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Research Article

Effects of temperature and N:P ratio on the invasion success of the cyanobacterium *Raphidiopsis raciborskii*

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Abstract

The tropical invasive freshwater cyanobacterium *Raphidiopsis raciborskii*, first recorded in Central Europe about two decades ago, is now a relatively widespread species, expanding its geographic range. Currently, however, there are no records of this species in Sweden. As a bloom-forming and toxin-producing species, future population growths of *R. raciborskii* could negatively affect local biodiversity and ecosystem services. Hence, there is an urgent need to understand the factors controlling its capability of establishment in Northern European lakes. We performed a laboratory experiment to study the competitive success of *R. raciborskii* when interacting with other phytoplankton from major taxonomic groups typically found in Scandinavian lakes (diatoms, green algae, and cyanobacteria). The experimental settings included three temperature conditions (17; 22; 26 °C) and three different nutrient conditions (N:P ratios 8:1; 16:1; 32:1). The experiment was performed in a semi-continuous culture setup to test the invasion success of *R. raciborskii*. *Raphidiopsis raciborskii* did not become the dominant species in any of the tested conditions; however, it was able to grow and maintain its biomass in all treatments, also in relatively low temperature (17 °C). Temperature played an important role in the phytoplankton community composition, especially for the cyanobacterial group. *Raphidiopsis raciborskii* was more successful than *Planktothrix agardhii*, but less dominant than *Microcystis aeruginosa*. Temperature is thus important in determining the potential survival and settlement of the invasive *R. raciborskii* in lakes.

Key words: invasive species, competition, semi-continuous culture, *Scenedesmus*, *Planktothrix*, *Microcystis*, *Chlamydomonas*

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Introduction

Climate change and eutrophication are the two main causes of increased cyanobacterial blooms (Burdick et al. 2020). These blooms negatively affect recreation, property values and drinking water, the latter affecting the health of humans as well as domesticated animals and wildlife (Ewing et al. 2020). Additionally, invasive species, defined as non-native species that have spread or have the potential to spread outside their native range, are considered a leading cause of biodiversity loss globally, often with severe ecological, economic and social repercussions (Amalfitano et al. 2015). The spread of microbes to new aquatic environments has the potential to alter community structure and ecosystem functioning by disrupting the balance of native species dynamics (Litchman 2010; Savadova-Ratkus et al. 2021). Microbial invasions are usually overlooked during assessments of invasive species in aquatic systems. Generally, invasive microbial species are harder to track as they are detected mainly when there is a noticeable impact, such as the formation of blooms (Acosta et al. 2015). Consequently, greater emphasis should be placed on invasive microbes as well as their effects on communities and ecosystems in freshwaters as they could potentially increase in the future (Litchman 2010).

One species of concern is the invasive freshwater cyanobacterium *Raphidiopsis raciborskii* (formerly *Cylindrospermopsis raciborskii*). *Raphidiopsis raciborskii* is a nitrogen-fixing, toxin-producing and bloom-forming filamentous cyanobacterium, known to impact local biodiversity and ecosystem services worldwide (Svirčev et al. 2014; Svirčev et al. 2016). *Raphidiopsis raciborskii* is a species of tropical origin and it develops in reservoirs, lakes, and rivers worldwide (Sinha et al. 2012; Yang et al. 2018). In south and central Europe, this species usually proliferates in shallow, turbid, and eutrophic/hypereutrophic lakes and it is currently expanding its range, migrating towards Northern parts of Europe, e.g. Poland and Lithuania (Kokociński et al. 2009; Kokociński and Soininen 2012; Kokociński et al. 2017). Currently, however, there are no records of this species in Scandinavia. In a recent study, we used species distribution models (SDMs) and regional bioclimatic environmental variables to identify suitable areas for the potential colonization of *R. raciborskii* in Scandinavia (Meriggi et al. 2022). The model predictions revealed areas with a high probability of occurrence of *R. raciborskii* in the south-eastern part of Sweden. However, the modelling approach does not account for physiological adaptations or interactions with other species, which is an important step towards more accurate predictions of an invasive species' range of expansion. Hence, there is an urgent need to understand the factors controlling its potential establishment in Scandinavia to predict its possible invasion.

