussion).

*Elemental analysis-isotope ratio mass spectrometry*
The GF/F filters were freeze-dried and decalcified overnight with the fumes of 37% HCl in a desiccator, packed into tin cups and analyzed by a Thermo Flash EA 1112 elemental analyzer coupled to an isotopic ratio mass spectrometer (Thermo Delta Plus XP, Thermo Fisher Scientific, Waltham, MA, USA) at MPI, Bremen, Germany. Caffeine was used as a standard for isotope correction and C/N quantification of bulk carbon and nitrogen assimilation.

*NanoSIMS analysis of microbial single cells*
NanoSIMS analysis of single cells was performed on gold–palladium-coated polycarbonate filters (0.22-μm pore size, GTTP type, Millipore) containing the chemically fixed cells. To assess the distribution of autotrophic bacteria, autofluorescent cells on the filters were marked using a laser microdissection microscope (Leica, Wetzlar, Germany) and then re-identified after loading filters into the nanoSIMS 50L instrument at the MPI Bremen (Cameca, Gennevilliers Cedex, France) using the internal charge coupled device, as described by Polerecky *et al.* (2012). The areas were presputtered with a primary $Cs^+$ ion beam of around 300 pA. During nanoSIMS analysis, the $Cs^+$ ion beam was focused to a nominal spot size <100 nm and a beam current between 0.8 and 1.2 pA was used. Secondary ion images for $^{12}C^-$, $^{13}C^-$, $^{12}C^{14}N^-$, $^{12}C^{15}N^-$, $^{31}P^-$ and $^{32}S^-$ were simultaneously recorded from the analysis area. To minimize interferences for $^{13}C^-$, the instrument was tuned with an average mass resolution of 7000. The image and data processing were performed as previously described (Polerecky *et al.*, 2012). An overlay analysis with the Look@NanoSIMS software confirmed the single-cellular correlation of previously detected autofluorescence and autotrophic uptake ratios of $^{13}$C (data not shown).

*SIMS analysis of Aphanizomenon and diatom cells*
As diatoms are difficult to analyze by nanoSIMS owing to their silicified cells walls, we used a large geometry SIMS instrument (NORDSIM facility, Swedish Museum of Natural History, Stockholm, Sweden) capable of a primary beam with higher energy output for diatom cells as compared with that of the nanoSIMS. Furthermore, we analyzed additional *Aphanizomenon* sp. cells on the SIMS. The filters containing fixed cells were cut into ca. $4\times4$ mm$^2$ pieces and mounted on a sample holder. The analysis was performed using an IMS 1280 instrument manufactured by Cameca (Gennevilliers, France) using a $Cs^+$ primary beam with a spatial resolution of ca. 1 μm. Each diatom cell was presputtered with a beam of 10 nA for <5 min to remove the silicified cell wall and then imaged using

a 40–80 pA primary beam for 200 cycles. For each cell chain, we recorded secondary ion images of $^{13}C^{14}N^-$ and $^{12}C^{14}N^-$ and $^{12}C^{15}N^-$ using a peak-switching routine at a mass resolution of ca. 6000 (M/ΔM). Image and data processing using the Cameca WinImage (Gennevilliers, France) software defined regions of interest corresponding to individual cells from which isotope ratios were calculated.

*Ammonium analysis*
Total $NH_4^+$ concentration in the surrounding water was measured on a fluorometer (Turner Designs, Trilogy, Sunnyvale, CA, USA) using the method by Holmes *et al.* (1999). $^{15}NH_4^+$ was added to the bulk water enriched with *Aphanizomenon* to a final labeling of 28% in 1-L Duran bottles, which were carefully shaken after addition of the stable isotope tracer. Triplicate bottles and one control were incubated for 0, 2 and 5 h in light. Triplicate sub-samples of 40 ml were transferred into acid-washed falcon tubes and 10 ml OPA solution was added immediately afterwards for $NH_4^+$ concentration analysis. Triplicate subsamples for isotopic analysis were transferred into 7.7 ml Exetainer, and biological activity was immediately stopped by adding 100 µl saturated $HgCl_2$ solution. The isotopic ratio of $^{15}NH_4^+$: $^{14}NH_4^+$ was analyzed after evacuation to remove $^{15}N_2$ and $^{14}N_2$ and subsequent chemical conversion of $NH_4^+$ into $N_2$ with alkaline hypobromite (NaOBr) (Warembourg, 1993, Preisler *et al.*, 2007). The isotopic ratios of $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ were analyzed by a gas chromatographic IRMS (VG Optima, Micromass, Manchester, UK) calibrated with known concentrations of $^{15}NH_4^+$ in the nM range (1–100 nM). The net $NH_4^+$ production rate was calculated from the change in $^{15}NH_4^+$ concentration over time (Holtappels *et al.*, 2011).

