RESEARCH ARTICLE

Seasonal dynamic of diazotrophic activity and environmental variables affecting it in the Gulf of Riga, Baltic Sea

Ineta Liepina-Leimane*, Ieva Barda, Iveta Jurgensone, Atis Labucis, Natalija Suhareva, Vendija Kozlova, Agita Maderniece and Juris Aigars

Latvian Institute of Aquatic Ecology, Agency of Daugavpils University, Voleru Street 4, Riga, Latvia, LV-1007

ABSTRACT

The semi-enclosed Baltic Sea experiences regular summer blooms of diazotrophic cyanobacteria. Previously, it has been conclusively demonstrated that in open nitrogen-limited parts of the Baltic Sea, cyanobacteria successfully fix atmospheric N₂. At the same time, diazotrophic activity is still poorly understood in Baltic Sea sub-regions where nitrogen and phosphorus are co-limiting primary production. To address this gap in research, we used the ¹⁵N tracer method for in situ incubations and measured the N₂-fixation rate of heterocyst-forming cyanobacteria and picocyanobacteria in the Gulf of Riga, Baltic Sea, from April to September. Physicochemical variables and phytoplankton community composition were also determined. Our results show that the dominant species of cyanobacteria for this region (*Aphanizomenon flos-aquae*) was present in the phytoplankton community during most of the study period. We also establish that the N₂-fixation rate has a strong correlation with the proportion of *A. flos-aquae* biomass containing heterocysts (*r* = 0.80). Our findings highlight the importance of a heterocyst-focused approach for an accurate diazotrophic activity evaluation that is one of the foundations for future management and protection of the Baltic Sea.

Keywords: *Aphanizomenon flos-aquae*; Baltic Sea; diazotrophic cyanobacteria; heterocysts; N₂-fixation

Introduction

N₂-fixing or diazotrophic cyanobacteria are considered the gatekeepers of bioavailable nitrogen and primary productivity in the open ocean. The estimated marine diazotroph contribution on the global scale is from 90 Tg (Kolber 2006) to 130–280 Tg (Konno et al. 2010, Eugster and Gruber 2012) of fixed nitrogen annually. Diazotroph-derived nitrogen assimilation of the dissolved atmospheric N₂ in temperate and coastal marine systems is also considerable. Nitrogen fixation in the Baltic Sea alone supplies 0.03 to 0.434 Tg of nitrogen annually (Rahm et al. 2000, Larsson et al. 2001, Wasmund et al. 2001, 2005, Rolff et al. 2007).

The wide range of estimated atmospheric nitrogen deposition and its subsequent utilization by marine organisms in the Baltic Sea reflects a disparity of measured N₂-fixation rates that could be as low as 0.17 nmol-N h⁻¹ L⁻¹ (Eigemann et al. 2019) or as high as 41 nmol-N h⁻¹ L⁻¹ (Ploug et al. 2011). There is a common perception that the temporal and spatial variation of the N₂-fixation rate is regulated by environmental conditions such as the availability of inorganic phosphorus...
(i.e. low inorganic nitrogen to phosphorus ratio), light intensity, temperature, turbulence and water-column mixing depth (Howarth et al. 1988). Furthermore, the cyanobacteria blooms are heterogeneous between Baltic Sea sub-basins in regard to the N₂-fixing taxa. Throughout the region, three predominant diazotrophic cyanobacteria—Nodularia spumigena, Aphanizomenon and Dolichospermum (formerly Anabaena)—are found in various proportions (Niemi 1979, Wasmund 1997). This factor of diazotrophic cyanobacteria species composition has been addressed in several recent studies (Wasmund et al. 2001, Ploug et al. 2011, Klawonn et al. 2016, Eigemann et al. 2019) when estimating N₂-fixation rates.

The variation of N₂-fixation rates makes extrapolation of values measured in short-term summer cruises across all cyanobacteria productivity season challenging. The attempts to use abundance (Degerholm et al. 2008) or biomass of filamentous cyanobacteria (Wasmund et al. 2001, Zilius et al. 2021) have been successful on some occasions. At the same time, the inability to estimate N₂-fixation from diazotrophic cyanobacteria biomass alone has also been stressed (Wasmund et al. 2001). Although Moisander et al. (1996) and Zilius et al. (2021) have demonstrated N₂-fixation rate dependence from the abundance of heterocysts—specialized cells for N₂-fixation—the work of Lindahl and Wallstrom (1985) has shown that their activity and therefore the N₂-fixation rate can vary. Furthermore, even although most common diazotrophic cyanobacteria found in the Baltic Sea form heterocysts, some unicellular picocyanobacteria (< 2 μm) have also been proposed to contribute to the fixed nitrogen (Wasmund et al. 2001).

