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Cell-specific nitrogen- and carbon-fixation of cyanobacteria in a temperate marine system (Baltic Sea)

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Summary

We analysed N_2- and carbon (C) fixation in individual cells of Baltic Sea cyanobacteria by combining stable isotope incubations with secondary ion mass spectrometry (SIMS). Specific growth rates based on N_2- and C-fixation were higher for cells of *Dolichospermum* spp. than for *Aphanizomenon* sp. and *Nodularia spumigena*. The cyanobacterial biomass, however, was dominated by *Aphanizomenon* sp., which contributed most to total N_2-fixation in surface waters of the Northern Baltic Proper. N_2-fixation by *Pseudanabaena* sp. and colonial picocyanobacteria was not detectable. N_2-fixation by *Aphanizomenon* sp., *Dolichospermum* spp. and *N. spumigena* populations summed up to total N_2-fixation, thus these genera appeared as sole diazotrophs within the Baltic Sea's euphotic zone, while their mean contribution to total C-fixation was 21%. Intriguingly, cell-specific N_2-fixation was eightfold higher at a coastal station compared to an offshore station, revealing coastal zones as habitats with substantial N_2-fixation. At the coastal station, the cell-specific C- to N_2-fixation ratio was below the cellular C:N ratio, i.e. N_2 was assimilated in excess to C-fixation, whereas the C- to N_2-fixation ratio exceeded the C:N ratio in offshore sampled diazotrophs. Our findings highlight SIMS as a powerful tool not only for qualitative but also for quantitative N_2-fixation assays in aquatic environments.

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Introduction

A substantial input of new nitrogen (N) to the marine environment is via N_2-fixation – the transformation of inert N_2 gas into bioavailable ammonium by a distinct group of microbes (Codispoti *et al.*, 2001; Galloway *et al.*, 2004; Gruber, 2004). N_2-fixing organisms are thus instrumental for element cycling, e.g. in the Baltic Sea. Extensive blooms of N_2-fixing cyanobacteria emerge during summer in the Baltic Sea, delivering several hundred kilotons of new nitrogen per year to the Baltic Proper (Larsson *et al.*, 2001). This magnitude of N-load equals the annual N-load by riverine discharge and is more than twice as much as the atmospheric N-deposition in this basin (Larsson *et al.*, 2001). N_2-fixation in the Baltic Sea is well-known to be carried out by *Aphanizomenon* sp., *Dolichospermum* spp. and *Nodularia spumigena* which are large, filamentous and colony-forming cyanobacteria with specialized cells for N_2-fixation (heterocysts); however, their genera-specific contribution to C- and N_2-fixation is largely unquantified. Non-heterocystous, filamentous cyanobacteria or picocyanobacteria may also contribute to N_2 assimilation. N_2-fixation has been suggested for *Pseudanabaena* sp. by molecular analyses (Farnelid *et al.*, 2009), whereas it has been excluded for unicellular picocyanobacteria (Ohlendieck *et al.*, 2007; Adam *et al.*, 2016) and has remained untested for colonial picocyanobacteria in the Baltic Sea.

Commonly, pelagic N_2-fixation in natural systems is quantified following stable isotope incubations by analysing the ^{15}N-incorporation into mixed phytoplankton communities (Montoya *et al.*, 1996; Mohr *et al.*, 2010). N_2-fixation is thereby revealed as a rate per plankton biomass or water volume, but the identity of N_2-fixing organisms and their

Table 1. Sampling dates, stations and depths.

Date	Station	Coordinates	Sampling depth (m)
Year 2012			
26/27-June			
17/18-July	B1(max.40 m)	N 58° 48′ 18 E 17° 37′ 52	1, 3, 5, 7
06/07-Aug			
Year 2013			
18/19-June			
17/18-July	BY31 (max. 459 m)	N 58° 35′ 00 E 18° 14′ 00	1, 4, 8, 12
14/15-Aug			

species-specific contribution to N_2-fixation remains largely unresolved. Secondary ion mass spectrometry (SIMS) is a recently introduced and promising technique to resolve this knowledge gap in aquatic microbiology. SIMS allows for the quantification of incorporated stable isotope tracers and thus metabolic activities of microbes within single cells (Musat *et al.*, 2012), e.g. N_2- and carbon (C-) fixation rates have been determined for single cells in natural, mixed plankton communities (Ploug *et al.*, 2010; Ploug *et al.*, 2011; Woebken *et al.*, 2012; Foster *et al.*, 2013; Krupke *et al.*, 2013).

Herein, we used two high resolution SIMS instruments (IMS 1280 and NanoSIMS 50L) in combination with conventional stable isotope incubations and microscopy to quantify cell-specific N_2- and C-fixation rates of Baltic Sea cyanobacteria. The NanoSIMS 50L instrument offers a high spatial resolution of >50 nm albeit a low sample throughput. Conversely, the IMS 1280 has a resolution of >1 μm but allows for a rather high sample throughput which enabled us to resolve cellular N_2- and C-fixation activities of large cyanobacteria at a rather high temporal and spatial resolution in their natural environment. Water sampling and *in situ* incubations encompassed a coastal and an offshore station, various water depths (0–12 m), day- and night-time, and consecutive summer months. Our objectives were (1) to assess variations in N_2- and C-fixation of *Aphanizomenon* sp., *Dolichospermum* spp. and *N. spumigena*, (2) to quantify their genera-specific contribution to C- and N_2-fixation at a coastal and an offshore station in the Northern Baltic Proper, and (3) to test the *in situ* N_2-fixation activity of *Pseudanabaena* sp. and colonial picocyanobacteria.

Results

Physicochemical setting

Water was sampled and incubated at a coastal station (B1) off the south-eastern coast of Sweden and at an offshore station (BY31, Landsort Deep) in the Baltic Proper during summer 2012 and 2013, respectively (Table 1, Supporting information Fig. S1). The upper mixed layer (UML) was separated from deeper water by a seasonal thermocline between 4 to 27 m (Supporting information Fig. S2). Light attenuation in water was 63–75% of air-irradiance at 1 m water depth at both stations, and 4–12% at 7 m (Stn B1) and 2–3% at 12 m (Stn BY31, Supporting information Fig. S3, Table S1). Water temperatures in the UML increased from ≤ 14.0°C in June to ≤ 18.4°C in August at both stations (Supporting information Table S1, Fig. S2). Concentrations of dissolved inorganic nitrogen (NH_4^+, NO_3^-/NO_2^-) were <0.5 μmol L^{-1} in the UML but >1 μmol L^{-1} below the UML at both stations. Phosphate concentrations reached up to 0.3 μmol L^{-1} in the UML at B1 but did not exceed 0.05 μmol L^{-1} in the UML at BY31 (Supporting information Fig. S4).

Phytoplankton composition and biomass

The depth-integrated phytoplankton C-biomass was similar at both stations ranging from 91 to 186 mg C m^{-3} (Supporting information Fig. S5). The C-biomass was dominated by Cyanophyceae (24–72%), Dinophyceae (5–48%) and Cryptophyceae (0–35%). Cyanophyceae consisted of the three orders Chroococcales (0–31% of cyanobacterial C-biomass, only colonial picocyanobacteria counted), Nostocales (61–99.8%) and Oscillatoriales (0.2–14%). The order Nostocales comprised *Aphanizomenon* sp. (11–97% of Nostocales C-biomass), *Dolichospermum* spp. (2–55%) and *N. spumigena* (0–58%); Chroococcales included *Aphanocapsa* sp., *Cyanodictyon* sp., *Aphanothece* sp., *Lemmermanniella* sp., *Merismopedia* sp. and *Woronichinia* sp., while Oscillatoriales were identified as *Pseudanabaena* sp. and *Planktolyngbya* sp.