Human activities have greatly increased the bioavailability of nitrogen (N) and phosphorus (P) in freshwaters and, combined with climate change, have increased the number of areas suitable for invasive cyanobacteria (Goyette et al. 2016), like the potential spread of *R. raciborskii* to Sweden. The invasion success of *R. raciborskii* worldwide has been primarily attributed to its phenotypic plasticity (intra-population strain variability) and ecological tolerances to light intensity and temperatures as well as its ability to fix atmospheric nitrogen (N_2) under N-depleted conditions and its strong affinity for phosphate (Padisák and Istvánovics 1997; Mehnert et al. 2010; Bonilla et al. 2012; Kokociński et al. 2017). These characteristics give *R. raciborskii* a competitive advantage over other bloom-forming cyanobacteria under nutrient limited conditions (Zheng et al. 2023). However, it is still unclear how the potential establishment of *R. raciborskii* in a newly invaded habitat could be affected by the presence of native species under different combinations of temperature or nutrient conditions. Here we address the potential risk of the invasive *R. raciborskii* to establish itself in Scandinavian freshwaters by experimentally testing its ability to compete with common phytoplankton species, native to many Scandinavian lakes. Understanding the behaviour of this invasive species, when interacting with native species, and using environmental conditions that are relevant to boreal regions is particularly important as both temperature and nutrients are rapidly changing due to climate-driven processes (Huisman et al. 2018). We therefore hypothesize that *R. raciborskii* would outgrow native phytoplankton species in high temperature and N-limited conditions.

Materials and methods

Study organisms

A laboratory competition experiment was performed with *R. raciborskii* (NIVA-CYA 399) and five phytoplankton species from three major functional groups: chlorophytes (*Chlamydomonas reinhardtii*, NIVA-CHL 152 and *Scenedesmus* sp., NIVA-CHL 114); cyanobacteria (*Planktothrix agardhii*, NIVA-CYA 429 and *Microcystis aeruginosa*, NIVA-CYA 123/1) and diatoms (*Asterionella formosa*, NIVA-BAC 3). These five phytoplankton species are native to many Scandinavian lakes and therefore likely to encounter *R. raciborskii* if/when it invades. All isolates were purchased from the Norwegian Culture Collection of Algae (NORCCA) and were not axenic. The phytoplankton were originally isolated from Scandinavian lakes except for *R. raciborskii* (Hungary) and *A. formosa* (UK) (Suppl. material 1). Regardless of differences in isolation location, we expect that competition between these strains will provide knowledge of potential interactions in natural systems (Ryan et al. 2017). Pre-experimental cultures were maintained at 22 °C in 250-ml batch cultures containing diluted Z8 medium (Suppl. material 1) with a silicate concentration (50-µmol L^{-1} medium) to meet species' requirements according to NORCCA (i.e. diluted media or silicate enrichment; Suppl. material 1). The cultures were exposed to 70–90 µE m^{-2} s^{-1} light in a 16:8 light/dark cycle and stirred regularly. The cultures were arbitrarily re-arranged to avoid any potential differences in light/temperature. At the end of the exponential growth phase, cultures were transferred into the experimental treatments.

Pilot experiment

The choice of experimental temperatures was based on a pilot study to identify the minimum, optimal and maximum growth temperature of *R. raciborskii* (NIVA-CYA 399) when exposed to a gradient of temperatures ranging from 3.5 °C to 35 °C. Biomass was measured daily as fluorescence in a TD-700 Fluorometer (Turner Designs, Sunnyvale, CA) with a 436 nm excitation filter and a 680 nm emission filter for 1 to 3 weeks depending on the growth rate at each temperature (Suppl. material 2). *Raphidiopsis raciborskii* had detectable growth at 17 °C and an optimal growth rate at 26 °C. *Raphidiopsis raciborskii* was able to grow at 35 °C, the maximum temperature tested (Suppl. material 2: fig. S1). Therefore, for the experimental study, we chose 17 °C as the lowest temperature, 22 °C as typical surface temperature in Swedish lakes and 26 °C as the highest temperature.