There is a long history of N₂-fixation studies in the Baltic Sea. However, there have been only a few studies targeting the whole productive season of Aphanizomenon that dominates the Baltic Sea central and coastal areas (Degerholm et al. 2008) and the highly productive Curonian Lagoon (Zilius et al. 2021). The attempts to estimate the contribution to N₂-fixation of non-heterocystous diazotrophs (i.e. picocyanobacteria) are even fewer (Wasmund et al. 2001). Therefore, in this study we aimed to investigate the seasonal pattern of heterocyst-forming cyanobacteria N₂-fixation rates as well as estimate N₂-fixation by picocyanobacteria in the coastal waters of the Gulf of Riga, Baltic Sea. We demonstrate that the parameters associated with diazotrophic activity are best explained by variables characterizing the Aphanizomenon flos-aquae population and that the N₂-fixation rate is linked to the proportion of heterocysts containing biomass of A. flos-aquae. We also provide empirical evidence that non-heterocystous picocyanobacteria cells are able to fix N₂.

Materials and Methods

Study area

The Gulf of Riga is a relatively shallow, semi-enclosed sub-basin of the Baltic Sea with an average depth of 26.2 m, water volume of 424 km³ and water residence time of 2 to 4 years (Yurkovskis et al. 1999, Purina et al. 2018). It is strongly influenced by freshwater runoff, because its drainage area significantly exceeds the Gulf of Riga surface area.

The phytoplankton seasonal succession in the Gulf of Riga follows the general pattern for temperate coastal waters with a spring bloom of diatoms that, in a later period, are taken over by dinoflagellates and ciliates. Summer blooms are characterized by cyanobacteria often accompanied by chlorophytes and cryptophytes, and afterwards follows the second peak of diatoms in autumn (Yurkovskis et al. 1999, Jurgensone et al. 2011, Labucis et al. 2017, Purina et al. 2018, Tünens et al. 2022).

For study purposes, a 20-m deep coastal station (Fig. 1) was chosen so that it is outside the direct influence of river discharge and represents coastal conditions.

Sampling

Seawater was collected with a Van Dorn water sampler 11 times during the period from April to September 2021 from the Latvian Institute of Aquatic Ecology Vessel “Ronis2” (see the sampling dates in Supplementary Table 1). Samples for nutrient concentration analysis were taken at depths of 0.5, 2.5 and 5 m, transferred to 1-liter plastic bottles and kept in the dark at 5°C until they were analyzed in the laboratory. Additionally, samples from depths of 0.5, 2.5 and 5 m were taken for chlorophyll a (Chl a) analysis, and phytoplankton community analysis as well as picoplankton biomass determination. The samples for Chl a analysis were filtered on glass fiber filters (Whatmann GF/F), placed in the excicator and kept in the freezer until further treatment. The samples (300 ml) for phytoplankton community analyses were fixed with Lugol’s solution, acid (final conc. 0.5%) immediately after sampling and stored in the dark until further analyses in the laboratory. Picoplankton samples (50 ml) were fixed with glutaraldehyde (final conc. 1%) immediately after sampling and stored in the dark until further analyses in the laboratory.

Profiles of water temperature and salinity were measured using a CTD probe (SBE 19plus Sea-Cat, Sea-Bird Scientific, USA) with a vertical resolution of 0.5 m. Profile of photosynthetically active radiation (PAR, 0-10 m) was measured with a LI-COR Data Logger and LI-190R Quantum Sensor. Turbidity (0-10 m) was profiled with a digital water quality meter (YSI ProDSS). These measurements of vertical profiles were repeated each hour throughout the incubation period to acquire average diurnal values. The real-time measurement database of the Skulte Port smart buoy situated 2 km from the sampling station was used to determine the current speed during the sampling occasions.

Analytical procedures

The concentration of Chl a was measured according to HELCOM Monitoring Guidelines (HELCOM 2017). Chl a was extracted from a glass fiber filter in 96% ethanol for 24 h and analyzed using a spectrophotometer (Cary 100 Conc UV–Visible Spectrophotometer). Oxygen concentrations were determined with a sensor before and after incubation (PreSens Fibox 4) and converted to carbon units according to the stoichiometry of the photosynthesis equation. Net primary production (NPP, gC m⁻² d⁻¹) was calculated by subtracting initial oxygen concentrations from the final oxygen concentrations at each sampling depth: 0.5, 2.5 and 5 m.