Heterocysts were more frequent in filaments of *Dolichospermum* spp. and *N. spumigena* compared to *Aphanizomenon* sp. (Supporting information Table S2). The heterocyst frequency of each genus was significantly higher, on average twice as high, at the coastal station compared to the offshore station (t-test, $p<0.001$, Supporting information Table S2). *Aphanizomenon* sp. had the highest heterocyst frequency in June compared to late summer (*t*-test, $p<0.01$), whereas the heterocyst frequencies in filaments of *Dolichospermum* spp. and *N. spumigena* were not significantly different between the months ($p>0.05$). Depth-distributions of *Aphanizomenon* sp., *Dolichospermum* spp. and *N. spumigena* were variable between each sampling occasion and station (Supporting information Fig. S6), with no significant distribution pattern.

Total N_2- and C-fixation (by the entire phytoplankton community, EA-IRMS-based approach)

Diel total C-fixation was maximally 17.0 μmol C L^{-1} day^{-1} at 1 m water depth and decreased to a minimum of 1.7 μmol C L^{-1} day^{-1} at 7 m during June, July and August

2012 at Stn B1. At BY31 in 2013, C-fixation was significantly lower than at B1 in 2012 ($p = 0.01$, $n = 36$, paired *t*-test) and decreased from a maximum of 5.3 μmol C L^{-1} day^{-1} at 1 m to a minimum of 0.7 μmol C L^{-1} day^{-1} at 12 m water depth (Supporting information Fig. S7). Areal C-fixation integrated over 0–12 m was 55 ± 23 mmol C m^{-2} day^{-1} ($n = 3$, June, July and August 2012) at Stn B1 and 34 ± 4 mmol C m^{-2} day^{-1} ($n = 3$, June, July and August 2013) at BY31. C-fixation from 9 PM to 9 AM accounted for $21 \pm 6\%$ ($n = 24$) of the diel C-fixation (9 AM–9 AM).

Diel N_2-fixation was maximally 1.45 μmol N L^{-1} day^{-1} (1 m) and minimally 0.05 μmol N L^{-1} day^{-1} (12 m) at Stn B1, whereas it was significantly lower at BY31 ($p < 0.001$, $n = 36$, paired *t*-test), ranging from 0.05 μmol N L^{-1} day^{-1} (1 m) to 0.01 μmol N L^{-1} day^{-1} (12 m). Areal N_2-fixation integrated over 0–12 m was 3.6 ± 2.6 mmol N m^{-2} day^{-1} at Stn B1 ($n = 3$, June, July, August 2012) and 0.4 ± 0.1 mmol N m^{-2} day^{-1} at BY31 ($n = 3$, June, July, August 2013). N_2-fixation from 9 PM to 9 AM contributed to $36 \pm 12\%$ ($n = 24$) of the diel N_2-fixation (9 AM–9 AM).

Specific N_2- and C-fixation of Aphanizomenon *sp.,* Dolichospermum *spp. and* N. spumigena

Mean cell volumes of *Aphanizomenon* sp., *Dolichospermum* spp. and *N. spumigena* were 122, 78 and 297 μm^3, respectively. The calculated cellular C- and N-contents were 2.13 pmol C and 0.34 pmol N per *Aphanizomenon* sp. cell, 1.36 pmol C and 0.22 pmol N per *Dolichospermum* spp. cell, and 5.20 pmol C and 0.82 pmol N per *N. spumigena* cell (see also Supporting information Table S3). Mean N-specific N_2-fixation (i.e. the N-specific growth based on N_2-fixation) of *Aphanizomenon* sp., *Dolichospermum* spp. and *N. spumigena* cells ranged from 0.002 to 0.097 h^{-1} in June–August 2012 at Stn B1 and from 0.0002 to 0.013 h^{-1} in June–August 2013 at BY31 (Fig. 1, for details see Supporting information Table S4a–c). These data were equivalent to N-based doubling times of 0.3–18.4 days and 2.2–125.7 days at B1 and BY31, respectively. N-specific N_2-fixation and C-specific C-fixation were highest for *Dolichospermum* spp. Diel N-specific N_2-fixation of *Dolichospermum* spp. was on average 1.4- and 1.2-times as much as for *Aphanizomenon* sp. and *N. spumigena*, respectively, and N-specific N_2-fixation of *N. spumigena* was 1.2-times that of *Aphanizomenon* spp. (Table 2). Mean C-specific C-fixation (i.e. the C-specific growth based on C-fixation) for cells of *Aphanizomenon* sp., *Dolichospermum* spp. and *N. spumigena* ranged from 0.001 to 0.043 h^{-1} at Stn B1 and from 0.002 to 0.026 h^{-1} at BY31 (Fig. 1, Supporting information Table S4a–c). C-specific doubling times were 0.7–27.5 days and 1.1–18.4 days at Stn B1 and BY31, respectively. C-specific C-fixation of *Dolichospermum* spp. was 1.4-times that of *Aphanizomenon* sp. and *N. spumigena*, which both had similar fixation rates (Table 2).

Diel N-specific N_2-fixation at the coastal station was approx. eight times as high as that at the offshore station. By comparison, C-specific C-fixation at both stations differed by a factor of 1.7 (Table 2). N_2- and C-fixation from 9 PM to 9 AM accounted for 36% and 28% of the diel N_2- and C-fixation, respectively (separately for *Aphanizomenon* sp., *Dolichospermum* spp. and *N. spumigena*). N_2- and C-fixation in *Aphanizomenon* sp. and *Dolichospermum* spp. were significantly greater in June compared to July and August ($p < 0.05$, Tukey's HSD test) although temperatures in June were 3–6°C less than in July and August. For *N. spumigena* rates tended to be highest in July and August (Fig. 1, see also significant ranking of mean fixation rates in Supporting information Table S4a–c).

Cell-specific fixation of *Aphanizomenon* sp., *Dolichospermum* spp. and *N. spumigena* ranged from 0.3 to 32.7 fmol N $cell^{-1}$ h^{-1} and from 1.4 to 108.5 fmol C $cell^{-1}$ h^{-1} at Stn B1, and from 0.1 to 5.3 fmol N $cell^{-1}$ h^{-1} and 2.1 to 97.7 fmol C $cell^{-1}$ h^{-1} at BY31 (Fig. 1, Supporting information Table S4a–c). Consistent with the higher cellular C- and N-content of *N. spumigena* compared to *Aphanizomenon* sp. and *Dolichospermum* spp., cell-specific C- and N_2-fixation for *N. spumigena* were three times (3.1 ± 1.4 $n = 80$, all incubations) as much as for *Dolichospermum* spp. and *Aphanizomenon* sp. while cell-specific C- and N_2-fixation were similar in *Dolichospermum* sp. and *Aphanizomenon* (relative difference of 0.9 ± 0.4, $n = 48$). The ratio of cell-specific C- to N_2-fixation (mol:mol) by the three diazotrophs over a diel cycle was significantly lower at Stn B1 (3.2 ± 0.9, mean ± s.d., $n = 32$) than at BY31 (13.3 ± 4.8, $n = 36$, $p < 0.001$).

Specific N_2- and C-fixation of Pseudanabaena *sp. and colonial picocyanobacteria*

NanoSIMS 50L and IMS1280 yielded similar N_2- and C-incorporation rates for cells of *Pseudanabaena* sp. and colonial picocyanobacteria (*Aphanocapsa* sp., *Cyanodictyon* sp. *Aphanothece paralleliformis*, data will be presented elsewhere). Cells were not or only slightly enriched above the natural ^{15}N-background after $^{15\text{-}15}N_2$ incubations, while mean N-specific N-assimilation never exceeded 0.004 h^{-1} (see Fig. 2 for an example). Rates of C-specific C-fixation in *Pseudanabaena* sp. and colonial picocyanobacteria ranged from 0.0002 to 0.035 h^{-1}, similar to those of active N_2-fixing cyanobacteria.