*C- and N-growth rates by cells*
SIMS and nanoSIMS measure the ratio of stable isotopes of the same element, whereas EA-IRMS measures atom percentage (AT%) of stable isotopes of the same element. The C-specific assimilation rates and N-specific assimilation rates, $k$(t), in the various organisms were calculated from the isotope ratios (IR) of solid matter after the incubation time ($T_1 - T_0$) with stable isotope tracers as:

$$k = \frac{\mathrm{IR}_{T_1} - \mathrm{IR}_{\mathrm{background}}}{F_{\mathrm{bulk}} \times (T_1 - T_0)} \quad (1)$$

where $F_{\mathrm{bulk}}$ is the fraction of labeled isotope in the bulk. The generation time was calculated assuming exponential growth, where $k$ is expressed by Equation 1.

*Model calculations of diffusion-limited NH4+ uptake and N-generation time by different phytoplankton types*
The size-specific cellular N-content of dinoflagellates and diatoms was calculated using empirical relations between cell size and C- and N-content reported by Menden-Deuer and Lessard (2000). Extrapolated to a cell size of 1 µm, the cellular N-content was similar to those reported in different *Synechococcus* strains (Bertilsson *et al.*, 2003). Diffusive boundary layers and the cellular N-content of *Aphanizomenon* colonies of different sizes were previously quantified (Ploug *et al.*, 2010). The equivalent spherical diameters of cells, cell chains, trichomes and colonies were calculated from their respective volume. The diffusion-limited $NH_4^+$ supply to these cells was calculated according to (Sherwood *et al.*, 1975):

$$Q = 4\pi D r_0 (C_\infty - C_0) \quad (2)$$

where $Q$ is the uptake rate (nmol N $s^{-1}$), $D$ is the molecular diffusion coefficient of $NH_4^+$ ($1.2 \times 10^{-5}$ $cm^2\,s^{-1}$ at 10 °C and a salinity of 6; Li and Gregory, 1974), $r_0$ is the average radius of the cell or colony and ($C_\infty - C_0$) is the concentration difference between that of the bulk (250 nM) and that at the cell or colony surface. The maximum diffusion-limited supply of $NH_4^+$ occurs when $C_0$ is zero at the cell surface. The minimum N-doubling time was calculated as the ratio between the cellular N-content of cells or colonies and the diffusion-limited $NH_4^+$ uptake.

## Results

*Single-cell analysis of $N_2$-fixation and transfer of newly fixed N to other organisms in the plankton community*
*Aphanizomenon* dominated the large cyanobacteria population (>99% of large cyanobacteria) as is usual during June in the Baltic Sea plankton community. After 12 h incubation with $^{13}C$-labeled bicarbonate and $^{15}N_2$ gas, the $^{15}N/^{14}N$ isotope ratio was enriched above that of the natural abundance within *Aphanizomenon*, autotrophic and heterotrophic bacteria attached to *Aphanizomenon* and free-living autotrophic and heterotrophic bacteria (Figure 1a). Diatom cells (*Chaetoceros* sp.), in addition to the other cell types from the same incubation, were analyzed after 24 h and showed a high enrichment of $^{15}N$ above that of the natural abundance (Figure 1b). The highest $^{15}N$-enrichment within the plankton community was measured in *Aphanizomenon* indicating a transfer of $^{15}N$ derived from recently fixed $^{15}N$ by *Aphanizomenon* to the other cells in the plankton community. Copepods were also enriched with $^{15}N$, after incubation with *Aphanizomenon* and diatoms during 24 h, demonstrating assimilation and transfer of newly fixed $^{15}N$ also to higher tropic levels (Table 1).