Nutrient concentrations were determined according to Grasshoff et al. (1999). The inorganic dissolved phosphate (DIP) and ammonium (NH₄⁺) concentrations were measured using the molybdenum blue and indophenol blue methods, respectively. Nitrite (NO₂⁻) and nitrate (NO₃⁻), after reduction to nitrite in a copper-coated cadmium column, were determined by nitrite reaction with an azo dye. Dissolved inorganic nitrogen (DIN) was expressed as the sum of ammonium, nitrite and nitrate.

Microscopy

For phytoplankton community determination, subsamples of 10 ml were analyzed using an inverted microscope. Individual
cells were counted according to HELCOM Monitoring Guidelines (HELCOM 2021). Subsamples were settled in a sedimentation chamber for 12 h and counted at 200x and 400x magnification (Uthermöl 1958). The number of counted cells in all subsamples exceeded 500. The wet weight of the phytoplankton biomass was expressed as mg m$^{-3}$ and calculated in accordance with Olenina et al. (2006). Phytoplankton organisms were identified to the lowest possible rank. Scientific names and classification were compiled with the accepted binomial nomenclature of the World Register of Marine Species (version 2021).

For picoplankton analyses, subsamples of 5 ml were filtered with $<10$ kPa vacuum onto an Irgalan black-stained 0.2-$\mu$m pore-sized Nuclepore polycarbonate filter and stained with proflavine (Hobbie et al. 1977). The filter was mounted on paraffin oil and picocyanobacteria cells were counted with an epifluorescence microscope at 100x oil immersion objective under green excitation light. At least 50 cells were counted on each filter. The volume of picocyanobacteria was expressed as the average of 50 individual cell measurements and the cell shape dimensions were used to calculate wet weight (mg m$^{-3}$, Edler 1979).

**Incubations with $^{15}$N$_2$-enriched water**

The collected seawater at 0.5, 2.5 and 5 m was prepared for two parallel series of incubation for each depth to acquire the total N$_2$-fixation rate and the picocyanobacteria N$_2$-fixation rate. For the total N$_2$-fixation rate, seawater was filtered through a 10-$\mu$m mesh stainless steel sieve (Humboldt, No. 850, frame diameter 25.4 cm) before being transferred to 500-ml Duran glass bottles. To ensure that filaments and individual cells of cyanobacteria were removed by filtration, samples from the filtered bulk volume were investigated using an inverted microscope. Both non-filtered and filtered sample series for each depth consisted of three replicate bottles. Thereafter, the bottles were sealed with bromobutyryl rubber stoppers and 50 ml of the seawater was replaced with 50 ml of MiliQ water enriched with $^{15}$N$_2$ through an injection. The enriched MiliQ water used to spike incubation bottles was pre-prepared based on adjusted methodological recommendations by Klawonn et al. (2015). For each sampling occasion, 900 ml of degassed MiliQ water (sonicated for 1 h, 60 $^\circ$C) was transferred to a Tedlar gas sampling bag (push lock valve, 1 L) and spiked with 9 ml of $^{15}$N$_2$ gas (Sigma-Aldrich, 98 atom% $^{15}$ N). The filled gas sampling bag was left for 24 h at 8 $^\circ$C before being used to dissolve the $^{15}$N$_2$ gas bubble in the MiliQ water. After enriching the seawater, the bottles were placed and secured in a stainless-steel frame that was deployed to the corresponding depth the sample was taken from. The samples were then incubated for 10–12 h corresponding to the diurnal time of the day. The $^{15}$ N atom% in the enriched samples was expressed as the sum of added and naturally abundant $^{15}$ N. First, the total of dissolved N$_2$ in the sample was calculated based on the seawater temperature, salinity and N$_2$ solubility coefficients ($\text{A}_0$, $\text{A}_1$, $\text{A}_2$, $\text{A}_3$, $\text{B}_0$, $\text{B}_1$, and $\text{B}_2$) provided by Hamme.
where $C$ is the $N_2$ concentration at equilibrium with the atmosphere, $T$ is the temperature (°C) and $S$ is the salinity. After that, the natural nitrogen ratio in the atmosphere ($^{15}N/^{14}N = 0.366$ atom%) was used to determine the $^{15}N$ content in the sample. As a last step, the $N_2$ solubility formula was used to calculate $^{15}N$ in the 50 mL of enriched water assumed to contain only $^{15}N_2$ because the water was degassed prior to enrichment and added to the $^{15}N$ content in the sample. However, it must be acknowledged that a theoretical estimation of $^{15}N_2$ gas dissolution can result in some underestimation of rates (White et al. 2020).