N_2- and C-fixation by the diazotrophic community (SIMS-based approach)

The contributions to C- and N_2-fixation by each diazotrophic genus varied between each month as a function of their relative biomass at both stations (Supporting

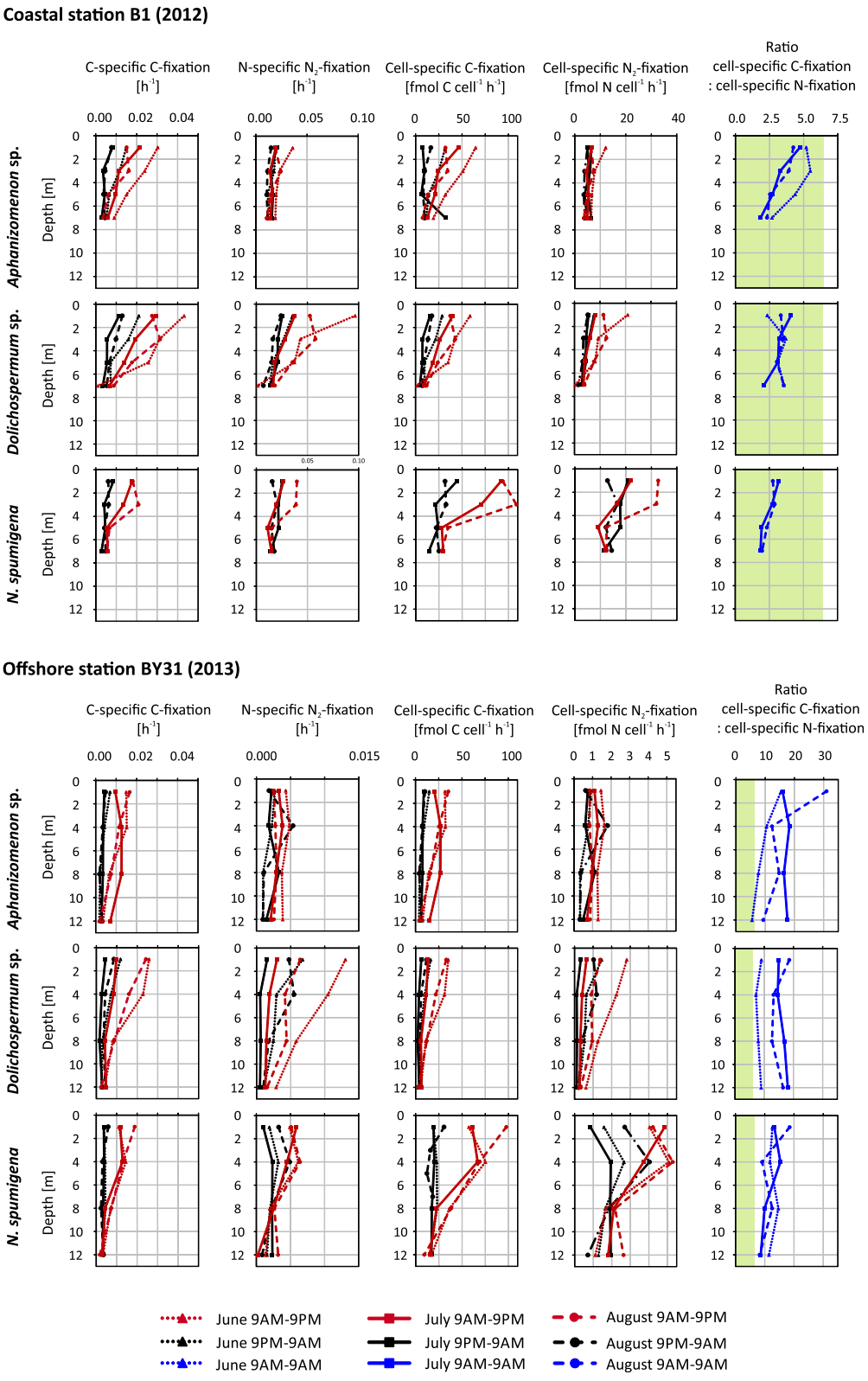

Fig. 1. N-specific N_2-fixation, C-specific C-fixation, and cell-specific C- and N_2-fixation in *Aphanizomenon* sp., *Dolichospermum* spp. and *N. spumigena* analysed by secondary ion mass spectrometry (IMS 1280). Please note, *x*-axes for N_2-fixation and the ratio of N_2- to C-fixation are different for the coastal and offshore station. Green areas highlight ratios of cell-specific C- to N_2-fixation below a cellular C:N ratio of 6.3 (mol:mol) which is typical for Baltic Sea diazotrophs. Data and more details are listed in Supporting Information Table S4a–c. N – nitrogen, C – carbon

Table 2. Relative differences of N-specific N_2-fixation and C-specific C-fixation rates among and between N_2-fixing genera incubated under different environmental conditions. The table reads as follows: e.g. 1st row 'N-specific N_2-fixation of *Dolichospermum* spp. was 1.4-times as high as N-specific N_2-fixation of *Aphanizomenon* sp.' The factor for the relative difference was calculated for diel N-specific N_2-fixation and C-specific C-fixation rates (9 AM–9 AM), except for the pairing 9 AM–9 PM vs 9 PM–9 AM. Data are given as mean ± s.d.

Lower fixation rates		Relative difference in fixation rates		Higher fixation rates
Genera/species (all incubations)				
Aphanizomenon sp.	N_2-fix	x 1.4 ± 0.7	$n = 24$	*Dolichospermum* spp.
	C-fix	x 1.4 ± 0.4	$n = 24$	
N. spumigena	N_2-fix	x 1.2 ± 0.6	$n = 20$	*Dolichospermum* spp.
	C-fix	x 1.4 ± 0.5	$n = 20$	
Aphanizomenon sp.	N_2-fix	x 1.2 ± 0.3	$n = 20$	*N. spumigena*
	C-fix	x 1.0 ± 0.2	$n = 20$	
Station (all three genera)				
Offshore (BY31)	N_2-fix	x 8.1 ± 4.0	$n = 32$	Coastal (B1)
	C-fix	x 1.7 ± 0.6	$n = 32$	
Time of day (all three genera)				
9PM–9AM	N_2-fix	x 1.8 ± 1.0	$n = 51$	9AM–9PM
	C-fix	x 2.6 ± 1.3	$n = 51$	
Water depth (all three genera)				
7 m (B1) or 12 m (BY31)	N_2-fix	x 2.7 ± 1.9	$n = 17$	1 m (B1 and BY31)
(i.e. 4–12% or 2–3% air-irradiance)	C-fix	x 3.7 ± 1.6	$n = 15$	(i.e. 63–75% air-irradiance)

information Fig. S8). The contribution to depth-integrated N_2- and C-fixation by the diazotrophic community was 50% and 49% by *Aphanizomenon* sp., 36% and 40% by *Dolichospermum* spp. and 14% and 11% by *N. spumigena*, respectively, during our incubations at Stn B1 in 2012 (total during June, July and August, see Supporting information Fig. S8). At Stn BY31, the contribution to N_2- and C-fixation by diazotrophs was 64% and 68% by *Aphanizomenon* sp., 8% and 8% by *Dolichospermum* spp. and 28% and 24% by *N. spumigena*, respectively.

We also used decadal-long monitoring data of the cyanobacterial biomass to extrapolate the putative genera-specific contribution to C- and N_2-fixation during 2000–2014. Nostocales biomass in June, July and August 2000–2014 were on average 37.5 mg C m^{-3} (B1) and 33.2 mg C m^{-3} (BY31), similar to 24–89 mg C m^{-3} during 2012/13. Nostocales comprised *Aphanizomenon* sp. to 70–89%, *Dolichospermum* spp. to 1–10% and *N. spumigena* to 10–21%. The contribution to total N_2-fixation was 79 and 68% by *Aphanizomenon*. sp., 15% and 23% by *N. spumigena*, and 6 and 9% by *Dolichospermum* spp. at the coastal and offshore station, respectively, during June–August (Fig. 3). C-fixation by N_2-fixing cyanobacteria was estimated to account for 21% of the total phytoplankton C-fixation (see next paragraph). Of the 21%, *Aphanizomenon* sp. accounted for 17% and 15%, *N. spumigena* for 3% and 4%, and *Dolichospermum* spp. for 1% and 2% at the coastal and offshore station, respectively.

