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Research Article

RESEARCH ARTICLE

High cell-specific rates of nitrogen and carbon fixation by the cyanobacterium *Aphanizomenon* sp. at low temperatures in the Baltic Sea

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One sentence summary: The cyanobacterium Aphanizomenon sp. is an important contributor to nitrogen fixation at low in situ temperatures in the early growth season in the Baltic Sea.
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ABSTRACT

Aphanizomenon is a widespread genus of nitrogen (N_2)-fixing cyanobacteria in lakes and estuaries, accounting for a large fraction of the summer N_2-fixation in the Baltic Sea. However, information about its cell-specific carbon (C)- and N_2-fixation rates in the early growth season has not previously been reported. We combined various methods to study N_2-fixation, photosynthesis and respiration in field-sampled Baltic Sea *Aphanizomenon* sp. during early summer at 10°C. Stable isotope incubations at *in situ* light intensities during 24 h combined with cell-specific secondary ion mass spectrometry showed an average net N_2-fixation rate of 55 fmol N cell^{-1} day^{-1}. Dark net N_2-fixation rates over a course of 12 h were 20% of those measured in light. C-fixation, but not N_2-fixation, was inhibited by high ambient light intensities during daytime. Consequently, the C:N fixation ratio varied substantially over the diel cycle. C- and N_2-fixation rates were comparable to those reported for *Aphanizomenon* sp. in August at 19°C, using the same methods. High respiration rates (23% of gross photosynthesis) were measured with ^{14}C-incubations and O_2-microsensors, and presumably reflect the energy needed for high N_2-fixation rates. Hence, *Aphanizomenon* sp. is an important contributor to N_2-fixation at low *in situ* temperatures in the early growth season.

Keywords: *Aphanizomenon*; SIMS; isotopes; O_2-microsensors; C-fixation; N_2-fixation

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INTRODUCTION

Cyanobacteria of the genus *Aphanizomenon* are widespread in inland lakes and estuaries. In the Baltic Sea, a nitrogen (N_2)-fixing *Aphanizomenon* sp. forms blooms every summer (Larsson *et al.* 2001; Walve and Larsson 2007). Here, it occurs mainly as colonies composed of bundles of trichomes (Fig. 1). The colonies, which can be several millimetres long, contain thousands of cells and belong to the most productive microenvironments known in aquatic systems (Ploug *et al.* 2010). The volumetric photosynthesis rate measured by O_2-microsensors in *Aphanizomenon* colonies is comparable to, or higher than, those measured in benthic cyanobacterial communities inhabiting shallow sediments, in microbial mats and in epilithic cyanobacterial communities (Jørgensen, Revsbech and Cohen 1983; Lassen, Ploug and Jørgensen 1992; Kühl *et al.* 1996). Colony formation may reduce grazing pressure by larger zooplankton (Engström, Viherluoto and Viitasalo 2001), and increase buoyancy control, thereby limiting sedimentation losses (Reynolds and Walsby 1975; Walsby *et al.* 1997). The peak population density of *Aphanizomenon* sp. often occurs as a subsurface maximum in the euphotic zone where the energy needed for N_2-fixation is provided by photosynthesis (Walsby *et al.* 1997; Hajdu, Höglander and Larsson 2007; Walve and Larsson 2007).

With trichome abundances of about 20 m L^{-1} (Hajdu, Höglander and Larsson 2007) and fixation rates of approximately 0.7 nmol N m^{-1} h^{-1} (Ploug *et al.* 2010), *Aphanizomenon* sp. is considered an important N_2-fixing organism in the Baltic Sea during summer (Degerholm *et al.* 2008; Ploug *et al.* 2010). Spring time N_2-fixation by the total cyanobacterial community in the Baltic Sea has been measured using the ^{15}N tracer method (Wasmund, Voss and Lochte 2001; Degerholm *et al.* 2008), but information about the cell-specific N_2-fixation rates of *Aphanizomenon* sp. in spring and early summer has not previously been reported. Based on CO_2 exchange and nitrogen budgets in the Baltic Sea, it has been suggested that N_2-fixation during spring may be higher than earlier anticipated (Schneider *et al.* 2009; Eggert and Schneider 2015). In support, isotopic signatures ($\delta^{15}N$) have shown that in the Baltic Sea *Aphanizomenon* sp. take up little or no combined N for most of the year, even at high dissolved inorganic nitrogen concentrations (Zakrisson, Larsson and Höglander 2014). The biomass of *Aphanizomenon* sp. usually increases in early June (Larsson *et al.* 2001; Walve and Larsson 2007). Therefore, high cell-specific rates of carbon (C)- and N_2-fixation, inducing growth, can be expected during this season.

N_2-fixation in *Aphanizomenon* sp. is performed by specialized cells, the heterocysts, containing the enzyme nitrogenase. The frequency of heterocysts in *Aphanizomenon* sp. varies over the year and usually peaks in the early growth season (May–June), before the biomass peak (Laamanen and Kuosa 2005; Walve and Larsson 2007; Zakrisson and Larsson 2014). *Aphanizomenon* sp. has a comparably low heterocyst frequency of 1–3% of the total cell number (Janson, Carpenter and Bergman 1994; Walve and Larsson 2007; Ploug *et al.* 2010; Mohlin *et al.* 2012), while frequencies of 5–10% have been reported for other heterocystous cyanobacteria, e.g. *Nodularia spumigena* (Janson, Carpenter and Bergman 1994; Mohlin *et al.* 2012). Nevertheless, the N assimilation per vegetative cell volume derived from N_2-fixation is similar in *Aphanizomenon* sp. and *N. spumigena* in the Baltic Sea during late summer (Ploug *et al.* 2010, 2011). Incubation studies using stable isotope tracers combined with a nanosecondary ion mass spectrometry (SIMS) technique have revealed fast transfer of fixed $^{15}N_2$ from heterocysts to all vegetative cells in trichomes of *Aphanizomenon* sp., despite the low heterocyst frequency (Ploug *et al.* 2010). The same study also showed that heterocysts were less enriched in fixed $^{15}N_2$ relative to vegetative cells and that recently fixed $^{15}N_2$ (e.g. cyanophycin) in vegetative cells can be detected after 3 h of incubation (Ploug *et al.* 2010).