After incubation, an aliquot subsample (50–100 mL) from each bottle and of the bulk volume used to fill incubation bottles was filtered on a pre-combusted (500 °C for 2 h) 13-mm diameter GF/F filter for isotopic signature analysis with an elemental analyser (EuroEA-3024, EuroVector S.p.A, Italy) coupled with a continuous flow stable isotope ratio mass spectrometer (Nu-HORIZON, Nu Instruments Ltd, UK). The isotope ratio mass spectrometry analysis was performed in the Laboratory of Analytical Chemistry, University of Latvia. Isotope ratios were reported relative to atmospheric nitrogen for $\delta^{15}N$ as parts per thousand (%):

$$\delta^{15}N = \left( \frac{^{15}N/^{14}N}_{\text{sample}} - 1 \right) \times 1000$$

where ($^{15}N/^{14}N$)atmosphere = 0.003676. To use a mass balance approach and determine the $N_2$-fixation rate as described by Montoya et al. (1996), the average of the three replicate $\delta^{15}N$ values was then converted to the absolute abundance ratio $A$ ($^{15}N$ atom%) of the $^{15}N$ enrichment of particulate N as follows:

$$A = 100 \times \left( \frac{(10^{-3} \delta^{15}N + 1)(^{15}N/^{14}N)_{\text{atmosphere}}}{1 + (10^{-3} \delta^{15}N + 1)(^{15}N/^{14}N)_{\text{atmosphere}}} \right)$$

The remainder of incubated seawater in the Duran bottles was used to filter a second batch of aliquot subsamples on pre-combusted (500 °C for 2 h) 24-mm diameter GF/F filters for N% analysis (Elementar Vario El III). In addition, 0.4–1.4 L aliquot of bulk volume used to fill incubation bottles was filtered on pre-weighted nitrocellulose membrane (Millipore, 45-mm diameter, 0.45-μm pore size) to determine suspended particulate matter (SPM). After drying (24 h, room temperature), the nitrocellulose filters were weighed again and SPM (mg L$^{-1}$) was calculated as follows:

$$\text{SPM} = \frac{m_{f+SPM} - m_f}{V}$$

where $m_{f+SPM}$ is the mass of filter and SPM, $m_f$ is the filter mass and $V$ is the volume of bulk seawater filtered through the nitrocellulose filter.

Diurnal $N_2$-fixation rate (NFR, nmol N L$^{-1}$) was then estimated using the following equation (Montoya et al. 1996):

$$\text{NFR} = \frac{A_{PN} - A_{PN(0)}}{A_{N(0)} - A_{N(0)}} \times PN$$

where $A_{PN(0)}$ is the $^{15}N$ enrichment of particulate N before the incubation, $A_{PN}$ is the $^{15}N$ enrichment of particulate N after the incubation and $A_{N(0)}$ is the sum of naturally abundant and added $^{15}N$. PN represents particulate nitrogen concentration at the end of the incubation period calculated based on the corresponding samples N% and SPM (transformed in nmol N L$^{-1}$). The heterocyst-forming cyanobacteria $N_2$-fixation rate was achieved by subtracting the picocyanobacteria $N_2$-fixation rate of pre-filtered seawater samples (~10 μm) from the total $N_2$-fixation rate. Because picocyanobacteria isotope signatures for week 32 and week 36 were not analyzed, the heterocyst-forming cyanobacteria $N_2$-fixation rate for these sampling occasions was obtained by subtracting the mean value of picocyanobacteria $N_2$-fixation rate throughout the whole sampling period (0.5 m = 3.2 nmol N L$^{-1}$, 2.5 m = 2.2 nmol N L$^{-1}$, 5 m = 0 nmol N L$^{-1}$) from the total rate.