We used two approaches to measure pelagic N_2- and C-fixation – the EA-IRMS- and SIMS-based approach. The EA-IRMS-based approach analysed the $^{15\text{-}15}N_2$ and ^{13}C-DIC incorporation into the total particulate organic matter on GF/F filters (Text S1 Eq. (1) and (2)), thus including all planktic organisms (> 0.7 μm). The SIMS-based approach quantified the $^{15\text{-}15}N_2$ and ^{13}C-DIC incorporation into single cells, and cell-specific fixation rates were multiplied by cell

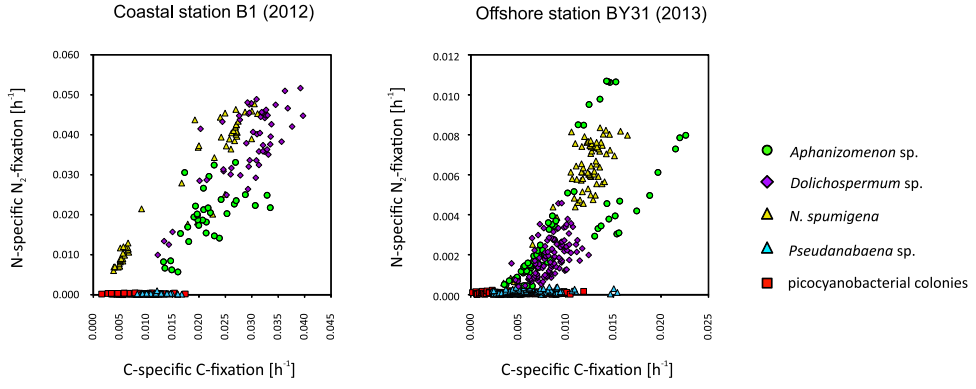

Fig. 2. N-specific N_2-fixation and C-specific C-fixation in cells of *Pseudanabaena* sp. and colonial picocyanobacteria in comparison to cells of *Aphanizomenon* sp., *Dolichospermum* spp. and *N. spumigena*, exemplary shown for incubations at 1 m in July 2012/13.

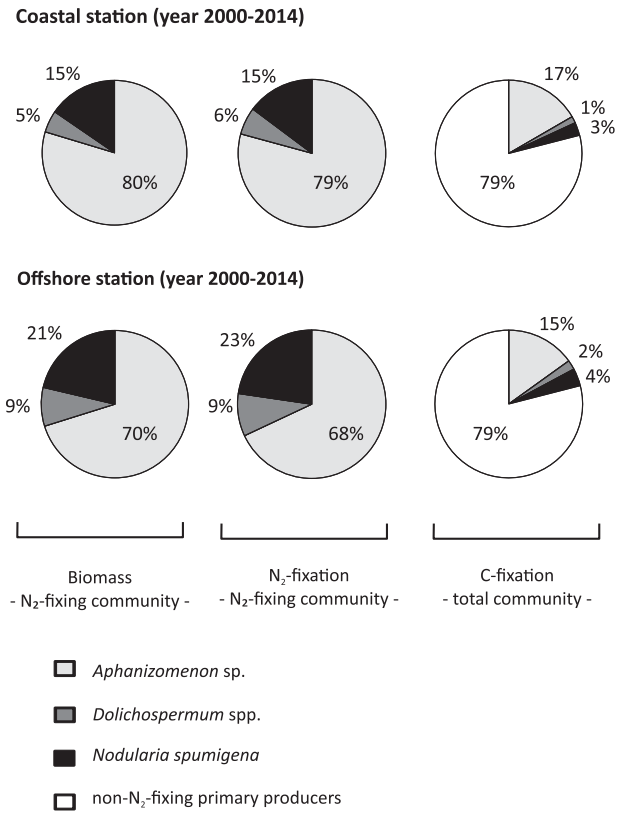

Fig. 3. Diazotrophic biomass and estimated contributions to areal N_2- and C-fixation by *Aphanizomenon* sp., *Dolichospermum* spp. and *N. spumigena* during June–August in 2000–2014. Genera-specific contributions were extrapolated from the diazotrophic biomass during June–August in 2000–2014 (Swedish Monitoring Program) and cell-specific fixation rates presented herein. Mean Nostocales biomass (depth-integrated for 0–20 m) in June, July and August 2000–2014 were on average 37.5 mg C m^{-3} (B1) and 33.2 mg C m^{-3} (BY31).

abundances, thus including only N_2-fixing cyanobacteria. The slope of the correlation line of total N_2-fixation rates measured with both approaches was ~1 at both stations (Fig. 4). The slope of the correlation line of C-fixation rates was <1 since C-fixation was processed by N_2-fixing cyanobacteria but also by other autotrophic organisms. Depth-integrated C-fixation of *Aphanizomenon* sp., *Dolichospermum* spp. and *N. spumigena* accounted for 21 ± 12% ($n = 6$, June, July and August 2012/13) of the total C-fixation at both stations.

Discussion

Detecting and quantifying metabolic activities of aquatic microbes and their contribution to ecosystems' element transformation processes are keystones in understanding global biogeochemical cycles. Advantageously, SIMS measurements combined with stable isotope tracers can reveal metabolic activities of microorganisms at a single-cell level in uncultured, mixed plankton communities (Musat *et al.*, 2012). The SIMS applicability on field-sampled plankton communities is of benefit given the great diversity of aquatic microbes whose vast majority cannot be isolated and grown in culture to date (Joint *et al.*, 2010).

Interestingly, C- and N_2-fixation rates of *Aphanizomenon* sp. and *Dolichospermum* spp. were highest in June when the temperature was still low (≤ 14°C); presumably due to a sufficient phosphorus (P) availability and at least for *Aphanizomenon* sp. an elevated heterocyst frequency in June compared to later summer (see also Walve and Larsson, 2007; Svedén *et al.*, 2015). N-specific N_2-fixation and C-specific C-fixation (i.e. the N- and C-specific growth based on N_2- and C-fixation, respectively) were highest at shallower water depths and during day-time, i.e. at high photosynthetic irradiance (Table 2, Fig. 1). Yet, N_2-fixation by *Aphanizomenon* sp., *Dolichospermum* spp. and *N. spumigena* was only slightly influenced by light and temperature (see also Moisander *et al.*, 2007; Svedén *et al.*, 2015) as compared to an eightfold difference in N-specific N_2-fixation between the coastal and offshore station (Table 2).

The eightfold difference in cell-specific N_2-fixation rates between stations resulted in considerably higher areal N_2-fixation at the coastal station, despite similar biomasses of N_2-fixing cyanobacteria at both stations. The substantial

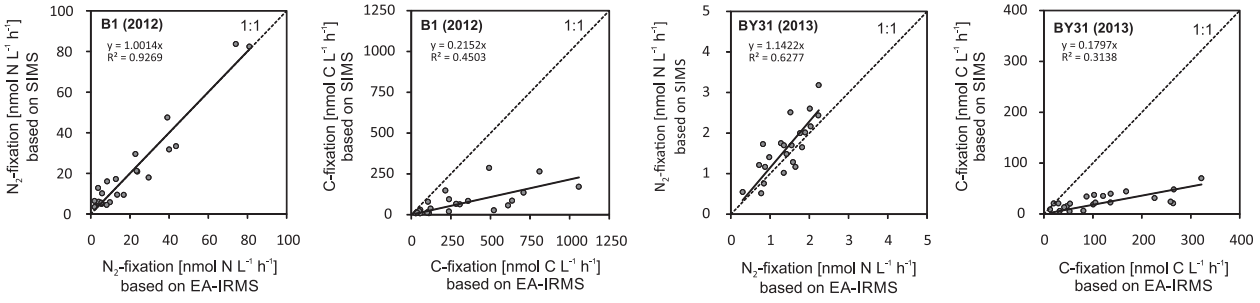

Fig. 4. Correlation of N_2- and C-fixation rates quantified by EA-IRMS and SIMS-based analyses (see text for details). The dashed line gives the 1:1 ratio of fixation rates measured by both approaches. EA-IRMS-based data accounted for all planktic organisms (> 0.7 μm on GF/F filter), whereas the SIMS-based data included only *Aphanizomenon* sp., *Dolichospermum* spp. and *N. spumigena*.

difference in N_2-fixation between stations co-occurred with higher PO_4^{3-} concentrations in the upper mixed layer at the coastal station (up to 0.3 μmol L^{-1}) compared to the offshore station (below 0.05 μmol L^{-1}, Supporting information Fig. S4). Thus, P-limitation presumably lead to a reduced heterocyst frequency and N_2-fixation in offshore diazotrophs compared to coastal ones (Supporting information Table S2, see also Moisander *et al.*, 2003; Moisander *et al.*, 2007). Nevertheless, N_2-fixation may not be consistently lower in offshore areas (e.g. see Degerholm *et al.*, 2008) since turbulent water mixing and upwelling events can affect Baltic Sea offshore regions (Lehmann and Myrberg, 2008), thus leading to occasional promotion of cyanobacterial N_2-fixation in the Central Baltic Proper (Wasmund *et al.*, 2012).