Cells >5 µm were removed by filtration, leaving small eukaryotic cells and the phototrophic and heterotrophic bacteria in the community. This smaller size fraction was incubated with $^{13}C$-labeled bicarbonate and $^{15}N_2$-tracer to examine whether they were able to fix dissolved inorganic C and $N_2$. Cells <5 µm were $^{13}C$-fixing (Figure 2), and the isotope enrichments corresponded to an average C-generation time of 1.4 days. However, no $^{15}N$ assimilation was detectable in this size fraction

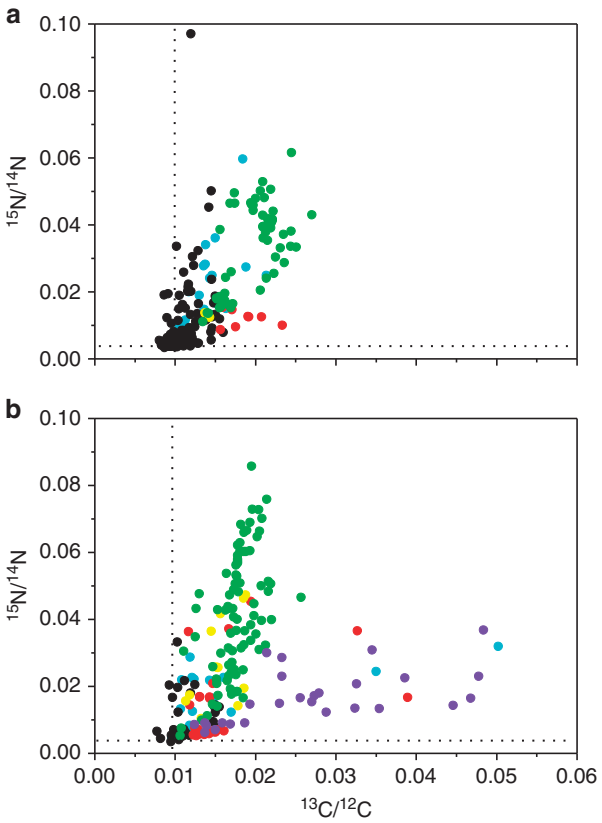


**Figure 1** Bulk sample enriched with *Aphanizomenon* sp. and incubated with $^{13}$C-labeled bicarbonate and $^{15}N_2$. Isotope ratios of $^{15}N/^{14}N$ versus $^{13}C/^{12}C$ in various organisms (**a**) incubated from 0300 to 1500 hours and (**b**) incubated from 0300 to 0300 hours: *Aphanizomenon* sp. vegetative cells (green symbols); autotrophic bacteria attached to *Aphanizomenon* sp. (yellow symbols); free-living autotrophic bacteria (red symbols); heterotrophic bacteria attached to *Aphanizomenon* sp. (cyan symbols); free-living heterotrophic bacteria (black symbols); and diatoms (violet symbols). The dotted lines represent the isotope ratios of the natural abundance of $^{15}N/^{14}N$ and $^{13}C/^{12}C$.

as the $^{15}N/^{14}N$ ratio remained stable at the natural abundance level (Figure 2). These observations were also confirmed by EA-IRMS analysis of particulate matter $<5$ µm, which did also not reveal any $^{15}N$ assimilation during incubations $\leqslant 24$ h (Table 1). The high cellular rates of $^{13}CO_2$ uptake in combination with the non-detectable $^{15}N$ uptake show that there were no active $N_2$-fixers in the $<5$ µm fraction. In contrast, when cells $<5$ µm were incubated together with *Aphanizomenon*, their $^{15}N/^{14}N$ ratios increased above that of the natural abundance owing to the uptake of released diazotrophic $^{15}N$.

*Ammonium release and uptake rates by different plankton organisms*

The net $^{15}NH_4^+$ release in the bulk water (tracked from $^{15}N_2$-fixation) is shown as a fraction of the $^{15}N_2$-fixation during the 24-h experiment (Figure 3). Its average value varied from $0.069 \pm 0.041$ to $0.005 \pm 0.003$ and it decreased throughout the 24-h (from 0200 to 0200 hours) experiment. Tracers of