Experimental set-up

We performed a 3×3 factorial experiment with three replicates for each treatment combination. The experimental settings included three temperature conditions (17 °C; 22 °C; 26 °C) and three N:P ratios to achieve N-limiting, optimal and P-limiting growth conditions (8:1; 16:1; 32:1 mole ratio of N and P, respectively). The individual nutrient ratios were achieved by modifying the N source from the Z8 growth medium and by keeping the P source constant (Suppl. material 3). We used 250-ml glass Erlenmeyer flasks. Each E-flask was prepared with 100 mL of its designated N:P ratio of the modified Z8 growth medium and inoculated with an approximately equal biovolume of each phytoplankton culture (total volume added 15 ml). At the start of the experiment, the algal assemblages were mixed and the flasks were placed in three temperature-controlled rooms (Fig. 1). A proportion of the growth media was replaced daily to achieve a semi-continuous culture with

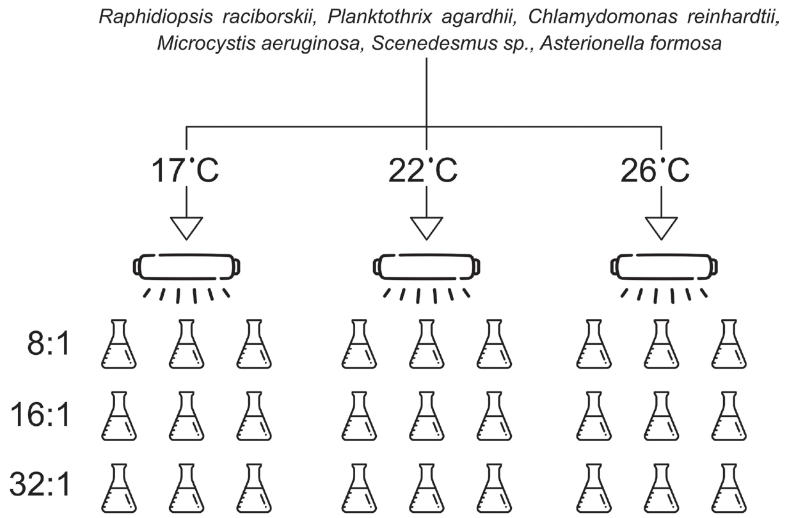

Figure 1. Experimental setup performed for a 3×3 factorial experiment with three replicates for each treatment combination. The cultures were exposed to several experimental conditions, including three temperature ranges (17 °C; 22 °C; 26 °C) and three different N:P mole ratio concentrations (8:1; 16:1; 32:1). The experiment was performed using an irradiance level between 70 and 90 µE m^{-2} s^{-1} and a 16:8 light/dark cycle.

a dilution rate of 0.1 day^{-1} (removing 10 ml/day) (*R. raciborskii*'s growth rate at 17 °C; Suppl. material 2: fig. S1). The experiment was performed using irradiance levels between 70 and 90 µE m^{-2} s^{-1} and a 16:8 light/dark cycle. Each flask was stirred gently by hand and re-arranged daily. No wall growth was observed over the course of the experiment. Every third day the volume of culture removed (10 ml/day) was preserved in Lugol's solution for later analysis of the phytoplankton species identification and biomass. This was done on 11 occasions during the 31-day experiment. Every tenth day the volume of culture removed (10 ml/day) was instead filtered (0.1 µm filter) and analysed to monitor the nutrient conditions.