**Statistical analysis**

R software v. 3.6.1 (R Core Team 2019) was used for data visualization and analysis of the environmental variables collected during the sampling period. An overview of the relationship between environmental variables was provided based on Spearman’s rank correlation with the coefficient significance set at $\alpha = 0.05$. For multivariate analysis, the dependent matrix of parameters associated with diazotrophic activity ($N_2$-fixation rate, heterocyst abundance, proportion of A. flos-aquae biomass containing heterocysts) was Hellinger-transformed. Detrended correspondence analysis was used to identify the appropriate response model for the dependent matrix of parameters associated with diazotrophic activity. Because the gradient length for all calculated axes was below 2, redundancy analysis (RDA) was applied. The explanatory variables or terms of both abiotic (PAR, DIN, DIP, NH$_4^+$, turbidity, current speed) and biotic (A. flos-aquae total biomass and filament length) drivers were included in a global model. To construct a simplified model, automatic forward selection was applied to the RDA global model by retaining variables that significantly increased the adjusted $R^2$. The significance for the models, axis and terms was measured by a permutation test ($n = 1000$). The observations of the last sampling occasion (week 45) were not included in the RDA due to a lack of $N_2$-fixation rate measurements.

**Results**

**Physicochemical variables**

The temperature and nutrient concentrations (Supplementary Table 1) generally followed the seasonal pattern for the Gulf of Riga. The water temperature reached the suggested limit (8–10 °C; Laamanen and Kuosa 2005, Mehnert et al. 2010) for filamentous cyanobacteria growth on 16 May (week 19). The water temperature indicated a stratification in the depth of up to 5 m from May (week 19) to July (week 28). Starting at the end of July (week 29) and until the last $N_2$-fixation rate measurements.

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The concentrations of DIP varied from below the detection limit (<0.04 μmol L$^{-1}$) to 0.69 μmol L$^{-1}$ and DIN varied from 0.14 μmol L$^{-1}$ to 33.43 μmol L$^{-1}$. As expected, concentrations of both DIP and DIN were relatively low throughout the productive season (i.e. from May to September), except for on 17 June (week 24), when the highest concentrations of DIN and elevated concentrations of DIP were observed. Overall, the DIN: DIP ratio in the surface water layer (0–5 m) corresponded to the phosphorus-limited marine system, except for on 4 August (week 31) and
During the experiment, the heterocyst formation by *A. flosaquae* accompanied by N\(_2\)-fixation was detected (Fig. 5) as early as May (week 19). However, the observed N\(_2\)-fixation rate was very low (0.1-1.2 nmol N L\(^{-1}\)) and remained so until mid-July (week 28), except for on 17 June (week 24), when a noticeable increase in the N\(_2\)-fixation rate was observed in the 0.5 m horizon. The gradual increase of N\(_2\)-fixation rates started in late July (week 29) and reached the highest rate (0.5 m, 36.9 nmol N L\(^{-1}\)) in August (week 32). Thereafter, a decline in N\(_2\)-fixation rates was observed, although it should be noted that the N\(_2\)-fixation rates observed in September were substantially higher than those recorded during June-July. The average total N\(_2\)-fixation rates (Supplementary Table 2) generally follow the same pattern as was observed for the number of heterocysts (Fig. 5). The peak N\(_2\)-fixation rate, however, does not coincide with maximum heterocyst abundance, with the latter offset to September (week 36). Similarly, a considerable increase in the number of heterocysts was observed on 17 June (week 24) at the 2.5- and 5-m horizons that did not result in an increase in the N\(_2\)-fixation rates.

Incubation experiments with pre-filtered seawater samples (<10 \(\mu\)m) of N\(_2\)-fixation by picoplankton were performed from late April to early September. Due to equipment failure, the samples taken during weeks 32 and 36 were not analyzed and only results from late April to early August are used for the purposes of this study. During this period, the N\(_2\)-fixation rates (Fig. 6) were sporadic. Measurable values were only detected in May and from the end of July to August at the depths of 0.5 and 2.5 m. N\(_2\)-fixation rate values were in the range of 30–33% of corresponding unfractonated samples, except for the 0.5-m layer in early August (week 31), which reached 42%. The observed steady increase in picocyanobacterial biomass from week 17 to week 32 did not, however, result in a corresponding increase in N\(_2\)-fixation rates. Due to the small size and lack of distinctive morphological features, microscopic examination yielded only picocyanobacterial biomass (Fig. 6). However, because the N\(_2\)-fixation rate was detected on only a few sampling occasions, it was not used as a variable for correlation purposes.