**Table 1** AT% of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ measured in the $<5$-µm size fraction and copepods (EA-IRMS), and in *Aphanizomenon* sp. and *Chaetoceros* sp., autotrophic bacteria analyzed by SIMS or nanoSIMS after incubation with $^{13}$C-labeled bicarbonate and $^{15}N_2$ tracers

| *Organism or cell size fraction* | *AT% $^{13}C/^{12}C$* | *AT% $^{15}N/^{14}N$* |
|---|---|---|
| $<5$-µm size fraction, $T_0$ ($n=3$) | $1.075 \pm 0.001$ | $0.3711 \pm 0.0011$ |
| $<5$-µm size fraction, $T_6$ ($n=3$) | $1.205 \pm 0.005$ | $0.3694 \pm 0.0002$ |
| $<5$-µm size fraction, $T_{12}$ ($n=3$) | $1.292 \pm 0.085$ | $0.3709 \pm 0.0002$ |
| $<5$-µm size fraction, $T_{24}$ ($n=3$) | $1.600 \pm 0.008$ | $0.3711 \pm 0.0003$ |
| Copepods, $T_{24}$ ($n=3$) | $1.195 \pm 0.021$ | $0.542 \pm 0.030$ |
| *Chaetoceros* sp., $T_{24}$ ($n=27$) | $2.7 \pm 1.1$ | $1.6 \pm 1.1$ |
| *Aphanizomenon* sp., $T_{24}$ ($n=85$) | $1.8 \pm 0.2$ | $4.2 \pm 1.8$ |
| Autotrophic bacteria att. to Aphan, $T_{24}$ ($n=10$) | $1.6 \pm 0.3$ | $2.7 \pm 1.3$ |
| Autotrophic bacteria, free-living, $T_{24}$ ($n=24$) | $1.6 \pm 0.6$ | $1.5 \pm 1.2$ |
| Autotrophic bacteria, $T_{24}$ ($n=40$)[a] | $2.4 \pm 0.7$ | $0.37 \pm 0.11$ |
| Bacteria att. to Aphan, $T_{24}$ ($n=15$) | $1.6 \pm 1.1$ | $1.7 \pm 0.8$ |
| Free-living bacteria, $T_{24}$ ($n=28$) | $1.2 \pm 0.2$ | $1.1 \pm 0.7$ |

Abbreviations: AT%, atom percentage; EA-IRMS, elemental analysis isotope ratio mass spectrometry; nanoSIMS, nanoscale secondary ion mass spectrometry; SIMS, secondary ion mass spectrometry.
[a]Incubated in the $<5$-µm size fraction.

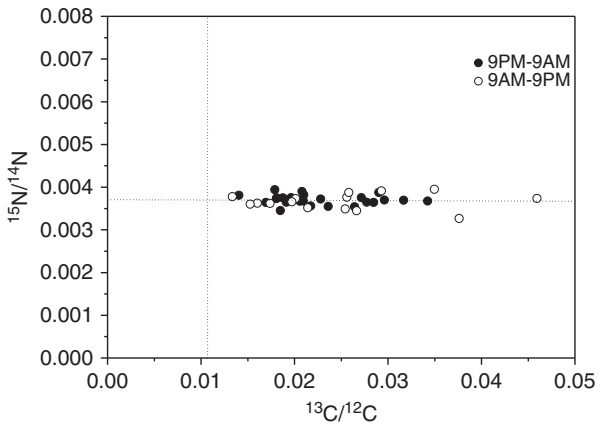


**Figure 2** Bulk ($<5$-µm fraction) sample incubated with $^{13}$C-labeled bicarbonate and $^{15}N_2$. Isotope ratios of $^{15}N/^{14}N$ versus $^{13}C/^{12}C$ in cells incubated from 2100 to 0900 hours (open symbols) and incubated from 0900 to 2100 hours (closed symbols). The dotted lines represent the natural abundance levels of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotope ratios.

$^{13}$C-labeled bicarbonate and $^{15}NH_4^+$ were added to the phytoplankton community to study C-fixation and $NH_4^+$ uptake rates by the different (micro)organisms and the $NH_4^+$ turnover time in the bulk water. All cell types, except *Aphanizomenon*, showed enrichment in their $^{15}N/^{14}N$ isotopic ratio above that of the natural abundance after 5 h (Figure 4). Thus, *Aphanizomenon* apparently did not take up $NH_4^+$ from the bulk water. However, bacteria attached to *Aphanizomenon* cells showed high isotope ratios of both $^{13}C/^{12}C$ and $^{15}N/^{14}N$. The high $^{15}N/^{14}N$ ratios observed in attached microbiota relative to that of *Aphanizomenon* were due to $^{15}NH_4^+$ uptake from the bulk water. Hence, attached and free-living microbiota showed much higher affinity to $^{15}NH_4^+$ in the nM concentration range than *Aphanizomenon* did.