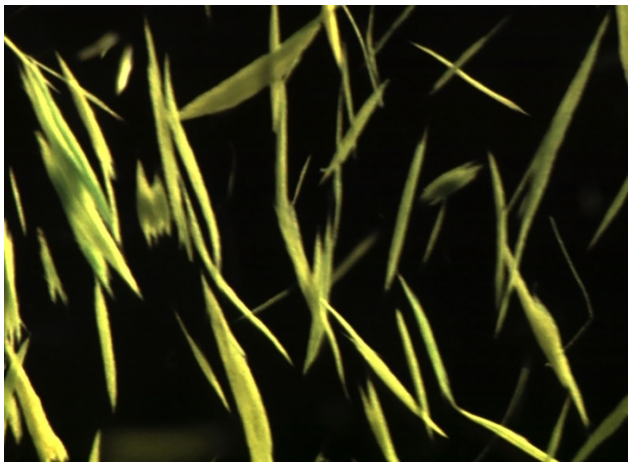

Figure 1. Colonies of *Aphanizomenon* sp. from the Baltic Sea. Photo: Helena Höglander.

The SIMS method is greatly increasing our understanding of the ecophysiology of microorganisms and biogeochemical fluxes on a single cell level, without the use of cultivation techniques (Wagner 2009). This technology allows for simultaneous determination of cell identity and activity within mixed microbial populations in field samples. In the present study we measured C- and N-fixation by *Aphanizomenon* sp. using stable isotope tracers ([^{13}C]bicarbonate and $^{15}N_2$) combined with SIMS and elemental analysis–isotope ratio mass spectrometry (EA-IRMS). Furthermore, we quantified the heterocyst frequency and elemental composition, and combined the use of ^{14}C-labeled radioisotopes and O_2-microsensors in *Aphanizomenon* sp. colonies to study early season photosynthesis and respiration. Our integrated methodological approach enabled us to investigate the activity of single cells and colonies, incubated at low *in situ* temperature (10°C), to gain new insights into C- and N_2-fixation by a field population of *Aphanizomenon* sp. in early summer.

MATERIALS AND METHODS

Sampling

In early June 2010, *Aphanizomenon* sp. colonies were sampled in the upper 10 m of the water column at station B1 (58° 48′ 28″ N, 17° 37′ 60″ E) in the southern Stockholm archipelago, using a plankton net (Hydrobios; diameter, 0.5 m; mesh size, 90 μm). Water was collected at 5 m depth by a water sampler (NMTech AB). The salinity was 6 (PSU) and the temperature was 10°C. Samples with concentrated colonies were brought to the laboratory within 30 min and placed in a light trap in a climate-controlled room at *in situ* temperature, to separate zooplankton from the cyanobacterial colonies. The light trap consisted of a funnel covered by black foil, except at its base where it was illuminated to attract zooplankton, while cyanobacterial colonies were allowed to float to the surface. Afterwards, the cyanobacteria were sampled for compositional analysis and heterocyst frequency, incubated with stable isotopes and radioactive ^{14}C, and O_2-microsensor measurements were made.

Volume specific carbon, nitrogen and phosphorous content of *Aphanizomenon* sp.

Aphanizomenon colonies collected from the light traps were washed in NaCl solution (7‰) through a sieve and then poured into a Petri dish and placed under a dissection microscope. Individual *Aphanizomenon* colonies were picked with a gold needle and washed in NaCl solution before being added to microcentrifuge vials, prefilled with 2 mL of NaCl solution. At least 100 colonies were collected in each vial, which was thoroughly shaken to obtain a homogeneous solution of individual *Aphanizomenon* filaments. One milliliter was transferred into another microcentrifuge vial to which 10 μL of Lugol's iodine solution (supplemented with acetic acid) was added. The remaining 1 mL subsample was frozen until analysis. Blanks were prepared by repeatedly washing a needle, without any colony on it, in NaCl solution before dipping it in a vial.

The frozen subsamples were analysed for total nitrogen and phosphorous (P) with a simultaneous oxidation of N- and P-compounds in a buffered persulphate solution (Walve and Larsson 2007). The derived nitrate and phosphate were determined on a segmented flow system, ALPKEM Flow Solution IV. The carbon content was calculated from a C:N (mol:mol) ratio of 6.31 ± 0.02 (n = 7) measured on a Leco CHNS-932 Analyzer, with EDTA as standard. The volume-specific cellular C-, N- and P-content were calculated from the total cell volume measured by microscopy analysis of the parallel subsample (see below).

Microscopy

The Lugol-preserved subsamples for the elemental analysis were analysed under an inverted microscope (Leica DMIRB) at ×100 magnification in order to measure total *Aphanizomenon* trichome length and heterocyst frequency. From each sample, 0.02–0.3 mL was transferred to a 1 mL counting chamber. The subsample was diluted to 1 mL with NaCl solution and was allowed to settle for 2 h before counting the total trichome length in the whole chamber, according to HELCOM (2013). If counting of the whole chamber did not result in a total trichome length of 0.1 m or more, a new subsample was prepared and counted. Average cell lengths and widths were measured at 1000-fold magnification under a fluorescence microscope (Olympus AH2) until the mean values were stable and the standard error was <0.001 of the mean value. Cell volume was calculated assuming a cylindrical form.

Incubations with stable isotopes

Water samples containing 1.1 × 10^8 *Aphanizomenon* sp. cells L^{-1} (±0.1 × 10^8 cells L^{-1}; standard error of the mean) were incubated in 250 mL serum bottles by adding colonies harvested by the plankton net to bulk water samples. The serum bottles were closed with rubber stoppers (butyl) through which [^{13}C]bicarbonate, and $^{15}N_2$ (as gas, ISOTECH no. CX0937) were injected to a final concentration of 9% and 36% labeling, respectively, except for three control bottles. Recently, it was shown that $^{15}N_2$ gas occasionally can be contaminated by $^{15}NH_4^+$ (Dabundo *et al.* 2014). In parallel with the present study, experiments were made in which the <5 μm cell fraction (no N_2 fixers) was incubated with [^{13}C]bicarbonate and $^{15}N_2$ (as gas) or $^{15}NH_4^+$ (Adam *et al.* 2015). Cells in this size fraction had high affinity for NH_4^+. However, no enrichment of ^{15}N was found after 12 h of incubation with $^{15}N_2$ (demonstrated by nanoSIMS and EA-IRMS), even though the cells in this size fraction indeed assimilated ^{13}C, showing that they were actively photosynthesizing. These results show that the $^{15}N_2$ used was not contaminated by $^{15}NH_4^+$, and that the data on N_2-fixation are reliable.