### Relationships between diazotrophic activity and environmental variables

RDA was applied to reveal the canonical relationship between environmental variables and the parameters associated with diazotrophic activity, that is, the N\(_2\)-fixation rate, heterocyst abundance and the proportion of *A. flosaquae* containing heterocysts. The environmental variables included in the global RDA model (\(P \geq 0.001\)) explain 67.8% of the variation of the parameters associated with diazotrophic activity from April to September when N\(_2\)-fixation was measured (Fig. 7A). Based on a permutation test of the global model, the first two canonical axes resulting from the RDA as well as five variables—current speed, DIP, PAR, total *A. flosaquae* biomass and filament length—are statistically significant (\(P \leq 0.05\)). After removing redundant environmental variables from the global model, only two—total *A. flosaquae* biomass and filament length—were selected for a simplified RDA model (Fig. 7B). The simplified model explains 67.4% of the variation of the parameters associated with diazotrophic activity. The permutation test indicates that the simplified model and the first two of its canonical axes are significant (\(P \geq 0.001\)).
In addition to the RDA, environmental variables measured at depths of 0.5, 2.5 and 5 m are presented in a Spearman’s rank correlation coefficient matrix (Fig. 8). Several positive statistically significant and strong correlations for the N₂-fixation rate and associated parameters are displayed, such as with heterocyst abundance, heterocyst containing *A. flosaquae* biomass and filament length. However, the strongest correlator for N₂-fixation rate was the proportion of *A. flosaquae* heterocyst containing biomass (Fig. 8).

Our results demonstrate a complex relationship between *A. flosaquae* biomass and heterocyst development. Generally, an increase in total *A. flosaquae* biomass is expected to indicate a higher biomass of *A. flosaquae* containing heterocysts. However, the portion of the heterocyst containing biomass does not correlate with the total *A. flosaquae* biomass. On the other hand, heterocyst proportion does correlate strongly with the filament and heterocyst length.

Only two physicochemical variables demonstrated a notable impact on the N₂-fixation rate or associated biological parameters. We established a positive correlation between the current speed and the *A. flosaquae* biomass containing heterocysts, the proportion of *A. flosaquae* heterocyst containing biomass, heterocyst abundance and N₂-fixation rate. By contrast, the correlation between ammonia concentration and N₂-fixation rate was negative. Additionally, PAR had a weak positive correlation with filament and heterocyst length.

**Discussion**

The N₂-fixation rates observed in the coastal waters of the Gulf of Riga during this study are in the range of those reported for the Baltic Sea in the last two decades (Table 1). The findings of Eigemann et al. (2019) also support the empirical evidence provided herein that non-heterocystous diazotrophs contribute to N₂-fixation. However, an earlier overview of N₂-fixation rates summarized by Wasmund et al. (2001) displays more conservative N₂-fixation rates 40–50 years ago.

A number of previous studies have also addressed a variety of physicochemical and biological variables that potentially regulate the N₂-fixation rate. Most of those earlier studies were conducted in late summer–early autumn when the temperature is not considered to limit N₂-fixation. However, in studies covering the whole productive season, such as this study, the temperature could be an important abiotic driver for N₂-fixation, as suggested by Laamanen and Kuosa (2005). The results herein confirm the previously established cyanobacteria ability to form heterocysts and perform N₂-fixation at temperatures below 10 °C (Zakrisson and Larsson 2014, Sveden et al. 2015). At the same
time, similar to the conclusion made by Stal and Walsby (2000), the light availability expressed as PAR (Fig. 7A) seems to be more important than temperature. The impact of PAR on N₂-fixation, however, is mostly indirect as PAR correlates with filament length (Fig. 8), which is one of two main factors affecting N₂-fixation (Fig. 7B).

The role of the DIN: DIP ratio in triggering heterocyst formation in diazotrophic cyanobacteria and consequently governing N₂-fixation in the Baltic Sea is still uncertain. Although it has been suggested that a low DIN: DIP ratio might give a competitive advantage to N₂-fixing cyanobacteria (Smith 1983, Laamanen and Kuosa 2005), some later studies have argued that nitrogen depletion (Zakrisson and Larsson 2014) or availability (Vintila and El-Shehawy 2007) does not affect the N₂-fixation rate. Furthermore, it has been proposed that rather than the DIN: DIP ratio, the DIP availability is the factor affecting the growth of diazotrophic cyanobacteria and N₂-fixation (Olofsson et al. 2016). In this study we did not find an increase of N₂-fixation under low DIN: DIP conditions or during periods of elevated DIP concentrations. The Gulf of Riga, especially its coastal areas, is a relatively shallow and dynamic water body periodically experiencing an influx of DIN and DIP enriched near-bottom water in the photic zone. Although it was expected that these events would affect the N₂-fixing capacity of diazotrophic cyanobacteria, our results indicate the opposite, because the highest N₂-fixation rates were observed during periods when the concentrations of both DIP and DIN were low. The low concentrations of DIP during periods of high N₂-fixation rates is most likely not the prerequisite, but rather a consequence, as cyanobacteria is incorporating DIP into biomass while assimilating N₂, thus depleting DIP from water media. Furthermore, because the biomass of A. flosaquae containing heterocysts did not exceed 50% on any of the sampling occasions, it can be concluded that a substantial fraction of the predominant species was not fixing N₂ but instead using DIN as an N source.