A large fraction of free-living bacteria showed high but variable $^{15}N/^{14}N$ ratios while the $^{13}C/^{12}C$ isotope ratio was close to the natural abundance of these isotopes, that is, these cells were most likely heterotrophic bacteria. Other free-living bacteria showed autofluorescence and their $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios were high. These cells were photoautotrophic relying on $NH_4^+$ from the bulk water.

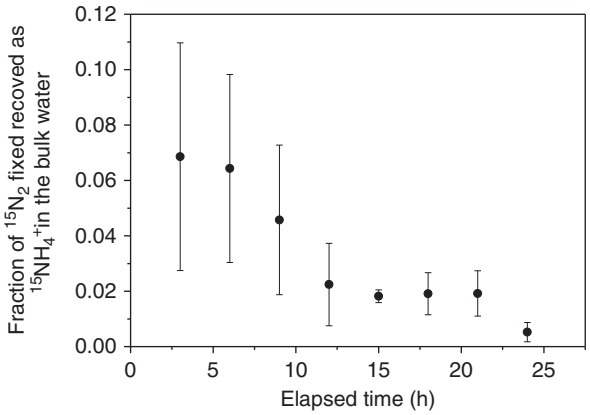


**Figure 3** Ratio of $^{15}NH_4^+$ tracked in the bulk water to $^{15}N_2$-fixation during the 24-h experiment. The symbols show the average value with the s.d. ($n = 3$) of the average value shown as bars.

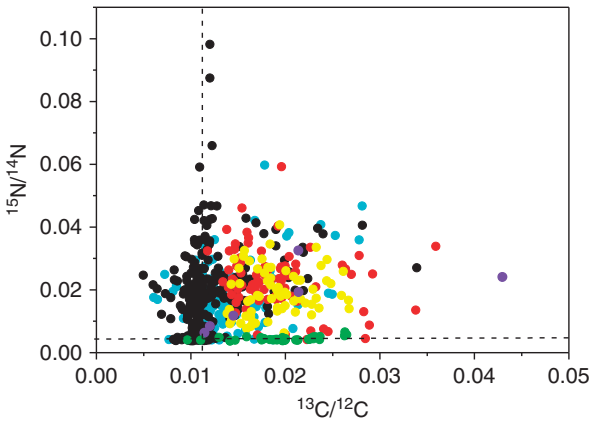


**Figure 4** Bulk sample enriched with *Aphanizomenon* sp. incubated with $^{13}C$-labeled bicarbonate and $^{15}NH_4^+$ during 5 h in light: isotope ratios of $^{15}N/^{14}N$ versus $^{13}C/^{12}C$ in various organisms: *Aphanizomenon* sp. vegetative cells (green symbols); autotrophic bacteria attached to *Aphanizomenon* sp. (yellow symbols); free-living autotrophic bacteria (red symbols); heterotrophic bacteria attached to *Aphanizomenon* sp. (cyan symbols); free-living heterotrophic bacteria (black symbols); and eukaryotes (violet symbols). The dotted lines represent the isotope ratios of the natural abundance of $^{15}N/^{14}N$ and $^{13}C/^{12}C$.

The isotopic composition of the various cell types was used to calculate average C-specific growth rates, N-specific growth rates based on $NH_4^+$ and N-doubling times (Table 2). *Aphanizomenon* showed C-specific growth rates similar to those of small autotrophic picoplankton cells, but its low N-specific growth rate based on $NH_4^+$ uptake resulted in a long N-doubling time of approximately 1 month with $NH_4^+$ from the surrounding water as the main N-source. All other cells showed similar N-doubling times of 1–2 days. Hence, the $NH_4^+$ uptake rate was insufficient to sustain the growth of *Aphanizomenon* and $N_2$-fixation must have been its major N-source.

The total $NH_4^+$ concentration and the $^{15}NH_4^+$ concentration in the surrounding water during the experiment are shown as a function of time after addition of $^{15}NH_4^+$ tracers (Figure 5). The total bulk $NH_4^+$ concentration was relatively stable during the entire 5 h of the incubation. However, the $^{15}NH_4^+$ concentration decreased linearly over time. Thus the $NH_4^+$ concentration of the bulk was at steady state, with $NH_4^+$ uptake being balanced by $NH_4^+$ production, that is, it was taken up by the organisms in the community at the same rate as it was produced. The turnover time of $NH_4^+$ in the bulk water was ~5.5 h.