Phytoplankton analysis

The samples were analysed by a FlowCam (VS1, Fluid Imaging Technologies Inc., Scarborough., ME, USA), which is a flow imaging microscope that combines imaging and laser light to detect particles from a fluid sample (Vorobjev and Barteneva 2016). The FlowCam produces photos of all passing particles and they are then sorted based on shape and size using the VisualSpreadsheet software (Version 4.15.1) to be able to analyse, classify and count each species separately in the experimental samples. Biovolume estimates ($µm^3$ /mL) of each species were calculated based on formula for their geometric shapes according Olrik et al. 1998 (Details in Suppl. material 4).

Nutrient concentration measurements

Phosphorous concentrations were measured as orthophosphate (PO_4-P) and analyzed photometrically according to Murphey et al. (1962), with discrete or continuous flow analysis depending on concentration (ISO 15923-1:2013, SS-EN ISO 15681-2:2018). Nitrogen concentration, as the sum of nitrate and nitrite measurements (NO_2 + NO_3-N), was analyzed with continuous flow analysis according to SS-EN ISO 13395 after reducing nitrate to nitrite according to Wood et al. (1967). All nutrient analyses were performed by the certified laboratory at the Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences.

Statistical analysis

Repeated-measures ANOVA was performed to test the effects of temperature, nutrient ratios and their combined effects on each individual species' biomass over the course of the experimental period. The statistical results presented used data from day 1, 10, 16, and 25, which give a good overview of the phytoplankton development over time. The last time point (day 31) was not taken into account as the FlowCam analysis was affected by bacterial growth. *Asterionella formosa* was present in low numbers at the beginning of the experiment but not recorded in any of the experimental treatments in later measurements, therefore it was excluded from the statistical analysis. The primary measurements prior to analysis were not transformed and statistical analyses was performed using the R version 4.2.1 (R Core Team 2022).

Results

Changes in nutrient concentrations

The evident increase in phytoplankton cell densities in all the treatments was reflected in declines in nutrient concentrations throughout the experiment (Figs 2, 3). Phosphorous concentrations were similar in all treatments at the beginning of the experiment and below detection limits on day 20 (Fig. 2). Differences in nutrient ratios were evident through the different N concentrations at the beginning of the experiment (Fig. 3). Nitrogen-limitation was most severe in N-limited treatment (8:1), noticeable already on day 11. In the optimum ratio treatment (16:1), N was depleted by the end of the experiment. In the P-limited treatment (32:1), P-limitation was evident as PO_4-P reached undetectable levels over time, whereas N (NO_2+NO_3-N) was available throughout the experiment.

Effect of temperature and nutrients on species' growth

Chlamydomonas reinhardtii and *M. aeruginosa* became the dominant species in all treatments (Fig. 4). *Chlamydomonas reinhardtii*'s biomass increased quickly at the beginning of the experiment. After day 10, it reached a growth limit in all treatments, without any increase in growth based on nutrient ratios. *Microcystis aeruginosa*'s biomass increased in all treatments over time, reaching a saturation level at the end of the experiment. Our target organism, *R. raciborskii*, had almost the same biomass in all treatments, corresponding to a growth close to the dilutions rate. *Planktothrix agardhii* was slowly diluted away, mainly at the lowest temperature (17 °C) and P-limiting (32:1) treatments. *Scenedesmus* sp. biomass increased over time in the low temperature treatment (17 °C) but decreased in high temperature (26 °C) and N-limiting (8:1) conditions.