The samples were incubated at 0.5 m depth in an outdoor mesocosm at ambient light and a constant flow of sea water at *in situ* temperature (10°C). Ambient light in air was measured using a LI-COR irradiance sensor. Three replicates and a control were incubated for each time point (0, 3, 6, 9, 12, 15, 18, 21 and 24 h) from the start of the experiment (3 a.m.). In addition, three dark replicates (serum bottles wrapped in aluminum foil) were incubated for all time points except 0 h. The actual labeling percentage is lower than the theoretical value, especially during the first 6 h of incubation, because of the long equilibrium time of $^{15}N_2$ gas in water (Mohr *et al.* 2010; Klawonn *et al.* 2015). In incubations >6 h and <24 h the underestimation of N_2-fixation is about 20% when injecting a $^{15}N_2$ bubble relative to adding $^{15}N_2$-enriched water (Klawonn *et al.* 2015). Incubations were stopped by filtration onto pre-combusted GF/F filters or by fixing subsamples with 2% paraformaldehyde for 24 h at 4°C. The GF/F filters were immediately frozen (−80°C) until further treatment. The samples fixed with paraformaldehyde were filtered onto gold–palladium coated GTTP filters (pore size, 0.22 μm; diameter, 25 mm; Millipore), washed, dried and stored at −20°C until SIMS analysis.

Secondary ion mass spectrometry analysis

The GTTP filters containing fixed cells were cut into *c.* 4 × 4 mm pieces and mounted on a sample holder. The analysis was performed using an IMS 1280 instrument manufactured by Cameca (Gennevilliers, France), the only large geometry ion microprobe performing both microscope and microprobe imaging. The imaging capabilities of the IMS 1280 can be used to map the distribution of major, minor and trace elements or isotopes at submicron lateral resolution. Using a Cs^+ primary beam with a spatial resolution of about 1 μm while ensuring a sputtering equilibrium, secondary ion images of $^{13}C^{14}N$, $^{12}C^{14}N$ and $^{12}C^{15}N$ were recorded using a peak-switching routine at a mass resolution of *c.* 6000 ($M/\Delta M$). Image and data processing were performed as previously described for nanoSIMS analysis using the CAMECA Winimage2 software (Musat *et al.* 2008). The $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios of up to 50 individual *Aphanizomenon* cells per time point were determined from the incubation periods of 3, 6, 9, 12, and 24 h (light only). The cellular net C- and N_2-fixation rates (fmol C or N $cell^{-1}$ h^{-1}) were calculated from the isotope ratio (IR) of individual cells after the incubation time ($T_1 - T_0$), the measured cellular volume (V, μm^3) and cellular C- or N-content ($C_{C/N}$, fmol μm^{-3}) as:

$$\frac{(IR_{T_1} - IR_{background}) \times V \times C_{C/N}}{F_{bulk} \times (T_1 - T_0)} \quad (1)$$

where F_{bulk} is the theoretical fraction of labeled isotope in the bulk, calculated from the standard solubility of N_2 at 10°C and a salinity of 6 (Colt 2012). An increasing number of non-adjacent cells (from three to eight different trichomes) were analysed until the mean values of the cellular C- and N_2-fixation were stable and the standard error of the mean was less than 10% of the mean value. An example is shown in Fig. 2. This mean value thus represented the average assimilation of fixed N per cell in the population.

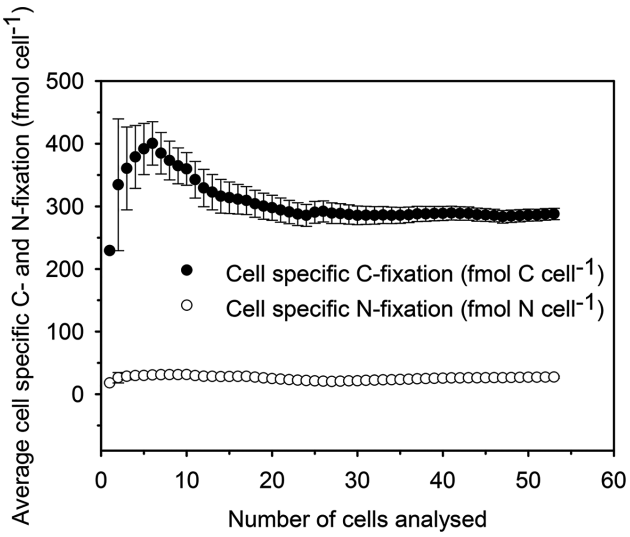

Figure 2. Number of cells analysed by SIMS. An increasing number of cells were analysed (secondary ion mass spectrometry, SIMS) until the mean values of the cellular C- and N-fixation rates were stable and the standard errors were less than 10% of the mean value (example from 9 h of incubation in light).

Elemental analysis–isotope ratio mass spectrometry

GF/F filters were freeze-dried, decalcified overnight in a desiccator with HCl (37%) fumes, packed into tin cups and analysed by an Thermo Flash EA 1112 elemental analyser coupled to an isotopic ratio mass spectrometer (Thermo Delta Plus XP, Thermo Fisher Scientific) at MPI, Bremen. Caffeine was used as a standard for isotope correction and C/N quantification. The C-specific C-assimilation and N-specific net N_2-fixation were calculated from the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ enrichment of each incubated bottle according to equation (1) but excluding cellular volume and cellular C- and N-content.