## Discussion

Using $^{13}C$- and $^{15}N$-tracers in combination with SIMS and nanoSIMS, we could directly demonstrate that recently fixed $N_2$ by *Aphanizomenon* is released as $NH_4^+$ to the surrounding water and consequently supports the primary production by other phytoplankton and the microbial as well as the eukaryotic food web in the Baltic Sea.

Very recently, it was reported that $^{15}N_2$ gas used for $N_2$-fixation studies can be contaminated with other $^{15}N$-labeled compounds, for example, $^{15}NH_4^+$ and $^{15}NO_3^-$ potentially leading to biased estimates of $N_2$-

**Table 2** C-specific C-fixation rate with respect to $^{13}C$-labeled bicarbonate uptake and N-specific $NH_4^+$-growth rate and N-doubling time of N-biomass calculated from the isotope ratios in the various cell types (Figure 4)

| *Cell type* | *C-specific C-growth rate ($h^{-1}$)* | *N-specific $NH_4^+$-growth rate ($h^{-1}$)* | *N-doubling time (days)* |
|---|---|---|---|
| *Aphanizomenon* sp. ($n = 39$) | 0.025 ± 0.008 | 0.0011 ± 0.0008 | 38 |
| Heterotrophic bacteria attached to Aphan ($n = 110$) | 0.009 ± 0.011 | 0.025 ± 0.012 | 1.7 |
| Free-living heterotrophic bacteria ($n = 264$) | 0.003 ± 0.007 | 0.026 ± 0.016 | 1.6 |
| Autotrophic bacteria attached to Aphan ($n = 68$) | 0.024 ± 0.007 | 0.028 ± 0.009 | 1.2 |
| Free-living autotrophic bacteria ($n = 84$) | 0.022 ± 0.009 | 0.035 ± 0.012 | 1.2 |
| Eukaryotes ($n = 6$) | 0.041 ± 0.025 | 0.032 ± 0.011 | 1.3 |

Autotrophic bacteria were identified by autofluorescence.

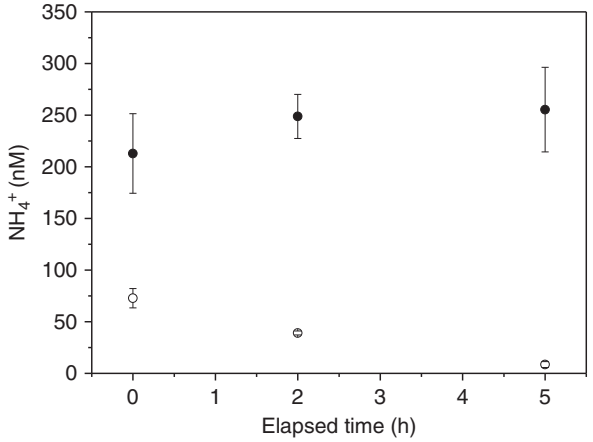


**Figure 5** Total $NH_4^+$ ($^{15}NH_4^+$ and $^{14}NH_4^+$) (closed symbols) in the bulk and the added tracer $^{15}NH_4^+$ (open symbols) measured as a function of time in light.

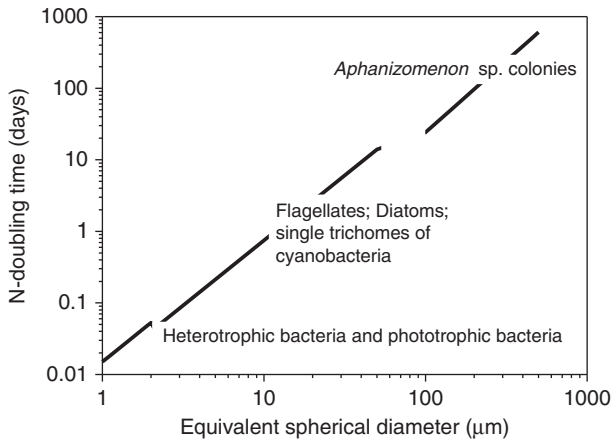


**Figure 6** The theoretical shortest time required to cover N-demand for one cell division at diffusion limitation at a $NH_4^+$ bulk concentration of 250 nM (for details, please see text).