Cell-specific N_2- and C-fixation for herein sampled coastal *Aphanizomenon* sp. and *N. spumigena* were in agreement with rates reported from the same station during previous years (Ploug *et al.*, 2010; Ploug *et al.*, 2011; Adam *et al.*, 2016) and during 2013 (Klawonn *et al.*, unpublished). Coastal areas may thus be more significant habitats for Baltic Sea N_2-fixation than commonly anticipated; and N_2-fixation in temperate coastal and estuarine areas should be taken into consideration more thoroughly for N budget calculations (see also Bentzon-Tilia *et al.*, 2015). We speculate that elevated P-availability in coastal areas, due to more common upwelling (Lehmann and Myrberg, 2008) and diffusive P-transport from sediments, may favour consistently high cell-specific N_2-fixation. However, the reasoning behind substantially higher coastal N_2-fixation compared to offshore N_2-fixation was not sufficiently documented in this study and should be targeted in future studies.

The ratio of cell-specific C- to N_2-fixation can indicate if the amount of instantaneously fixed C and N was in balance with the cellular C:N ratio of *Aphanizomenon* sp., *Dolichospermum* spp. and *N. spumigena*. The mean ratio of C-specific C-fixation to N-specific N_2-fixation over a diel cycle was above the molar C:N ratio in populations of cyanobacterial cells at the offshore station in 2013 and clearly below this ratio at the coastal station in 2012 (Fig. 1, Supporting information Table S4a–c). We therefore conjecture that N_2-fixing cyanobacteria at the offshore station did not cover their N-demand for growth by only N_2-fixation and potentially relied on other N-sources and/or stored excess C in their cells, at least on a short-term. In contrast, N_2-fixing cyanobacteria at the coastal station exceeded their cellular N-demand by N_2-fixation (relative to their C-growth), suggesting that fixed N may later be released from N_2-fixing cyanobacteria (Ploug *et al.*, 2010; Ploug *et al.*, 2011) and potentially transferred to other members of the plankton community as shown by Adam *et al.* (2016). Adam *et al.* (2016) detected a substantial N-transfer already after 12 h when using a higher $^{15\text{-}15}N_2$-labelling percentage as herein. In contrast, the release and transfer of newly fixed N was hardly indicated during our 12 h incubations, as the fraction of ^{15}N-labelled ammonium was likely diluted in the ambient water due to high rates of ammonium regeneration (Klawonn *et al.*, unpublished). Moreover, a 1:1 fit of N_2-fixation rates measured with the single-cell approach (SIMS-based, only diazotrophs) and the total-community approach (EA-IRMS-based; Fig. 4) indicated that newly fixed N largely remained in the diazotrophic cells, and non-diazotrophic cyanobacteria were only marginally enriched in ^{15}N, even at the coastal station (Fig. 2).

No N_2-fixation was detected in *Pseudanabaena* sp. and colonial picocyanobacteria (*Aphanocapsa* sp., *Cyanodictyon* sp. and *Aphanothece paralleliformis*; Fig. 2). Interestingly, some Baltic Sea strains of *Pseudanabaena* sp. have been reported to possess *nifH* genes (Stal *et al.*, 2003; Acinas *et al.*, 2009; Farnelid *et al.*, 2009) which encode for a subunit of nitrogenase, the enzyme complex that mediates N_2-fixation. *NifH* expression was even shown for *Pseudanabaena* sp. which were sampled in parallel to our incubations at BY31 in 2013 (Muenster Happel, 2014). This contradiction of *nifH* expression and actual N_2 assimilation in *Pseudanabaena* sp. asks for combining molecular analyses with rate measurements in future studies. At the coastal station, low ^{15}N-enrichments in *Pseudanabaena* sp. and colonial picocyanobacteria after $^{15}N_2$ incubation can be mistaken for low N_2-fixation rates up to 0.004 h^{-1} but most likely indicate an uptake of ^{15}N-compounds that were released by cyanobacteria fixing N in excess of their N-demand (see above). The reason why solely heterocystous cyanobacteria but no non-heterocystous cyanobacteria appear to be able to assimilate N_2 in the temperate, brackish Baltic Sea is not fully understood (but see Staal *et al.*, 2003).

To put N_2-fixation activity of Baltic Sea cyanobacteria into a perspective of global N_2-fixing microorganisms, we compiled literature data on N_2- and C-fixation of individual diazotrophs of different species/genera. Cell-specific N_2- and C-fixation of heterocystous *Aphanizomenon* sp., *Dolichospermum* spp. and *N. spumigena* in temperate, brackish waters are similar to those of non-heterocystous *Trichodesmium* spp. found in subtropical and tropical marine systems (Fig. 5, Supporting information Table S5). The herein presented rates of N-specific N_2-fixation and C-specific C-fixation of coastal *Dolichospermum* spp. are the highest (up to 0.097 h^{-1}) of previous reported rates for filamentous cyanobacteria (Fig. 5, Supporting information Table S4). Cell-specific N_2- and C-fixation were mostly higher for large cells than for small cells due to their higher C- and N-content. However, N-specific N_2-fixation and C-specific C-fixation rates and thus the N- and C-based growth of symbiotic cyanobacteria are in the same range as those of free-living filamentous cyanobacteria, whereas the N-growth appeared to be even higher for small unicellular cyanobacteria (*Crocosphaera* spp., *Cyanothece* spp.