Incubations with ^{14}C

Incubations with ^{14}C were performed in a climate-controlled room. For this incubation, glass exetainers (7.7 mL; Exetainers, Labco Scientific, High Wycombe, UK) were filled with 7 mL of seawater, and 0.25 mL cyanobacterial suspension and 50 μL $Na^{14}CO_3$ solution were added. Nine exetainers were immediately covered with aluminum foil and used as controls to correct for autotrophic dark fixation of ^{14}C. The exetainers were incubated horizontally under saturating (for photosynthesis) artificial light at 300 μmol photons m^{-2} s^{-1}, and at ambient temperature (10°C). Random triplicate exetainers were sampled after 1, 3, 6 and 9 h of incubation in light. After 9 h of incubation, the remaining exetainers were covered in aluminum foil and sampled after additional 1, 3, 6 and 9 h of incubation in darkness. When sampled, time was noted for each exetainer, and the content filtered onto a 60 μm sieve, rinsed five times with seawater, and transferred to a Petri dish. Colonies of *Aphanizomenon* sp. were transferred with a needle to a dish containing a NaCl solution, prior to being moved to 2-mL microcentrifuge vials filled with NaCl solution. Blank microcentrifuge vials were prepared by repeated dipping of the needle in the dish and vials. The samples were frozen (−20°) for later analysis.

Upon analysis, the thawed vials were shaken to obtain a homogeneous solution of single *Aphanizomenon* filaments. From each vial 1 mL was used for total N-analysis (see above). For analysis of ^{14}C-uptake, 1 mL of Lumagel Safe scintillation cocktail (Lumac LSC B.V.) was added to the remaining 1 mL sample and analysed in a Tri-Carb 1600 TR (Packard Corp.) scintillation counter. Total carbon uptake was calculated from the ^{14}C-uptake equation of Parsons, Maita and Lalli (1984), without correction for respiration during incubation, using a total dissolved inorganic C-concentration of 1.29 mmol L^{-1} (as calculated from pH, salinity and temperature). Specific carbon uptake (h^{-1}) was calculated from measured total N and the measured cyanobacterial C:N ratio.

Direct measurements of O_2-fluxes in colonies by microsensors

Freshly sampled colonies were transferred to a Petri dish coated by a 3 mm thick agar layer (1% w:w) at the bottom, prepared from the water of the sampling site and covered by filtered (0.2 μm) water from the sampling site. The diffusion coefficients of gases, ions and solutes in 1% agar are close to those in (sea) water (Revsbech 1989). The physical/chemical microenvironment of the *Aphanizomenon* colony in this set-up is therefore close to that of a neutrally buoyant colony being suspended in sea water. The Petri dish was placed in a thermostated container at *in situ* temperature. Oxygen concentrations were measured at the colony–water interface using a Clark type O_2-microsensor (Revsbech 1989) attached to a micromanipulator. The current was measured by a picoamperemeter (Unisense, PA2000) connected to a strip-chart recorder (Kipp and 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%. The oxygen microelectrode tip was 2 μm wide and its position was observed under a dissection microscope with a calibrated ocular micrometer. The electrode was manually moved towards the colony until it visually touched the surface. Oxygen concentration gradients at the colony–water interface were measured at steady-state at 50 μm step increments. Three to four replicates of the O_2-concentration gradient were measured in light and in dark. The light source was a Schott lamp (KL 1500 LCD) equipped with an infra-red cut-off filter and calibrated using a LI-COR scalar irradiance sensor. The light intensity was 300 μmol photons m^{-2} s^{-1}. The fluxes of oxygen were calculated from Fick's first law of diffusion, according to Ploug *et al.* (2010), but with a diffusion coefficient of O_2 in the bulk water of 1.55×10^{-5} cm^2 s^{-1} at a salinity of 6 and 10°C (Broecker and Peng 1974). All three dimensions of individual colonies were measured under the dissection microscope using a calibrated ocular micrometer. Surface area and volume was calculated as for ellipsoids (Mass 1994): volume $= 4/3\pi \times a \times b \times c$, where a, b and c are the half-axes in the three dimensions.

Cell-specific O_2 net photosynthesis and respiration were calculated from the distribution of O_2 net photosynthesis and respiration as a function of colony volume, divided by the number of cells in *Aphanizomenon* colonies as a function of colony volume, according to Ploug *et al.* (2010). Carbon-specific net photosynthesis and respiration were calculated from the measured values of cellular C-content.

RESULTS AND DISCUSSION

High cellular assimilation of fixed N at 10°C

SIMS analysis identified *Aphanizomenon* sp. as an organism with the capacity to perform high rates of N_2-fixation at 10°C (Table 1 and Fig. 3). The incubation period from 3 a.m. to 6 a.m. showed a very high net C:N fixation ratio, which was likely an effect of the

Table 1. Net cell-specific C- and N_2-fixation in *Aphanizomenon* sp. over different time periods determined from stable isotope tracer incubations and SIMS analysis.

	3 a.m.–6 a.m. (3 h)	3 a.m.–9 a.m. (6 h)	3 a.m.–12 p.m. (9 h)	3 a.m.–3 p.m. (12 h)	3 a.m.–3 p.m. (24 h)
C-fixation (fmol C cell^{-1})	56 ± 32	58 ± 19	319 ± 73	315 ± 79	215 ± 65
N_2-fixation (fmol N cell^{-1})	0.5 ± 0.3	3 ± 2	19 ± 8	45 ± 13	55 ± 19
C:N fixation ratio (cell^{-1})	148 ± 117	29 ± 26	25 ± 22	8 ± 3	4 ± 2

All values represent the mean ± SD.