Even although physicochemical conditions could not be directly linked to the N₂-fixation rate, they remain important environmental drivers to look into as they likely affect biological variables that were directly linked to N₂-fixation rates. Previous studies have also addressed biological variables such as abundance or biomass of cyanobacteria (Wasmund et al. 2001, Degerholm et al. 2008) and abundance of heterocysts (Moisander et al. 1996, Zilius et al. 2021). Zilius et al. (2021) were even able to demonstrate a consistent relationship between biomass of phytoplankton, expressed as chlorophyll a, and N₂-fixation rate, because the phytoplankton biomass increase over the summer season was primarily driven by an increase in biomass of N₂-fixing species. By contrast, in this study, only on one sampling
Figure 5. Heterocyst-forming cyanobacteria N$_2$-fixation rate seasonal dynamic and the corresponding heterocyst abundance in A. flos-aquae.

Figure 6. Seasonal dynamic of N$_2$-fixation rate and biomass of picocyanobacteria.
Table 1. Overview of reported N2-fixation rates in the Baltic Sea.

| Sub-basin            | N2-fixation rate | Heterocyst-forming species                      | Sampling period          | Reference                  |
|----------------------|------------------|-------------------------------------------------|--------------------------|---------------------------|
| Eastern Gotland Sea  | Min: 0.21 nmol N L−1 h−1 | Aphanizomenon sp., except in August (Nodularia sp. 38.5%) | May–November 1997        | Wasmund et al. (2001)     |
|                      | Max: 23.6 nmol N L−1 h−1 | Aphanizomenon sp., except in July–August (Nodularia sp. 39.1%–61.5%) | February–November 1998  |                           |
| Stockholm archipelago| 26.7 ng N L−1 h−1 (1.91 nmol N L−1 h−1) | Aphanizomenon sp. (98%) | August 2000              | Degerholm et al. 2008     |
| Offshore, Baltic Proper | 125.5 ng N L−1 h−1 (8.95 nmol N L−1 h−1) | Aphanizomenon sp. (95%) | August 2008              | Ploug et al. 2010         |
| Stockholm archipelago| 15 nmol N L−1 d−1 | Aphanizomenon sp. | August 2009              | Ploug et al. 2011         |
| Stockholm archipelago| 41 nmol N L−1 h−1 | Aphanizomenon sp., Anabaena sp., Nodularia sp. | August 2009              |                           |
| Stockholm archipelago| 1.45 μmol N L−1 d−1 (1 m); 0.05 μmol N L−1 d−1 (12 m) | 50% Aphanizomenon sp., 32% Dolichospermum sp., 18% Nodularia sp. | June–August 2012         | Klawonn et al. 2016       |
| Offshore, Baltic Proper | 0.05 μmol N L−1 d−1 (1 m); 0.01 μmol N L−1 d−1 (12 m) | 50% Aphanizomenon sp., 36% Dolichospermum sp., 14% Nodularia sp. | June–August 2013         |                           |
| Baltic Proper        | 0.17–1.03 nmol N L−1 h−1 | Aphanizomenon sp. | August 2015              | Eigemann et al. 2019      |
| Curtonian Lagoon     | 0.18–0.87 nmol N L−1 h−1 | Nodularia sp. | August–September 2018    | Zilius et al. 2021        |
| Danish Strait        | 3.0–4.6 μmol N L−1 d−1 (northern site); <0.4 μmol N L−1 d−1 (central site) | Dolichospermum sp., Aphanizomenon sp. | August 2015              |                           |
| Gulf of Riga         | Diurnal: 0.1–36.9 nmol N L−1 | dominated by Nodularia A. flosaquae (~90%) | September 2019           | Reeder et al. 2021        |
|                      | Nightly: 2.8–30.6 ng N L−1 | | April–September 2021     |                           |
|                      |                 | | This study               |                           |