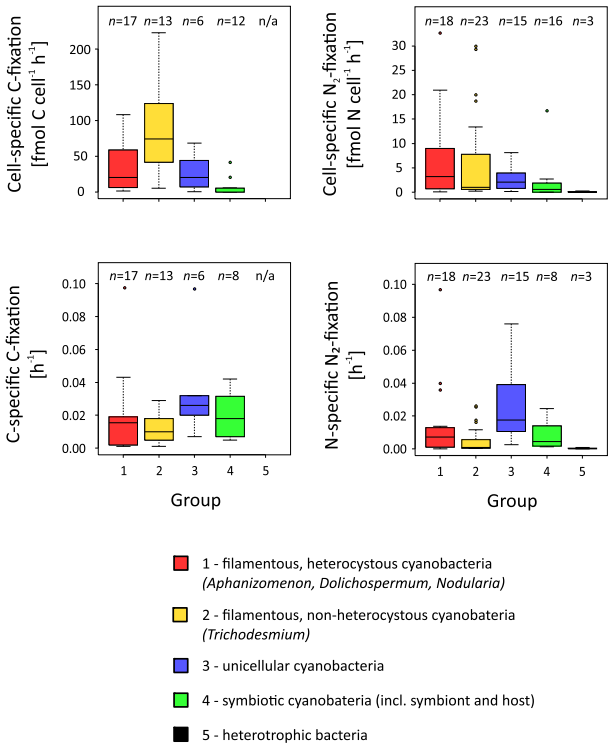

Fig. 5. Compiled literature data on N_2- and C-fixation of diazotrophic organisms. Data on *Trichodesmium* resulted mainly from monocultures and field-sampled populations (acetylene reduction assay and cell counts assuming that *Trichodesmium* is the sole diazotroph in the plankton community). Data on Baltic Sea cyanobacteria and tropical/sub-tropical symbiotic cyanobacteria derive mainly from field-sampled populations analysed by SIMS. C- and N_2-fixation for unicellular cyanobacteria and heterotrophic bacteria (only N_2-fixation) have been measured in monocultures. Data are listed in Table S5 and compiled from Bentzon-Tilia *et al.* (2015), Berthelot *et al.* (2015), Capone (2001), Carpenter and Price (1977), Carpenter and Romans (1991), Carpenter (1993), Czerny *et al.* (2009), Dron *et al.* (2012), Foster *et al.* (2011), Garcia *et al.* (2013), Goebel *et al.* (2008), Kitajima *et al.* (2009), Kromkamp *et al.* (1997), Krotzky and Werner (1987), Krupke *et al.* (2015), Mague *et al.* (1977), Martínez-Pérez et al. (2016) Masuda *et al.* (2013), McCarthy and Carpenter (1979), Mulholland and Bernhardt (2005), Orcutt *et al.* (2001), Ploug *et al.* (2010), Ploug *et al.* (2011), Popa *et al.* (2007), Reddy *et al.* (1993), Rodier and Le Borgne (2008), Svedén *et al.* (2015) and this study. Boxes show the median, 25th and 75th percentiles, error bars represent the 10th and 90th percentiles and circles the outliers. n/a not analysed.

and UCYN-A). Data on heterotrophic N_2-fixation are rare but suggest slow N-based growth due to N_2-fixation. Heterotrophic bacteria, however, may still add substantially to marine N_2-fixation due to their high abundances and wide distribution (Riemann *et al.*, 2010; Halm *et al.*, 2012; Bombar *et al.*, in press).

We validated the applicability of secondary ion mass spectrometry (SIMS) to qualitatively and quantitatively study the contribution of natural cyanobacterial communities to total pelagic N_2-fixation. A 1:1 fit of N_2-fixation rates quantified with the single-cell approach (SIMS-based) and the conventional ^{15}N-tracer assay (EA-IRMS-based) indicated that N_2-fixation was fully accounted for by *Aphanizomenon* sp., *Dolichospermum* spp. and *N. spumigena* (Fig. 4). In contrast, these cyanobacteria contributed 21% to total C-fixation. *Dolichospermum* spp. showed the highest N-specific N_2- and C-specific C-fixation, and thus their N- and C-based growth was higher (on average 1.4-times) than that of *Aphanizomenon* sp. and *N spumigena* (Table 2). However, the respective contributions to total N_2- and C-fixation were largely determined by the genera's biomass (Fig. 3). The N_2-fixing C-biomass was dominated by *Aphanizomenon* sp. during 2000–2014, especially in June and July (including our sampling years) at both stations in the Northern Baltic Proper. Hence, *Aphanizomenon* sp., which commonly receives little public perception, accounted for the major part (more than two-third) of N_2-fixation in this area (Fig. 3). On the contrary, the conspicuous *N. spumigena*, which is widely recognized due to its surface scums and toxicity (Karjalainen *et al.*, 2007), did contribute to maximally one-fifth of areal N_2-fixation. Similar patterns may also apply to the Bothnian Sea, Gulf of Finland and Gulf of Riga where the cyanobacterial biomass is dominated by *Aphanizomenon* sp., whereas in southern areas of the Baltic Sea such as the Eastern Gotland Sea, Bornholm Basin and Arkona Sea *N. spumigena* can dominate at times (Wasmund *et al.*, 2014). Cell-specific C- and N_2-fixation, however, are expected to vary in different Baltic Sea basins. Thus, similar diazotrophic biomasses found in different regions do not necessarily equate to similar volumetric N_2-fixation rates as presented herein for the offshore and coastal station.

Our study demonstrates that the SIMS-based approach can be powerful for not only qualitative and mechanistic studies (Finzi-Hart *et al.*, 2009; Woebken *et al.*, 2012; Mohr *et al.*, 2013) but also for quantitative estimates of pelagic N_2-fixation. Thereby, the main contributors in C- and N_2-fixation can be revealed, given that the number of cells analysed by (nano)SIMS are representative mean values (Svedén *et al.*, 2015). The spectrum of aquatic N_2-fixing organisms has been greatly expanded during the last decade (Zehr, 2011), but the contribution of single diazotrophic species to global N_2-fixation is largely unquantified. SIMS-based N_2-fixation analyses e.g. in the oligotrophic ocean, are therefore predicted to not only identify but also to quantify the importance of single diazotrophic groups (up to species level) for global N_2-fixation as recently shown by Martínez-Pérez et al (2016).

Experimental procedures

Study area and water sampling

Water samples were taken at a coastal station (B1) off the south-eastern coast of Sweden once in June, July and August 2012, and at an offshore station (BY31) at the Landsort Deep in the Baltic Proper once in June, July and August 2013

(Supporting information Fig. S1; Table 1). At Stn B1 water was sampled from 1, 3, 5 and 7 m water depth and transported to the Marine Research Station Askö Laboratory where the water was prepared for stable isotope incubations. Due to the lower light attenuation in water at Stn BY31, we chose deeper sampling and incubation depths at BY31 to ensure similar light intensities during incubations at both stations. Accordingly, water was sampled at 1, 4, 8 and 12 m water depth at BY31 during a cruise on M/F Fyrbyggaren and incubations were prepared on board the research vessel.

Physicochemical setting

Depth profiles of salinity, temperature, dissolved oxygen and photosynthetically active radiation (PAR) were recorded with a CTD-probe equipped with an irradiance and oxygen sensor (Sea-Bird Electronics, WA, USA). PAR profiles were used to calculate the light attenuation coefficient in water. Data on photosynthetic irradiance in air were extracted from the database of the Swedish Meteorological and Hydrological Institute (SMHI, http://strang.smhi.se/extraction). Photosynthetic irradiance at the different water depths were calculated by using the hourly-integrated irradiance (SHMI) and according light extinction coefficients.

Nutrient concentrations of phosphate (PO_4^{3-}), ammonium (NH_4^+) and nitrate + nitrite ($NO_3^- + NO_2^-$), and the ratios of both ($NH_4^+ + NO_3^- + NO_2^-$) to PO_4^{3-} and total N to total P were extracted from the SHARK database at the Swedish Meteorological and Hydrological Institute (Swedish National Marine Monitoring Program, analyses by the Department of Ecology, Environment and Plant Sciences, Stockholm University). Sampling by the monitoring program was conducted at the same day as our sampling at BY31 and maximum one week before or after our sampling at B1.

Water incubations for N_2- and C-fixation

Four Duran® glass bottles (1 L) were filled to the brim with water from each sampling depth. Thereafter, ^{15}N-labelled N_2 gas (364584-1 L, Aldrich, 98 atom% ^{15}N) and ^{13}C-labelled sodium bicarbonate (372382 Aldrich, 98 atom%) were added to three out of the four bottles. The ^{15}N-labelled N_2 gas was added as pre-dissolved $^{15\text{-}15}N_2$ following Klawonn *et al*. (2015, see also Text S1). The ^{15}N-atom% excess in the final incubation volume was 1–1.5% in 2012 and 10–11% in 2013 as assessed by membrane-inlet mass spectrometry (MIMS; GAM200, IPI) at the MPI in Bremen or by using gas chromatography isotope-ratio mass spectrometry (GC-IRMS; Thermo Delta V, Thermo Fisher Scientific Inc.) at the Stable Isotope Laboratory, Department of Geological Sciences, Stockholm University. The ^{13}C-atom% excess was 5% in both years as analysed by GC-IRMS (UC Davis, California, US). After the addition of pre-dissolved $^{15\text{-}15}$N- and ^{13}C-label, the bottles were closed headspace-free, gently inverted 20-times by hand and incubated at *in situ* temperature and light by fixing the bottles horizontally in floating arrays at different depths (B1: 1, 3, 5, 7 m; BY31: 1, 4, 8, 12 m). Water without $^{15\text{-}15}N_2$- and ^{13}C-label was sampled at time zero and at the incubations' end to serve as non-labelled controls.