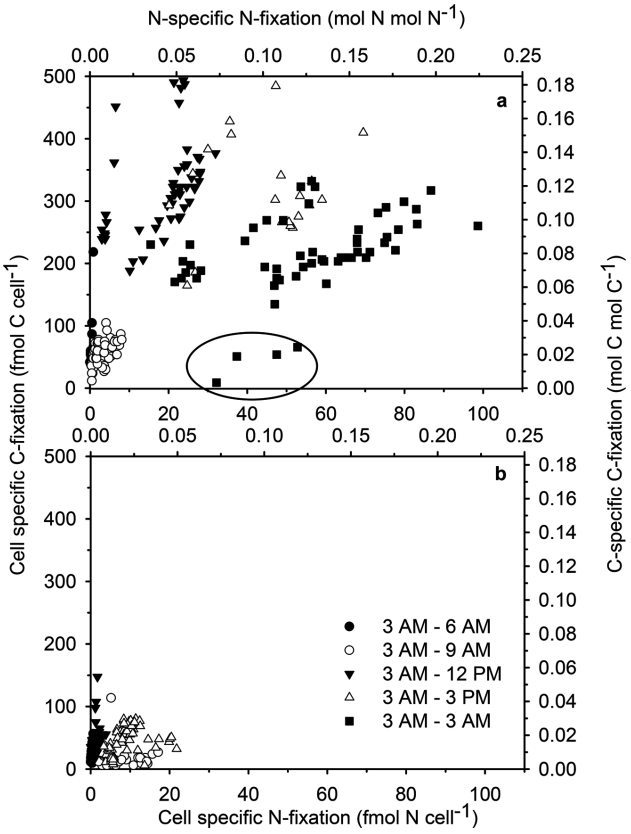

Figure 3. C-fixation and N_2-fixation in light and darkness measured by SIMS. C-fixation (fmol C cell^{-1} and mol C mol C^{-1}) and N_2-fixation (fmol N cell^{-1} and mol N mol N^{-1}) in (**a**) light and (**b**) darkness measured by secondary ion mass spectrometry (SIMS). Circled cells are likely to be heterocysts.

slow equilibration time of the $^{15}N_2$ gas in water, during the first hours (Mohr *et al.* 2010). The C:N fixation ratio was considerably lower in cells incubated from 3 a.m. to 9 a.m. than during the previous period (3 a.m. to 6 a.m.). From 9 a.m. to 12 p.m., net cell-specific fixation of C and N increased fivefold. From noon and in the early afternoon, the accumulated average C-assimilation did not change whereas N_2-fixation increased twofold. Hence, C-assimilation appeared to be strongly inhibited from 12 p.m. while N_2-fixation continued. The light intensity (incident irradiance in air) was >1000 μmol photons m^{-2} s^{-1} from 10 a.m., reaching its maximum of 1600–1800 μmol photons m^{-2} s^{-1} from 12 p.m. to 3 p.m., and photosynthesis was probably inhibited by these high light intensities. However, the N_2-fixation rate was relatively constant independent of irradiance, as has previously been measured in the Baltic Sea (Moisander *et al.* 2007). Consequently, photoinhibition of photosynthesis but not of N_2-fixation led to lower C:N fixation ratios (Table 1). Under natural field conditions *Aphanizomenon* sp. may partly escape the photoinhibiting irradiance of the surface waters during the middle of the day by vertical migration to deeper water layers (Heiskanen and Olli 1996).

After 24 h of incubation the average net C-fixation was 215 ± 65 fmol C cell^{-1} and the average net N_2-fixation was 55 ± 19 fmol N cell^{-1} (±standard deviation). Net N_2-fixation in dark bottles during 12 h was 9 ± 4 fmol N cell^{-1}, or 20% of that fixed during light. The N_2-fixation rate per hour (6 a.m. until 12 p.m.) was only 36% lower than reported for *Aphanizomenon* sp. at 19°C (August), using the same methods, whereas the C-fixation was very similar (Ploug *et al.* 2010). The 19°C study was performed under constant saturating (for photosynthesis) light of 300 μmol photons m^{-2} s^{-1}, which further emphasizes the high rates of photosynthesis and N_2-fixation seen at 10°C, despite photoinhibiting ambient light conditions. Nitrogenase activity in Baltic Sea cyanobacteria has earlier been shown to be inhibited by irradiances above 1000 μmol photons m^{-2} s^{-1} (Evans *et al.* 2000). Hence, although N_2-fixation continued under higher irradiances than photosynthesis, the net N_2-fixation rates of the present study might not be at their maximum.

Uncoupling of C- and N_2-fixation

Our study suggests that C- and N_2-fixation are not strictly coupled and that the energy for N_2-fixation can be partly provided by respiration of storage products. N_2-fixation continued both in the very high irradiance that inhibited C-fixation and in darkness. After 24 h, the net cellular C:N fixation ratio was lower than the biomass C:N ratio, due to limited C-fixation during the afternoon and night combined with continuous N_2-fixation during day and night. Previous studies in the Baltic Sea have also shown temporary uncoupling of C- and N_2-fixation, with variable C:N fixation ratios deviating from the Redfield ratio during the early bloom (Gallon *et al.* 2002; Ohlendieck *et al.* 2007). Seasonally, however, the cellular biomass C:N ratio of *Aphanizomenon* sp. varies less than 10% (Walve and Larsson 2007). The deviation in C:N fixation ratio from the cellular C:N molar mass ratio (6.31) is probably explained by N-storage (e.g. as cyanophycin) of recently fixed N_2, release of dissolved organic carbon, and respiration of C. Storage and release of excess products are short-term control mechanisms to manage the C:N imbalance caused by different responses of C- and N_2-fixation to irradiance (Stal and Walsby 1998). Stored N may later be released as ammonium. *Aphanizomenon* sp. from the Baltic Sea has been shown to release up to half of the recently fixed N as ammonium, which is used by co-existing phytoplankton (Ploug *et al.* 2010; Adam *et al.* 2015).

Values of assimilated ^{13}C and ^{15}N (measured with SIMS) varied considerably between cells and when measured after different time periods of the incubation (Table 1 and Fig. 3). This heterogeneity on a cellular level has previously been observed with nanoSIMS in *Aphanizomenon* sp. (Ploug *et al.* 2010) but also in, for example, *Trichodesmium* (Finzi-Hart *et al.* 2009), unicellular cyanobacteria (Foster, Sztejrenszus and Kuypers 2013; Mohr *et al.* 2013) and anaerobic phototrophic bacteria (Musat *et al.* 2008).

Transmission electron microscopy studies have shown that *Aphanizomenon* sp. vegetative cells, within the same colony, can be highly heterogeneous in terms of vacuoles and nutrient storage granules (Janson, Carpenter and Bergman 1994). This variation may thus partly reflect the (functional) heterogeneity between cells.