fixation rates as well as of diazotrophic $^{15}NH_4^+$ transferred to other organisms (Dabundo *et al.,* 2014). We detected low contaminations of $^{15}NH_4^+$ and $^{15}NO_3^-$ in our samples, which corresponded to $<1\%$ and $<15\%$ of total $^{15}NH_4^+$ release and transfer from $^{15}N_2$-fixation, respectively. The *in situ* $NO_3^-$ concentration was low ($<100$ nM; Swedish monitoring program, SMHI), and phytoplankton is known to preferentially use excreted and remineralized $NH_4^+$ as N-sources above $NO_3^-$ at low concentrations (McCarthy and Goldman, 1979).The detection of $^{13}C$-fixation but no enrichment of $^{15}N$ from $^{15}N_2$-fixation (or uptake of contaminated $^{15}NH_4^+$ and $^{15}NO_3^-$) by cells $<5$ μm after 12–24 h incubation confirmed that our results were not biased by contaminated $^{15}N_2$ gas (Figure 2, Table 1). When 70 nM $^{15}NH_4^+$ (final concentration) was added as tracer to the natural bulk water ($<250$ nM) the community showed a very fast uptake rates by cells $<5$ μm as directly demonstrated by nanoSIMS (Figure 4, Table 2). These cells had on average 2% enrichment of $^{15}N/^{14}N$ above that of the natural abundance after 5 h incubation time. Hence, the cells $<5$ μm showed a high affinity for $^{15}NH_4^+$, and these cells would quickly have assimilated $^{15}NH_4^+$ and shown an enrichment of $^{15}N/^{14}N$ above that of the natural background if exposed to $^{15}N_2$ gas contaminated by this nutrient. The same $^{15}N_2$ gas bottles used for $N_2$-fixation measurements in the $<5$-μm cell fraction were also used for all transfer experiments. We conducted and partly repeated our experiments during 4 years, with highly reproducible results between different years. Hence, we consider our data to be robust and not compromised by contaminations in the added $^{15}N_2$ gas.

Simultaneous measurements of C- and $N_2$-fixation rates in the Baltic Proper have suggested that $N_2$-fixation supports 5–37% of the N-demand for the measured C-fixation by the whole phytoplankton community assuming a C:N molar ratio of 6.6 in phytoplankton (Karlson *et al.*, 2015). In the present study, $NH_4^+$ release by *Aphanizomenon* was substantial although we did not measure any high accumulation of $^{15}NH_4^+$ in the bulk water during our $^{15}N_2$-fixation experiments. $NH_4^+$ was produced and consumed within the phytoplankton community at a faster rate than previously measured by tracking $^{15}NH_4^+$ from $^{15}N_2$-fixation rates in the bulk water (Ploug *et al.*, 2010, 2011). The average net $N_2$-fixation rate was 31 nM N $h^{-1}$ in samples enriched with *Aphanizomenon*. At steady state of the $NH_4^+$ concentration in the bulk water, the net $NH_4^+$ production and consumption rate of 45 nM $h^{-1}$ implies an average release rate equal to 59% of the gross $N_2$-fixation rate. The $N_2$-fixation has been estimated to contribute about as much to the new production in the Baltic Proper as riverine sources (Wasmund *et al.*, 2001). Hence, $NH_4^+$ release by $N_2$-fixing cyanobacteria and its uptake by other phytoplankton may therefore correspond to about half or more of the riverine input to the Baltic Sea Proper.

Interestingly, nanoSIMS analysis demonstrated $NH_4^+$ uptake by bacteria attached to *Aphanizomenon* cells while no $NH_4^+$ assimilation was detected in the *Aphanizomenon* cells (Figure 4, Table 2). The $NH_4^+$ and $NO_3^-$ concentrations are generally $<250$ nM during summer in the Baltic Sea (Swedish Monitoring Program, Stockholm University and SMHI). A 3-year, large-scale field study in the Baltic Sea demonstrated that *Aphanizomenon* colonies show persistently low $\delta^{15}N$-values owing to high $N_2$-fixation independent of variations in concentrations of combined inorganic nitrogen in the surrounding water (Zakrisson *et al.*, 2014). Theoretical N-doubling time in biomass of various organisms at diffusion limitation by $NH_4^+$ from the bulk water depends on cell or colony size (Figure 6). Larger colonies, for example, chain-forming diatoms or bundles of trichomes of *Aphanizomenon*, will have much longer theoretical N-doubling times than small cells such as picocyanobacteria at