Chlamydomonas reinhardtii was significantly and positively affected by nutrients ($F_{(2, 18)} = 9.41$, $p < 0.05$), temperature ($F_{(2, 18)} = 5.01$, $p < 0.05$), interactions between nutrients-time ($F_{(6, 54)} = 4.68$, $p < 0.05$) and temperature-time ($F_{(6, 54)} = 4.30$, $p < 0.05$). The post-hoc analysis showed that on day 25, *C. reinhardtii*'s biomass was significantly higher in the N-limiting treatment (8:1) ($p < 0.005$) compared to the optimal (16:1) and P-limiting ones (32:1). Additionally, on day 25, *C. reinhardtii* biomass was significantly higher at 17° ($p < 0.05$) compared to 22 °C. *Microcystis aeruginosa* increased significantly by the interaction between temperature-time ($F_{(6, 54)} = 8.600$, $p < 0.001$). The post-hoc analysis showed that on day 10, 16, 25 *M. aeruginosa*'s biomass was significantly lower

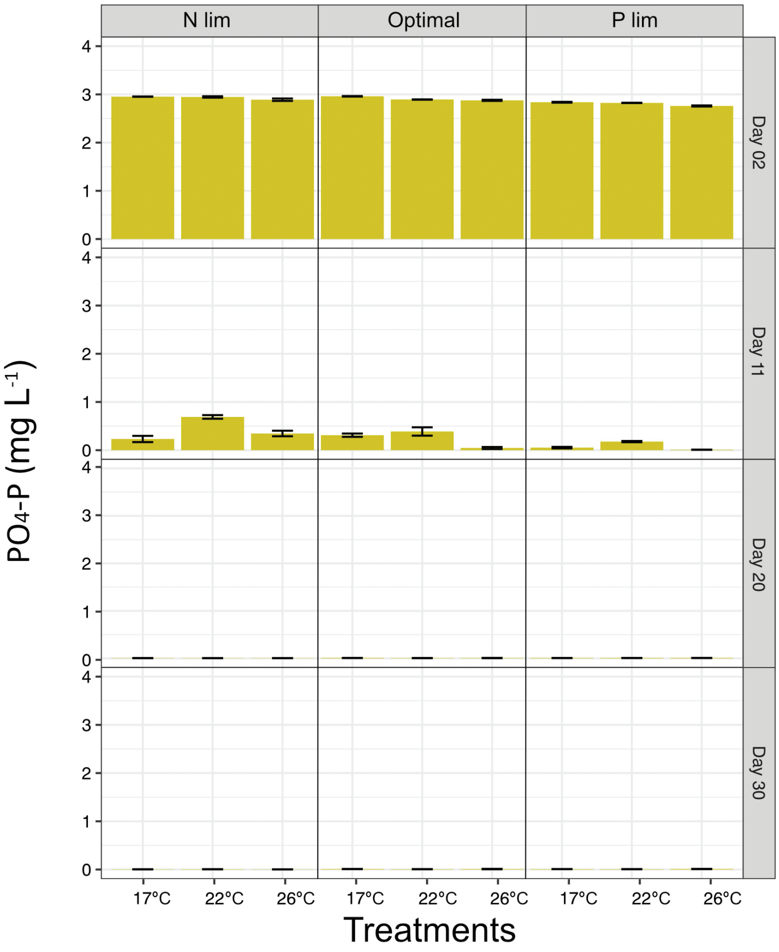

Figure 2. Phosphate levels throughout the experiment (sampled approximately every 10th day, starting from day 2). From day 20 the phosphate levels were under the detection limit (below 0.004 mg L^{-1}). The error bars represent SE.