Incubations were done at 9 AM–9 PM and 9 PM–9 AM. The following sub-samples were taken from each bottle at the incubations' end. (1) *^{15}N- and ^{13}C-labelling percentage* – Triplicate sub-samples were filled headspace-free into 12 mL Exetainer® vials and preserved with 100 μL saturated $ZnCl_2$ solution. (2) *Specific N_2- and C-fixation* – 50 mL sub-samples were preserved with 2% paraformaldehyde for 24 h at 4°C and thereafter filtered onto polycarbonate membrane filters (GTTP, pore-size 0.22 μm; diameter 25 mm; Merck Millipore Ltd., IRL) and washed with 0.2 μm-filtered seawater. The filters were stored at room temperature in the dark until (nano)SIMS analyses. (3) *Phytoplankton community* – A 50–100 mL subsample was preserved with Lugol's solution (L6146 Sigma) and stored at 4°C in the dark until microscopy. (4) *N_2- and C-fixation of total planktic community* – 400–700 mL were filtered onto pre-combusted GF/F filters (25 mm, Whatman) and frozen at −80°C to quantify the amount of ^{15}N and ^{13}C incorporated into biomass. The filters were later freeze-dried, fumed over concentrated HCl, pelletized into tin cups and analysed by an elemental analyser interfaced to a continuous flow isotope-ratio mass spectrometer (EA-IRMS; Stable Isotope Facilities, U. California Davis, USA).

N_2- and C-fixation rates were calculated after Montoya *et al*. (1996) and Großkopf *et al*. (2012), see also Text S1). Diel C- and N_2-fixation (9 AM–9 AM) were calculated as cumulative fixation during day and night. Some $^{15}N_2$ gas bottles from Sigma–Aldrich have been shown to be contaminated with $^{15}NO_3^-$, $^{15}NO_2^-$ and $^{15}NH_4^+$ (Dabundo *et al*., 2014). False N_2-fixation rates due to $^{15}NO_3^-$, $^{15}NO_2^-$ or $^{15}NH_4^+$ assimilation could be excluded for our incubations since we could not detect any suspicious ^{15}N-enrichment in non-diazotrophic cyanobacteria after SIMS analyses (see Results). Moreover, the gas was tested negative for contaminations with ammonium, and we used the same gas bottles for water sampled and incubated at 30 m, resulting in insignificant N_2-fixation rates.

Phytoplankton composition and biomass

Lugol-preserved water samples were settled in 25 mL Utermöhl sedimentation chambers (Hydrobios, Germany). Phytoplankton species and genera were counted and allocated to size classes under an inverted NIKON Eclipse Ti-U light microscope at 150–400x magnification. Only cells >2 μm and colony forming picocyanobacteria were counted and identified. The ciliate *Mesodinium rubrum* was included in the phytoplankton biomass due to its autotrophy. Biovolume and carbon biomass (C-biomass) were calculated following the HELCOM guidelines (2014) and Olenina *et al*. (2006). Dimensions of single cells, filaments and the heterocyst frequencies of *Aphanizomenon* sp., *Dolichospermum* spp. and *N. spumigena* were measured at 600x magnification. Depth-specific C-biomass was linearly interpolated between sampling depths and integrated over 0–7 m depth for Stn B1, and over both 0–7 m and 0–12 m for BY31.

Cell-specific N_2- and C-fixation (large-geometry SIMS and NanoSIMS)

Large-geometry SIMS. The ^{13}C-DIC and $^{15\text{-}15}N_2$ incorporation into single cells of *Aphanizomenon* sp., *Dolichospermum*

spp., *N. spumigena*, *Pseudanabaena* sp. and colonial picocyanobacteria was analysed using secondary ion mass spectrometry (SIMS) on an IMS 1280 (Cameca, Gennevilliers, France) at the Natural History Museum in Stockholm, Sweden. Paraformaldehyde-preserved GTTP filters were cut into 4 × 4 mm pieces, glued onto C-covered glass slides and coated with a 5 nm-thick gold layer. Areas of interest (90 × 90 μm) on the filters were pre-sputtered with a primary caesium-ion (Cs^+) beam (3 nA) for 100 sec and then imaged using a 40–60 pA Cs^+ beam with a spatial resolution of ca 1 μm for 100 cycles. Secondary ion images (256 × 256 pixel) were recorded for $^{13}C^{14}N$, $^{12}C^{14}N$ and $^{12}C^{15}N$ using a peak-switching routine at a mass resolution of 6000 (M/ΔM) and an ion counting electron multiplier. Image and data processing were done using the CAMECA WinImage2 software. Regions of interest (ROIs) were defined corresponding to individual cells from which the cell-specific isotope ratios $^{15}N/^{14}N$ and $^{13}C/^{12}C$ were calculated. Cell analyses were aimed to continue until the mean value stabilized and the standard error was ≤ 5% which was possible in > 90% of our analyses. We analysed more than 10,000 cells with $n = 16$–139 per mean value for cells of single incubations.

NanoSIMS. Cells of *Pseudanabaena* sp. and colonial picocyanobacteria (*Aphanocapsa* sp., *Cyanodictyon* sp. *Aphanothece paralleliformis*) are smaller compared to cells of *Aphanizomenon* sp., *Dolichospermum* spp. and *N. spumigena*, and were therefore additionally analysed on a NanoSIMS 50L instrument (Cameca, Gennevilliers, France) at the MPI, Bremen. Cells on GTTP filters were DAPI-stained to distinguish between phototrophic cyanobacteria (autofluorescent and DAPI-stained) and heterotrophic bacteria (= non-autofluorescent but DAPI-stained) which colonized the phototrophic cyanobacterial cells. GTTP filters were coated with a nm-thin gold-palladium layer and cut into 5 mm-circles. Cells to be analysed with NanoSIMS were pre-marked using a laser microdissection microscope (LMD, Leica, Wetzlar, Germany) and traced back after loading the filter into the NanoSIMS 50L. The areas of interest (15 × 15 μm to 25 × 25 μm) were pre-sputtered with a primary Cs^+ ion beam (300 pA). During NanoSIMS analysis the Cs^+ ion beam (0.8–1.2 pA) was focused to a nominal spot size of < 100 nm. The mass resolution was 8000 (M/ΔM). Secondary ion images (256 × 256, 512 × 512 pixels) for ^{12}C, ^{13}C, $^{12}C^{14}N$ and $^{12}C^{15}N$ were recorded simultaneously from the areas of interest for 40–60 cycles. The images and data were processed with the software Look@nanoSIMS (Polerecky *et al.*, 2012) including drift correction and accumulation for every field of view.

Calculation of specific fixation rates and comparison of total N_2- and C-fixation determined by SIMS and EA-IRMS. Fixation rates of single cells are presented as N-specific N_2-fixation and C-specific C-fixation (h^{-1}), and as cell-specific N_2- and C-fixation (fmol $cell^{-1}$ h^{-1}). C-specific C-fixation and N-specific N-fixation rates relate to the cellular C-turnover through C-fixation and the N-turnover through N_2-fixation, respectively. In other words, these fixation rates relate to N- and C-based growth rates independent of their cell sizes (Montoya *et al.*, 1996; Zehr and Montoya, 2007), e.g. N-specific N_2-fixation rates of 0.01 h^{-1} imply that 1% of the cellular N-content is fixed per hour. In contrast, cell-specific fixation rates account for cell sizes, i.e. the N- or C-content per cell. Cell-specific N_2- or C-fixation in combination with cell abundances were used to extrapolate fixation rates from single cells to the diazotrophic communities. Please note, N_2-fixation rates are presented per cell without differentiating between heterocystous and vegetative cells, but in fact N_2 gas is assimilated in heterocysts of *Aphanizomenon* sp., *Dolichospermum* and *N. spumigena* and rapidly transferred and incorporated into vegetative cells. N_2-fixation rates are thus *de facto* defined as net N-incorporation based on N_2-fixation.