occasion did N2-fixing species exceed 50% of the total biomass of phytoplankton. Consequently, there was no correlation between N2-fixation rate and the total biomass of phytoplankton. Furthermore, even correlation between total biomass of the most abundant N2-fixing species A. flosaquae and N2-fixation rate was relatively weak. At the same time, a significant strong positive correlation of heterocyst abundance, and by association biomass of A. flosaquae filaments that contains heterocysts, with N2-fixation rate, corresponded to previous findings by Moisander et al. (1996) and Zilius et al. (2021). The consistently low A. flosaquae filament biomass that developed heterocysts as well as the high variability of proportion of A. flosaquae biomass containing heterocysts, is most likely the reason why in this study no profound correlation between the total A. flosaquae biomass and N2-fixation rate was established.

Although the correlation between heterocysts and N2-fixation rate emphasizes the crucial role of heterocysts in N2-fixation in the Gulf of Riga, the relationship is substantially weaker than in previous reports (Findlay et al. 1994, Zilius et al. 2021). This suggests that a simple abundance of heterocysts is not the most accurate indicator of the actual N2-fixation rate in the Gulf of Riga because their activity varies, as previously reported by Lindahl and Wallstrom (1985).

The heterocyst formation is a terminal and a relatively fast process (Kumar et al. 2010), which for Aphanizomenon sp. is not characterized by a developmental pattern along the filaments like for the Dolichospermum sp. and Nodularia sp. (Yoon and Golden 2001; Voss et al. 2013). It could be argued that there are short-term or localized physicochemical conditions that trigger the formation of heterocysts in a small proportion of the A. flosaquae population. Thereafter, the conditions shift and heterocysts become redundant and less active. However, if environmental conditions that promote the formation of heterocysts persist for a longer period, a larger proportion of the diazotrophically active A. flosaquae population is able to form. As our results show, the proportion of A. flosaquae biomass containing heterocysts is a more precise proxy of the N2-fixation rate than heterocyst abundance in ecosystems that are not N-limited.

This assumption at the present stage is still speculative, however, it does explain some of the differences when comparing N2-fixation rates between aquatic systems. In the Curonian Lagoon (Zilius et al. 2021), the excess DIP throughout the summer season facilitates A. flosaquae cell growth depending on the available P supply (Degerholm et al. 2006, Chen et al. 2020), while in the Gulf of Riga nutrient conditions change relatively fast and seem to limit the development of the A. flosaquae population. Similar findings have been reported in a study of Clear Lake, North America, that concluded that the annual N2-fixation is best described by the proportion of heterocysts to vegetative cells in A. flosaquae (Horne and Goldman 1972).

Conclusions

This study illustrates that the diazotrophic activity in the Gulf of Riga has a seasonal pattern that broadly corresponds to the heterocyst abundance in A. flosaquae filaments and that the N2-fixation rate reaches its peak during the cyanobacteria summer bloom. We were also able to confirm that picocyanobacteria are capable of fixing N2 at a rate comparable with that exhibited by heterocysts-forming species, but it is overall limited to the surface water layer. The data acquired in this study, however, do not indicate any definite physicochemical variables that affect the diazotrophic activity. Nevertheless, these physicochemical drivers are expected to be involved in regulating biological parameters—heterocysts and a proportion of A. flosaquae
Figure 7. Ordination diagram based on RDA between diazotrophic activity parameters (NFR = N₂-fixation rate, heterocysts = heterocyst abundance, %Aph-Fix = proportion of A. flos-aquae biomass containing heterocysts). (A) Global model of all environmental variables (current = current speed, turb. = turbidity, biomassAph = total A. flos-aquae biomass, biomassAphFix = biomass of A. flos-aquae containing heterocysts, %AphFix = proportion of A. flos-aquae biomass containing heterocysts, current = current speed, NH₄ = ammonium). Significant environmental variables and RDA axes are labeled accordingly: *** - 0.001 ≤ P < 0.001; ** - 0.01 ≤ P < 0.05.

Figure 8. Spearman’s rank correlation matrix containing statistically significant coefficients (NFR = N₂-fixation rate, heterocysts = heterocyst abundance, biomassAph = total A. flos-aquae biomass, biomassAphFix = biomass of A. flos-aquae containing heterocysts, %AphFix = proportion of A. flos-aquae biomass containing heterocysts, current = current speed, NH₄ = ammonium).

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Supplementary data
Supplementary data are available at FEMSEC online.

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