at 17 °C compared to 22 °C and significantly lower at 17 °C compared to 26 °C ($p < 0,05$). *Raphidiopsis raciborskii* was predominantly influenced by temperature ($F_{(2, 18)} = 4.52$, $p < 0.05$) and by the interaction between temperature-time ($F_{(6, 54)} = 8.600$, $p < 0.001$). Post-hoc analysis showed that on day 10, 16, 25, *R. raciborskii*'s biomass was significantly lower at 17 °C compared to 22 °C and on day 16 it was significantly lower at 17 °C compared to 26 °C ($p < 0.05$). For *Scenedesmus* sp., temperature ($F_{(2, 18)} = 13.377$, $p < 0.05$) and several other interactions between variables significantly affected its biomass, specifically, the interaction between temperature-time ($F_{(6, 54)} = 6.443$, $p < 0.001$), nutrients-time ($F_{(6, 54)} = 3.216$, $p < 0.05$), and the combination of temperature-nutrients-time ($F_{(12, 54)} = 3.063$, $p < 0.05$). The post-hoc analysis showed that on day 16 and 25 *Scenedesmus* sp. biomass was significantly higher at 17 °C compared to 26 °C ($p < 0.05$) and on day 25 its growth was significantly higher at 22 °C compared to 26 °C. On day 25 at 26 °C *Scenedesmus* sp. biomass was significantly higher in the N-limiting (8:1) treatment compared to the optimal one (16:1) ($p < 0.05$) and its growth was significantly lower at the optimal (16:1) treatment compared to the P-limiting one (32:1). The biomass of *P. agardhii* was significantly affected by temperature ($F_{(2, 17)} = 3.59$, $p < 0.005$) and the post-hoc analysis showed that on day 16 its growth was significantly lower at 17 °C compared to 26 °C and significantly lower at 22 °C compared to 26 °C ($p < 0.05$).

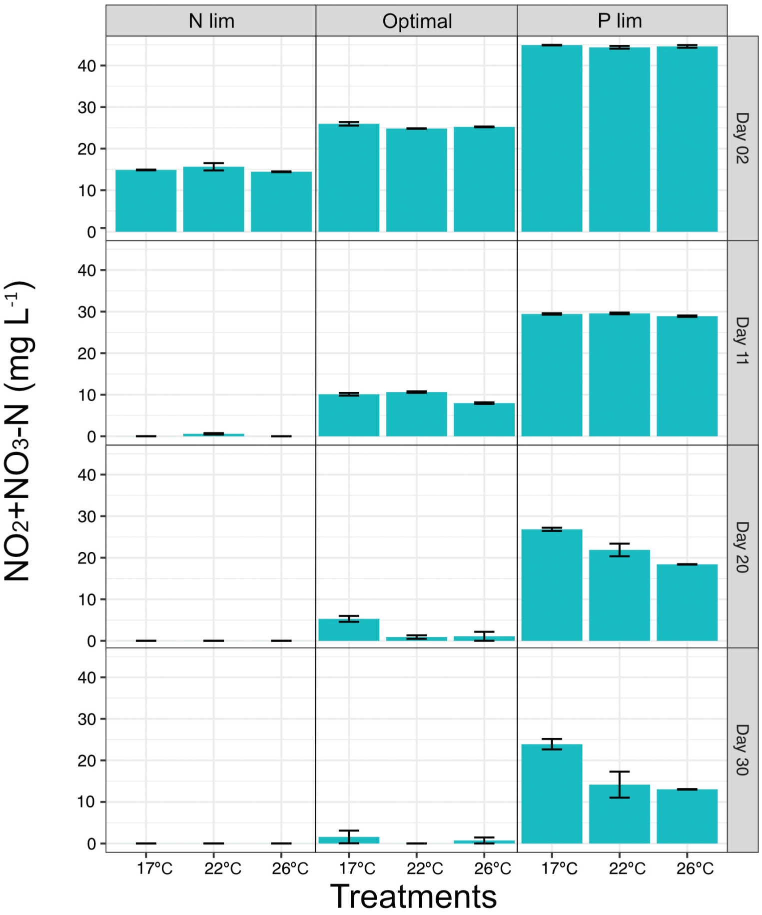

Figure 3. Nitrogen levels throughout the experiment (sampled approximately every 10th day, starting from day 2). Nitrogen-limitation was most severe in the N-limited treatment (below detection limit of 0.003 mg L^{-1} at day 11). In the optimal treatment, N levels were low at and after day 20. In P-limited treatments, nitrogen was available in high levels throughout the experiment. The error bars represent SE.