diffusion limitation owing to larger diffusion distances and lower surface area-to-volume ratio of colonies relative to those of single cells. The calculated N-doubling times with respect to biomass must be considered as the theoretical lower limit at low bulk $NH_4^+$ concentrations ($<250$ nM) observed in the Baltic Sea during summer. Small, autotrophic bacteria can theoretically cover their N-demand for one cell division within hours owing to their small size and large surface area:volume ratio. This is faster than the measured rates of about 1 day (Table 2). The $NH_4^+$ uptake rates by these small cells may likely be reaction-limited (partly due to temperature limitation at 10 °C) by enzymatic activities rather than by diffusion-limited transport of nutrients toward the cell surface. Using the frequency of dividing cells as a proxy for growth rates, the minimum generation time was estimated to 0.7 days for the $<3$-μm cell fraction at 15 °C during summer in the same area of the Baltic Sea (Larsson and Hagström, 1982). Larger phytoplankton (flagellates, diatom chains and single trichomes of large cyanobacteria) with diameters of 10–100 μm need several days to cover their N-demand for one cell division owing to their larger size and lower surface area:volume ratio. This was also confirmed by our observation of the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios in diatoms, which on average corresponded to C-generation times of 3–4 days (Figure 1). The *Aphanizomenon* colonies are represented by sizes between 100 and 1000 μm, and cells within such colonies would need about 1 month to cover their N-demand for one cell doubling from $NH_4^+$ uptake from the surrounding water as also indicated by our tracer experiments (Table 2). Colonies normally contain thousands of densely packed cells, which must share the low $NH_4^+$ flux to the whole colony at low bulk concentrations (Ploug *et al.*, 2010). Thus, given the low $NH_4^+$ flux at bulk concentrations $<250$ nM, colony-formation and generation times on the order of days would be impossible in *Aphanizomenon* colonies without $N_2$-fixation. Low $NH_4^+$ uptake capacity, however, may also lead to its release from colonies as $NH_4^+$ is the first product during $N_2$-fixation.

Release of newly fixed $N_2$ may appear as a poor growth strategy because $N_2$-fixation is a highly energy-demanding process. However, commensalism and mutualism are well-documented life strategies within plankton communities. In cyanobacterial colonies, attached bacteria may benefit from $NH_4^+$ release from the large cyanobacteria while the heterotrophic bacteria through respiration may change the $O_2$ and pH microenvironment for carbon acquisition and trace metal (Fe) speciation to improve the growth conditions for the cyanobacteria (Ploug, 2008). Interestingly, we detected a high $NH_4^+$ uptake by heterotrophic bacteria (Table 2, Figure 4), which suggest that $NH_4^+$ released from cyanobacteria was quantitatively more important for heterotrophic bacterial growth than other N-sources, that is, the concentration of dissolved organic matter was low and/or its C:N ratio was high. Hence, the present study showed that $NH_4^+$ released by $N_2$-fixing cyanobacteria can be a key nutrient source for pelagic production in N-limited environments during a cyanobacteria bloom in addition to remineralization. We could demonstrate N-transfer to very different functional plankton types encompassing heterotrophic and autotrophic bacteria and picoeukaryotes as well as diatoms and zooplankton. Transfer of diazotrophic nitrogen to diatoms and zooplankton has profound consequences for both the classical food web, including fish, as well as for large-scale biogeochemical carbon and nitrogen fluxes. The average C-specific growth rate by *Chaetoceros* sp. was higher than that of *Aphanizomenon.* However, its abundance was low, possibly due to preferential grazing on diatoms by copepods because their feeding appendices tend to be clogged by cyanobacterial colonies (Engström *et al.*, 2001). Diatom frustules act as ballast in sinking zooplankton fecal pellets and marine snow aggregates, which comprise a large fraction of the fast-sinking flux of organic matter in the ocean (Ploug *et al.*, 2008). Thus our study demonstrates that diazotrohpic nitrogen is not only re-distributed to other organisms during the decay and remineralization of cyanobacterial blooms. Instead, a substantial release and transfer of recently fixed diazotrophic nitrogen during the early bloom can stimulate pelagic production, microbial and classical food webs as well as large-scale $CO_2$ sequestration and export production more directly and at much shorter timescales (hours to days) than previously thought.

## Conflict of Interest

The authors declare no conflict of interest.

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