Heterocyst frequency, cellular dimensions and volume-specific C-, N- and P-content

Microscopy showed that the number of heterocysts comprised only 2.0% of the total cell number. Consequently, the heterogeneity displayed in the C:N fixation ratio among cells within the same incubation periods was mainly due to heterogeneity among vegetative cells. The four cells with very low assimilation of fixed C and N_2 after 24 h were likely heterocysts (Fig. 3a, circled), as demonstrated in an earlier study using nanoSIMS (Ploug *et al.* 2010). SIMS does not allow for as high a spatial resolution as nanoSIMS, and heterocysts cannot with certainty be visually recognized by this technique. However, considering that a total number of 230 cells incubated in light were analysed by SIMS, and that heterocysts represented 2.0% of the total cell number, a number of four heterocysts is expected for statistical reasons. In the present study the heterocyst frequency was higher than in August at 19°C (1.5%; Ploug *et al.* 2010). The higher heterocyst frequency often observed in early summer, compared to late summer (Laamanen and Kuosa 2005; Walve and Larsson 2007; Zakrisson and Larsson 2014), may compensate for colder temperatures and concomitant reduced activity of the enzymes (nitrogenase), and thus partly explain the high N_2-fixation rates observed at 10°C (Zakrisson and Larsson 2014).

The average dimensions and elemental composition of single *Aphanizomenon* cells are presented in Table 2. The volume specific C-content resulting from these data (0.21 pg C μm^{-3}) was higher (~24%) than that of *Aphanizomenon* sp. in the HELCOM PEG biovolume file (0.16 pg C μm^{-3}; HELCOM 2013), which was estimated from relationships between cellular C-content and volume measured in diatoms and flagellates (Menden-Deuer and Lessard 2000). A high specific C-content is in accordance with the observation that preservation with Lugol's solution causes a 15–30% decrease in cell volume of other filamentous cyanobacteria, e.g. *Dolichospermum* sp. (Hawkins *et al.* 2005). For live cells, the C- and nutrient content per cell volume may therefore be similar to those of diatoms and flagellates. Our measurements of the cellular C- and N-content are, to our knowledge, the first of their kind reported on a Baltic Sea *Aphanizomenon* sp. field population, and suggest that for samples preserved with Lugol's solution, the use of the conventional conversion factors may considerably underestimate *Aphanizomenon* carbon biomass.

Table 2. Mean cell size, C:N ratio, cellular C-, N-, and P-content and heterocyst frequency in *Aphanizomenon* sp. colonies.

Mean cell width (μm)	5.0 ± 0.1
Mean cell length (μm)	7.9 ± 1.1
Mean cellular volume (μm^3)	155
Cellular C:N (mol:mol) ratio	6.31 ± 0.02
Cellular C content (pmol C $cell^{-1}$)	2.7 ± 0.3
Cellular N content (pmol N $cell^{-1}$)	0.43 ± 0.1
Cellular P content (pmol P $cell^{-1}$)	0.042 ± 0.013
Heterocyst frequency (mm^{-1})	2.6 ± 0.03

All values represent the mean ± SD.

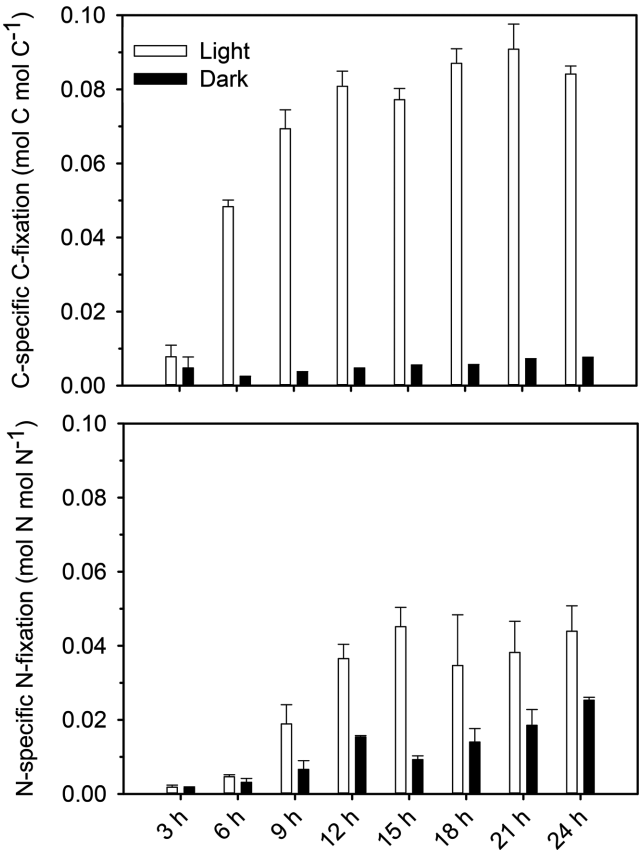

Figure 4. C-specific C-fixation and N-specific N_2-fixation measured by EA-IRMS. The cumulative C-specific C-fixation and N-specific N_2-fixation (mol mol^{-1}) for time intervals (3–24 h) from 3 a.m. to 3 a.m. (incubations in light and darkness) measured by elemental analysis-isotope ratio mass spectrometry (EA-IRMS). Error bars represent standard error of the mean.

Rates of C-assimilation and N_2-fixation measured by EA-IRMS

Aphanizomenon sp. dominated the biomass of the incubation due to enrichment with net-collected colonies. It constituted 99% of the filamentous cyanobacterial community, with only a few *Dolichospermum* spp., at the time of the study. The SIMS measurements provide cell-specific values for *Aphanizomenon* sp. whereas ^{13}C- and ^{15}N-assimilation measured by EA-IRMS include other autotrophic and heterotrophic organisms in the bulk sample. The C-specific C-assimilation of the bulk sample in light increased almost 10-fold from 6 a.m. to 12 p.m., whereas the dark C-assimilation was consistently low (Fig. 4). In the following 9–24 h of the incubation, the average C-specific C-assimilation remained at about 0.08–0.09 mol C mol C^{-1}. Hence, C-fixation was strongly reduced after noon, similar to what was observed in the SIMS analysis of single cells. The C:N fixation ratio of the bulk sample after 24 h was about 12 in light and 2 in darkness.