Discussion

This experiment showed an increase in species' biomass with an increase in temperature in all treatments, except for *Scenedesmus* sp., which showed the opposite trend. In our study, by using a gradient of temperatures similar to those found in Scandinavian lakes during summer, we were able to show that none of the three cyanobacteria grew well at the lowest temperature tested (17 °C), especially *P. agardhii*. *Raphidiopsis raciborskii* was able to maintain a relatively stable biomass throughout the experiment even at the lowest temperature (17 °C). These results imply that if *R. raciborskii* establish viable populations in Swedish lakes, it might be able to outcompete a common filamentous cyanobacteria, like *P. agardhii*, by growing earlier in the season when lake temperatures are colder.

Chlamydomonas reinhardtii and *Scenedesmus* sp. were the only two species affected by nutrients. *Chlamydomonas reinhardtii* had a positive response to the different nutrient ratios, being mostly successful in the N-limiting treatment (8:1). *Scenedesmus* sp., towards the end of the experiment, was also positively affected by N-limiting conditions (8:1). We could speculate that *C. reinhardtii*, due to its high biomass, controlled the nutrient availability, creating a severely nutrient limited environment for all other species influencing their growth, despite the daily exchange of media.

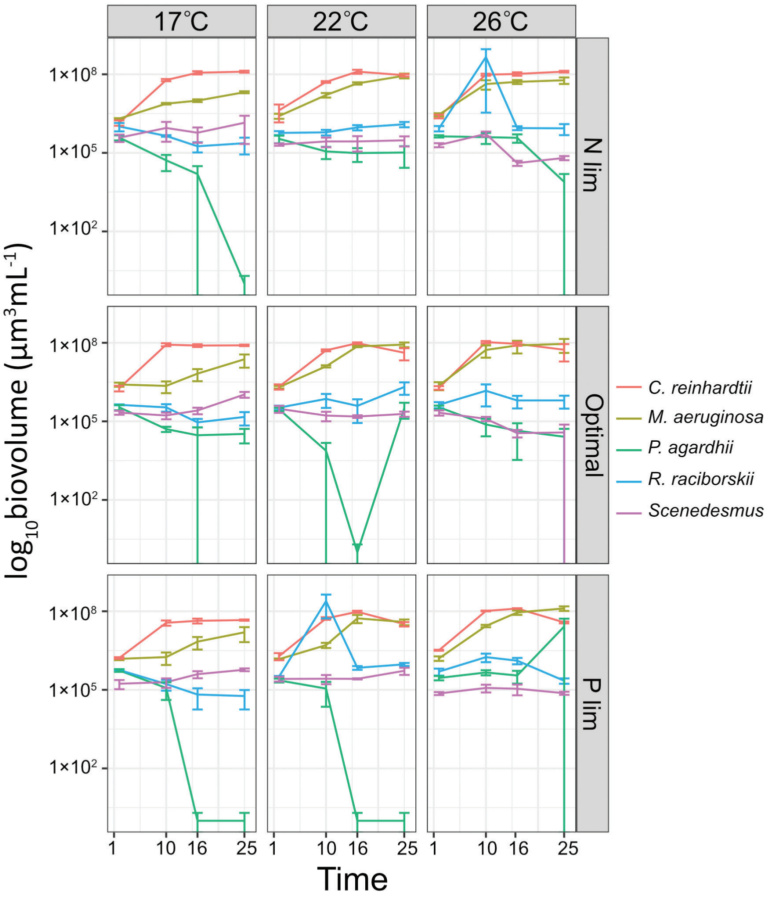

Figure 4. Species' growth (log-transformed biovolume in µm^{3}mL^{-1}) over time (days 1, 10, 16, 25) for each experimental treatment. The last time point (day 31) was removed as the FlowCam analysis was affected by bacterial growth. The most dominant species reached saturation from day 10. The error bars represent SE.