Specific C-and N_2-fixation, measured in single cells, were calculated as

$$\text{C-specific C-fixation } [h^{-1}] = \frac{^{13}\text{C-atom\% excess}_{(\text{cell})}}{^{13}\text{C-atom\% excess}_{(\text{DIC})} \times \Delta \text{ time}} \quad (1)$$

$$\text{N-specific } N_2\text{-fixation } [h^{-1}] = \frac{^{15}\text{N-atom\% excess}_{(\text{cell})}}{^{15}\text{N-atom\% excess}_{(N_2)} \times \Delta \text{ time}} \quad (2)$$

with the *^{15}N and ^{13}C atom% excess* in the cyanobacterial *cell* and in the total dissolved *N_2* or *DIC* pool, respectively (adapted from Montoya *et al.*, 1996; Zehr and Montoya, 2007). C- and N-based doubling times were calculated as ln(2)/(C-specific C-fixation) and ln(2)/(N-specific N_2-fixation), respectively. Cell-specific C- and N_2-fixation were calculated as

$$\text{Cell-specific C-fixation [fmol C cell}^{-1}\text{ h}^{-1}] = \text{C-specific C-fixation} \times \text{C-content cell}^{-1} \quad (3)$$

$$\text{Cell-specific } N_2\text{-fixation [fmol N cell}^{-1}\text{ h}^{-1}] = \text{N-specific } N_2\text{-fixation} \times \text{N-content cell}^{-1} \quad (4)$$

The cellular C- and N-content for *Aphanizomenon* sp. in the Baltic Sea have been shown to be 17.5 fmol C μm^{-3} and 2.77 fmol N μm^{-3} cell volume (Svedén *et al.*, 2015). These volumetric C- and N-contents are in agreement with Verity *et al.* (1992) and were also applied for *Dolichospermum* and *N. spumigena*. Cell dimensions as measured under the microscope were used to calculate cell volumes. The C:N ratio in Baltic Sea diazotrophs has been shown to be relatively stable over a long-term sampling period, with C:N ratios ranging from 6 to 7 from April through September 1999 and 2000 with an average value of 6.3 (Walve and Larsson, 2007; Svedén *et al.*, 2015), which we applied in this study.

Statistical differences between mean N_2- and C-fixation were computed with the Student's t-test (Excel2010, for two-sample comparisons) or the Tukey's test (HSD.test in R 3.2.0, for multiple comparisons). Genera-specific proportions of N_2- and C-fixation by *Aphanizomenon* sp., *Dolichospermum* spp. and *N. spumigena* were calculated by multiplying their depth-specific cell abundances with cell-specific N_2- and C-fixation rates. Fixation rates were then linearly interpolated between depths and depth-integrated (0–12 m) for each genus to obtain areal fixation rates on our sampling dates in 2012/13. We further estimated relative contributions of *Aphanizomenon* sp., *Dolichospermum* spp. and *N. spumigena* to total C- and N_2-fixation on a longer term based on

monitoring data of cyanobacterial biomass during 2000–2014 at station B1 and BY31 (Swedish National Marine Monitoring Program) and herein presented cell-specific fixation. Monitoring biomass data were therefore related to biomass-specific N_2- and C-fixation of each genus as measured in 2012/13.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Text S1. Preparation of $^{15\text{-}15}N_2$ water and calculation of N_2- and C-fixation rates.

Fig. S1. Sampling station B1 was located in the coastal area off south-eastern Sweden and station BY31 offshore in the Baltic Proper at the Landsort Deep.

Fig. S2. Depth profiles of light, temperature, salinity and sigma at station B1 and BY31. The depth-axes are different for light profiles than for the other profiles.

Fig. S3. Photosynthetic irradiance in air and water (at incubation depths). The light extinction coefficient was 0.30–0.46 m^{-1} at B1 and 0.29–0.32 m^{-1} at BY31. The incubations from 9PM to 9AM are referred to as night incubations but in fact these were not at complete darkness due to sunrises a few hours before 9AM during summer in the high-latitude Northern Hemisphere.

Fig. S4. Depth profiles of nutrients and nutrient ratios at station B1 (upper panel) and BY31 (lower panels). Total N and P included inorganic and organic compounds in the dissolved and particulate fractions, except N_2. Data were provided by the Swedish National Marine Monitoring Program.

Fig. S5. Particulate C- and N-content (POC, PON analysed by EA-IRMS), and phytoplankton biomass and composition (analysed by microscopic counts). The C-biomass constituted of Cyanophyceae (24–72%), Dinophyceae (5–48%), Cryptophyceae (0–35%), Ciliophora (0.4–8%, only *Mesodinium rubrum* as autotrophic ciliate included), Bacillariophyceae (0–5%), Chlorophyta (0–0.5%), Prymnesiophyceae (0–5%) and Zoomastigophora (0–7%). The non-cyanophyceae species and genera were mainly identified as *Gymnodinium* spp., *Heterocapsa* sp., *Dinophysis* spp. (Dinophyceae); *Teleaulax* spp., *Plagioselmis* sp. (Cryptophyceae); *Cyclotella* sp., *Chaetoceros* spp., *Actinocyclus/Coscinodiscus* spp., *Skeletonema marinoi* (Bacillariophyceae); *Planctonema lauterbornii*, *Oocystis* spp. (Chlorophyta); cf *Chrysochromulina* (Prymnesiophyceae); *Ebria tripartita* and *Bicosoeca* sp. (Zoomastigophora). The ciliate *Mesodinium rubrum* was included on the phytoplankton biomass due to its autotrophy. *Others* include Prymnesiophyceae, Chlorophyta, and Zoomastigophora. Only colonial but not unicellular picocyanobacterial were included in the microscopic counts. Data are depth-integrated for 0–7 m. At Stn BY31, the depth-integrated biomass at 0–7 m accounted for 61% of that at 0–12 m in accordance with a well-mixed surface layer of at least 12 m depth.

Fig. S6. Depth distributions of *Aphanizomenon* sp. *Dolichospermum* spp. and *N. spumigena*. The percent C-biomass was calculated as fraction of the cumulative biomass in

0–7 m (B1) or 0–12 m (BY31). The depth-distribution was highly variable between day- and night-time, and between different months, i.e. distribution pattern were not significant. *Aphanizomenon* sp. seemed to be rather evenly distributed from 0 to 12 m at Stn BY31 but less evenly distributed at B1 where its C-biomass peaked at 5 m (Figure S6). *Dolichospermum* spp. had their greatest C-biomass at 1 m at both stations. *N. spumigena* was most abundant at 1 m at Stn B1, but its C-biomass at 12 m even exceeded that at 1 m at BY31. Data are given as mean ± s.e. ($n = 6$).

Fig. S7. Depth profiles of diel C- and N_2-fixation by the entire phytoplankton community.

Fig. S8. Biomass, and N_2- and C-fixation by heterocystous cyanobacteria at station B1 in 2012 and BY31 in 2013. The genera-specific contributions to N_2- and C-fixation (only by diazotrophs) are given as percentage.

Table S1. Light intensities and temperatures during *in situ* incubations.

Table S2. Heterocyst frequencies (mean ± s.d., $n \geq 20$) in filaments of diazotrophic cyanobacteria. n.p. not present.

Table S3. Cell sizes, volumes and C-/N-contents. Cellular C- and N-contents were calculated after Svedén et al (2015).

Table S4. Specific C- and N_2-fixation rates for cells of (a) *Aphanizomenon* sp., (b) *Dolichospermum* spp. and (c) *N. spumigena*. Statistical differences of mean C- and N_2-fixation were computed with the Tukey's (HSD-) test and ranked according their value and significant difference. If two means share at least one letter, they are not significantly different ($p > 0.05$). Significant differences were tested separately for coastal and offshore N_2-fixation and coastal and offshore C-fixation. Lowercase letters were used for coastal fixation rates and uppercase letters for offshore ones due to substantial differences in fixation rates between both stations.

Table S5. Compiled literature data on specific N_2- and C-fixation of diazotrophs. ARA – Acetylene reduction assay, $^{15}N/^{13}C/^{14}C$- isotope incubations, n.m. not mentioned.