The N-specific N-fixation of the bulk sample (measured by EA-IRMS) increased about 10-fold from 6 a.m. to 3 p.m., in both light and dark bottles (Fig. 4). The EA-IRMS analysis showed lower N-specific N-fixation rates compared to SIMS (single cells), despite the *Aphanizomenon* sp. dominance in the bulk sample. This is probably explained by the non-N_2-fixing organisms and other sources of particulate organic N also present in the bulk water. Dark fixation rates of about 50% of the light fixation rates were measured in the bulk sample. The N_2-fixation by single

Aphanizomenon cells during darkness (measured by SIMS) was substantially lower than 50% of the value in light. Relatively low assimilation of fixed N_2, and high NH_4^+ release, by *Aphanizomenon* cells during darkness has previously been observed (Ploug *et al.* 2010). This apparently higher dark N_2-fixation in the bulk sample, compared to that of single *Aphanizomenon* cells, may thus be explained by a release of fixed $^{15}N_2$ from *Aphanizomenon* sp., in the form of ammonium ($^{15}NH_4$), which is subsequently taken up by other organisms in the bulk water, e.g. diatoms, and autotrophic and heterotrophic bacteria (Ploug *et al.* 2010; Adam *et al.* 2015).

Contribution from other N_2-fixers, in addition to *Aphanizomenon* sp., cannot be excluded. In our study, though, microscopy revealed very few filaments of *Dolichospermum* spp. (~1%) and none of *N. spumigena* amongst the *Aphanizomenon* sp. filaments. Studies have suggested that diazotrophic picocyanobacteria may occur in the Baltic Sea (Wasmund, Voss and Lochte 2001; Farnelid, Öberg and Riemann 2009). Additionally, N_2-fixing heterotrophic bacteria have been detected below the chemocline (Farnelid et al. 2013). However, N_2-fixation by other phytoplankton than *Aphanizomenon* sp., *Dolichospermum* spp. and *N. spumigena* in the upper mixed layer of the Baltic Sea has not yet been documented. In a parallel study, the <5 μm cell fraction (which did not include any filamentous heterocystous cyanobacteria) was incubated with $^{15}N_2$, and no enrichment was found after 12 h (Adam *et al.* 2015).

Photosynthesis and respiration by *Aphanizomenon* sp. colonies at 10°C in constant light

The C-specific C-assimilation increased linearly with ^{14}C incubation time in light-exposed colonies (Fig. 5). At this constant light intensity, the colonies did not experience the same inhibiting irradiances as in the parallel outdoor mesocosm incubations. The linear increase was described by: $P = 0.034\ (\pm 0.001) \times t + 0.014\ (\pm 0.005)$, where photosynthesis ($P$) is measured as μmol C μmol C^{-1} h^{-1} and t is time in hours ($r^2 = 0.98$). The C-specific assimilation decreased immediately after the samples were exposed to darkness. It showed a relatively large variation, but the average decrease per unit time, assumed to be respiration (R), was described by: $R = 0.0074\ (\pm 0.0016) \times t$ ($r^2 = 0.44$). The large variation possibly reflected that not only newly synthesized carbon sources but also older storage products were respired during darkness. The high abundance of cyanobacterial cells in colonies, combined with their photosynthesis and respiration rates, resulted in significant O_2 gradients at the colony–water interface during darkness, measured by an O_2-microsensor. *Aphanizomenon* sp. colonies occur as flat bundles of trichomes, limiting self-shading (Fig. 1). The O_2 concentration at the surface of colonies varied between 300 μmol L^{-1} in darkness and 428 μmol L^{-1} in light. The O_2 concentration at the colony surface was slightly below air-saturation during darkness, and anoxia was never detected in any colony (data not shown). The net O_2 fluxes were calculated from the steady-state O_2 gradients measured during light and darkness. Both photosynthesis and respiration were linear functions of colony volume (Fig. 6). The photosynthesis (P) was described by: $P = 126\ (\pm 13) \times V_{colony} - 0.34\ (\pm 0.29)$ ($r^2 = 0.75$; $n = 29$), where photosynthesis is measured in nmol O_2 h^{-1} and colony volume (V_{colony}) is measured in mm^3. Respiration was described by: $R = 34\ (\pm 5) \times V_{colony} - 0.13\ (\pm 0.12)$ ($r^2 = 0.72$; $n = 19$). Paired measurements of dark respiration and gross photosynthesis in individual colonies of the present study showed a ratio of dark respiration to gross photosynthesis of 0.23 ± 0.09 at 10°C. An earlier study of a late-summer *Aphanizomenon* sp. bloom revealed a ratio of dark respiration to gross photosynthesis of 0.16 ± 0.06 at 20°C (Ploug *et al.* 2010). By comparison, the ratio of dark respiration to gross photosynthesis at saturating light intensities in other colony-forming, non-N_2-fixing phytoplankton, which primarily use nitrate as an N source, e.g. *Phaeocystis* sp., is 0.058 ± 0.020 at 9°C (Ploug *et al.* 1999). The high respiration of *Aphanizomenon* sp. presumably reflects the energy requirements for N_2-fixation (Staal *et al.* 2003).

The ^{14}C-method is supposed to describe a value in between the gross and net C-assimilation during photosynthesis. It is expected to be closer to gross photosynthesis during short incubations and closer to net photosynthesis during long incubations (Laws 1991; Kirk 1994). In the present study, the average C-specific assimilation in *Aphanizomenon* sp. was closer to the net O_2 photosynthesis rate than to the gross O_2 photosynthesis rate (Table 3). The C-specific respiration estimated by the ^{14}C-method and that calculated from the O_2-fluxes were similar. Hence, the two methods showed consistent values of C-specific photosynthesis and respiration by these colonies at a light intensity of

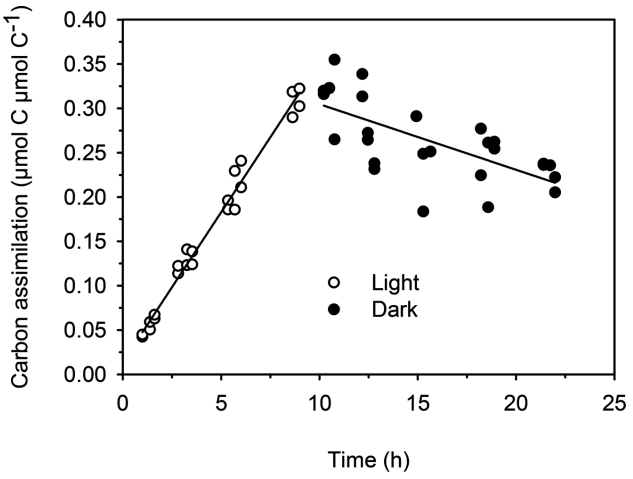

Figure 5. C-specific C-assimilation using the ^{14}C-method. The C-specific C-assimilation measured in light (open symbols) and during a subsequent dark period (closed symbols) as a function of time using the ^{14}C-method.