Comparison between *R. raciborskii* and native cyanobacteria

Filamentous cyanobacteria like *P. agardhii*, *R. raciborskii*, and the colonial *M. aeruginosa* are usually the most successful bloom-forming cyanobacteria in temperate shallow lakes (Scheffer et al. 1997; Wiedner et al. 2007; Bonilla et al. 2012). The coexistence between *M. aeruginosa* and *R. raciborskii* is commonly observed in some subtropical and tropical freshwaters. Variations in their dominance have been related to different environmental conditions such as nutrient levels, temperature, light conditions, selective grazing and allelopathy (Lei et al. 2020). Many studies have been conducted to understand the competition and co-occurrence of these two bloom-forming cyanobacteria (Chislock et al. 2014; Yang et al. 2017; Bai et al. 2020). Zheng et al. (2023) showed a result similar to our study, and discussed that *R. raciborskii* could invade into *M. aeruginosa* dominated lakes but it would not be able to take over as a bloom forming species, instead it would be able to survive with a relatively low biomass. It is not possible to predict whether *R. raciborskii* would eventually take over *M. aeruginosa* dominated lakes, as the competition and co-occurrence between these two bloom-forming cyanobacteria is strain-dependent (Zheng et al. 2023).

Raphidiopsis raciborskii and *P. agardhii* have similar phenotypical traits and morphology, indicating that they might be functionally equivalent and occupy a similar ecological niche (Bonilla et al. 2012). However, *R. raciborskii* is able to fix atmospheric nitrogen through heterocysts, giving it a competitive advantage over

P. agardhii in N-depleted environments (Bonilla et al. 2012). Co-occurrence of *R. raciborskii* and *P. agardhii* has been observed in a subtropical reservoir (Jenhani et al. 2012). *P. agardhii* occurred as a permanent species and dominated the phytoplankton community, whereas the presence of *R. raciborskii* was noticeable only for a short period, when temperatures and light conditions were very high (Jenhani et al. 2012). Bonilla et al. (2012), in an extensive field and experimental study, confirmed that *R. raciborskii* exhibited competitive advantages over *P. agardhii* in environments with both high light intensity, high temperature and low P conditions, whilst *P. agardhii* dominated phytoplankton communities in high P and low light availability conditions, mainly due to turbidity. In our study, *P. agardhii* probably suffered from the presence of the other taxa, as it was not able to outcompete *R. raciborskii* in conditions that are known to favour its growth.

Raphidiopsis raciborskii did not dominate in any of the treatments; however, it was able to maintain a stable biomass throughout the experimental period. This suggests a potential high capacity of *R. raciborskii* for coexistence with other species, supporting the hypothesis of its plasticity (Kokociński et al. 2010; Bonilla et al. 2012). If *R. raciborskii* was able to surpass the natural/ecological barriers, eventually spread, and settle in Scandinavian lakes, it would probably be found first in small warmer eutrophic lakes and ultimately be able to survive in low biomass and overcome winters as dormant resting stages.

Conclusions

Differences between experimental conditions and natural ecosystems are unavoidable. Species-traits, environmental conditions and presence of grazers affect competition and invasion outcomes within aquatic systems. Our experimental study showed that the combination of temperature and nutrients ratio controlled the growth of each species within the experiment, providing an overview of species' niches in a controlled environment. The dominant species did not inhibit the growth of *R. raciborskii* and its presence was consistent in all treatments. The results show that *R. raciborskii* might also be able to survive in relatively low temperatures and be able to grow despite competition from native phytoplankton. This means that, based on our experimental conditions, *R. raciborskii* might be able to establish and survive at relatively high latitudes, like in Scandinavian lakes.

Authors' contribution

C.M., R.K.J., A.T.L. and S.D. designed the study. C.M. ran the experiment, performed FlowCam analysis and measurements. C.M. performed data analyses and interpreted the results. C.M. wrote the manuscript. All authors read and approved the manuscript.

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Supplementary material 1

Species’ details purchased from the Norwegian Culture Collection of Algae (NORCCA)

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Supplementary material 2

R. raciborskii growth rate and temperature choice for the experiment

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Supplementary material 3

Z8 media stock solutions

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Supplementary material 4

Flowcam analysis

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