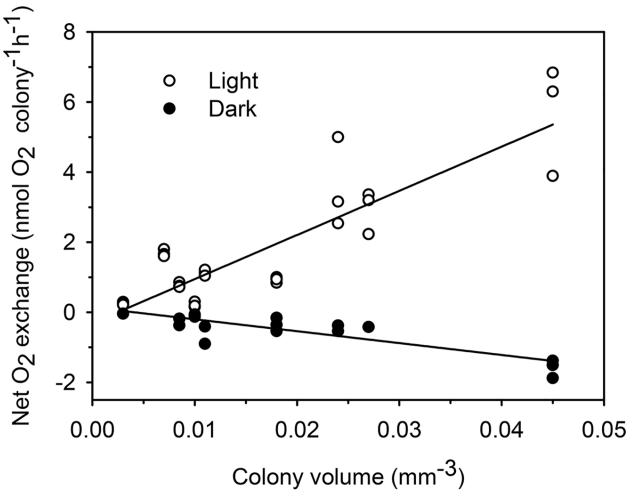

Figure 6. Net O_2 exchange as a function of colony size. The net O_2 exchange measured at the colony–water interface at a light intensity of 300 μmol photons m^{-2} s^{-1} (open symbols) and in darkness (closed symbols) as a function of colony size.

Table 3. C-specific gross and net O_2 production, C-specific net C-assimilation, and respiration measured by microsensors and the ^{14}C-method, and calculated from O_2 fluxes and mol C in the sample.

Method	Gross O_2 production (mol O_2 mol C^{-1} h^{-1})	Net C-assimilation (mol C mol C^{-1} h^{-1})	Net O_2 production (mol O_2 mol C^{-1} h^{-1})	C-respiration (mol C mol C^{-1} h^{-1})
^{14}C		0.034 ± 0.001		0.0074 ± 0.0016
O_2	0.040 ± 0.003[a]		0.032 ± 0.002	0.0085 ± 0.0007[b]

All parameters represent the mean ± SEM.
[a]Calculated from the sum of net photosynthesis and dark respiration.
[b]Assuming a respiratory quotient of 1.

300 μmol photons m^{-2} s^{-1}. In the natural day light regime at higher light intensities (from 6 a.m. to 12 p.m.), the ^{13}C-method combined with SIMS showed an average value of 0.016 ± 0.004 μmol C μmol C^{-1} h^{-1}. Using the ^{14}C-method, the C-specific uptake has previously been shown to vary between 0.010 and 0.034 μmol C μmol C^{-1} h^{-1} in the Baltic Sea at light intensities >200 μmol photons m^{-2} s^{-1} (Walve and Larsson 2007). These net C-based uptake rates are similar to the highest uptake rates observed for *Anabaena* sp. colonies of 0.027 μmol C μmol C^{-1} h^{-1} (Chan *et al.* 2004) as well as to those of *Trichodesmium* spp. colonies (0.005 to 0.045 μmol C μmol C^{-1} h^{-1}) growing in the Caribbean Sea (Carpenter and Roenneberg 1995). Hence, the C-specific assimilation rate by *Aphanizomenon* sp. at 10°C is similar to those of other colony-forming cyanobacteria growing in the tropics at temperatures >25°C.

High cell-specific C- and N_2-fixation rates at 10°C may mediate biomass increase during early summer

Our integrated methodological approach showed high rates of N_2-fixation, photosynthesis and respiration in *Aphanizomenon* sp. cells and colonies at 10°C. Assuming exponential growth, the potential doubling time based on N_2-fixation in the present study ranged between 3 and 14 days (6 days on average). This growth rate at low temperatures appears unique among cyanobacteria in temperate brackish and fresh water systems (Paerl and Otten 2013). In fact, the ^{15}N-assimilation rates presented in our study are probably underestimated by 20%, considering the underestimation of $^{15}N_2$-enrichment when injecting $^{15}N_2$ as a bubble compared to injecting ^{15}N-enriched water (Mohr *et al.* 2010; Grosskopf *et al.* 2012; Klawonn *et al.* 2015). Furthermore, *Aphanizomenon* sp. releases 30–50% of its recently fixed N_2 to the surrounding water and its gross fixation rates are thus likely in the order of 100 fmol N $cell^{-1}$ day^{-1} (Ploug *et al.* 2010; Adam *et al.* 2015). Long term data on *Aphanizomenon* sp. biomass and water temperature (downloaded from the SHARK database, available at www.smhi.se), from national monitoring stations B1 (same as in this study) and BY31 (Northwestern Baltic Proper, 58° 35′ N, 18° 14′ E), show the initiation of the biomass increase at about 10°C (see Supplementary Fig. S1), confirming the potential for a particularly high cellular activity at this temperature.

High cell-specific N_2-fixation rates in relatively cold water temperatures is an advantage, both during late spring and during late summer in deeper and colder waters, where inorganic P concentrations are replete. *Aphanizomenon* sp. has the widest depth range of the filamentous cyanobacteria in the Baltic Sea, and has occasionally been observed down to 50 m depth in summer (J. Walve, personal observation). Furthermore, its tolerance for cold water might be beneficial during upwelling events. The adaptation of *Aphanizomenon* sp. to exploit nutrient pulses as part of a niche separation has been discussed by Grönlund *et al.* (1996), Kononen, Kuparinen and Mäkelä (1996), Degerholm *et al.* (2006) and De Nobel *et al.* (1997). Using SIMS, we have been able to demonstrate directly that *Aphanizomenon* sp. fixes N_2 at high rates in early summer, comparable to those measured in late summer. The high cell-specific N_2-fixation rates at 10°C indicate that *Aphanizomenon* sp. is an important player in Baltic Sea biogeochemical N-fluxes early in its growth season.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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